

**EFFECTS OF VETERINARY ANTIBIOTICS ON ATRAZINE DEGRADATION  
IN SOIL**

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## ABSTRACT

The presence of veterinary antibiotics (VAs) in manure applied to agricultural lands may decrease agrichemical degradation by inadvertently altering soil microbial communities or function. Reduced soil microbial degradation of the commonly used herbicide atrazine (ATZ) could increase frequency of detection and concentration of ATZ in water resources. Therefore, the objectives of this study were to investigate the influence of two VAs, sulfamethazine (SMZ) and oxytetracycline (OTC), on ATZ degradation and activities of the soil microbial enzymes dehydrogenase (DH) and  $\beta$ -glucosidase ( $\beta$ -glu) in soil and soil amended with 5% swine manure. Sandy loam soil and swine manure were used to conduct two side-by-side incubation experiments, one to analyze for  $^{14}\text{C}$ -ATZ degradation and the other to measure enzyme activity (no radio-labeled ATZ). Samples were incubated in the dark at 25°C and destructively sampled over a 96 day incubation period. No significant differences between treatments in the soil incubation study were observed for the quantity of ATZ remaining in soil. The distribution of ATZ and its metabolites remaining in the soil were slightly, but not significantly different. After 96 days, approximately half as much ATZ was mineralized to  $^{14}\text{CO}_2$  in samples treated with 100  $\mu\text{g}$  SMZ  $\text{kg}^{-1}$  relative to the ATZ only control. The apparent half-life of ATZ in the soil incubation experiment ranged from 10.6 to 13.5 days.

The addition of manure dramatically changed the behavior of ATZ in soil. In the presence of manure, ATZ degradation decreased by nearly 20% and increased the half-life of ATZ by approximately 20 days. The addition of manure resulted in over 50% more ATZ remaining, a reduction in DDA by nearly half, and the complete absence of DEA in

soil. Atrazine mineralization was reduced by nearly 50% in manure-amended soil. However, the VA treatment did not significantly affect ATZ degradation in manure-amended soil.  $\beta$ -glu activity was significantly influenced by VA type and concentration in the soil incubation study; the least overall  $\beta$ -glu activity was observed in 100  $\mu\text{g kg}^{-1}$  SMZ treated soil and the greatest activity was observed in 100  $\mu\text{g kg}^{-1}$  OTC treated soil.  $\beta$ -glu activity was repressed in manure-amended soils and elevated in VA treated soils (no ATZ or manure) relative to the ATZ only control. Veterinary antibiotics did not significantly influence DH activity in soil, yet the addition of manure stimulated DH activity. A complicated interaction effect between treatment and time was observed for both  $\beta$ -glu and DH enzymatic activity in soil and manure amended soil. Microbial turnover, utilization of manure, VAs, and ATZ as carbon sources, as well as sensitivity of different groups within microbial consortia to ATZ/VAs are possible explanations for the interaction of treatment and time. It appears the application of VAs to agricultural fields does not significantly reduce ATZ degradation in soil at the investigated concentrations. However, the input of manure significantly increased the length of time ATZ will remain in soil. Further research investigating different VA types and concentrations, additional manure sources, and ATZ adapted soils is warranted. The results of this research will influence management decisions which could mitigate negative impacts associated with ATZ and VA co-application to soils.

# **CHAPTER 1: INTRODUCTION, OBJECTIVES, AND LITERATURE REVIEW**

## **1.1 Introduction**

The release of veterinary antibiotics (VAs) into the environment from animal agriculture has become a topic of growing concern. The presence of VAs in the environment has been linked to the development of antimicrobial resistant bacteria and VAs may influence nutrient cycling and pollutant degradation by altering soil microbial activity and communities. Manure containing VAs is often applied to agricultural land treated with herbicides. Many studies have observed how VAs interact in soil and manure but little research has been performed to investigate VA-herbicide interactions. One of the environmental processes VAs may influence is herbicide degradation. Because VAs are intended to suppress microbial activity and herbicide degradation is largely driven by soil microorganisms, there is concern that the presence of VAs may decrease herbicide degradation.

In this research, atrazine (ATZ), one of the most commonly used herbicides in the United States, was selected for study. Degradation of ATZ in soil is important because ATZ has been linked to serious water quality issues. The health risks associated with VAs and environmental problems caused by ATZ make it essential to understand potential interactions of these agrichemicals in soil.

Recognizing the relationship between VAs and herbicides is crucial, yet information on the subject is very limited. Varying lab conditions and protocols make it hard to draw any conclusions from the current data as to how VAs influence microbial

populations. Additionally, soil chemical, physical, and biological properties differ widely in the environment. These differences could have a dramatic impact on how VAs behave in soil and, therefore, the interactions that take place between VAs and herbicides. For example, VA sorption to soil changes with VA chemical structure, VA solubility, and soil chemical and physical properties (e.g., pH, soil organic matter, and clay content). Due to these varying properties, it is near impossible to make generalizations about how VAs effect herbicide degradation in soil.

## **1.2 Objectives and Hypotheses**

A few studies have established a positive correlation between soil microorganisms and herbicide degradation. However, there is a large knowledge gap regarding how VAs may influence herbicide degradation. Therefore, additional research on this subject is warranted. The specific objectives and hypotheses of this study are as follows:

***Objective 1.*** Compare ATZ degradation rates in soil amended or not amended with manure in the presence of SMZ or OTC at environmental concentrations ranging from moderate to extreme.

***Hypothesis 1.*** The rate of ATZ degradation will be reduced in soils containing VAs due to decreased microbial activity compared to control samples containing no VAs. The decrease in degradation rate will be most evident in soils with high VA concentrations. Atrazine degradation in soil containing manure will not be as affected by VAs as soil alone due to increased herbicide and VA sorption to manure.



**Objective 2.** Investigate changes in soil microbial enzymatic activity, specifically the activity of  $\beta$ -glucosidase ( $\beta$ -glu) and dehydrogenase (DH), as a function of time following application of SMZ and OTC to soils amended with ATZ.

**Hypothesis 2.** Enzyme activities will be reduced in soils amended with VAs relative to controls due to antimicrobial properties of the VAs. The decrease will be more pronounced in soils with greater VA concentrations compared to soils containing lesser VA concentrations.

## **1.3 Literature Review**

### **1.3.1 Antibiotic Use in Animal Agriculture and Human Medicine**

Antibiotics are naturally occurring, semi-synthetic, and synthetic compounds that prevent or decrease growth of microorganisms such as bacteria, fungi, and protozoa (Kemper, 2008; Kümmerer, 2009a). Human medicine, animal agriculture, aquaculture, and high value crops utilize antibiotics to control pathogenic microorganisms (Kümmerer, 2004). Veterinary antibiotics (VAs) have been used in animal agriculture in the United States since 1949 (Kumar et al., 2005). The United States is the largest consumer of VAs using over 11,000 tons annually (Kim et al., 2011). The quantity of VAs used in animal husbandry in the United States is eight times greater than the amount of antibiotics used in human medicine (Kümmerer, 2004). Within animal agriculture, swine production is the largest consumer of VAs followed by poultry production (Kim et al., 2011). A remarkable 92.7% of hogs are fed antimicrobials during the finishing stage of production (Mellon et al., 2001).

Historically, VAs were commonly used in animal agriculture to treat and prevent animal illness as well as promote increased animal growth (Wegener 2003; Sarmah et al.,

2006; Wang and Yates., 2008; Lertpaitoonpan et al., 2009). In December 2013, the FDA announced it was implementing a voluntary program to restrict the use of VAs in animal agriculture. Participating animal pharmaceutical companies have three years to change product labeling so to exclude growth promotion and feed efficiency as approved uses. After these changes have been made, the use of the affected VAs in animal agriculture will require veterinary supervision (U.S. FDA, 2013). The dosage levels and variety of VAs used in animal husbandry tend to be greater in large scale animal farms compared to smaller operations (Li et al., 2013). If an animal exhibits signs of illness, VAs are administered to all animals within concentrated animal feeding operations (CAFOs), even if they do not all show symptoms, in order to prevent an outbreak (Mellon et al., 2001). Preventative dosages of VAs are also administered before shipment because animals tend to be stressed and more susceptible to disease (Mellon et al., 2001). Additionally, subtherapeutic levels of VAs help the animal reach market weight faster and counteract the unsanitary conditions found within CAFOs (Accinelli et al., 2006; Kumar et al., 2005). Veterinary drugs can be administered to animals via feed or water, by injection, implant, drench, paste, orally, topically, pour on, or bolus (Sarmah et al., 2006).

### **1.3.2 Commonly Used Veterinary Antibiotics**

Today more than 150 antimicrobial drugs are available for use in animal agriculture and human medicine (Khachatourians, 1998). Antibiotics are grouped into classes based on their chemical structure and mode of action (Kümmerer, 2009a). There are twenty classes of antibiotics and common classes of VAs used in animal agriculture include tetracyclines, sulfonamides, aminoglycosides, fluoroquinolones, penicillins, macrolides, fenicoles, cephalosporins, and arsenicals (Sarmah et al., 2006; Kemper

2008). The main targets of action for VAs include cell walls (bacitracin, cephalosporins, and penicillins), cell membranes (polymyxins), nucleic acids and protein synthesis (aminoglycosides, chloramphenicol, and tetracyclines), and folate synthesis (methotrexate and sulfonamides) (Khachatourians, 1998).

### **1.3.3 Veterinary Antibiotic Uses and Rates**

A complete inventory of antimicrobial use in the United States is not readily available (Mellon et al., 2001). However, it is estimated that 9 to 16 million kg of VAs are used in animal agriculture annually (Mellon et al., 2001; Sarmah et al., 2006). Tetracyclines, sulfonamides, and macrolides are the most commonly used families of VAs (Kim et al., 2011).

Tetracyclines are a broad spectrum group of antibiotics used widely for therapeutic purposes as well as growth promotion in the United States (Sarmah et al., 2006). From 1985 to the late 1990s a 15% increase in tetracycline use in swine production was observed (Mellon et al., 2001). Tetracycline (TTC), chlortetracycline (CTC), and oxytetracycline (OTC) are some of the most commonly used VAs (Kulshrestha et al., 2004). Oxytetracycline [4S,4Ar,5S,5Ar,6S,12Ar)-4-(dimethylamino)-1,5,6,10,11,12a-hexahydroxy-6-methyl-3,12-dioxo-4,4a,5,5a-tetrahydrotetracene-2-carboxamide] is used to treat intestinal and respiratory diseases in animal agriculture (Arikan et al., 2007; Migliore et al., 2012). When administered via medical feed dosages are in the  $\text{g kg}^{-1}$  range (Migliore et al., 2012). Tetracyclines account for 48% of VA use during the swine finishing stage. They are used in poultry production to prevent disease and often combined with sulfonamides to treat cattle before entering feedlots (Mellon et

al., 2001). Oxytetracycline may also be used as a pesticide on high value fruit and vegetable crops (Mellon et al., 2001).

There are 20 to 30 different commercially available sulfonamides (Shelver et al., 2010). Sulfonamides, sometimes called sulfa drugs, are a broad spectrum class of synthetic medicines used against most gram-positive and gram-negative bacteria (Accienlli et al., 2005; Ross-Flanigan and Uretsky, 2006). As of 2004, sulfonamide use in humans was mostly limited to sulfisoxazole, trimethoprim/sulfamethoxazole, sulfadiazine, and sulfasalazine for the treatment of certain infections (Ross-Flanigan and Uretsky, 2006). Sulfonamides are more commonly used in animal agriculture. Sulfamethazine (SMZ), 4-amino-N-(4,6-dimethylpyrimidin-2-yl)-benzenesulfonamide, is the most prevalent sulfonamide used in animal agriculture (Lee et al., 2007). It is commonly used in swine and cattle production to treat and prevent infections as well as increase animal growth (Mellon et al., 2001; Lertpaitoonpan et al., 2009). Annually about  $3.63 \times 10^5$  kg of SMZ are used in animal agriculture (Mellon et al., 2001).

Macrolides are a well-established class of antibiotics with naturally occurring and semi-synthetic derivatives used in both human and veterinary medicine (Kirst, 2002). Tylosin, 2-[(4R,5S,6S,7S,9R,11E,13E,15R,16R)-6-[(3R,5S)-5-[(2S,5S,6S)-4,5-dihydroxy-4,6-dimethyloxan-2-yl]oxy-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-16-ethyl-4-hydroxy-15-[[[(2R,3S,5R,6R)-5-hydroxy-3,4-dimethoxy-6-methyloxan-2-yl]oxymethyl]-5,9,13-trimethyl-2,10-dioxo-1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde, is in the macrolide group of antibiotics produced by *Streptomyces fradiae* (Sarmah et al., 2006; National Center for Biotechnology Information). It is used exclusively in veterinary medicine as a broad-spectrum antibiotic and works against most

gram-positive as well as some gram-negative bacteria (Kirst, 2002; Sarmah et al., 2006). Tylosin is the second most commonly used antimicrobial agent in animal agriculture, the first being ionophores (Mellon et al., 2001). Cattle, chicken, and swine production all utilize tylosin as a growth promoter and therapeutic medicine (Kumar et al., 2005). During the feedlot stage of cattle production, 43% of operations in the United States treat animals with tylosin (Mellon et al., 2001).

Only about 10% of antibiotics used in animal agriculture are intended to treat infection; approximately 90% are administered to prevent illness or increase animal growth (Khachatourians, 1998). Antibiotics used for growth promotion are selected because they are poorly absorbed by the gastro-intestinal wall (Nelson et al., 2011). Bacitracin, a peptide antibiotic, is one of the most widely used feed additive VAs in the world (Sarmah et al., 2006). Another common VA, virginiamycin, is used during all stages of swine production in the United States (Mellon et al., 2001). Subtherapeutic levels of ampicillin, arsenilic acid, bacitracin, bambermycin, chlortetracycline, dihydrostreptomycin, efrotomycin, lalalocid, monensin, oleandomycin, penicillin, roxarsone, spectinomycin, tylosin, and virginiamycin were formally approved as growth promoters in animal agriculture in the U.S. (Teuber, 2001). A 3-5% increase in the rate of animal weight gain is observed when animals are provided feed enhanced with low levels of antibiotics (Khachatourians, 1998). However, the use of VAs for growth promotion has been banned in countries such as Sweden and Switzerland due to environmental and health concerns (Haller et al., 2002). In December 2013, the FDA announced a voluntary plan to phase out the use of VAs as growth promoters by 2016 (U.S. FDA, 2013). The widespread use of VAs in animal agriculture is concerning because it facilitates their

release into the environment. Some threats that antibiotics pose to the environment include: potential entry into the food web; contamination of ground and surface waters; adverse effects on soil microbial communities; and the development of anti-microbial resistant bacteria (Kumar et al., 2005; Dolliver et al., 2007; Lee et al., 2007; Igel-Egalon et al., 2011; Chu et al., 2013).

### **1.3.4 Veterinary Antibiotics in the Environment**

#### **1.3.4.1 Veterinary Antibiotics in Manure**

Animal waste may contain high VA concentrations because a significant portion of an administered VA is not absorbed by the animal (Sarmah et al., 2006; Lee et al., 2007). However, the amount excreted varies based on the antibiotic, mode of action, animal, and time since the animal was treated (Kemper, 2008). Antibiotics undergo little to no metabolism and are therefore still biologically active upon excretion (Yang et al., 2009). Additionally, some antibiotic metabolites can be converted back to the parent compound within manure (Sarmah et al., 2006; Wang and Yates, 2006). Typical concentrations of VAs in manure are between 1 to 10 mg kg<sup>-1</sup>, although levels can reach up to 200 mg kg<sup>-1</sup> (Kumar et al., 2005).

Animals may excrete 25-75% of tetracyclines in the active form (Kulshrestha et al., 2004). In some cases, tetracycline metabolites are more toxic than the parent compound which may result in ecotoxic effects (Zhao et al., 2010). Concentrations of CTC, OTC, and TTC in manure are in the mg kg<sup>-1</sup> range (Yang et al., 2009; Migliore et al., 2012). As much as 90% of sulfonamides are excreted by the animal after consumption (Thiele-Bruhn et al., 2004). Concentrations of SMZ in manure and manure

slurry typically ranges from 0.835 to 9.74 mg kg<sup>-1</sup> (Haller et al., 2002; Burkhardt et al., 2005; Kumar et al., 2005; Shelver et al., 2010).

#### **1.3.4.2 Veterinary Antibiotics in Soil**

On an annual basis, cattle, swine, and poultry production in the United States creates 132 million metric tons (dry weight) of manure (Dolliver et al., 2007). Approximately 9.2 million hectares of land are treated with manure in the United States (Dolliver et al., 2007). Land application of manure is an effective technique for manure disposal that has the added benefit of fertilizing agricultural land. However, the impact of VAs in manure on soil microorganisms and environmental processes could prove to be harmful. Upon entry to a soil, VAs can persist for a few to several hundred days depending on the compound, sorption processes, and environmental conditions (Dolliver et al., 2007; Igel-Egalon et al., 2011). Hamscher et al. (2004) found that tetracyclines could persist at concentrations of 160 µg kg<sup>-1</sup> for two years after repeated application to soil. Haller et al. (2002) found that sulfonamide concentrations in manure treated fields could reach levels similar to pesticide application, up to 1 kg ha<sup>-1</sup>. Tylosin has a short half-life (<48 hours) and is therefore not usually detected in solid manure or soil samples (Aust et al., 2008).

#### **1.3.4.3 Veterinary Antibiotic Transport and Transformation in Soil**

Biotic and non-biotic processes are responsible for the dissipation of VAs in the environment. The three most significant factors for determining VA transport in soil-water systems are sorption, leaching, and degradation (Sarmah et al., 2006). Biotic processes include biodegradation of VAs by bacteria and fungi (Kümmerer, 2009a), and penicillins, macrolides, and aminoglycosides are easily degraded in the environment

(Haller et al., 2002). Biological degradation is the driving force in OTC removal from manure (Wang and Yates, 2008). Aerobic conditions facilitate faster biological dissipation of tetracyclines in soil and manure (Wang and Yates, 2006, 2008; Yang et al., 2009). Sulfonamides are resistant to biological degradation and more likely to be transported to water resources due to lower solid-to solution partition coefficients ( $K_d$  values) (Haller et al., 2002; Chu et al., 2013). Sorption, hydrolysis, photolysis, oxidation, and reduction are non-biotic processes through which VAs are eliminated or dissipated (Kümmerer, 2009a). Quinolones, tetracyclines, sulfonamides, tylosin, and nitrofurantoin antibiotics are sensitive to light and will degrade more readily at the soil surface (Kümmerer, 2009a; Zhao et al., 2010). Arikan et al. (2007) found that OTC concentrations were significantly reduced during manure composting. The reduction in OTC could be a result of degradation, mineralization, or sorption of OTC to the solid matrix (Arikan et al., 2007). The fate and transport of organic contaminants such as VAs in the environment is strongly influenced by their chemical properties and interaction with soil (Sarmah et al., 2006; Chu et al., 2013).

#### **1.3.4.4 Veterinary Antibiotic Sorption to Soil**

The solid-to-solution partition coefficient,  $K_d$ , is a reversible measure of the sorptive exchange of chemicals between the solid-phase sorbent and the aqueous phase (Tolls, 2001). The  $K_d$  value is expressed as the concentration of the compound sorbed to the solid phase ( $C_s$ ) over the concentration of the compound dissolved in the water ( $C_w$ ) (Tolls, 2001). In general,  $K_d$  values can be used to estimate the behavior of a compound in the soil. Veterinary antibiotics with greater  $K_d$  values are more likely to be sorbed to soil and organic matter while VAs with smaller  $K_d$  values are more available for



degradation and transport. Soil properties such as cation exchange capacity (CEC), iron oxide content, clay type and content, pH, and organic matter content all influence VA sorption to soil (Yang et al., 2009; Kong et al., 2012). Sorption of VAs can occur at clay mineral surfaces; thus, the clay mineral content of a soil will influence the sorption coefficient (Tolls, 2001). Due to physical and chemical properties, the clay minerals montmorillonite and vermiculite tend to correspond to greater  $K_d$  values for VAs, kaolinite is associated with the lowest  $K_d$  values, and illite has intermediate values (Tolls, 2001).

Tetracyclines and quinolone carboxylic acid antibiotics have  $K_d$  values (70-5000 L kg<sup>-1</sup>) large enough to consider the VAs relatively immobile in soil (Tolls, 2001). Oxytetracycline and CTC are generally immobile in soil because they are strongly sorbed and are not readily desorbed (Kulshrestha et al., 2004; Laak et al., 2005; Yang et al., 2009). For this reason, OTC and CTC persist in soil and they are unlikely to leach into ground or surface waters (Hamscher, et al., 2004; Kulshrestha, et al., 2004). Cation bridges formed by multivalent cations such as Fe<sup>3+</sup> and Al<sup>3+</sup> may further enhance the sorption of tetracyclines (Tolls, 2001; Rubert et al., 2009); thus, reducing bioavailability through sorptive processes and metal complexes. However, these processes do not actually remove OTC from the environment and may inhibit degradation of the compound (Kulshrestha et al., 2004; Rubert et al., 2009). Transport of tetracycline in soil is limited to macropore flow or enabled by adsorption to dissolved organic matter (Kemper, 2008).

Veterinary antibiotics with intermediate  $K_d$  values have moderate mobility in soils. Avermectin, tylosin, and efrotomycin have intermediate  $K_d$  values ranging from 7 to

300 L kg<sup>-1</sup> (Tolls, 2001). Sorption of these VAs is influenced by the physical properties of the soil. Tylosin was found to be immobile in a clay loam soil and relatively mobile in a loamy sand (ter Laak et al., 2005). Zhang et al. (2011) found that tylosin was moderately mobile in three test soils, with 86% to 97% of the total mass of the chemical being sorbed on the solid soil phase. While tylosin is more likely to be sorbed to soil than VAs with smaller  $K_d$  values, little to no desorption hysteresis suggests that tylosin could be readily desorbed from soil and released into the aqueous phase (Zhang et al., 2011).

Sulfonamides are highly mobile in soils with  $K_d$  values ranging from 0.9 to 10 L kg<sup>-1</sup> (Thiele-Bruhn, 2003; Chu et al., 2013). Their mobility in soil is related to high polarity and water solubility (Shelver et al., 2010). Compounds such as SMZ and sulfachloropyridazine with low  $K_d$  values (0.2-3.9 L kg<sup>-1</sup>) are more likely to be transported to ground or surface waters (ter Laak et al., 2005; Lertpaitoonpan et al., 2009). Frequent detection of SMZ in water resources may be attributed to weak sorption to soil, which increases the likelihood that SMZ will remain in solution and move from soil to water sources. However, Henderson et al. (2009) found that SMZ has the potential to sorb to manure making it important in determining antibiotic fate in soil. The VAs olaquinox metronidazole, and chloramphenicol ( $K_d$  values ranging from 0.2-2 L kg<sup>-1</sup>) are also considered highly mobile and not likely to sorb to soil (Tolls, 2001).

Sorption of VAs in the environment is highly dependent on soil pH. For this reason, VA sorption at different pH values is necessary for environmental risk assessment (ter Laak et al., 2005). Veterinary antibiotics may experience protonation or deprotonation in the environment if they have pK<sub>a</sub> values within the soil pH range (Tolls, 2001). Sulfonamides have pK<sub>a</sub> values corresponding with pH values found in most soil

solutions (pH 4.5-6.5) (Chu et al., 2013). As a result, cationic, neutral and zwitterionic, and anionic sulfonamides may all be present in soil solution (Sakurai and Ishimitsu, 1980; Shelver et al., 2010). Tetracyclines are predominantly zwitterions in the environment, although they may also exist as cations or anions (Jones et al., 2005; Sarmah et al., 2006). The addition of manure to the system will, in part, influence sorption because of its effect on pH (ter Laak et al., 2005). Soil pH values can be vastly different due to manure application and transformation within the soil (ter Laak et al., 2005). Initially the addition of manure containing ammonium will make soil solution more basic. However, as soil microbes convert the ammonium to nitrate, protons will be released into soil solution causing pH to decrease (ter Laak et al., 2005).

### **1.3.5 Environmental Challenges Associated with Veterinary Antibiotics**

#### **1.3.5.1 Effect on Food Webs**

There is concern that VAs may accumulate in plants and adversely affect plant health (Dolliver et al., 2007; Sarmah et al., 2006; Kim et al., 2011). Oxytetracycline is of environmental concern because phytotoxic effects have been observed in higher plants (Brain et al., 2009). Additionally, the uptake of VAs by food crops or VA residues in animal-based food products could cause VA entry into the food chain (Dolliver et al., 2007). Humans may also be exposed to antibacterial resistant organisms by ingesting food products containing resistant bacteria (Haller et al., 2002). In addition to spreading antibiotic resistant bacteria, uptake of VAs by food plants could adversely affect digestive function and promote allergic reactions, acute toxicity, and chronic toxic effects due to long term exposure (Dolliver et al., 2007; Kemper 2008; Wang and Yates, 2008). Sulfamethazine is taken up by plants more easily than other antibiotics due to its low

molecular weight and low sorption potential (Dolliver et al., 2007). This is problematic because studies conducted in 1988 by the National Center for Toxicology found that SMZ is a carcinogen and has been shown to cause thyroid tumors in rats and mice after receiving doses  $\geq 2.4 \text{ mg kg}^{-1}$  SMZ in their diet over the course of two years (Lertpaitoonpan et al., 2009). Human health could be negatively impacted if food products containing VA residues are consumed.

### **1.3.5.2 Effects on Water Quality**

Numerous antibiotics have been detected in wastewater from hospitals, wastewater treatment plants, and facilities producing antibiotics as well as agricultural wastewater, surface runoff, ground water, and drinking water (Wen et al., 2009). Agricultural areas can contaminate receiving water bodies via leaching, subsurface flow, and surface runoff (Meyer et al., 2010). Certain antibiotics such as bacitracin, tylosin, sulfonamides, and to some extent tetracyclines are very soluble in water (Sarmah et al., 2006; Shelver et al., 2010). Sulfonamides are very mobile in soil and likely to be transported to water resources (Lertpaitoonpan et al., 2009). Sulfamethazine has been detected in surface waters throughout the United States (Henderson et al., 2009). Concentrations of SMZ detected in surface runoff from manured plots can reach 2.5 to  $6.8 \mu\text{mol L}^{-1}$  (Burkhardt et al., 2005; Kreuzig et al., 2005). Furthermore, sulfonamides may leach from agricultural fields and enter groundwater (Hamscher et al., 2004; Shelver et al., 2010). In addition to having poor water quality, water near agricultural sites, especially near CAFOs, has high levels of single and multi-drug resistant bacteria compared to water from wastewater treatment plants (West et al., 2011). However, there

are no regulations related to antibiotic resistant bacteria in waters of the United States (West et al., 2011).

### **1.3.5.3. Effects on Soil Microbial Communities**

Soil bacteria may experience reduced activity or mortality if they are sensitive to VAs applied to agricultural fields via manure application (Nelson et al., 2011). Even though VAs are quickly adsorbed and thus removed from soil solution, soil bound VAs are still bioactive (Kemper, 2008) and have the ability to interact with soil microorganisms. Veterinary antibiotics exhibit short term effects on the soil microbial community (Nelson et al., 2011; Unger et al., 2012). Wang and Yates (2006) observed a decrease in microbial activity with increasing antibiotic concentration in manure amended soils. An initial decline in activity followed by a recovery of soil microorganisms exposed to VAs was indicated by enzyme assays (Unger et al., 2012). Nelson et al. (2011) observed differences in microbial community structure based on the absence or presence of CTC in manure amended soil. Changes in bacterial and actinomycetes community structure were observed with the addition of  $\leq 10 \text{ mg kg}^{-1}$  OTC (Yang et al., 2009). The change in composition and diversity of soil microbial communities could disrupt the natural cycles of decomposition, energy flow, and nutrient transformation (Wang and Yates, 2008).

The presence of VAs may decrease important biotransformation processes such as denitrification, nitrogen fixation, and degradation of organic compounds such as soil applied pesticides because these processes are facilitated by soil microorganisms (Accinelli et al., 2006; Wen et al., 2009). Kong et al. (2006) found that bacteria responsible for degradation of polymers and carbohydrates appear to be very sensitive to

OTC. Yang et al. (2009) observed sensitivity of alkaline phosphatase, the enzyme that catalyzes transformation of organic phospholipids into inorganic phosphate, to OTC at concentrations ranging from 10-30 mg kg<sup>-1</sup>. This suggests the application of animal manure to soils may decrease processes facilitated by soil microorganisms such as residue decomposition.

Even though changes in microbial communities appear to be temporary, the long term effects of repeated VA application to soils are unknown (Nelson et al., 2011). A study in northeastern China found that OTC posed the largest threat to soil microorganisms due to an extremely high hazard quotient of 15.75 for the VA (a quotient  $\geq 1$  is considered hazardous) (Li et al., 2013). However, it is difficult to predict if antibiotics will negatively impact soil microorganisms. Igel-Egalon et al. (2011) found that VA concentration and length of exposure caused different effects on various soil enzymes. Additionally, soil microorganisms can naturally produce antibiotics such as  $\beta$ -lactams, streptomycins, and aminoglycosides (Kümmerer, 2009a; Igel-Egalon et al., 2011). As a result, some soil bacteria are naturally resistant to antibiotics (Kemper, 2008).

#### **1.3.5.4 Development of Antibiotic Resistant Bacteria**

The greatest risk antibiotics in the environment pose is the development of antimicrobial resistant bacteria. Antibiotics are used extensively to treat diseases in humans and animals. For this reason, the emergence of antimicrobial resistant bacteria is a global concern from a public health and veterinary health perspective (West et al., 2011). Bacteria can develop primary or secondary resistance to antibiotics (Kümmerer, 2009b). Primary resistance refers to resistance inherited by organisms of the same species

through cell division (Kümmerer, 2009b). This kind of resistance naturally occurs in microorganisms and no contact with the antibiotic is necessary (Mellon et al., 2001; Kümmerer, 2009b). Secondary resistance develops after exposure to an antibiotic but can eventually be lost. Unlike primary resistance, secondary resistance can cause horizontal gene transfer, the transfer of resistant genes, between different bacterial species (Kümmerer, 2009b).

In the soil, bacteria may become resistant to antimicrobials through genetic mutation or the transfer of genetic material from one bacterium to another (Lee et al., 2007). Low concentrations of VAs in soils may cause increased selection pressure of VA-resistant bacteria in the environment (Nygaard et al., 1992; Kümmerer 2004) However, resistant bacteria in soil is normally stimulated in manure and then transferred to the soil by application to agricultural fields (Kemper, 2008). Antibiotic resistant microorganisms are frequently found in the intestinal tracts of animals and their manure (Haller et al., 2002). The presence of VAs in the soil may induce resistant populations, maintain these populations after development, alter beneficial bacterial activity, and increase the ease of resistant gene transfer between bacterium (Henderson et al., 2009). This would be especially harmful if resistance developed for antibiotics such as OTC and SMZ which are used to treat diseases in humans and animals (Yang et al., 2009). Within animal agriculture, it is possible for bacteria to develop resistance to antibiotics they have not been exposed to if there are significant structural similarities between the employed VA and the outside antibiotic (Khachatourians, 1998; Mellon et al., 2001). Once a microorganism develops resistance to a specific VA, it is normally resistant to the entire family of drugs regardless of exposure (Mellon et al., 2001).

## **1.4 Atrazine**

### **1.4.1 Atrazine Use in Agriculture**

Atrazine (ATZ), a triazine class of herbicide, is one of the most commonly used broadleaf weed control products in the United States (Galluzzo et al., 1999; USDA, 2007). It has been used as a major agricultural herbicide in the U.S. since the 1960s (Ribaudó et al., 1994). Atrazine is sold under the brand names Aatrex, Astram, Atratol, and Gesaprim, and can also be sold in combination with other agrichemicals (Agency for Toxic Substances and Disease Registry, 2003; Prostko, et al. 2009). Nearly all ATZ in the environment is due to crop application although a small portion is due to manufacturing (Agency for Toxic Substances and Disease Registry, 2003).

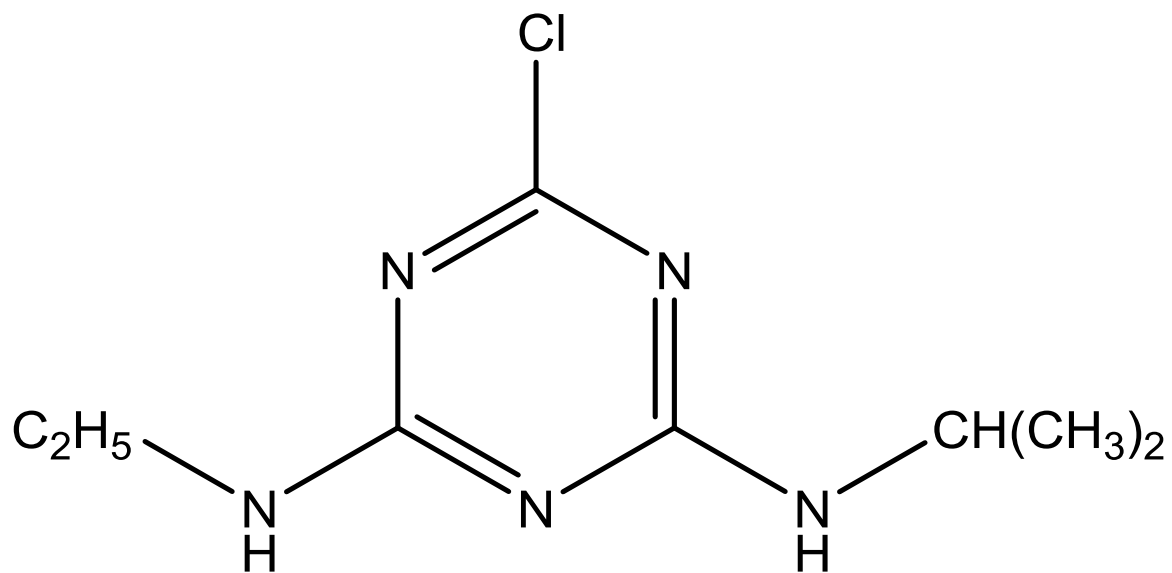
Atrazine can be applied pre-planting, pre-emergence, or post-emergence (Ribaudó et al., 1994). Application rates for this triazine herbicide range from 2.2 to 4.5 kg ha<sup>-1</sup> (Mudhoo and Garg, 2011), and the application rate required for effective weed control is determined by soil texture and climate. During the years 1990 to 2005, 78 to 95% of all acres planted to corn were treated with ATZ (USDA- National Agricultural Statistics Service, 2011). In 2010, approximately 23 million kilograms of ATZ were applied across 18 states making it one of the most widely used herbicides in the nation (USDA- National Agricultural Statistics Service, 2011). Even though it is most commonly used on corn, ATZ can also be used to control weeds for other crops such as sugarcane, sorghum, and pineapples (Agency for Toxic Substances and Disease Registry, 2003). Atrazine may also be used in forestry plantations where it is applied less frequently but application rates used in forestry are four times greater than in agricultural settings (Kookana et al., 2010).



### 1.4.2 Atrazine Properties and Mode of Action

Atrazine [6-chloro-N-ethyl-N<sup>2</sup>-(1-methylethyl)-triazine-2,4-diamine] (Fig. 1.1) is a synthetic, white, odorless powder which will dissolve in water and is very soluble in organic solvents (Agency for Toxic Substances and Disease Registry, 2003). It is a chlorinated, N-hybrid aromatic ring xenobiotic compound with ethylamino- and isopropylamino-constituents at the 4 and 6 positions, respectively (Galluzzo et al., 1999). Atrazine is the most commonly used *s*-triazine herbicide in the United States (Mandelbaum et al., 1993). It is much more mobile and persistent in the environment compared to other pesticides such as metolachlor and alachlor because of the *s*-triazine ring structure (Mandelbaum et al., 1993; Wietersen et al., 1993). Atrazine is also more persistent compared to other *s*-triazine herbicides because it is chlorinated and heavily substituted (Mandelbaum et al., 1993).

Atrazine in soil solution is available for uptake by plant roots. Once the herbicide is absorbed by the plant, ATZ prevents photosynthesis in the shoots and leaves; thus, killing the plant (Agency for Toxic Substances and Disease Registry, 2003). Disruption of photosynthesis is accomplished through ATZ binding to the quinone-binding protein in photosystem II which, in turn, prevents photosynthetic electron transport (Wackett et al., 2002). Certain plants, such as corn, are able to metabolize concentrations of ATZ that are lethal to many weeds before it can prevent photosynthesis (Wackett et al., 2002; Agency for Toxic Substances and Disease Registry, 2003). This makes ATZ particularly important in agriculture because it is able to effectively kill weeds without harming the desired crop.



**Figure 1.1** Atrazine [6-chloro-N-ethyl-N<sup>1</sup>-(1-methylethyl)-triazine-2,4-diamine]

### 1.4.3 Atrazine in the Environment

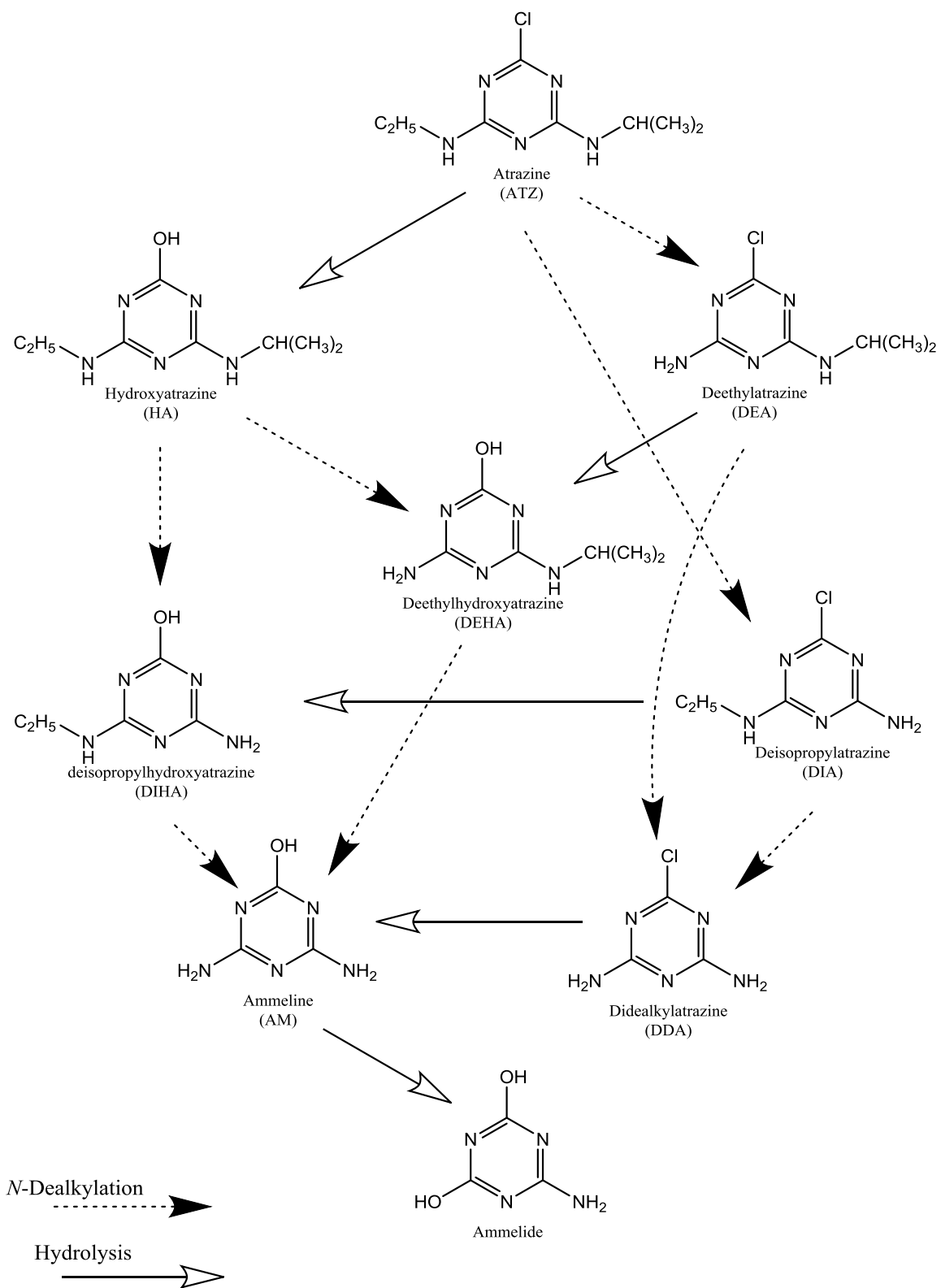
#### 1.4.3.1 Atrazine in Soil

Soil organic matter, clay particles, oxides and hydroxides are the predominant surfaces where ATZ will sorb within soil (Mudhoo and Garg, 2011). Fluctuations in pH may affect ATZ sorption because soil organic matter, clay minerals, and organic pollutants may be influenced by pH (Mudhoo and Garg, 2011). Clay and Koskinen (1990) observed that ATZ sorption to soil was greater at more acidic pH conditions. Additionally, ATZ was more readily desorbed in soils with more basic pH levels compared to acidic soils (Clay and Koskinen, 1990). Atrazine sorption to soil is positively correlated to organic matter content (Binet et al., 2006). When ATZ sorbs to soil and organic matter the bioavailability decreases and ATZ is less susceptible to biodegradation (Binet et al., 2006). Atrazine's hydroxylated degradation products sorb to soil through mixed-mode binding mechanisms of cation exchange and hydrophobic interaction (Lerch et al., 1997). The metabolite hydroxyatrazine (HA) [4-(ethylamino)-6-(isopropylamino)-1,3,5-triazin-2-ol] is strongly and irreversibly bound to soil and soil organic matter, and HA accounts for 5 to 25% of applied ATZ several months after application (Clay and Koskinen, 1990; Mandelbaum et al. 1995; Mudhoo and Garg, 2011; The European Bioinformatics Institute). The metabolites deethylatrazine (DEA) [6-chloro-*N*-(propan-2-yl)-1,3,5-triazine-2,4-diamine] and deisopropylatrazine (DIA) [6-Chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine] are much more mobile in soil compared to HA and account for only 2 to 10% of soil applied ATZ (Panshin et al., 2000; Mudhoo and Garg, 2011; National Center for Biotechnology Information; The European Bioinformatics Institute).

Atrazine is persistent in cool and dry environments where there is little fluctuation in pH (Graymore et al., 2001). Atrazine's water solubility ( $33 \text{ mg L}^{-1}$  at  $27 \text{ }^\circ\text{C}$ ) and low dissociation constant ( $\text{pK}_a = 1.68$ ) makes it less likely to adhere to soil particles, and reported  $K_d$  values range from 0.19 to  $2.46 \text{ L kg}^{-1}$  (Senesi, 1992; Solomon et al., 1995; Mudhoo and Garg, 2011). These qualities make ATZ fairly mobile in soil and increase the possibility of leaching, especially after heavy rainfall events (Graymore et al., 2001). However, ATZ flow within the soil matrix is retarded compared to bromide transport (Kazemi et al., 2008), and ATZ leaching occurs predominantly through macropore flow (Mudhoo and Garg, 2011). As a result, ATZ tends to stay in the surface soil where it is degraded rather than leaching to lower depths (Kookana et al., 2010). Although ATZ transport in the aqueous phase is more likely, transport of sorbed ATZ with eroded soil particles is possible (Graymore et al., 2001).

#### **1.4.3.2 Atrazine Degradation**

Atrazine remaining in soil will degrade over a period of days to months (Agency for Toxic Substances and Disease Registry, 2003). In rare cases, ATZ can persist in soil for several years (Agency for Toxic Substances and Disease Registry, 2003). Atrazine has five major degradation products which include the N-dealkylated metabolites, DEA and DIA, and the hydroxylated metabolites, hydroxyatrazine (HA), deethylhydroxyatrazine (DEHA), and deisopropylhydroxyatrazine (DIHA) (Fig. 1.2) (Lin et al., 2008). The chlorinated metabolites DEA and DIA are nearly as toxic as the parent compound (Kaufman and Kearney, 1970). Hydrolysis, sorption, volatilization, photodegradation, and microbial degradation determine the rate of ATZ dissipation in soil (Graymore et al., 2001; Kim et al., 2010). Biodegradation of ATZ by bacterial and



**Figure 1.2** The major atrazine degradation pathway adapted from Lin et al. (2008).

capable of completely mineralizing ATZ have been isolated, degradation is usually the result of microbial consortia (Mandelbaum et al., 1993, 1995; Kolić et al., 2012). Fungal populations is the primary mode of ATZ degradation in nature (Galluzzo et al., 1999; Ostrofsky et al., 2002). Behki and Khan (1986) observed that 50-60% of ATZ biodegradation could be attributed to nonfungal microbial species. Although individual species

The metabolic pathway for ATZ mineralization follows six successive steps: dechlorination; dealkylation; ring cleavage; biuret deamination; and allophanate hydrolysis (Kolić et al., 2012). Hydroxyatrazine is a nonphytotoxic ATZ metabolite formed by hydrolysis of the parent compound where the chlorine is replaced with an –OH group (Kaufman and Kearney, 1970; Clay and Koskinen, 1990). Following dechlorination, either the N-isopropyl or N-ethyl side group is removed and results in the formation of N-ethylammelide or N-isoproplamelide (Boundy-Mills et al., 1997; Smith et al., 2005; Kolić et al., 2012). Deamination of ATZ creates cyanuric acid as a product which is eventually transformed into biuret by the cyanuric acid ring cleavage (Galluzzo et al., 1999; Smith et al., 2005; Seffernick et al., 2012). After ring cleavage, deamination of biuret releases ammonia which is a possible nitrogen source for microorganisms (Kolić et al., 2012). Biuret can also be hydrolyzed to allophanate which is subsequently transformed into carbon dioxide and ammonia (Martinez et al., 2001; Cheng et al., 2005; Sharpir et al., 2005, 2006). Complete mineralization of ATZ results in the formation of carbon dioxide and ammonia (Galluzzo et al., 1999).

### 1.4.3.3 Effects on Soil Microorganisms

Several review papers outlining biodegradation of ATZ and the evolution of ATZ degrading microorganisms are available (Wackett et al., 2002; Kolić et al., 2012). During the early decades of ATZ use, biodegradation in soil was very slow (Sheets, 1970; Erickson and Lee, 1989) because the structure of the *s*-triazine ring is dissimilar to naturally occurring compounds (Esser et al., 1975). It was believed that dechlorination of ATZ in soil was a solely chemical process (Wackett et al., 2002) and the major metabolites resulting from biodegradation were DEA and DIA (Kolić et al., 2012). However, microorganisms adapt to the input of xenobiotics in the environment by developing mechanisms to utilize them as energy sources (Kolić et al., 2012). Horizontal gene transfer can occur in communities experiencing high selection pressure (Kolić et al., 2007), which has increased the ability of soil microorganisms to degrade ATZ.

Intensive application of ATZ to agricultural soil has caused increased selection pressure for the evolution of microbial ATZ metabolism (Sadowsky et al., 1998). The genes *atzA*, *atzB*, and *atzC* encode for enzymes involved in the upper degradation pathway where ATZ is transformed to HA and then cyanuric acid following removal of the isopropylamine and ethylamine groups (Kolić et al., 2007). Atrazine chlorohydrolase (AtzA) is the enzyme responsible for the formation of HA which was formerly credited to chemical hydrolysis alone (Wackett et al., 2002). Hydroxyatrazine in soil can be converted to cyanuric acid via hydroxyatrazine hydrolase (AtzB) (Wackett et al., 2002). The formation of cyanuric acid can also be accomplished by N-isopropylammelide isopropylaminohydrolase (AtzC), an enzyme capable of transforming N-isopropylammelide to cyanuric acid and isopropylamine (Sadowsky et al., 1998). The

lower degradation pathway involves the *atzD*, *atzE*, and *atzF* genes which encode for metabolism of the byproducts cyanuric acid, biuret, and allophanate, respectively (Wackett et al., 2002; Kolić et al., 2007). There are also a number of enzymes such as triazine hydrolase (TrzN) that catalyze the degradation of triazine herbicides such as ATZ (Kolić et al., 2012). Unlike soils that have a long history of annual ATZ application, no measurable biodegradation is observed in soils that had never been exposed to ATZ (Behki and Khan, 1986).

#### **1.4.3.4 Atrazine in Water**

Agricultural runoff is the largest source of ATZ found in aquatic environments (Lerch and Blanchard, 2003; Fortin et al., 2008). The runoff potential of an agricultural field is determined by soil texture and topography, land and herbicide management, climate, and the chemical and physical properties of the herbicide (Lerch and Blanchard, 2003). Runoff losses of ATZ from agricultural fields are usually less than 5% (Johnson et al., 1996). However, Lerch et al. (2011) found that claypan soils are especially vulnerable to ATZ transport with as much as 14% of applied ATZ lost annually. High levels of ATZ in surface water are at their peak in the corn belt of the Midwest immediately following spring and summer ATZ application (Solomon et al., 1996; Lerch and Blanchard, 2003). These high levels occur in pulses following rain events with spikes that can last for several days (Fortin et al., 2008).

Atrazine is the most frequently detected pesticide chemical in ground, surface, and drinking water (Solomon et al., 1996). It is detected in U.S. groundwaters up to twenty times more frequently than any other herbicide (Graymore et al., 2001). Atrazine has relatively slow hydrolysis and photolysis rates, thus it is somewhat persistent in the



water column (Solomon et al., 1996). The widespread use of ATZ in agriculture has resulted in ATZ concentrations in surface and groundwater that often exceed the authorized limits (Kolić et al., 2012). Lerch et al. (2011) found ATZ concentrations the USEPA considers harmful to aquatic ecosystems (maximum contamination levels of 10 to 20  $\mu\text{g L}^{-1}$ ) in the Goodwater Creek Experimental Watershed for 10 of the 15 years studied. Hydroxylated ATZ degradation products, such as HA, were also detected in the Goodwater Creek Experimental Watershed (Lerch et al., 1995). The presence of these hydroxylated metabolites may be the result of surface runoff, desorption from stream sediment, or ATZ degradation in the water column (Lerch et al., 1995).

The ability of ATZ to be transported to ground or surface water is a serious environmental risk. Best management practices can reduce ATZ transport to water resources. Observing buffer zones where herbicide is not applied near water resources can reduce likelihood that the herbicide will be transported to surface waters (Johnson et al., 1996). Additionally, several studies have evaluated the potential of vegetative buffers and phytoremediation to mitigate atrazine transport to ground and surface waters (Belden and Coats, 2004; Henderson et al., 2007; Lin et al., 2008, 2011; Albright et al., 2013; Murphy and Coats, 2011; Willett et al., 2013).

#### **1.4.4 Health Effects Associated with Atrazine**

Atrazine's herbicidal properties may inadvertently cause problems for many aquatic species. Algal production may be reduced when the herbicide reaches water resources and impacts the aquatic ecosystem. Reduced water quality and food availability in aquatic ecosystems may result from ATZ effects on aquatic flora (Graymore et al., 2001). Shifts in ecosystem composition are possible because of changes to nutrient

supply ratios, temperature, and biological controls (Graymore et al., 2001). Atrazine decreases primary productivity in water and results in reduced dissolved oxygen content (Solomon et al., 1996; Graymore et al., 2001). A decrease in primary productivity is also correlated with higher concentrations of dissolved inorganic carbon and decreases in dissolved and particulate organic carbon (Graymore et al., 2001). Aquatic organisms such as zooplankton, gastropods, crustacean, and insects that rely on algae as a food source may experience reduced reproduction and growth or changes in abundance, species richness, and diversity following decreases in food supplies (Dewey, 1986; Graymore et al., 2001).

In addition to the indirect impacts on aquatic ecosystems, the release of ATZ in the environment can have direct effects on wildlife and human health. Atrazine is considered slightly to moderately toxic to humans and other animals (Galluzzo et al., 1999). Absorption of ATZ into the bloodstream is possible via oral, dermal, or inhalation exposure (Galluzzo et al., 1999). The EPA classifies ATZ as a group C- possible carcinogen based on limited animal studies and inadequate human data (Galluzzo et al., 1999). MacLennan et al. (2002) observed a 1.8-fold increase in prostate cancer incidence among *s*-triazine herbicide manufacturing workers. However, increased medical surveillance of employees compared to the general public is a possible explanation for the greater number of documented cancer cases. Numerous studies have associated ATZ with reduced success of aquatic and terrestrial species following exposure. Fortin et al. (2008) observed that environmentally relevant concentrations of ATZ affected osmotic control in mummichog larvae. The presence of hyperhydrated and dehydrated larvae coupled with no change in mean percentage of water suggests that individual larvae have

varying sensitivity to ATZ (Fortin et al., 2008). Wiegand et al. (2001) concluded that the additional energy required to detoxify ATZ may affect the growth of zebrafish embryos. Of all the health concerns associated with ATZ, the most widely recognized is its ability to act as an endocrine disrupting chemical

Transport to surface water could cause detrimental impacts to wildlife populations because ATZ is a potential endocrine disruptor at environmentally relevant concentrations. Timing of ATZ application in the spring frequently corresponds to amphibian and fish reproduction and development (Wiegand et al., 2001; Hayes et al., 2003). Exposure at sub-part per billion levels, which are often exceeded in water resources including precipitation, can affect male African clawed frogs and American leopard frogs (Hayes et al., 2002, 2003). Testicular oocytes were observed in male frogs exposed to ATZ in field and laboratory settings (Hayes et al., 2003). Demasculinization and complete feminization of genetic male African clawed frogs was observed at ATZ levels as low as  $0.1 \mu\text{g L}^{-1}$  (Hayes et al., 2010). Males exposed to ATZ were unable to compete for female mates and showed decreased fertility. Additionally, the impact on population dynamics could be catastrophic because sex reversed males can only produce genetic male offspring (Hayes et al., 2010). Environmentally relevant concentrations of ATZ ( $0.5 \mu\text{g L}^{-1}$ ) were observed to significantly affect reproductive output in the fathead minnow (Tillit et al., 2010). Atrazine appeared to have a threshold effect on the fathead minnow resulting in reduced egg production, decreased number of spawning events, and changes in oocyte maturation processes (Tillit et al., 2010). However, Bringold et al. (2004) did not find significant evidence that ATZ acts as an endocrine disrupting chemical in a short-term reproductive study of the fathead minnow. The endocrine

disrupting effects of ATZ may extend beyond aquatic organisms exposed to ATZ via runoff. Swan et al. (2003) found that semen quality may be reduced in men with exposure to pesticides such as ATZ compared to men that have not been exposed to agrichemicals.

The endocrine disrupting effects caused by ATZ do not appear to be caused by ATZ binding to the estrogen receptor (Cooper et al., 2000; Hayes et al., 2003). Continuous exposure to ATZ inhibited lutenizing hormone and prolactin secretion in female rats leading to deregulation of ovarian function (Cooper et al., 2000). The resulting endocrine effects appeared to be due to disruption of the central nervous system and pituitary function and not because of estrogenic properties (Cooper et al., 2000). Male Atlantic salmon showed decreased olfactory response to a priming pheromone in the presence of ATZ and another *s*-triazine herbicide, simazine (Moore and Lower, 2001). Atrazine induced aromatase activity resulted in production of endogenous estrogen in male American leopard frogs (Hayes et al., 2003).

## **1.5 Veterinary Antibiotics and Atrazine**

One of the environmental processes VAs may influence is herbicide degradation. Because VAs are intended to suppress microbial activity and herbicide degradation is largely driven by soil microorganisms, there is concern that the presence of VAs may decrease herbicide degradation. Understanding the relationship between VAs and herbicides is crucial, yet information on the subject is very limited. Accinelli et al. (2006) found that SMZ in the  $\mu\text{g kg}^{-1}$  range did not have any noticeable impact on metolachlor degradation or sorption. Another study by Kim et al. (2010) investigated influence of the antibiotics monensin, narasin, and salinomycin used in poultry production on ATZ degradation. It was found that ATZ concentrations were significantly greater in soils

treated with antibiotics compared to the control (Kim et al., 2010). Additionally, the half-lives of ATZ for the antibiotic treatments were significantly longer than the control treatment without antibiotics (Kim et al., 2010). Ostrofsky et al. (2002) and Levanon et al. (1993) found that VAs reduced the degradation of herbicides in soil. However, the concentrations of VAs [3-5 g kg<sup>-1</sup> (Ostrofsky et al., 2002) and 2.5-5.85 g kg<sup>-1</sup> Levanon et al., 1993] used in these studies were intended to sterilize the soil, thus the results are not relevant to this study. Decomposition of VAs in soil could reduce their antimicrobial properties and in turn serve as C or N sources for soil microorganisms. If these soil microorganisms are involved in ATZ mineralization, the additional nutrient sources could lead to enhanced attenuation of ATZ in soil (Ostrofsky et al., 2002).

Varying lab conditions and protocols make it hard to draw any conclusions from the current data regarding how VAs influence microbial populations (Sarmah et al., 2006). Additionally, soil chemical, physical, and biological properties are widely different. These differences could have a dramatic impact on how VAs behave in soil and, therefore, the interactions that take place between VAs and herbicides. Sorption to soil changes with VA chemical structure, VA solubility, soil pH, soil organic matter, and clay content of the soil (Kumar et al., 2005). Because of these varying properties, it is near impossible to make generalizations about how VAs effect herbicide degradation in soil.

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## **CHAPTER 2: INFLUENCE OF VETERINARY ANTIBIOTICS ON ATRAZINE DEGRADATION IN SOIL AND MANURE-AMENDED SOIL**

### **ABSTRACT**

The presence of veterinary antibiotics (VAs) in manure applied to agricultural lands may decrease agrichemical degradation by inadvertently altering soil microbial communities or function. Reduced soil microbial degradation of the commonly used herbicide atrazine (ATZ) could increase frequency of detection and concentration of ATZ in water resources. Therefore, the objectives of this study were to investigate the influence of two VAs, sulfamethazine (SMZ) and oxytetracycline (OTC), on ATZ degradation and activities of the soil microbial enzymes dehydrogenase (DH) and  $\beta$ -glucosidase ( $\beta$ -glu) in soil and soil amended with 5% swine manure. Sandy loam soil and swine manure were used to conduct two side-by-side incubation experiments, one to analyze for  $^{14}\text{C}$ -ATZ degradation and the other to measure enzyme activity (no radio-labeled ATZ). No significant differences between treatments in the soil incubation study were observed for the quantity of ATZ remaining in soil. The distribution of ATZ and its metabolites remaining in the soil were slightly different, but these differences were not statistically significant. After 96 days, approximately half as much ATZ was mineralized to  $^{14}\text{CO}_2$  in samples treated with  $100\ \mu\text{g SMZ kg}^{-1}$  relative to the ATZ only control. The addition of manure dramatically changed the behavior of ATZ in soil. In the presence of manure ATZ degradation decreased by nearly 20% and increased the half-life of ATZ by approximately 20 days. An increase in the percent of the parent compound remaining relative to metabolites after 96 days was also observed. Atrazine mineralization was



reduced by nearly 50% in manure-amended soil. However, the VA treatment did not significantly affect ATZ degradation in manure-amended soil.  $\beta$ -glu activity was significantly influenced by VA and manure amendments. A complicated interaction effect between treatment and time was observed for both  $\beta$ -glu and DH enzymatic activity in soil and manure amended soil. Microbial turnover, utilization of manure, VAs, and ATZ as carbon sources, as well as sensitivity of different groups within microbial consortia to ATZ/VAs are possible explanations for the interaction of treatment and time. It appears the application of VAs to agricultural fields does not significantly reduce ATZ degradation in soil at the investigated concentrations. However, the input of manure significantly increased the length of time ATZ will remain in soil. The results of this research will influence management decisions which could mitigate negative impacts associated with ATZ and VA co-application to soils.

## 2.1 Introduction

Veterinary antibiotics (VAs) have been utilized in U.S. animal agriculture since 1949 (Kumar et al., 2005), and over 11,000 tons are administered annually in the U.S. to treat and prevent animal illness and promote increased animal growth (Wegener 2003; Sarmah et al., 2006; Wang et al., 2008; Lertpaitoonpan et al., 2009; Kim et al., 2011). Tetracycline class antibiotics have broad spectrum efficiency and are commonly employed for therapeutic and growth enhancement purposes (Sarmah et al., 2006). Oxytetracycline (OTC), [4S,4Ar,5S,5Ar,6S,12Ar)-4-(dimethylamino)-1,5,6,10,11,12a-hexahydroxy-6-methyl-3,12-dioxo-4,4a,5,5a-tetrahydrotetracene-2-carboxamide], is a member of the tetracycline family that is used to treat intestinal and respiratory diseases in animals (Arikan et al., 2007; Migliore et al., 2012). Sulfonamides represent a broad

spectrum family of antibiotics that are effective against most gram-positive and gram-negative bacteria (Accinelli et al., 2006; Ross-Flanigan and Uretsky, 2006).

Sulfamethazine (SMZ), 4-amino-N-(4,6-dimethylpyrimidin-2-yl)-benzenesulfonamide, is the most widespread sulfonamide used in animal agriculture (Lee et al., 2007) for therapeutic, prophylactic, and growth promoting purposes (Mellon et al., 2001; Lertpaitoonpan et al., 2009).

The extensive use of VAs in animal agriculture increases the likelihood VAs will be released into the environment. High concentrations of biologically active VAs may be present in animal waste due to poor absorption and metabolism within the gastrointestinal tract of livestock (Sarmah et al., 2006; Lee et al., 2007; Yang et al., 2009). Land application of manure is a commonly used method to dispose of unwanted manure that has the added advantage of fertilizing agricultural lands. However, this may directly release VAs into the environment where they can persist (Dolliver et al., 2007; Igel-Egalon et al., 2011). Veterinary antibiotics dissipate via biotic degradation, sorption, hydrolysis, photolysis, and reduction (Sarmah et al., 2006; Kümmerer, 2009a, 2009b). Tetracyclines have high solid-to-solution partition coefficients ( $K_d$  values) ranging from 290-2060 L kg<sup>-1</sup>; thus, they are more likely to be sorbed to soil and organic matter. In contrast,  $K_d$  values for sulfonamides are substantially less (0.9 to 10 L kg<sup>-1</sup>) resulting in greater availability for degradation and transport (Tolls, 2001; Thiele-Bruhn, 2003; Chu et al., 2013).

Entry into food webs, contamination of water resources, negative impacts to soil microbial communities, and an increase in anti-microbial resistant bacteria are possible outcomes associated with VAs in the environment (Kumar et al., 2005; Dolliver et al.,

2007; Lee et al., 2007; Igel-Egalon et al., 2011; Chu et al., 2013). Veterinary antibiotics may enter the food chain due to VA uptake by food crops or VA residues present in animal-based food products, which could result in adverse health effects (Haller et al., 2002; Dolliver et al., 2007; Kemper 2008; Wang et al., 2008). Antibiotics are detected in wastewater as well as surface runoff, groundwater, and drinking water (Wen et al., 2009). Due to their antimicrobial properties, VA application to agricultural fields may cause reduced activity or mortality of soil bacteria (Nelson et al., 2011). Low concentrations of VAs in soil may increase selection pressure of VA-resistant bacteria, maintain these populations once they have developed, and increase the ease of resistant gene transfer between bacterium (Nygaard et al., 1992; Kümmerer 2004; Henderson et al., 2009).

Atrazine (ATZ), [6-chloro-N-ethyl-N'-(1-methylethyl)-triazine-2,4-diamine], is the most prevalent triazine herbicide in the U.S. and commonly used to control broadleaf weeds (Mandelbaum et al., 1993; Galluzzo et al., 1999; USDA 2007). Microbial degradation is the primary means of ATZ dissipation in soil, although hydrolysis, sorption, volatilization, and photodegradation are also important (Galluzzo et al., 1999; Graymore et al., 2001; Ostrofsky et al., 2002; Kim et al., 2010). Atrazine has five major degradation products categorized by the substituent at the 2- position of the triazine ring: chlorinated metabolites - deethylatrazine (DEA) [6-chloro-N-(propan-2-yl)-1,3,5-triazine-2,4-diamine] and deisopropylatrazine (DIA) [6-Chloro-N-ethyl-1,3,5-triazine-2,4-diamine]; and hydroxylated metabolites – hydroxyatrazine (HA) [4-(ethylamino)-6-(isopropylamino)-1,3,5-triazin-2-ol], deethylhydroxyatrazine (DEHA) [2-hydroxy-4-ethylamino-6-amino-s-triazine], and deisopropylhydroxyatrazine (DIHA) [4-amino-6-(ethylamino)-1,3,5-triazin-2-ol] (Lin et al., 2008; National Center for Biotechnology

Information; The European Bioinformatics Institute). Atrazine's water solubility (33 mg L<sup>-1</sup> at 27 °C), dissociation constant (pK<sub>a</sub> = 1.68), and solid to solution partition coefficients (*K<sub>d</sub>* values of 0.19 to 2.46 L kg<sup>-1</sup>) make it fairly mobile in soil and increase the likelihood that it will be transported to water resources, especially after heavy rainfall events (Senesi, 1992; Solomon et al., 1995; Graymore et al., 2001; Mudhoo and Garg, 2011). In addition to its mobility, ATZ is moderately stable in soils, with a half-life of 13 to 402 days (USDA-ARS, 2007), and therefore, it is the most frequently detected pesticide in ground, surface, and drinking water in the U.S. and is detected up to twenty times more frequently in groundwater than any other herbicide (Solomon et al., 1996; Graymore et al., 2001). Levels of ATZ in surface and groundwater often exceed maximum contamination levels established by the USEPA (Lerch et al., 2011; Kolić et al., 2012). Several studies have cited ATZ as a possible cause of negative health effects including prostate cancer (MacLennan et al., 2002), reduced success of aquatic species (Wiegand et al., 2001; Fortin et al., 2008), and endocrine disrupting effects in humans and aquatic organisms (Cooper et al., 2000; Moore and Lower, 2001; Hayes et al., 2003, 2010; Swan et al., 2003; Tillit et al., 2010).

Together VAs and herbicides are concerning because VAs are intended to suppress microbial growth and when applied to soil may inhibit soil microbial activity, resulting in decreased herbicide degradation. The interaction between VAs and herbicides will vary depending on VA and soil properties (Kumar et al., 2005). Understanding how VAs influence herbicide degradation is essential, yet information on the subject is very limited. Accinelli et al. (2006) found that SMZ in the µg kg<sup>-1</sup> range did not have an apparent influence on metolachlor degradation or sorption. Kim et al. (2010) found that

ATZ concentrations in soils treated with the VAs monensin, narasin, and salinomycin were significantly greater and ATZ half-lives were significantly longer compared to the control treatment without antibiotics. Two additional studies found that high concentrations of VAs used to sterilize soil reduced degradation of herbicides (Levanon et al. 1993; Ostrosky et al. 2000). Conversely, decomposition of VAs in soil could reduce their antimicrobial properties and allow microorganisms to utilize them as C or N sources, which may enhance the rate of herbicide dissipation in soil (Ostrosky et al., 2002).

Soil enzymes are a relatively quick and cost effective way to measure biochemical processes in soil.  $\beta$ -glucosidase ( $\beta$ -glu) catalyzes the final step in cellulose degradation and can therefore provide useful information about management effects and carbon cycling (Knight and Dick, 2004).  $\beta$ -glu activity can also be useful in determining ATZ degradation and can indicate the presence of N-dealkylation of ATZ (Lin et al., 2011). Dehydrogenase (DH) activity is a good indicator of metabolic activity in soil (Casida, 1977) and a positive correlation between DH activity and ATZ mineralization has been observed (Lin et al. 2011). Therefore, objectives of this study were to (1) compare ATZ degradation rates in soil amended or not amended with manure in the presence of SMZ or OTC and (2) investigate changes in soil microbial enzymatic activity, specifically the activity of  $\beta$ -glu and DH, as a function of time following application of SMZ and OTC to soils amended with ATZ.

## 2.2 Materials and Methods

### 2.2.1 Experimental Design

Soil was collected from a fallow field (0-10 cm depth) in Osage Co., MO that had not received ATZ application for at least 25 years (Fig. 2.1). This site is a Kaintuck fine sandy loam (coarse-loamy, siliceous, superactive, nonacid, mesic Typic Udifluvents) located in a floodplain landscape position with 0-1% slope. Vegetation consisted primarily of coolseason grasses (*Festuca* and *Agropyron* species); the field was last cultivated for sweet corn (*Zea mays* L.) with no herbicides in 1989. Soil characterization data for samples used in the study is presented in Table 2.1. The soil was used to conduct two sets of incubation experiments. The primary incubation experiment focused on moderate and extreme VA concentrations in soil while the second aimed to discern any effect manure may have on VA-ATZ interactions. Each incubation contained two side-by-side experiments, one to analyze for  $^{14}\text{C}$ -ATZ degradation and the other to measure enzyme activity (no radio-labeled ATZ).

# Map



Figure 2.1 Location of soil sampling site in Osage Co. Missouri.

**Table 2.1** Mean soil characterization data for soils collected from a fallow field in Osage Co., MO.

<b>Soil Properties</b>	<b>Mean <math>\pm</math> 95% CI</b>
Texture	Fine Sandy Loam
Clay (g kg <sup>-1</sup> )	120 $\pm$ 15
Silt (g kg <sup>-1</sup> )	340 $\pm$ 15
Sand (g kg <sup>-1</sup> )	540 $\pm$ 10
OC (g kg <sup>-1</sup> )	24 $\pm$ 0.6
Total N (g kg <sup>-1</sup> )	1.9 $\pm$ 0.08
CEC (cmol <sub>c</sub> kg <sup>-1</sup> ) <sup>†</sup>	19 $\pm$ 1.1
Base Saturation	87 $\pm$ 0.0
pH <sub>w</sub> <sup>‡</sup>	6.9 $\pm$ 0.0
pH <sub>s</sub> <sup>§</sup>	7.1 $\pm$ 0.0

<sup>†</sup>CEC (cation exchange capacity)

<sup>‡</sup>pH water

<sup>§</sup>pH salt

The ATZ concentration chosen for evaluation was determined based on ATZ concentrations measured in sandy soils of Missouri from 0 to 120 days after ATZ application (Kazemi et al., 2008). The two VAs, SMZ and OTC, were chosen for investigation due to vast differences in the sorption of these VAs to soil; thus, the availability of these VAs to interact with soil microbes is likely to be very different. The concentrations of SMZ and OTC chosen for investigation represent moderate and extreme values reported by Kumar et al. (2005).

### **2.2.2 Soil Incubation Experiment**

All soil samples were amended with 500  $\mu\text{g}$  ATZ kg<sup>-1</sup> and the following VA treatments: (1) control (0  $\mu\text{g}$  kg<sup>-1</sup> VA); (2) 100  $\mu\text{g}$  SMZ kg<sup>-1</sup>; (3) 1000  $\mu\text{g}$  SMZ kg<sup>-1</sup>; (4) 100  $\mu\text{g}$  OTC kg<sup>-1</sup>; and (5) 1000  $\mu\text{g}$  OTC kg<sup>-1</sup>. A sufficient number of samples were prepared to permit destructive sampling of three replicate samples for each treatment over



a 96 day incubation period. A period of 96 days was selected to ensure several half-lives of ATZ degradation were observed which would help to detect treatment differences

Before VA/ATZ application, 60 g (oven-dry equivalent) of soil was packed into polypropylene snap cap vials to a bulk density of  $1.2 \text{ g cm}^{-3}$ . On day zero of the incubation experiment, samples were spiked with 6.93 mL deionized water, 1 mL of the designated VA solution [1 mL of either SMZ or OTC solution at high concentration ( $1000 \mu\text{g VA kg}^{-1}$ ), SMZ or OTC solution at low concentration ( $100 \mu\text{g VA kg}^{-1}$ ), or 1 mL of ultrapure water ( $0 \mu\text{g VA kg}^{-1}$ )], and 2 mL of the ATZ solution. The VA solutions were prepared using >99% purity SMZ and >95% oxytetracycline hydrochloride (Sigma-Aldrich; St. Louis, MO). For samples intended to track ATZ degradation, samples were inoculated with a solution made of  $^{14}\text{C}$ -ATZ and non-radioactive ATZ combined to achieve  $500 \mu\text{g kg}^{-1}$  ATZ and  $1.0 \mu\text{Ci } ^{14}\text{C}$ -ATZ per sample. The  $^{14}\text{C}$ -ATZ solution was prepared with  $0.1 \text{ mCi}$  of  $^{14}\text{C}$ -ATZ with a specific activity of  $160 \text{ mCi mmol}^{-1}$  [ring- $^{14}\text{C}$  (U); American Radiolabeled Chemicals, Inc., St. Louis, MO]. The non-radioactive ATZ solution for the VA degradation and enzyme analysis study was prepared ( $500 \mu\text{g kg}^{-1}$  ATZ) using > 95% purity ATZ (Sigma-Aldrich; St. Louis, MO). A total of 9.93 mL of solution was added to each sample, achieving a desired 60% water-filled pore space.

The spiked samples were placed in glass wide-mouthed pint size mason jars with a 10 mL alkali trap (2M NaOH) to capture evolved  $^{14}\text{CO}_2$ . Jars were sealed and incubated at  $25^\circ\text{C}$  in the dark in an environment-controlled growth chamber (Environmental Growth Chambers GC72 walk-in unit, Chagrin Falls, OH). The samples were destructively sampled over the course of the experiment at 0, 1, 2, 7, 14, 28, and 96 days. At the end of a designated incubation period, 60 mL of 100% HPLC grade methanol was

added to the  $^{14}\text{C}$ -ATZ samples to stop the degradation reaction. Samples were then sealed and frozen. Non-radioactive samples were sealed and stored at  $4^\circ\text{C}$  for future enzyme analyses.

Alkali traps were removed with samples at times 1, 2, and 7 days. Starting at day seven, NaOH traps were replaced and soil moisture content was adjusted with deionized water biweekly for all samples remaining in the incubator to maintain 60% water-filled pore space. Traps from the  $^{14}\text{C}$ -ATZ spiked samples were sealed and saved for analysis of ATZ mineralized to  $^{14}\text{CO}_2$ . Alkali traps from the non-radioactive samples were discarded.

### **2.2.3 Chemical Analysis**

#### **2.2.3.1 Soil Extractions**

At the end of the incubation period, frozen samples containing 60 mL of 100% methanol were individually transferred to 250 mL polypropylene (PP) bottles. An additional 90 mL of 100% methanol was added to each PP bottle (150 mL total) followed by shaking for 1 h followed by 30 min of sonication. The suspension was centrifuged for 12 min at 3500 rpm and the supernatant was collected. The extraction was repeated with 100 mL of 90% methanol and the supernatant extracts were combined. The volume of the combined supernatant sample was recorded so a partial mass balance could be calculated. The supernatant sample was allowed to sit overnight to permit the settling of sediments. Floating organic matter was removed from the supernatant, and supernatant samples were individually transferred to TurboVac evaporation flasks. The supernatant was concentrated on a Caliper Life Sciences TurboVap II (Hopkinton, MA) with a bath

temperature of 35°C until ~5 to 10 mL of water remained. An additional 5 mL of HPLC grade water was added to the concentrate to increase liquid-liquid extraction efficiency.

### **2.2.3.2 Liquid-liquid Extractions**

To perform a liquid-liquid extraction the concentrate and water were added to a 500 mL separation funnel. Five milliliters of chloroform was used to wash the TurboVac glassware and the wash solvent was transferred into the separation funnel. An additional 45 mL of chloroform (50 mL total) was added to the separation funnel. Samples were then shaken by hand three times for 15 seconds and the organic phases were allowed to separate. The chloroform portion was collected in a glass jar and the liquid-liquid extraction was performed an additional two times. The 150 mL of chloroform which contained <sup>14</sup>C- ATZ and its chlorinated metabolites was transferred to TurboVac glassware and concentrated to 5 mL. The concentrated samples were then transferred to test tubes and the TurboVac glassware was washed with a few milliliters of chloroform to remove all residues. The wash solvent was added to the test tubes. Samples were evaporated to dryness using an Organomation Associates, Inc. N<sub>2</sub> gas-evaporator (Berlin, MA) at a water bath temperature of 40°C. Acetonitrile (1.5 mL) was added to each sample followed by sonication and vortex mixing to resuspend the samples. Samples were then centrifuged at 3500 rpm for 10 minutes. Lastly, the acetonitrile solution was filtered into chromatography vials using a 10 mL Norm-Ject LS syringe with a Life Sciences Bulk Acrodisc CR 13 mm syringe filter with 0.45 µm PTFE membrane (Pall Life Sciences, Ann Arbor, MI). Chromatography vials were capped and frozen until HPLC analysis could be performed.

### 2.2.3.3 High-Performance Liquid Chromatography

The  $^{14}\text{C}$ -ATZ and its degradation products deethylatrazine (DEA), deisopropylatrazine (DIA), and didealkylatrazine (DDA) were analyzed using a high-performance liquid chromatography (HPLC) method based on the method used by Lin et al. (2011). Samples dissolved in acetonitrile were injected into a SCL-10Avp HPLC system (Shimadzu, Columbia, MD). A silica-based Columbus C8 column (4.6 mm by 250 mm, 5  $\mu\text{m}$ ; Phenomenex, Torrance, CA) was used to separate  $^{14}\text{C}$ -ATZ and ATZ degradation products. The radioactivity was detected by an in-line IN/US ScinFlow  $\beta$ -Ram Model 3 (Tampa, FL) flow scintillation analyzer (HPLC-FSA). Sample injection volume was 10  $\mu\text{L}$  with a mobile phase flow rate of 1.25  $\text{mL min}^{-1}$ . The  $^{14}\text{C}$ -ATZ and its metabolites were eluted with a two-part mobile phase gradient. Mobile phase A and B consisted of 0.1%  $\text{H}_3\text{PO}_4$  buffer (pH = 2.1) and 100% acetonitrile, respectively. The gradient began at 30% B for 3 min and ramped linearly to 50% B at 15 min, 80% B at 30 min, 30% B at 31 min, and was held at 30% B for 9 min. Atrazine was identified by comparing the retention time of  $^{14}\text{C}$ -ATZ standards based on HPLC-FSA detection. The standards DEA, DIA, and DDA were purchased through Sigma-Aldrich (St. Louis, MO). Retention times of unlabeled standards based on HPLC-UV detection at 220 nm were used to identify ATZ metabolites in the FSA chromatographs.

Retention times were approximately 2.5, 3.9, 5.8, and 14.5 minutes for DDA, DIA, DEA, and ATZ respectively. Unlike the ATZ, DEA, and DIA standards, the retention time of DDA did not behave as expected. A slight delay in retention time is observed when using HPLC-FSA compared to HPLC with a UV detector. As a result, the cold standards would be expected to have retention times that are slightly shorter than the

radio-labeled samples. This held true for all standards except DDA, which has a retention time approximately 0.5 minutes before the observed sample retention time of 2.5 min. Nonetheless, we are confident these peaks represent DDA because the liquid-liquid extraction protocol rules out the possibility of detecting hydroxylated metabolites. Additionally, the time off-set was consistent and the early retention time was indicative of the polar DDA metabolite.

A mass balance of ATZ was not possible due to liquid-liquid extraction protocol. The hydroxylated metabolites were not examined in this study because they are much more polar than the chlorinated metabolites and will preferentially stay in the aqueous phase during liquid-liquid extractions. A partial mass balance including the parent compound, the chlorinated metabolites, and ATZ mineralized to  $^{14}\text{CO}_2$  was calculated. The chlorinated metabolite DDA is more polar than its counterparts DEA and DIA. As a result, some DDA was likely partitioned between the chloroform and aqueous phases after liquid-liquid extraction. Even though the amounts reported may be underestimated, the DDA peaks were well above the detection limit, and the metabolite was quantified. A signal to noise ratio of 0.002 mVolts was observed for the HPLC-FSA data; the limit of detection was considered to be three times the signal to noise ratio (0.006 mVolts) and a quantification limit ten times the signal to noise ratio (0.02 mVolts) was used to quantify ATZ and its metabolites. For the primary incubation study the extraction efficiencies of ATZ and the metabolites (DEA and DIA) from soil were >85% and >90%, respectively. The extraction efficiency for ATZ was reduced to >45% for the second incubation experiment. The concentration of ATZ, DEA, and DIA remaining in the soil was

corrected using the appropriate extraction efficiency. Due to the aforementioned reasons the metabolite DDA was not corrected for extraction efficiency.

#### **2.2.3.4 Liquid Scintillation**

One milliliter of each NaOH trap was added to a scintillation vial with 4 mL of Ultima Gold™ LSC cocktail. The  $^{14}\text{CO}_2$  evolved from samples due to  $^{14}\text{C}$ -ATZ mineralization during incubation was determined by measuring the radioactivity of the evolved  $^{14}\text{CO}_2$  using a Beckman LS600 liquid scintillation counter (Beckman, Fullerton, CA). A calibration curve was established which allowed us to calculate  $^{14}\text{CO}_2$  in  $\mu\text{Ci mL}^{-1}$  using the number of counts per minute recorded for each sample.

#### **2.2.4 Microbial Enzyme Activity**

The enzymes DH and  $\beta$ -glu were selected for this study because they are general indicators of microbial activity and Lin et al. (2011) found they can provide useful information regarding ATZ degradation in the soil. Gravimetric soil moisture of each sample was determined prior to enzyme analysis, thus permitting enzyme analyses to be expressed on an oven-dry mass basis.

Soil  $\beta$ -glu activity was measured according to procedures described by Dick et al. (1996). To perform the  $\beta$ -glu assay, 1.0 g of moist soil was placed in a 50 mL Erlenmeyer flask and treated with 0.25 mL toluene, 4.0 mL of MUB (pH 6.0), and 1.0 mL of 10 mM  $p$ -nitrophenyl  $\beta$ -D-glucoside. The flask was swirled, stoppered, and incubated for 1 h at 37°C. After incubation 1.0 mL of 0.5M  $\text{CaCl}_2$  and 4.0 mL of 0.1M THAM buffer (pH 12) were added to the flask. Flask contents were swirled and filtered through Whatman #2 filter papers using a vacuum source. The filtrate was measured on a spectrophotometer at 410 nm. It should be noted that for environmental and health reasons toluene was

removed from the procedure after three-quarters of the samples had been analyzed. However, comparative tests showed the absence of toluene did not affect the results.

DH activity was determined according to procedures described by Gerba and Bredecke (1995). Six grams of moist soil was weighed and placed in a test tube. One milliliter of 3% TTC (2,3,5-triphenyltetrazolium chloride) and 3.0 mL of CaCO<sub>3</sub> solution was added and then test tubes were capped and incubated at 37°C for 24 h. After the incubation the soil was washed with methanol and filtered. It was then brought to 50 mL with methanol and measured on a spectrophotometer at 485 nm.

### **2.2.5 Manure-Amended Soil Incubation Experiment**

A secondary incubation experiment was conducted to assess how the presence of manure changes the interaction between ATZ and VAs. Soil was collected from the same site as soil used in the primary incubation experiment. Swine manure was collected from a farm located in Osage Co., MO. The manure was from a barn with a deep straw bedding and animals had not been treated with any VAs. Characterization data of manure is displayed in Table 2.2. After collection, manure was dried and ground using a Wiley mill to ensure even distribution within soil samples. Samples containing soil only were amended with the following treatments: (1) 500 µg ATZ kg<sup>-1</sup>; (2) 100 µg SMZ kg<sup>-1</sup>; and (4) 100 µg OTC kg<sup>-1</sup>. The group of samples containing only ATZ (no manure or VAs) was used to determine if results from this incubation could be compared to the first incubation results. Soil containing 100 µg VA kg<sup>-1</sup> (no ATZ or manure) was used to discern if changes in soil enzymes from the first experiment could be related to VA rather than ATZ exposure. Samples containing 5% manure were amended with the following treatments: (6) control (0 µg kg<sup>-1</sup> VA and 0 µg kg<sup>-1</sup> ATZ); (7) 500 µg ATZ kg<sup>-1</sup>; (8) 100 µg

SMZ kg<sup>-1</sup>; (9) 100 µg OTC kg<sup>-1</sup>; (10) 500 µg ATZ kg<sup>-1</sup> and 100 µg SMZ kg<sup>-1</sup>; (11) 500 µg ATZ kg<sup>-1</sup> and 100 µg OTC kg<sup>-1</sup>. A sufficient number of samples were prepared to permit destructive sampling of three replicate samples for each treatment over a 96 day incubation period. Sample sets 1, 7, 10, and 11 were doubled so both <sup>14</sup>C-ATZ degradation and enzyme activity (no radio-labeled ATZ) could be analyzed.

For samples containing only soil (no manure), 60 g (oven-dry equivalent) of soil was packed into polypropylene snap cap vials to a bulk density of 1.2 g cm<sup>-3</sup>. In order to best replicate VA-manure co-application to agricultural fields, manure was spiked with VAs prior to the incubation experiment. Dried, antibiotic-free manure was inoculated with either SMZ or OTC using a similar procedure to Wang and Yates (2006; 2008). Briefly, 12.6 µg SMZ or OTC dissolved in acetone was added to 126 g manure to achieve a concentration of 100 µg VA kg<sup>-1</sup> manure. The acetone was allowed to evaporate so only the VA was left in the manure. Three grams of manure (oven dry equivalent) was combined with 57 g (oven dry equivalent) of soil to achieve 100 µg VA kg<sup>-1</sup> and 5% manure per sample. Soil was then packed in a snap top vial to achieve a bulk density of 1.2 g cm<sup>-3</sup>. The ATZ and aqueous VA solutions were prepared according to the methods previously described. On day zero of the incubation experiment, samples were spiked with the appropriate ATZ solution and/or VA solution (VA samples with no manure), and deionized water. A total of 2.48 mL was added to each sample to achieve a desired 60% water-filled pore space. The incubation, maintenance, and harvest of NaOH traps and samples followed procedures described previously. Enzyme activity and <sup>14</sup>C-ATZ degradation were measured following the same protocol used for the first



**Table 2.2** Mean characterization data for swine manure collected from a swine farm in Osage Co., MO.

<b>Element</b>	<b>Mean <math>\pm</math> 95% CI</b>
	$\text{g kg}^{-1}$
C	$340 \pm 4$
N	$31 \pm 1.3$
S	$2.50 \pm 0.40$
Al	$2.19 \pm 0.50$
Ca	$7.56 \pm 2.92$
K	$10.3 \pm 1.04$
Mg	$5.43 \pm 2.42$
Fe	$1.16 \pm 0.34$
Na	$1.8 \pm 0.12$
P	$9.4 \pm 4.26$
	$\text{mg kg}^{-1}$
B	$14 \pm 1.1$
Cu	$86 \pm 39.4$
Mn	$120 \pm 37$
Mo	$0.20 \pm 0.43$
Ni	$6.0 \pm 2.81$
Zn	$190 \pm 76$

incubation experiment. The results were compared to the initial findings to see if manure influenced the interaction between VAs and ATZ in soil.

### 2.2.7 Modeling Kinetics of Degradation

The degradation of ATZ was fit using a kinetic model. The reaction was found to follow a first order rate law, and can be expressed as  $\ln[A] = -kt + \ln[A_0]$  where  $[A]$  is the concentration of reactant  $A$ ,  $k$  is the first-order rate constant, and  $t$  is time. A plot of the natural log of  $[A]$  versus  $t$  was used to confirm the reaction was indeed first-order;  $R^2$  values averaged 0.8563 and 0.9791 for the soil and manure-amended soil incubations, respectively. The slope of the line for each kinetic model is equal to the rate constant,  $k$ . This information was then used to calculate the half-life,  $t_{1/2} = 0.693/k$ , of the reaction.

### 2.2.8 Statistical Analysis

The PROC MIXED module in SAS Enterprise Guide Software Version 9.3 was used to evaluate the effects of VA treatments on soil enzymes and ATZ degradation (SAS Institute Inc. Cary, NC, USA). The MIXED module was initially used to complete an analysis of variance on all data collected in the primary and secondary incubation experiments. It was determined that the ATZ only controls were significantly different for all parameters of interest between incubation studies. For this reason, comparisons were limited to treatments within a given incubation study. The mixed linear statistical model for the soil incubation was a two-way factorial design with VA treatment (i.e., 100  $\mu\text{g OTC kg}^{-1}$ , 100  $\mu\text{g SMZ kg}^{-1}$ , 1000  $\mu\text{g OTC kg}^{-1}$ , 1000  $\mu\text{g SMZ kg}^{-1}$ , and 0  $\mu\text{g VA kg}^{-1}$  control) and time (i.e., 0, 1, 2, 7, 14, 28, and 96 days) as the main factors. Therefore, main effects and their interactions could be assessed. Each variable for enzyme activity (DH and  $\beta$ -glu) and ATZ degradation (ATZ, DDA, DEA, DIA, and  $^{14}\text{CO}_2$ ) was analyzed separately. Least Squared Means were calculated and compared to distinguish significant differences between treatments. A macro program was utilized to convert mean separation output to letter groupings (Saxton, 1998). Since ATZ metabolites were below the limit of detection at the beginning of the incubation experiment, the time variable for these analyses (DDA, DIA, and DEA) was adjusted to include only time 7, 14, 28, and 96 days. A residual analysis in SAS indicated the residuals were normally distributed for all datasets excluding DEA and DIA. Log, natural log, square root, exponent, cosine, sine, tangent, and box cox transformations failed to correct the uneven residual distribution. This is most likely because data was below the limit of detection resulting in zero values at several points within the dataset. Future analyses using a different model may correct

for the non-normal residuals within the data. However, because the metabolites accounted for such a small portion of the applied ATZ no further action was taken.

Statistical analysis for the manure-amended soil incubation experiment closely resembled that of the primary incubation. The mixed linear statistical model for the two-way factorial analysis of the enzyme activity data included the ATZ-VA treatment (i.e., soil with 500  $\mu\text{g ATZ kg}^{-1}$ , 100  $\mu\text{g SMZ kg}^{-1}$ , or 100  $\mu\text{g OTC kg}^{-1}$ , or manure amended soil with 0  $\mu\text{g kg}^{-1}$  VA and 0  $\mu\text{g kg}^{-1}$  ATZ, 500  $\mu\text{g ATZ kg}^{-1}$ , 100  $\mu\text{g SMZ kg}^{-1}$ , 100  $\mu\text{g OTC kg}^{-1}$ , 500  $\mu\text{g ATZ kg}^{-1}$  and 100  $\mu\text{g SMZ kg}^{-1}$ , or 500  $\mu\text{g ATZ kg}^{-1}$  and 100  $\mu\text{g OTC kg}^{-1}$ ) and time (i.e., 0, 1, 2, 7, 14, 28, and 96 days). The model used for ATZ degradation was identical except treatments that did not contain ATZ were excluded. Once again, analysis of the chlorinated ATZ metabolites excluded days 0, 1, and 2. A residual analysis in SAS indicated the residuals for  $\beta$ -glu activity, DH activity, and ATZ degradation were normally distributed. The residuals were not normally distributed for the ATZ degradation products (DDA, DIA, DEA, and  $^{14}\text{CO}_2$ ). Transformations of the data including log, natural log, square root, exponent, cosine, sine, tangent, and box cox did not correct the normality of the residuals. It is possible a linear model is not the most appropriate choice for analyzing this data. Zero values within data set are also likely contributing to non-normality of the residuals. Once again, it is possible the use of a different model would correct for these discrepancies. However, because the ATZ metabolites represented such a small fraction of applied ATZ, no further action was taken.

Differences between ATZ half-lives for VA treatments was discerned using SAS. The PROC MIXED program was used to create a mixed linear statistical model for a one

analysis of variance with half-life as the main effect. Differences in half-lives for the soil incubation and manure-amended incubation were analyzed separately. The PROC CORR module in SAS Enterprise Guide Software Version 9.3 was used to develop a Pearson correlation for evolved  $^{14}\text{CO}_2$ ,  $\beta$ -glu, and DH enzyme activity (SAS Institute Inc. Cary, NC, USA). Correlations for the soil incubation and manure-amended incubation data were analyzed separately.

## 2.3 Results and Discussion

An initial analysis of all data collected during the two incubations indicated statistically significant differences between the two ATZ only controls. For this reason it was determined that results between incubations were not comparable. Consequently, data for the soil and manure-amended soil incubation experiments were analyzed separately. Statistical results displayed in Tables 2.3 and 2.6 summarize the PROC MIXED analysis results of main effects on ATZ degradation and soil microbial enzymes for soil and manure-amended soil incubation studies, respectively.

Table 2.3 indicates no significant treatment effect (trt) or interaction effect (trt\*time) for ATZ degradation and the chlorinated metabolites at the  $\alpha = 0.05$  level. However, an effect of treatment was observed for  $^{14}\text{C}$ -ATZ mineralization and  $\beta$ -glu enzyme activity. A significant interaction effect between treatment and time was also observed for  $^{14}\text{C}$ -ATZ mineralization and both  $\beta$ -glu and DH enzyme activities. As expected, all parameters of interest were significantly affected by time in the soil incubation study (Table 2.3). Table 2.6 indicates that treatment and time were significant main effects for all analytes studied in the manure-amended incubation study. The interaction of treatment and time was significant for all parameters except for DDA.

**Table 2.3** Analysis of soil only incubation experiment data. Type III tests evaluating treatment (trt), time, and the interaction effects (trt\*time) for the dependent variables: atrazine and metabolite concentrations; mineralized <sup>14</sup>C atrazine (<sup>14</sup>CO<sub>2</sub>); β-glucosidase enzyme activity (β-glu); and dehydrogenase enzyme activity (DH).

**Analysis of soil only incubation experiment data.**

	Analyte						
	-----p-values-----						
Source	ATZ <sup>†</sup>	DDA <sup>‡</sup>	DIA <sup>‡</sup>	DEA <sup>‡</sup>	<sup>14</sup> CO <sub>2</sub>	β-glu	DH
<b>Trt</b>	0.9163	0.4477	0.3854	0.3166	<b>0.0337</b>	<b>0.0115</b>	0.0691
<b>Time</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
<b>Trt*Time</b>	0.5299	0.3579	0.5196	0.9117	<b>0.0025</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>

<sup>†</sup>ATZ = atrazine; DDA = didealkylatrazine; DIA = deisopropylatrazine; DEA = deethylatrazine.

<sup>‡</sup>Indicates days 0, 1, and 2 were excluded during analysis.

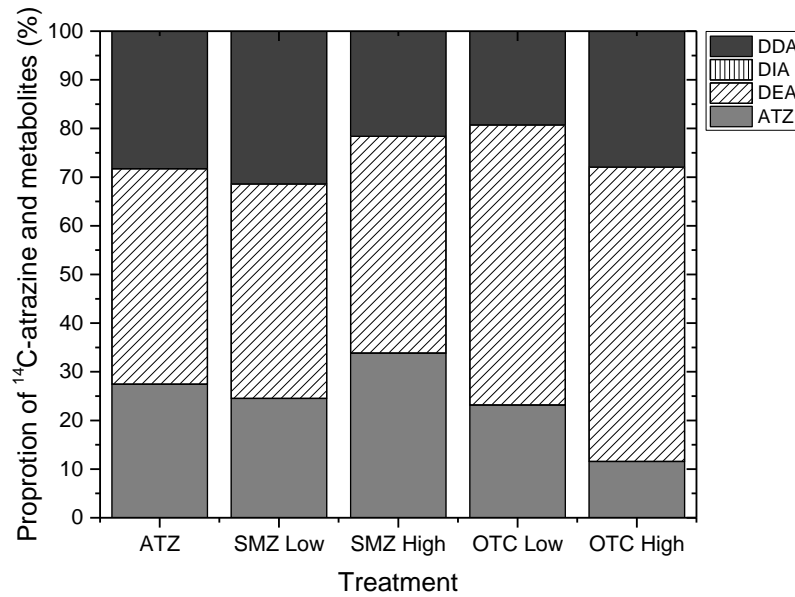
Bold type indicates significant treatment differences ( $\alpha = 0.05$ ).

### 2.3.1 Atrazine Degradation

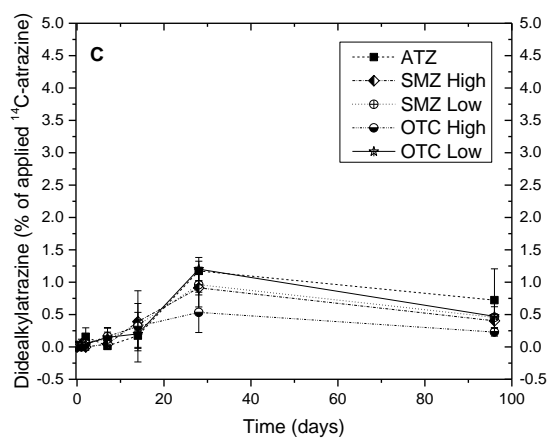
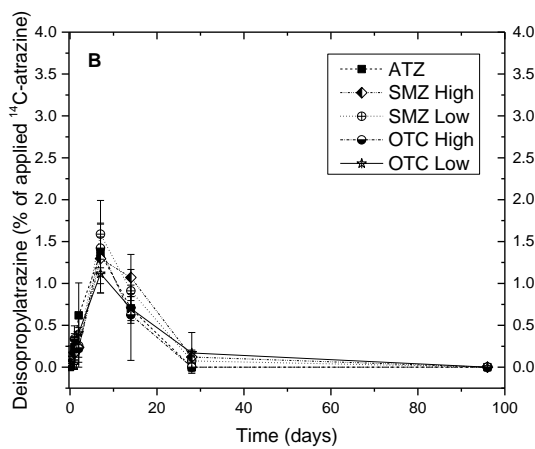
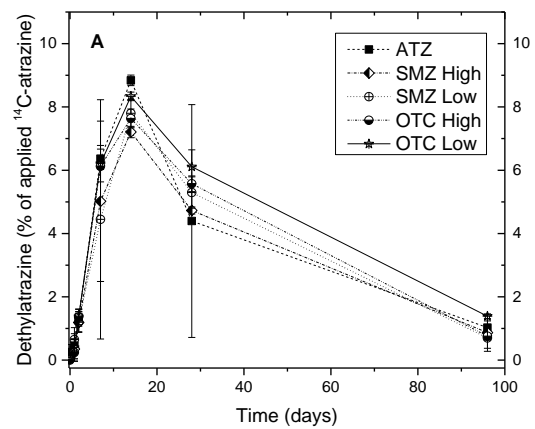
#### 2.3.1.1 Soil Incubation Study

The dissipation of ATZ in soil was not significantly affected by VA treatments in the soil incubation experiment (Table 2.3). No treatment effect was observed for the concentrations of chlorinated metabolites (DDA, DIA, DEA) beginning at day 7, suggesting the VA type or concentration did not significantly influence the degradation rate or pathway of ATZ (Table 2.3). However, nominal differences in the proportion of ATZ and ATZ metabolites remaining in the soil after 96 days of incubation can be observed in Figure 2.2. In general, slightly less ATZ and slightly greater DEA were observed in the OTC treatments, although the differences are not significant. The formation of chlorinated metabolites over time is illustrated in Figure 2.3. Formation of metabolites early in the incubation experiment is followed by further decomposition and an increase in <sup>14</sup>CO<sub>2</sub>. The percent of applied <sup>14</sup>C-ATZ and metabolites recovered after

14, 28, and 96 days of incubation are displayed in Table 2.4. The low recovery rate suggests a large portion of ATZ was converted to the hydroxylated degradation products or unextractable ATZ residues. Lerch et al. (1997) found aqueous-methanol extractions similar to those used in this study left behind 42.8% of bound ATZ residues, which when extracted with  $\text{KH}_2\text{PO}_4$  and  $\text{CH}_3\text{CN}$  revealed majority of the residues were hydroxylated atrazine degradation products.



**Figure 2.2** Distribution of extractable  $^{14}\text{C}$ -atrazine and its metabolites remaining in soil after 96 d incubation with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high concentrations ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) of sulfamethazine (SMZ) and oxytetracycline (OTC). Control represents ATZ amendment only.



**Figure 2.3** Formation of (A) deethylatrazine, (B) deisopropylatrazine, and (C) didealkylatrazine in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high (100 and 1000  $\mu\text{g kg}^{-1}$ ) sulfamethazine (SMZ) or oxytetracycline (OTC) concentrations during a 96 d incubation period. Control represents ATZ amendment only. Error bars represent one standard deviation

**Table 2.4** Atrazine (ATZ),  $^{14}\text{C}$ -atrazine, deethylatrazine (DEA) deisopropylatrazine (DIA), and didealkylatrazine (DDA) recovered after 14, 28, and 96 days represented as a percent of applied  $^{14}\text{C}$ -ATZ in soil amended with  $500 \mu\text{g kg}^{-1}$  and:  $0 \mu\text{g kg}^{-1}$  VA;  $100 \mu\text{g kg}^{-1}$  sulfamethazine (SMZ);  $1000 \mu\text{g kg}^{-1}$  SMZ;  $100 \mu\text{g kg}^{-1}$  oxytetracycline (OTC);  $1000 \mu\text{g kg}^{-1}$  OTC.

**Percent of Applied  $^{14}\text{C}$  atrazine (%)**

**Day 14**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>SMZ Low</b>	<b>SMZ High</b>	<b>OTC Low</b>	<b>OTC High</b>
<b>DDA</b>	0.26	0.50	0.59	0.30	0.48
<b>DIA</b>	1.05	1.37	1.61	1.05	0.94
<b>DEA</b>	13.26	11.70	10.81	12.50	11.45
<b>ATZ</b>	19.48	14.29	15.43	17.97	15.63
<b>Recovery (%)</b>	34.06	27.86	28.43	31.82	28.50
<b><math>^{14}\text{C}</math></b>	8.06	8.48	13.99	9.07	9.14

**Day 28**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>SMZ Low</b>	<b>SMZ High</b>	<b>OTC Low</b>	<b>OTC High</b>
<b>DDA</b>	1.08	0.58	0.68	0.71	0.30
<b>DIA</b>	0.00	0.11	0.18	0.26	0.00
<b>DEA</b>	6.59	7.94	7.09	9.17	8.36
<b>ATZ</b>	5.89	4.20	3.83	4.70	4.85
<b>Recovery (%)</b>	13.56	12.84	11.79	14.83	13.51
<b><math>^{14}\text{C}</math></b>	9.00	8.26	12.88	10.45	11.24

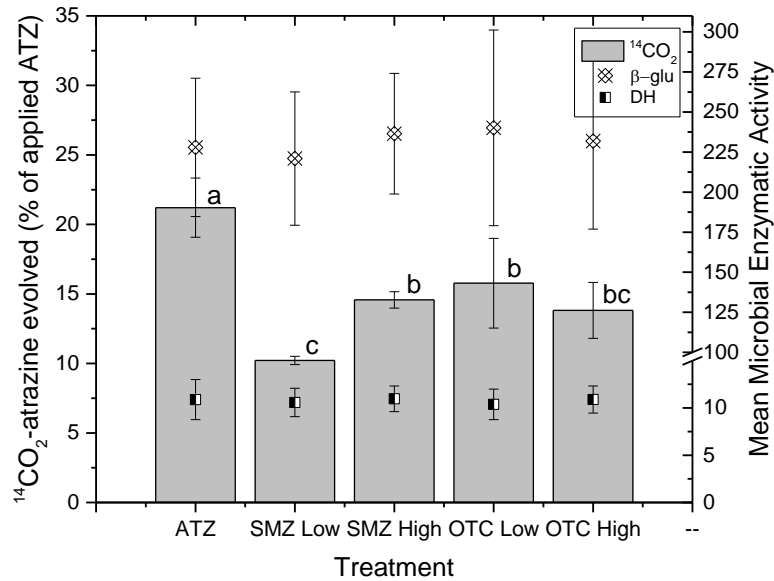
**Day 96**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>SMZ Low</b>	<b>SMZ High</b>	<b>OTC Low</b>	<b>OTC High</b>
<b>DDA</b>	0.01	0.01	0.01	0.01	0.00
<b>DIA</b>	0.00	0.00	0.00	0.00	0.00
<b>DEA</b>	0.02	0.01	0.01	0.02	0.01
<b>ATZ</b>	0.01	0.01	0.01	0.01	0.00
<b>Recovery (%)</b>	3.55	2.32	2.92	3.62	1.74
<b><math>^{14}\text{C}</math></b>	21.20	10.21	14.57	15.77	13.82

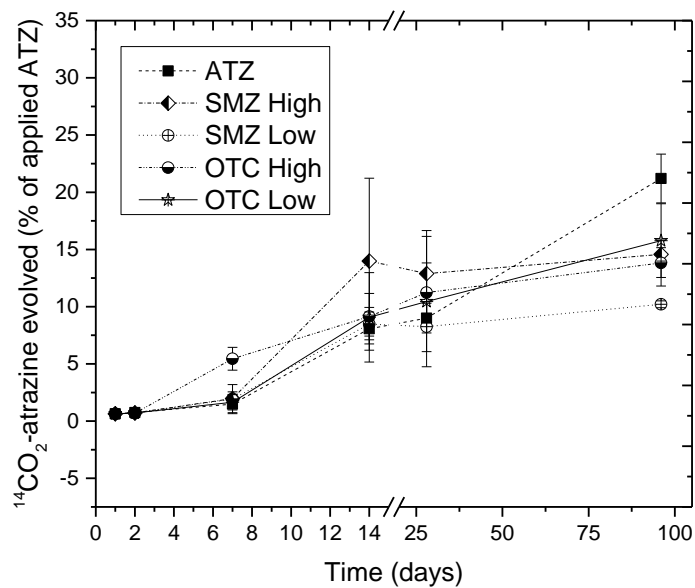


At the end of 96 days the total mineralization of  $^{14}\text{C}$ -ATZ was shown to be significantly reduced by all of the VA treatments (Figure 2.4). The  $100\ \mu\text{g}\ \text{kg}^{-1}$  SMZ treatment decreased mineralization to the greatest extent, with only 10% of the applied  $^{14}\text{C}$ -ATZ mineralized to  $^{14}\text{CO}_2$  compared to an average of 16% for the other treatments. However, there was a complex interaction effect between treatment and time for ATZ mineralized to  $^{14}\text{CO}_2$  (Figure 2.5). Due to their antimicrobial properties we would expect decreases in mineralization to be most evident at high VA concentrations and least apparent (closest to control) at low VA concentrations. However, the observed percentages of mineralized  $^{14}\text{C}$ -ATZ did not reflect the hypothesized pattern. Gutiérrez et al. (2010) found that DH activity inhibition was greatest at low sulfonamide concentrations and inhibition actually decreased with increasing sulfonamide concentrations. This suggests the ATZ mineralizing microorganisms may be more sensitive to low SMZ concentrations in soil relative to the other VA treatments. This interaction illustrates that an increase in ATZ mineralized to  $^{14}\text{CO}_2$  is observed with time, but this increase is dependent on treatment.

To our knowledge, only two studies have investigated the influence of VAs on herbicide degradation, and neither specifically looked at ATZ mineralization. Kim et al. (2010) found the presence of VAs commonly used in poultry production significantly slowed the rate of ATZ degradation in a sandy soil. These results do not generally agree with the findings from this study. Even though the  $100\ \mu\text{g}\ \text{kg}^{-1}$  SMZ application had significantly less ATZ mineralized to  $^{14}\text{CO}_2$ , VA treatment did not significantly affect the concentration of ATZ and its chlorinated metabolites. However, it is important to note the proportion of metabolites remaining in the soil at the end of the incubation varied by



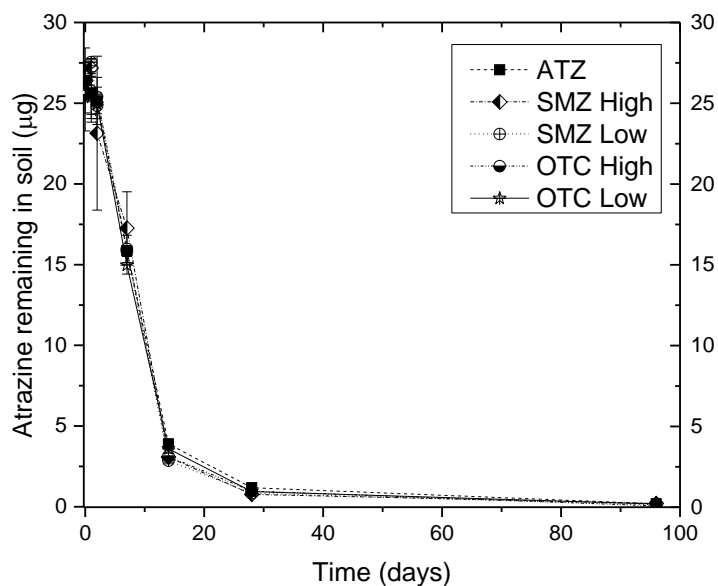
**Figure 2.4** Total mineralization of  $^{14}\text{C}$ -atrazine and mean of enzyme activities in soil amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high concentrations ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) of sulfamethazine (SMZ) and oxytetracycline (OTC). Control represents ATZ amendment only. Enzyme activity units are mg PNP or mg TPF released  $\text{g}^{-1}$  dry soil  $\text{h}^{-1}$  for  $\beta$ -glu and DH, respectively. Error bars represent one standard deviation.



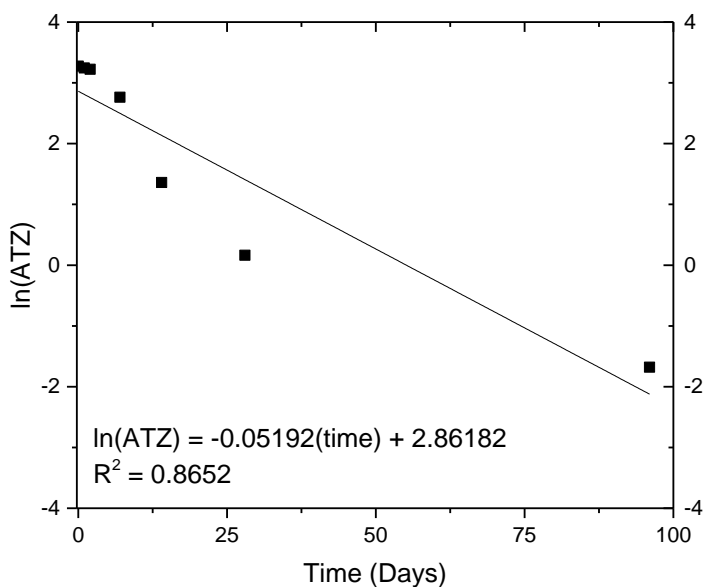
**Figure 2.5** Total mineralization of  $^{14}\text{C}$ -atrazine soil amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high concentrations ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) of sulfamethazine (SMZ) and oxytetracycline (OTC) over a 96 d incubation. Control represents ATZ amendment only. Error bars represent one standard deviation.

treatment (not statistically significant). Similarly, Accinelli et al. (2006) did not observe a significant difference in metolachlor degradation or sorption in the presence of SMZ. Experimental procedure varied for each study which could have resulted in dissimilar interactions between herbicides and VAs.

Atrazine degradation showed first-order kinetics for all treatments, but no significant treatment differences in the mass of ATZ remaining with time (Figures 2.6 and 2.7). Degradation of ATZ was detected by day 2 and was followed by a rapid decrease in ATZ concentration from days 2 to 14; by day 96 the concentration of ATZ in the soil was nearly zero. Figure 2.7 depicts the relationship between time (days) and the natural log of ATZ concentration in  $\mu\text{g}$  for the control soil (ATZ only). The coefficient of determination ( $R^2$ ) for the kinetic models ranged from 0.7982 to 0.9173 (Table 2.5). The calculated half-lives were similar and ranged from 10.6 to 13.5 days (Table 2.5). An analysis of variance indicated no significant differences between half-lives for each of the treatments in the soil incubation study. The USDA-ARS reported a field half-life of ATZ ranging from 13 to 402 days (USDA-ARS, 2007). The apparent half-life of ATZ in this experiment is at the very low end, or in some instances, below this reported range. This could be a result of laboratory conditions which are much more controlled and conducive to ATZ degradation relative to field environments. Conditions favorable to microbial activity (constant temperature and 60% water filled pore space) were maintained in this experiment, which would have facilitated more rapid ATZ degradation. Nevertheless, it is surprising that the ATZ dissipated so quickly given that the soil had not received ATZ for at least 25 years.



**Figure 2.6** Atrazine remaining in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ) and low and high (100 and 1000 µg kg<sup>-1</sup>) sulfamethazine (SMZ) or oxytetracycline (OTC) concentrations during a 96 d incubation period. Control represents ATZ amendment only. Error bars represent one standard deviation.



**Figure 2.7** Relationship between time (days) and the natural log of atrazine (ATZ) concentration in µg for the control soil (ATZ only).

**Table 2.5** Degradation kinetics of atrazine in soils amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high (100 and 1000  $\mu\text{g kg}^{-1}$ ) sulfamethazine (SMZ) or oxytetracycline (OTC) concentrations over a 96 d incubation period. Control represents ATZ amendment only. Half-lives followed by the same letter do not differ significantly from each other at the  $\alpha=0.05$  level.

<b>Treatment</b>	<b>t<sub>1/2</sub></b>	<b>First-order reaction</b>	<b>R<sup>2</sup></b>
ATZ	13.4A	$\ln Y = -0.0519x + 2.8618$	0.8652
SMZ Low	12.2A	$\ln Y = -0.0568x + 2.8103$	0.8513
SMZ High	13.5A	$\ln Y = -0.0514x + 2.7772$	0.7982
OTC Low	13.1A	$\ln Y = -0.0531x + 2.8149$	0.8498
OTC High	10.6A	$\ln Y = -0.0651x + 2.8997$	0.9173

Since soil was collected from an alluvial area, adapted microorganisms and their genes were most likely transported from fields where ATZ is regularly applied and deposited at the site through flooding and erosion. It also possible the soil microbial community composition had shifted to include proliferation of groups with the ability to metabolize ATZ during previous annual ATZ applications, allowing ATZ degrading genes to persist within the microbial genome and facilitate rapid dissipation of ATZ upon reintroduction into this soil. Several studies have shown soils that have long term ATZ exposure are able to degrade ATZ more readily (Behki and Khan, 1986; Ostrofsky et al., 1997; Yassir et al., 1999; Abdelhafid et al., 2000; Krutz et al., 2009). A similar scenario has been reported for development of adapted glyphosate-degrading microbial communities in soils under long-term glyphosate application (Dick et al., 2010). Still, it would be remarkable if ATZ degrading genes were able to persist in the soil for such an extended period. Yassir et al., (1999) did not see enhanced ATZ degradation persist in soils in which herbicide had not been applied for just three years. However, several studies reported rapid ATZ degradation in soils with no previous ATZ exposure.

Zablotowicz et al. (2006) found rapid ATZ dissipation in a soil that had not received ATZ for at least 10 years. It was speculated that the rapid dissipation in the untreated soil was caused by movement of soil from sites treated with ATZ via farm equipment (Zablotowicz et al., 2006). Zablotowicz et al. (2007) found that enhanced ATZ degradation could develop within a year of exposure for soils in the Mississippi Delta, even if the soils did not have previous ATZ exposure. It is also possible soil physical properties influence its ability to degrade ATZ. Krutz et al. (2010) reported that 90% of known *s*-triazine adapted soils fit into specified groups based on the textural triangle, including sandy loams, similar to the soil of interest in this study. Krutz et al. (2010) also found that 90% of *s*-triazine adapted soils had total organic carbon levels between 1 and 2.8% and a pH range from 5.8 to 8.1. The soil used in this study contained 2.4% total organic carbon and had a water pH of 7.1. As a result, it is possible the soil properties were such that ATZ enhanced degradation was able to develop rapidly.

The similarities between the calculated half-lives and the fact that three of the four VA treatments had shorter half-lives compared to the ATZ only control supports the conclusion that VA treatment at the investigated concentrations do not negatively affect ATZ degradation in soil. To our knowledge only two previous studies have investigated the interaction of VAs and herbicide degradation. Similar findings were reported by Accinelli et al. (2006), who found that SMZ in the  $\mu\text{g kg}^{-1}$  range did not have any noticeable impact on metolachlor degradation. These results contradict the findings of Kim et al. (2010) who observed significantly longer ATZ half-lives in the presence of the antibiotics monensin, narasin, and salinomycin. Differences in soil chemical, physical,

and biological properties as well as chemical and physical properties of the VAs and herbicides studied could account for these differences.

### 2.3.2.1 Manure-Amended Soil Incubation Experiment

Unlike the soil incubation experiment, there was a significant effect of treatment, time, and the interaction of treatment and time for ATZ degradation in the manure-amended soil incubation experiment (Table 2.6). The interaction indicates the amount of ATZ in soil is dependent on time, but the dissipation in soil will also depend on treatment. The primary treatment effect on ATZ concentration remaining in soil was the presence or absence of manure and not the addition of either VA (Figure 2.8). The same

**Table 2.6** Analysis of manure-amended soil incubation experiment data. Type III tests evaluating treatment (trt), time, and the interaction effects (trt\*time) for the dependent variables: atrazine and metabolite concentrations; mineralized <sup>14</sup>C atrazine (<sup>14</sup>CO<sub>2</sub>); β-glucosidase enzyme activity (β-glu); and dehydrogenase enzyme activity (DH).

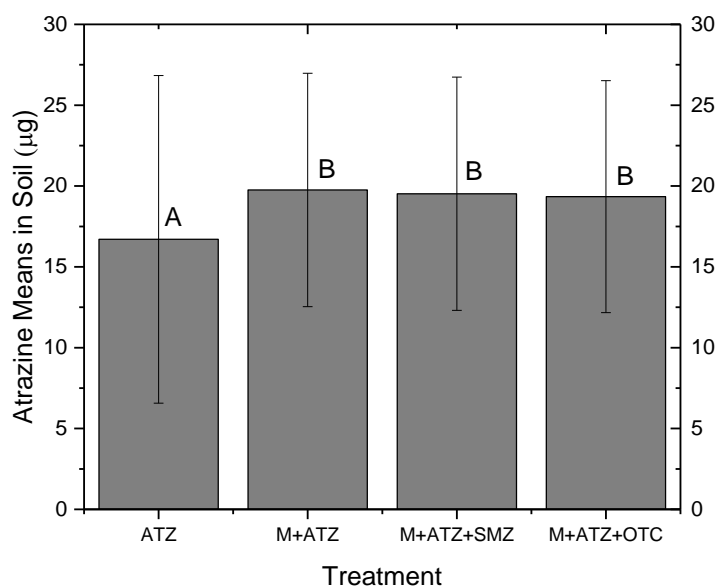
#### Analysis of manure-amended soil incubation experiment data.

	Analyte						
	-----p-values-----						
	-----						
Source	ATZ <sup>†</sup>	DDA <sup>‡</sup>	DIA <sup>‡</sup>	DEA <sup>‡</sup>	<sup>14</sup> CO <sub>2</sub>	β-glu	DH
<b>Trt</b>	<b>&lt;0.0001</b>	<b>0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
<b>Time</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>
<b>Trt*Time</b>	<b>&lt;0.0001</b>	0.0691	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0066</b>

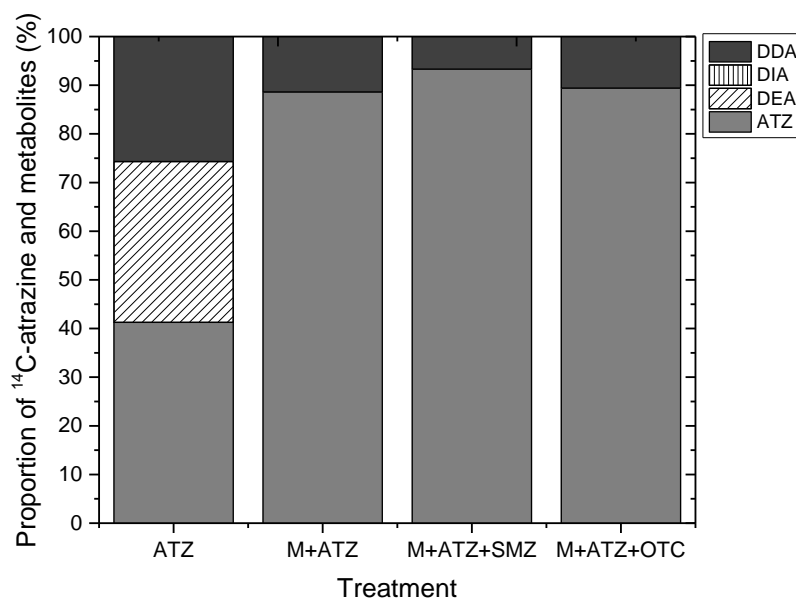
<sup>†</sup>ATZ = atrazine; DDA = didealkylatrazine; DIA = deisopropylatrazine; DEA = deethylatrazine.

<sup>‡</sup>Indicates days 0, 1, and 2 were excluded during analysis.

Bold type indicates significant treatment differences ( $\alpha = 0.05$ ).



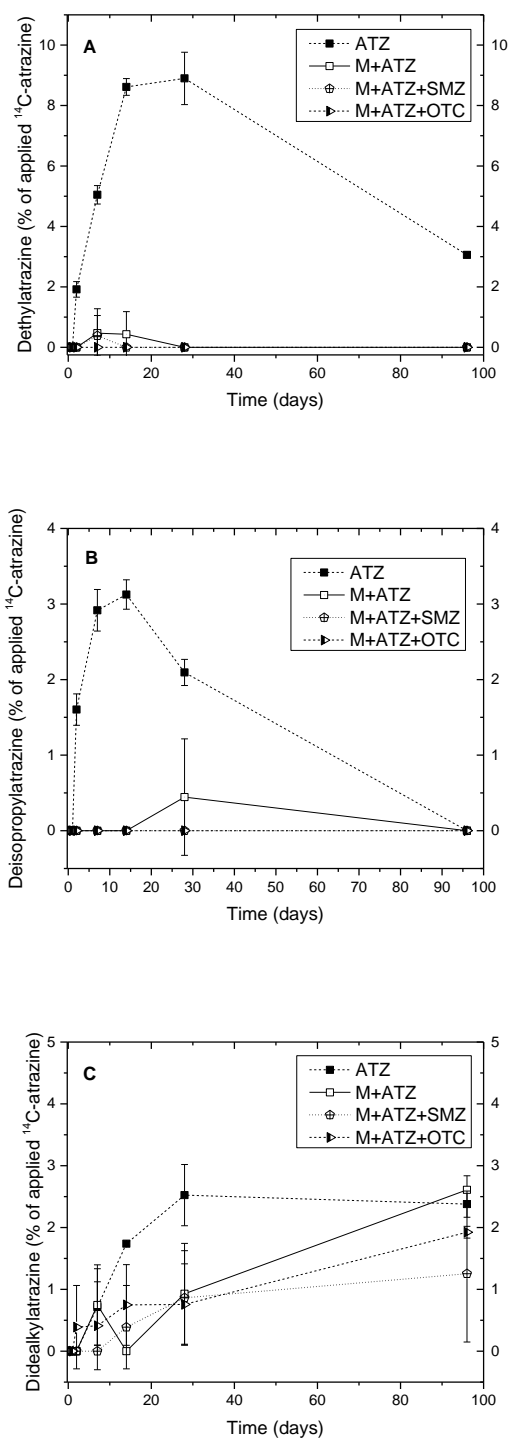
**Figure 2.8** Mean values of atrazine (ATZ) remaining in soil amended with 500 µg ATZ kg<sup>-1</sup> and 5% swine manure (M) and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC). Error bars represent one standard deviation.



**Figure 2.9** Distribution of extractable <sup>14</sup>C-atrazine and ATZ metabolites in soil after 96 d incubation with 500 µg ATZ kg<sup>-1</sup> and 5% swine manure (M) and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC).



holds true for the metabolites DDA, DIA, and DEA. The ATZ only control in the manure-amended soil incubation experiment had a composition of ATZ and metabolites similar to those observed in the primary (soil only) incubation experiment after 96 days (Figures 2.9 and 2.2, respectively). Unlike the samples containing soil only, the manure-amended samples had a much greater proportion of the parent compound remaining in the soil at the end of the incubation and the metabolite DEA was not detected (Figure 2.9). This demonstrated that the presence of manure significantly decreased ATZ degradation and may have influenced the degradation pathway. Figure 2.10 depicts the presence of the chlorinated metabolites over the 96 day incubation period. The percent of applied  $^{14}\text{C}$ -ATZ and metabolites recovered after 14, 28, and 96 days of incubation are displayed in Table 2.7. The addition of manure decreased the formation of all three metabolites and reduced the accumulation of DEA and DIA. The ATZ control (soil only) had significant amounts of DEA and DIA formed and degraded during the 96 day incubation. In contrast, VA treatments had no detectable DIA, and the M+ATZ+OTC treatment had no detectable DEA. All treatments had detectable levels of DDA, and the manure-amended samples mirrored the nonamended (soil only) control more closely compared to the other two metabolites. The formation of DDA requires a two-step dealkylation, so the formation of either DEA or DIA is necessary before DDA can be produced. This suggests DEA and DIA were formed and then converted to DDA more rapidly for manure-amended soils compared to the ATZ (soil only) control. The metabolites indicate a clear difference in ATZ degradation between manure-amended and nonamended soils (Figures 2.3 and 2.10). A subtle VA treatment effect is indicated by the



**Figure 2.10** Formation of (A) deethylatrazine, (B) deisopropylatrazine, and (C) didealkylatrazine in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations (100  $\mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

**Table 2.7** Atrazine (ATZ),  $^{14}\text{C}$ -atrazine, deethylatrazine (DEA) deisopropylatrazine (DIA), and didealkylatrazine (DDA) recovered after 14, 28, and 96 days represented as a percent of applied  $^{14}\text{C}$ -ATZ in soil amended with  $500 \mu\text{g kg}^{-1}$  ATZ, 5% swine manure (M),  $100 \mu\text{g kg}^{-1}$  sulfamethazine (SMZ) and  $100 \mu\text{g kg}^{-1}$  oxytetracycline (OTC).

**Percent of Applied  $^{14}\text{C}$  atrazine (%)**

**Day 14**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>M+ATZ</b>	<b>M+ATZ+SMZ</b>	<b>M+ATZ+OTC</b>
<b>DDA</b>	2.61	0.00	0.58	1.12
<b>DIA</b>	4.69	0.00	0.00	0.00
<b>DEA</b>	12.92	0.65	0.00	0.00
<b>ATZ</b>	64.55	92.23	100.88	94.60
<b>Recovery (%)</b>	84.76	92.87	101.46	95.72
<b><math>^{14}\text{C}</math><math>\text{O}_2</math></b>	2.06	1.26	1.26	1.26

**Day 28**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>M+ATZ</b>	<b>M+ATZ+SMZ</b>	<b>M+ATZ+OTC</b>
<b>DDA</b>	3.79	1.39	1.29	1.14
<b>DIA</b>	3.14	0.67	0.00	0.00
<b>DEA</b>	13.35	0.00	0.00	0.00
<b>ATZ</b>	5.77	30.53	26.07	24.58
<b>Recovery (%)</b>	26.05	32.59	27.36	25.71
<b><math>^{14}\text{C}</math><math>\text{O}_2</math></b>	4.15	1.89	1.89	1.89

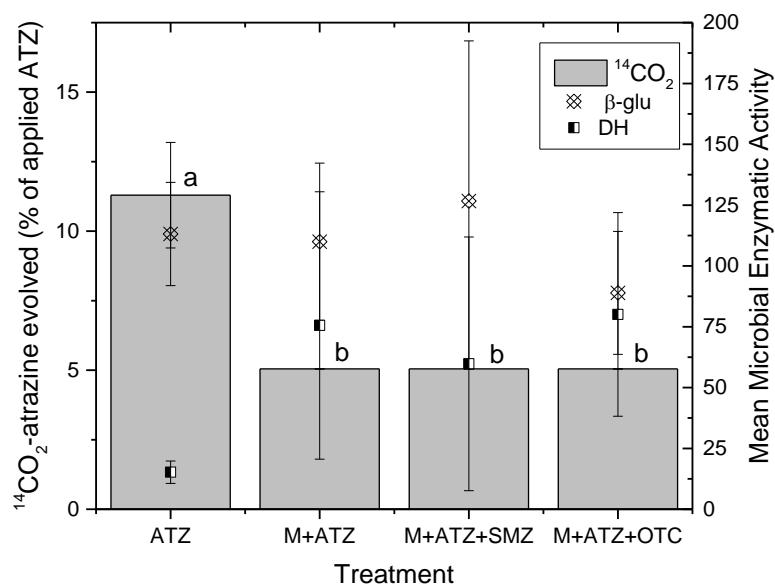
**Day 96**

<b>Atrazine and Metabolites</b>	<b>ATZ (Control)</b>	<b>M+ATZ</b>	<b>M+ATZ+SMZ</b>	<b>M+ATZ+OTC</b>
<b>DDA</b>	3.57	3.92	1.88	2.89
<b>DIA</b>	0.00	0.00	0.00	0.00
<b>DEA</b>	4.59	0.00	0.00	0.00
<b>ATZ</b>	5.77	30.53	26.07	24.58
<b>Recovery (%)</b>	13.93	34.44	27.94	27.46
<b><math>^{14}\text{C}</math><math>\text{O}_2</math></b>	11.29	5.04	5.04	5.05

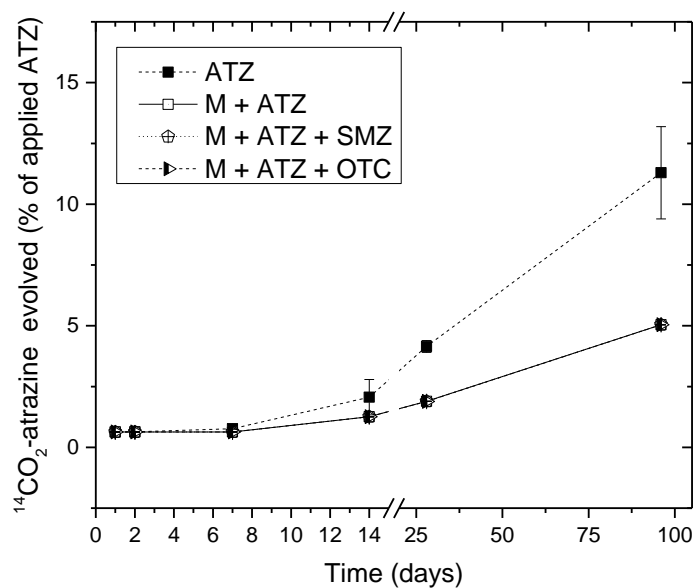
changes in metabolite formation for VA and manure amendments relative to the M+ATZ treatment.

Several studies have found that the addition of manure can decrease ATZ degradation in soil. Houot et al. (1998) found that the addition of composted straw and municipal compost both decreased ATZ mineralization and increased the formation of non-extractable ATZ residues. The type of organic amendment influenced how ATZ behaved; the addition of composted straw favored hydroxyatrazine production and municipal compost preserved extractable ATZ (Houot et al., 1998). Abdelhafid et al. (2000) found the addition of organic amendments in soils with no previous ATZ exposure favored ATZ dealkylation rather than triazine ring mineralization. However, the addition of organic amendments did not influence ATZ degradation in adapted soils (soils with long-term ATZ exposure) (Abdelhafid et al., 2000). Conversely, a number of studies have found that organic amendments such as manure can actually facilitate rapid degradation of ATZ, most likely due to enhanced microbial activity and increased ATZ bioavailability (Topp et al., 1996; Aguilera et al., 2009; Mukherjee, 2009).

The amount of  $^{14}\text{C}$ -ATZ mineralized to  $^{14}\text{CO}_2$  in the manure-amended soil was approximately 50% of ATZ mineralized for soil only control samples included in the second incubation experiment (Figure 2.11). It appears that the addition of manure protects ATZ from degradation in soil and as a result more of the parent compound remains in soil with less conversion to  $\text{CO}_2$  during the 96 day incubation (Figure 2.12). Possible reasons for this include increased ATZ sorption to soil because of additional soil organic matter (due to manure amendment), preferential microbial use of manure as a C



**Figure 2.11** Total mineralization of <sup>14</sup>C-atrazine and mean of enzyme activity in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC). Enzyme activity units are mg PNP or mg TPF released g<sup>-1</sup> dry soil h<sup>-1</sup> for β-glu and DH, respectively. Error bars represent one standard deviation.



**Figure 2.12** Total mineralization of <sup>14</sup>C-atrazine soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) over a 96 d incubation. Error bars represent one standard deviation.

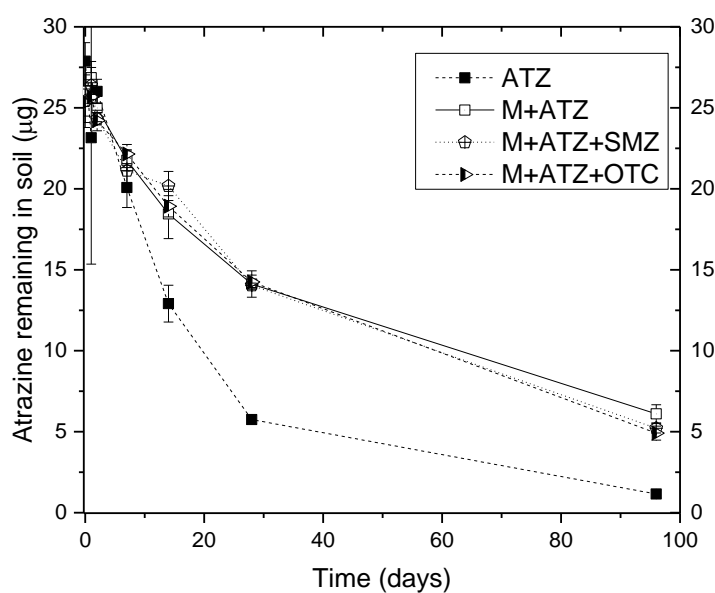
phenomenon has been described for  $\beta$ -glu repression and cellobiose degradation if and N source, and changes in soil pH as a result of manure addition to the soil. Atrazine sorption to soil is positively correlated with organic matter content (Binet et al., 2006), so the addition of manure to soil could have increased sorption and decreased ATZ bioavailability to microbes. It should be noted that organic amendments will not always increase sorption because sources such as liquid cow manure which are high in dissolved organic carbon have been shown to increase bioavailability of ATZ (Aguilera et al., 2009). Atrazine degradation may also be reduced because the addition of manure to soil provides a relatively available source of C and N which would be preferentially utilized by soil microbes. For example, Abdelhafid et al. (2000) found the addition of mineral N from organic amendments decreased ATZ mineralization in adapted and non-adapted soils. The presence of soluble C and N from manure could have suppressed synthesis of ATZ degrading enzymes because consuming ATZ as an energy source was no longer necessary. A similar glucose sources are readily available (Sternberg et al., 1976; Freer and Detroy, 1985).  $\beta$ -glu activity appears to be suppressed in manure amended soils (see section 2.3.2.2), suggesting enzyme activity specific to ATZ degradation may have been inhibited as well. Additionally, Martin-Laurent et al. (2004) found a slight correlation between the level of expressed ATZ degrading genetic potential in ATZ degrading organisms and the biotic and abiotic factors within the soil environment. It is possible the ATZ would not need to be metabolized as an energy source until all of the nutrients from the manure had been depleted. Still, the presence of nutrients could increase microbial activity and therefore facilitate rapid ATZ degradation (Topp et al., 1996; Aguilera et al., 2009; Mukherjee, 2009). Furthermore, the addition of manure to soil could change the pH

of the system, which in turn could affect the availability of nutrients and also the availability of organic contaminants to soil microorganisms. Houot et al., (2000) found that ATZ may degrade more slowly in acidic soils compared to alkaline soils, as demonstrated by increased ATZ mineralization above pH 6. Initially the addition of manure containing ammonia will make soil conditions more basic. However, as the ammonia is converted to nitrate, release of protons will cause pH to decrease (ter Laak et al., 2005). It is possible the pH decreased during the course of the incubation experiment causing ATZ to degrade more slowly in manure-amended soils relative to the soil only control.

Just as in the first incubation experiment, the main effect time was significant and ATZ dissipation followed first-order kinetics (Figures 2.13 and 2.14 and Table 2.6). No significant differences in ATZ degradation were observed between the treatments at days 0, 1, and 2 of the incubation. By day 14, significantly less ATZ remained in the samples containing unamended soil compared to all manure-amended treatments. However, ATZ remaining at 96 d was similar for all treatments. Once again, the VA treatment did not appear to significantly influence ATZ degradation. The relationship between time and the natural log of ATZ concentration for the ATZ and M+ATZ treatments showed a highly significant relationship, with coefficients of determination ( $R^2$ ) ranging from 0.958 to 0.995 (Figure 2.14). These  $R^2$  values are greater than the values obtained during the first incubation experiment, indicating that there was a better fit for the degradation kinetics in this study. The calculated half-life of ATZ for the soil only treatment was 21.1 days. This value is greater than in the initial incubation experiment, but still within the range of

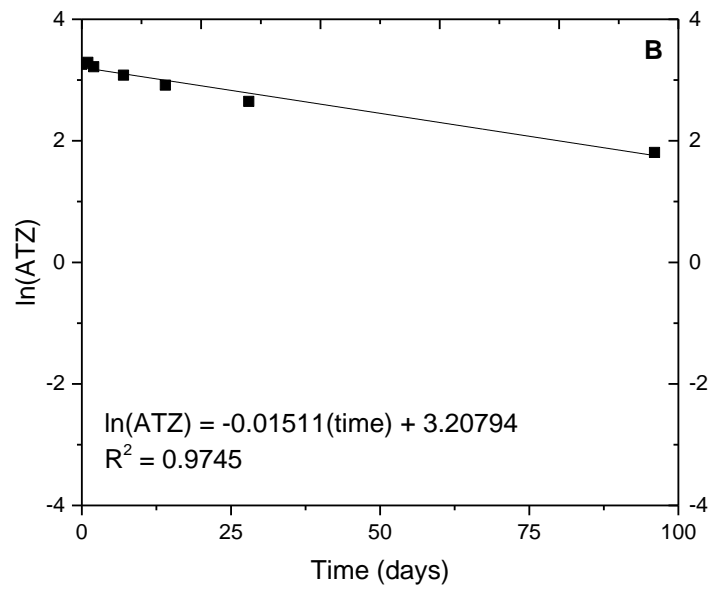
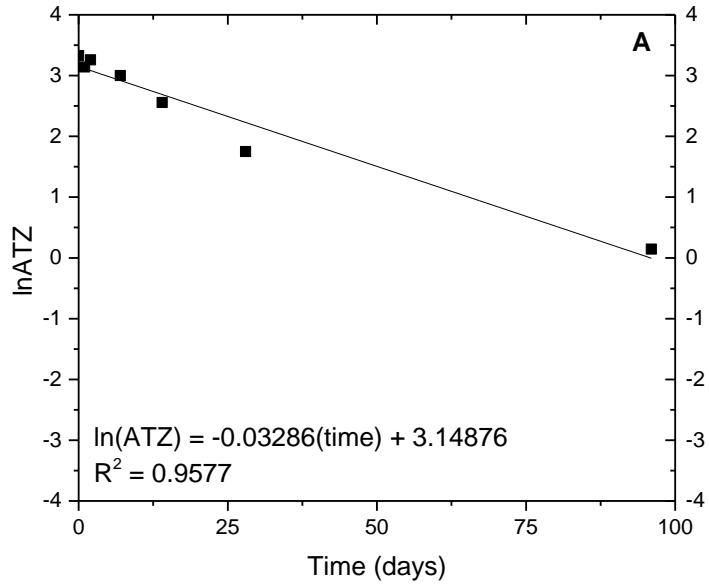
**Table 2.8** Degradation kinetics of atrazine in soils amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations (100  $\mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) over a 96 d incubation period. Control represents ATZ amendment only. Half-lives followed by the same letter do not differ significantly from each other at the  $\alpha=0.05$  level

Treatment	$t_{1/2}$	First-order reaction	$R^2$
ATZ	21.1C	$\ln Y = -0.0329x + 3.1488$	0.9577
M + ATZ	45.9A	$\ln Y = -0.0151x + 3.2079$	0.9745
M + ATZ + SMZ	41.7AB	$\ln Y = -0.0166x + 3.2150$	0.9892
M + ATZ + OTC	40.4B	$\ln Y = -0.0172x + 3.2126$	0.9950



**Figure 2.13** Atrazine remaining in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations (100  $\mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.





**Figure 2.14** Relationship between time (days) and the natural log of atrazine (ATZ) concentration in  $\mu\text{g}$  for (A) soil only and (B) manure-amended soil.

reported field half-lives (USDA-ARS, 2007). The calculated half-lives for the manure-amended soils were 45.9, 41.7, and 40.4 days for M+ATZ, M+ATZ+SMZ, and M+ATZ+OTC, respectively. These values are approximately double that of the soil only samples, but still well within the range of expected ATZ half-life in field settings. An analysis of variance indicated the half-life of ATZ was significantly less for the ATZ control (soil only) compared to all manure-amended samples. The half-life of ATZ was significantly shorter in the M+ATZ+OTC treatment relative to the M+ATZ treatment, depicting a subtle VA treatment effect.

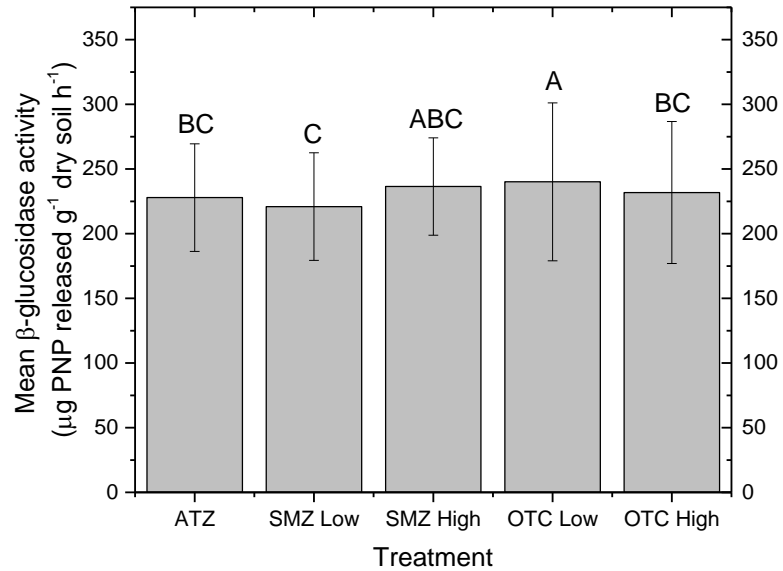
Just as with the primary incubation experiment, the calculated half-life for the laboratory experiment may be shorter than what would be found in a field setting because conditions for maximum microbial activity were maintained throughout the study. Regardless, these results clearly showed that the input of manure significantly decreased the rate of ATZ degradation in soil. These findings are consistent with results reported by Houot et al. (1998) and Abdelhafid et al. (2000). The interaction of treatment and time for ATZ, DEA, DIA, and  $^{14}\text{CO}_2$  makes it difficult to determine the relative significance of the two variables independently. The interaction term most likely illustrates that, with time, ATZ decreases and  $^{14}\text{C}$  mineralization, DEA, and DIA increase, and these changes are dependent on treatment. This trend is generally illustrated in Figures 2.10, 2.12, and 2.13. The confounded nature of these variables could be a result of VA/ATZ sensitivity among different members of the microbial consortia. It is possible the various bacteria have dissimilar sensitivity to SMZ and OTC, and as a result each step of the ATZ degradation pathway is affected differently. Additionally, the presence of manure

influences sorption and the metabolic preference of the soil microorganisms, which drives the synthesis of ATZ degrading enzymes in soil. Nonetheless, the addition of either SMZ or OTC did not significantly reduce ATZ degradation in manure amended soil at the studied concentrations.

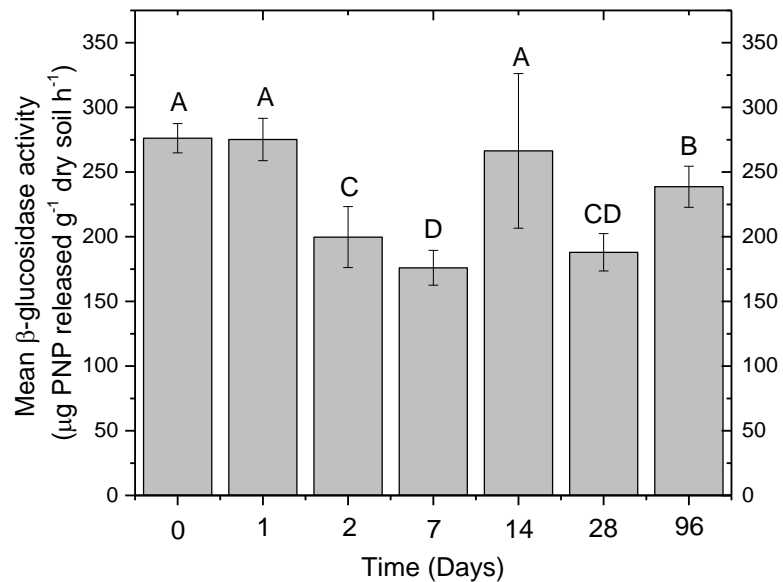
## **2.3.2 $\beta$ -glucosidase**

### **2.3.2.1 Soil Incubation Experiment**

$\beta$ -glucosidase activity exhibited a significant treatment effect in the first (soil only) incubation study and differences in  $\beta$ -glu activity amongst the treatments can be observed in Figure 2.15. The least overall  $\beta$ -glu activity was observed in 100  $\mu\text{g kg}^{-1}$  SMZ treated soil and the greatest activity was observed in 100  $\mu\text{g kg}^{-1}$  OTC treated soil. The control (ATZ only) soil had significantly less  $\beta$ -glu activity compared to the 100  $\mu\text{g kg}^{-1}$  OTC treated soil. The 1000  $\mu\text{g kg}^{-1}$  OTC treatment exhibited the second greatest activity, but the mean was not significantly different from any of the treatments with exception for the 100  $\mu\text{g kg}^{-1}$  OTC treatment. The 100  $\mu\text{g kg}^{-1}$  SMZ treatment exhibited the least  $\beta$ -glu activity as well as the least  $^{14}\text{C}$ -ATZ mineralization, so it is possible there is a connection between the two variables. Lin et al. (2011) established a positive correlation between  $^{14}\text{C}$ -ATZ mineralization and  $\beta$ -glu activity. However, the overall correlation between mean treatment values of  $\beta$ -glu activity and total  $^{14}\text{C}$ -ATZ mineralization was not significant for this study ( $r=0.10691$  and  $p=0.3159$ ; Figure 2.3). In contrast, Lin et al. (2011) found a significant positive correlation between the two variables ( $r=0.57$  and  $p=0.004$ ).



**Figure 2.15** Mean  $\beta$ -glucosidase activity in soils based on treatment. Soils were amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) concentrations of sulfamethazine (SMZ) or oxytetracycline (OTC). Control represents ATZ amendment only. Error bars represent one standard deviation.

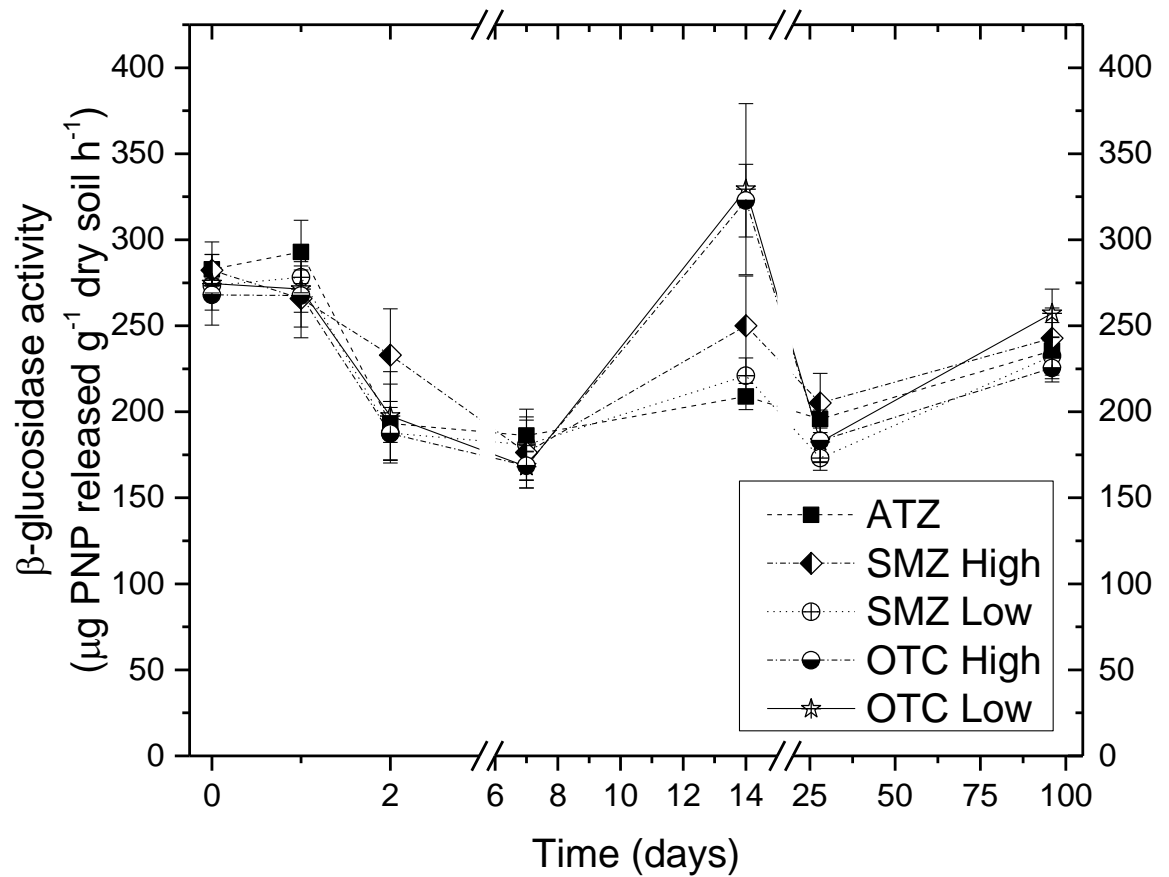


**Figure 2.16** Mean  $\beta$ -glucosidase activity in soils based on time (days). Soils were amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and  $0$ ,  $100$ , or  $1000 \mu\text{g kg}^{-1}$  of sulfamethazine (SMZ) or oxytetracycline (OTC). Error bars represent one standard deviation.

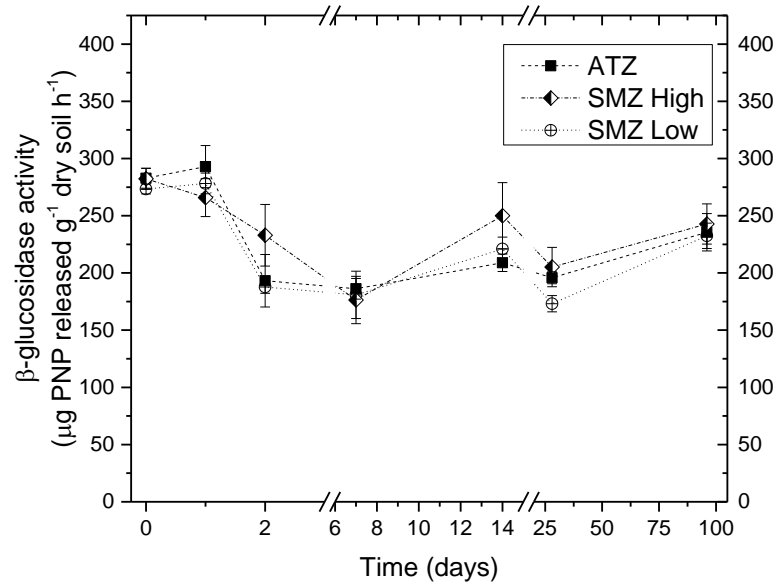
$\beta$ -glu activity was also significantly affected by time during the primary incubation study. Figure 2.16 depicts the mean  $\beta$ -glu activity, averaged over all treatments, as a function of incubation time. Activity was the greatest at days 0, 1, and 14 of the incubation experiment;  $\beta$ -glu activity was observed to decrease at day 2 and activity reached a minimum at day 7. A recovery in  $\beta$ -glu activity at day 14 resulted in activity levels that were statistically equivalent to the day 0 and 1 levels. Following the recovery at day 14, another decrease in activity was observed at day 28. By day 96 of the incubation experiment,  $\beta$ -glu activity began to recover but did not reach  $\beta$ -glu activity observed at the beginning of the incubation study.

It is difficult to determine the relative significance of the treatment and time effects because a complex interaction effect between treatment and time was observed. Figure 2.17 depicts  $\beta$ -glu activity for each of the five treatments over the course of the incubation study, and Figures 2.18 and 2.19 display  $\beta$ -glu activity over time for SMZ and OTC treatments, respectively. Initially the control sample (ATZ only) exhibited nominally greater  $\beta$ -glu activity compared to the VA treated soils. Thereafter, trends generally follow those shown in Figure 2.16 which was described previously.

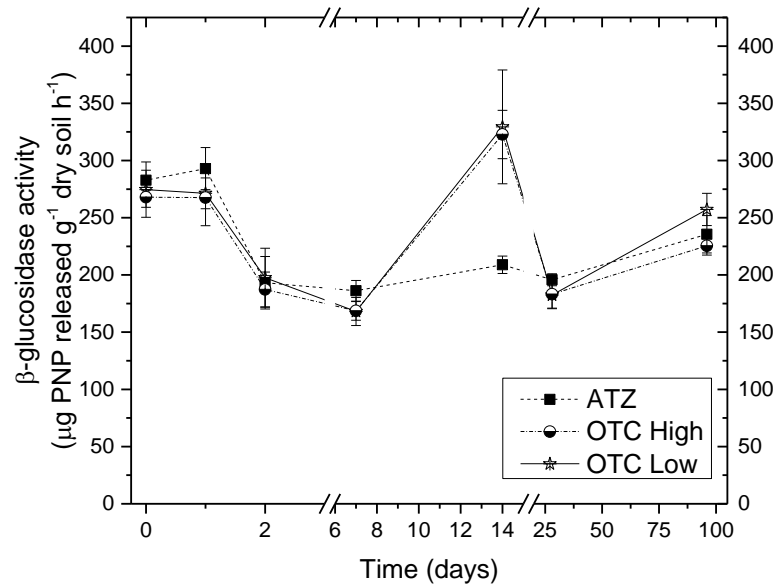
Since  $\beta$ -glu is indicative of C cycling, inferences about soil microbial activity can be made based on  $\beta$ -glu activity. Initial microbial mortality as a result of VA/ATZ application is a possible explanation for the change in  $\beta$ -glu activity over time. Unger et al. (2013) observed a similar decline and recovery pattern for the enzymes fluorescein diacetate and DH in soils treated with OTC and lincomycin. It was speculated that the VAs were adsorbed very quickly, and most active against soil microbes for a short period



**Figure 2.17** β-glucosidase enzyme activity in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ) and low and high (100 and 1000 µg kg<sup>-1</sup>) concentrations of sulfamethazine (SMZ) or oxytetracycline (OTC). Control represents ATZ amendment only. Error bars represent one standard deviation.



**Figure 2.18**  $\beta$ -glucosidase enzyme activity in soil amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) concentrations of sulfamethazine (SMZ). Control represents ATZ amendment only. Error bars represent one standard deviation.



**Figure 2.19**  $\beta$ -glucosidase enzyme activity in soil amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high ( $100$  and  $1000 \mu\text{g kg}^{-1}$ ) concentrations of oxytetracycline (OTC). Control represents ATZ amendment only. Error bars represent one standard deviation.

of time at the beginning of the study (Unger et al., 2013). Based on the observed  $\beta$ -glu activity, this appears to be true for the initial incubation study as well. A decrease in  $\beta$ -glu activity at day 2 and 7 of the incubation experiment could have resulted from microbial mortality if the populations were sensitive to the VAs or ATZ. After the microbes died they would begin to decompose and could be utilized as an energy source, during a process known as turnover (Wolf and Wagner, 2005), by the surviving microbes that were less sensitive to the agrichemicals. The sharp increase in  $\beta$ -glu activity at day 14 followed by a decline in activity at day 28 could also be a result of microbial metabolism of the VAs or ATZ as a C source. If microbes that are not sensitive to VAs were able to partially degrade the VAs so they were no longer toxic to more sensitive organisms, the VAs could serve as an energy source for the soil microorganisms. Topp et al. (2013) isolated a SMZ-degrading *Microbacterium* sp. that had developed accelerated biodegradation potential after continuous exposure to SMZ. This strain was apparently able to utilize SMZ as a sole C source. Similarly, Yanze-Kontchou and Gschwind (1994) isolated a microbial strain from the true pseudomonad group that was able to utilize ATZ as its sole C source. It is also possible the functional microbes were robust to the present agrichemical concentrations and adapted as indicated by the increased  $\beta$ -glu activity, as suggested by Unger et al. (2013). The decline in activity that followed could be a result of a mortality event if the soil microbe population reached unsustainably high levels at day 14. Comparison of PLFA markers between a non-treated soil control and the treated soils after 96 days show that ATZ, SMZ, and OTC all have significant effects on the size of microbial groups, some of which are likely  $\beta$ -glu producers (unpublished data). However, ATZ effects were most pronounced and no differences between SMZ and OTC were



detected for total PLFA markers. Presence of ATZ in the soil reduced fungi, arbuscular mycorrhizal fungi, eukaryotes, actinobacteria, and gram negative, gram positive, and anaerobic bacteria markers. Sulfamethazine did not exhibit an effect on arbuscular mycorrhizal fungi or gram positive bacteria. Neither VA significantly reduced eukaryotes or actinobacteria relative to the soil only control.

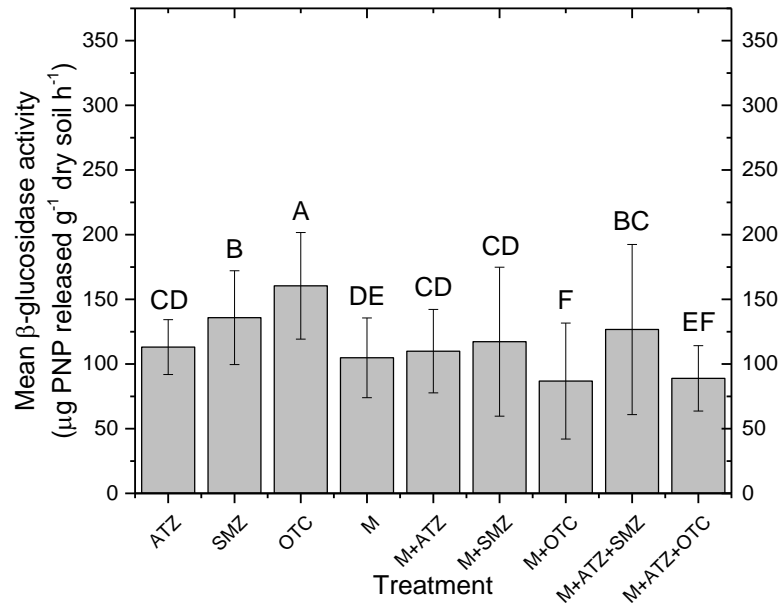
### **2.3.2.2 Manure-Amended Soil Incubation Experiment**

There was a significant treatment effect for  $\beta$ -glu activity in the secondary incubation experiment (Table 2.6). Differences in mean values of  $\beta$ -glu activity among the treatments indicate that the addition of ATZ and VAs may have induced dissimilar effects on soil microorganisms in the presence and absence of manure (Figure 2.20). The 100  $\mu\text{g kg}^{-1}$  OTC and SMZ treated soils (no manure) exhibited the two greatest  $\beta$ -glu activity levels overall. Additionally, greater  $\beta$ -glu activity was exhibited by soils treated with 100  $\mu\text{g kg}^{-1}$  OTC in the first and second incubation experiments (OTC+ATZ for first and OTC only for the second). Given the antimicrobial nature of VAs, it is surprising that an increase rather than a decline in  $\beta$ -glu activity was observed for these treatments. A decrease in  $\beta$ -glu activity was observed for soil containing ATZ relative to the VA only treatments. This suggests the soil microbes were unaffected or negatively affected by the presence of ATZ in soil but not the presence of VAs. Soil amended with manure only (no ATZ or VAs) exhibited less  $\beta$ -glu activity than soil treated with SMZ or OTC only. Since manure is an additional C source for soil microbes, it was interesting that an increase in  $\beta$ -glu activity, and therefore microbial activity, was not observed upon manure addition. However, this may be attributable to  $\beta$ -glu repression resulting from readily available C in the manure. The swine manure used in this study had a C:N ratio of 11:1

(Table 2.1), indicating that it was a labile source of organic C and N. Swine manure may also contain up to 1.7 g soluble C kg<sup>-1</sup> (Paul and Beauchamp, 1989), so it can act as a readily available energy source for soil microorganisms. Freer and Detroy (1985) found  $\beta$ -glu activity was repressed in the presence of high concentrations of glucose. The soil organisms did not begin to metabolize the carbon source, cellobiose, until all the glucose had been exhausted (Freer and Detroy, 1985). Similarly, Sternberg et al. (1976) found  $\beta$ -glu activity was stimulated following removal of inhibitory levels of cellobiose. After the manure input, the focus of microbial activity most likely switches from producing  $\beta$ -glu to metabolizing simple sugars and organic acids that comprise the labile C component; thus, the decrease in  $\beta$ -glu activity may not be reflective of an overall decrease in microbial activity. Additionally, the presence of manure could have changed the soil pH and nutrient availability, which could alter microbial activity. The addition of ATZ or VAs did not significantly change the  $\beta$ -glu activity compared to the manure treatment (M) except for the M+OTC and M+ATZ+SMZ samples. It is, however, interesting to note that the manure-amended soils treated with OTC (OTC only and ATZ+OTC) had the least observed  $\beta$ -glu activity, and this is contrary to findings in the first incubation study. This could be because OTC is suppressing a specific group of microorganisms in the manure that is not as prevalent in the soil. Hammesfahr et al. (2008) found sulfadiazine had pronounced, long-term effects on the microbial community structure in manure amended soil. In this case, the addition of OTC could have caused a change in community structure which led to a decrease in  $\beta$ -glu activity. Additionally, Gutiérrez et al. (2010) found sulfonamides only reduced enzymatic activities when soil microbial growth was stimulated by the addition of readily available C. It is possible that microbial

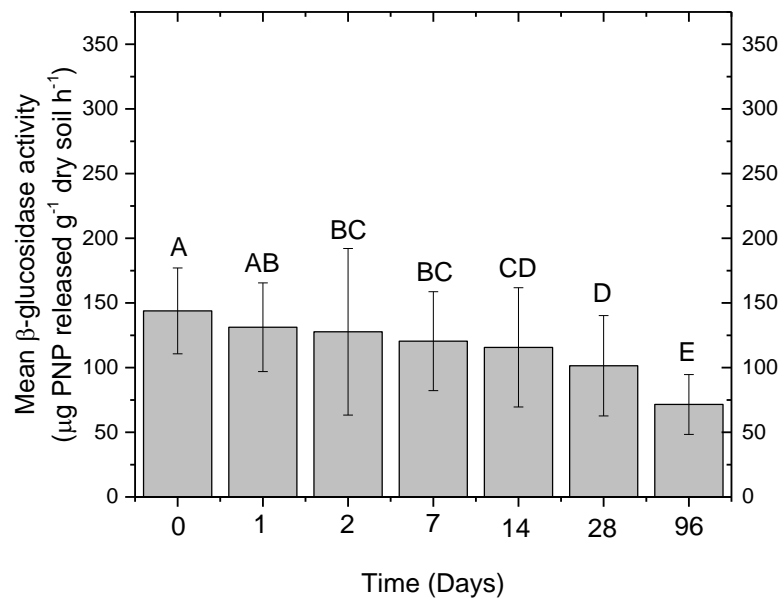
growth must be stimulated before VA effects can be observed. A Pearson correlation between  $\beta$ -glu activity and mineralized  $^{14}\text{C}$ -ATZ indicated a significant negative linear correlation was present between the two variables during the manure-amended incubation ( $r=-0.28325$  and  $p=0.0159$ ). Lin et al. (2011) also found a significant correlation between the two variables, although samples were not amended with manure and the linear correlation observed was positive.

Time also played a significant role in  $\beta$ -glu activity during the second incubation experiment (Table 2.6). Unlike the sharp declines and recoveries observed in the primary incubation experiment, the activity of  $\beta$ -glu steadily declined over the course of the second incubation study (Figure 2.21). The greatest activity was observed at day 0 of the



**Figure 2.20** Mean  $\beta$ -glucosidase activity in soils based on treatment. Soils were amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations ( $100 \mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

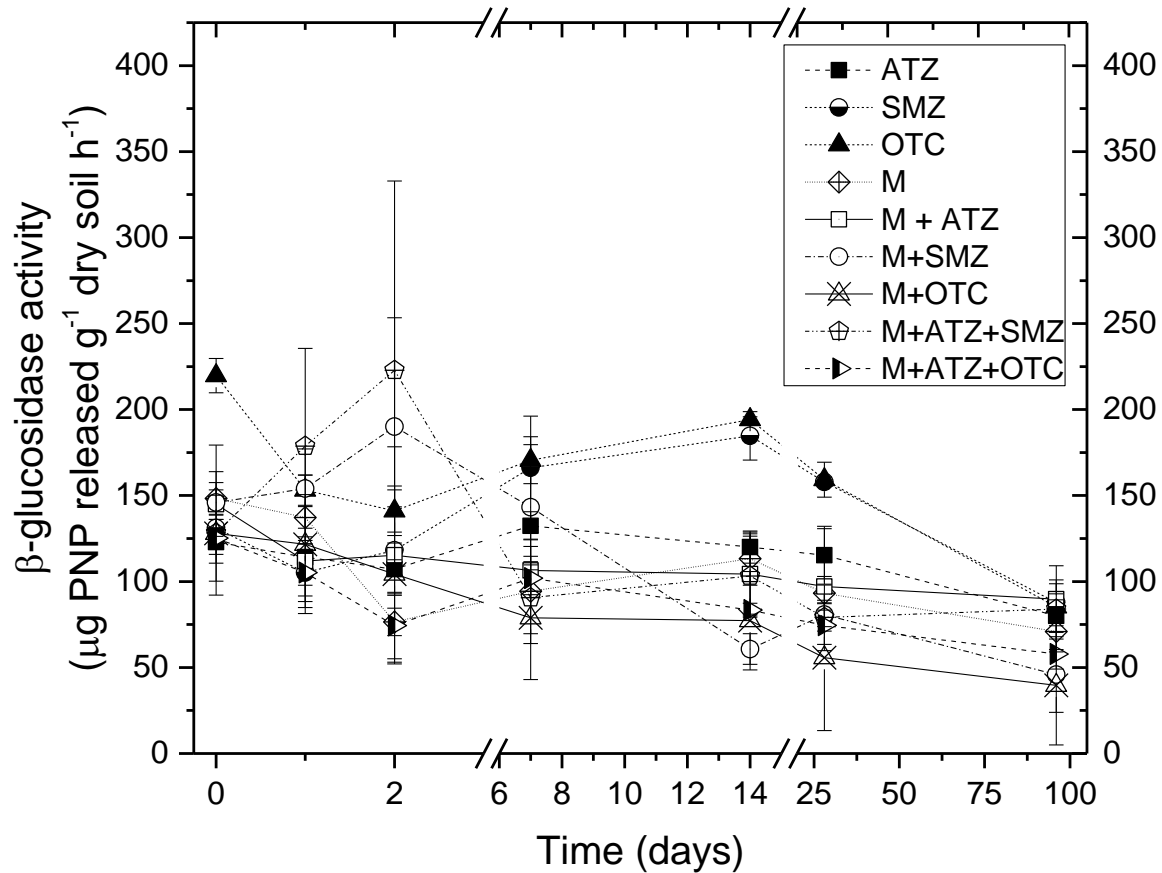
incubation experiment. A slight decline in activity was observed on days 1, 2, and 7 relative to day 0. The activity observed at day 14 decreased slightly from day 2 and 7. Another decrease was observed at day 28 and again at day 96 of the incubation. Thus, it appears that the conditions of the secondary incubation study were not favorable for  $\beta$ -glu activity. It should be noted that soil used for the soil incubation and manure-amended soil incubation were collected from the same site at two different times. Soil for the soil only incubation was collected in dry conditions during July 2011. Alternatively, soil for the manure-amended incubation was collected during a very wet period of March 2013. It could be that the environmental conditions caused the initial differences in  $\beta$ -glu activity, as the mean activity for samples in the manure-amended experiment at day 0 is almost



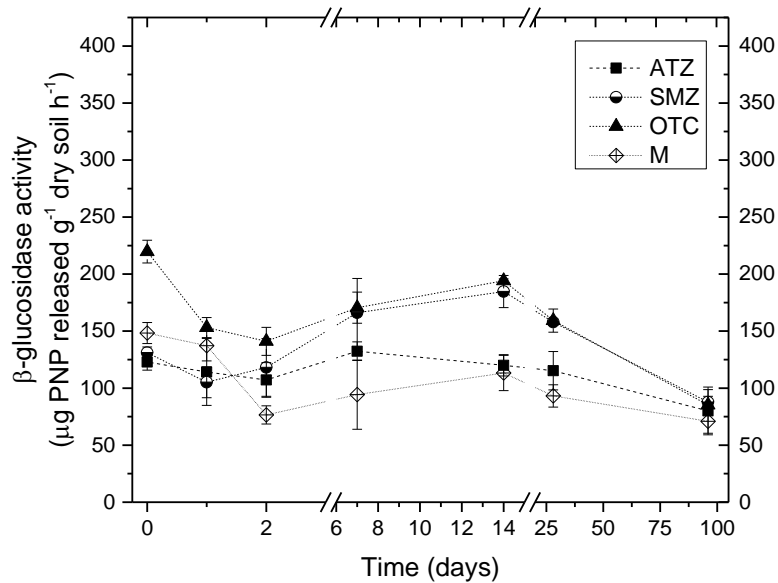
**Figure 2.21** Mean  $\beta$ -glucosidase activity in soils based on time (days). Soils were amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations ( $100 \mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

50% lower than the for the soil incubation. Larger concentrations of glucose could have been present in the soil due to differences in environmental conditions at time of soil collection. Initial conditions coupled with the manure amendment could have caused glucose, the byproduct of  $\beta$ -glu activity, to accumulate in the soil over the course of the incubation. These factors may have repressed  $\beta$ -glu activity, resulting in the general decrease through time, especially for manure-amended samples.

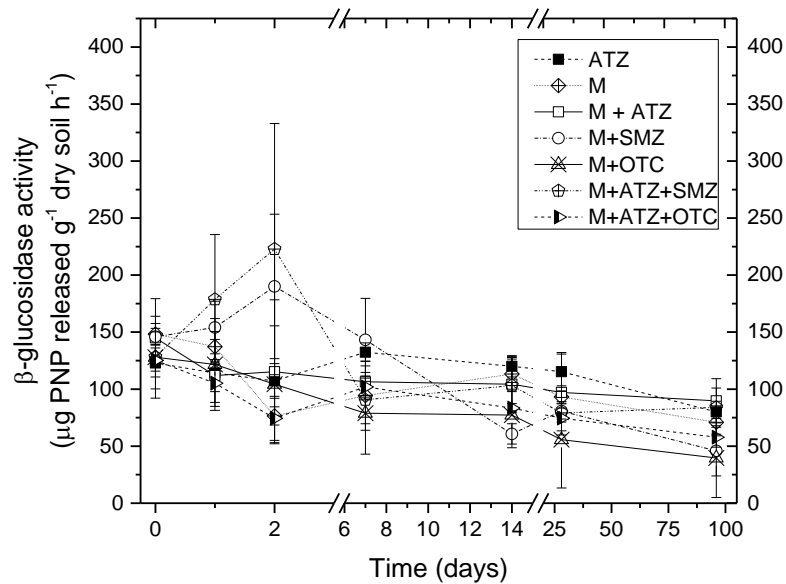
Once again, it is difficult to determine the relative significance of the treatment and time effects on  $\beta$ -glu activity because there was a complex interaction effect between treatment and time (Table 2.6). Figures 2.22 through 2.24 depict  $\beta$ -glu activity for each treatment over the 96 day incubation period. Similar to the first incubation experiment,  $\beta$ -glu activity for each treatment experiences minimums and maximums over the course of the incubation experiment. However, unlike the first incubation experiment, the timing of the decline and recovery for activity is different for each treatment, and there is an overall decrease in  $\beta$ -glu activity over time. The activity of soil-only treatments in the second incubation experiment appears to mimic the activity observed during the first incubation experiment (Figures 2.17 and 2.23). However, the spikes in activity observed at day 14 are less pronounced in the second incubation experiment, especially for the OTC treatments. This could be because the greater concentrations of VAs and combined application of VAs plus ATZ were not used in the second incubation experiment. If VAs and ATZ were used by some microorganisms as carbon substrates, lower inputs of the organic agrichemicals may have limited their use as potential energy sources by soil microorganisms, which could cause a decrease in overall activity. This may also explain the more subtle maximums observed and the overall decrease in activity with time.



**Figure 2.22**  $\beta$ -glucosidase enzyme activity in soil amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations ( $100 \mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.



**Figure 2.23**  $\beta$ -glucosidase enzyme activity in soil amended with either 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), or low concentrations (100  $\mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.



**Figure 2.24**  $\beta$ -glucosidase enzyme activity in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ), 5% manure (M), and low concentrations (100  $\mu\text{g VA kg}^{-1}$ ) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

Microbial turnover was not as evident in these samples because changes in activity over time were not as extreme as in the first incubation experiment.

Unlike the soil only incubation experiment, samples in the manure-amended study seem to exhibit much less  $\beta$ -glu activity and an overall decrease with time. The activities of manure-amended soils as well as the ATZ-only reference soil over time are displayed in Figure 2.24. Unlike in the primary incubation experiment, no real spikes in activity are observed at day 14 of the incubation. However, the M+SMZ and M+ATZ+SMZ treatments did exhibit increased  $\beta$ -glu activity at day 2, perhaps indicating a period of adaptation. Even though activity experienced alternating minimums and maximums over the course of the incubation period, the overall trend for manure-amended samples is a decrease in activity over time (Figure 2.24). As previously discussed, this is most likely because  $\beta$ -glu activity was repressed following the addition of labile C within the manure (Sternberg et al. 1976; Freer and Detory, 1985; Paul and Beauchamp, 1989). Additionally, changes in the soil possibly related to environmental factors at time of collection also influenced  $\beta$ -glu activity for both amended and unamended samples in the second incubation study relative to the initial soil only incubation experiment.

### **2.3.3 Dehydrogenase**

#### **2.3.3.1 Soil Incubation Experiment**

Dehydrogenase activity was not statistically significant in the first (soil only) incubation experiment (Table 2.6). Dehydrogenase activity comprises cumulative activities of many microbial DHs involved in the oxidation of a multitude of organic substances during microbial respiration, and is therefore an indicator of nonspecific intracellular enzyme activity (Subhani et al., 2001; Prosser et al., 2011). No significant differences between treatments suggests VA treatment did not have an impact on the overall microbial biomass. The lack of significance in

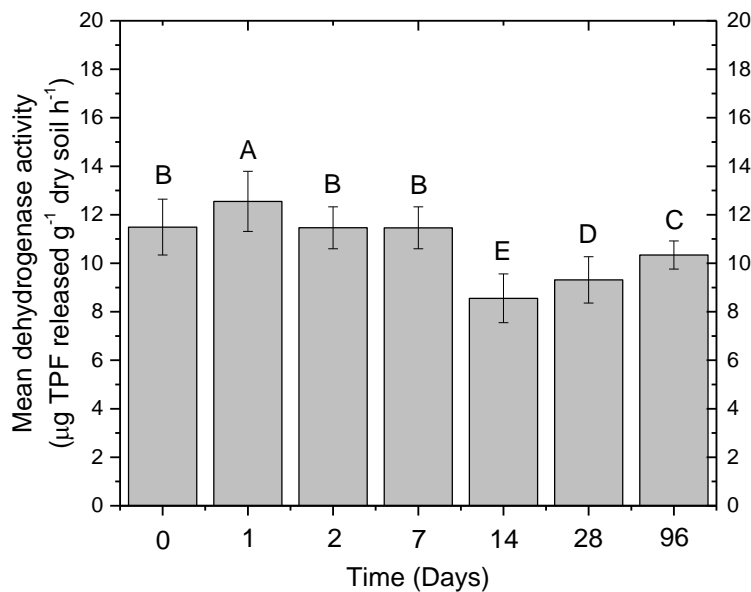


DH activity versus the significance of  $\beta$ -glu activity in the soil incubation experiment is most likely due to differences between the two enzymes. Since DH is an intracellular enzyme and a good indicator of microbial oxidative activities in soil (Subhani et al., 2001), a decrease in overall metabolic activity is reflected in DH activity. In contrast, the  $\beta$ -glu enzyme is extracellular, thus assays do not always reflect microbial activity (Skujins, 1978; Dick, 1994). A large portion of  $\beta$ -glu activity may be the result of abiotic enzymes (Hayano and Tubaki, 1985; Hope and Burns 1987), or enzymes of biological origin that are no longer associated with a living cell (Skujins, 1976). Within the soil these free cells have the potential to be sorbed to the soil and complex with humic substances (Stott et al., 2010), which could neutralize potential activity. The differences between these two enzymes could account for the contrast in apparent microbial activity in soil. Even though DH activity was not significantly affected by the treatments, a significant correlation between DH activity and mineralized  $^{14}\text{C}$ -ATZ was observed. A Pearson correlation between the two variables exhibited a significant negative correlation ( $r=-0.52786$  and  $p<0.0001$ ). These results contrast with findings by Lin et al. (2011), where no significant correlation between DH and ATZ mineralization was observed ( $r=0.38$  and  $p=0.065$ ).

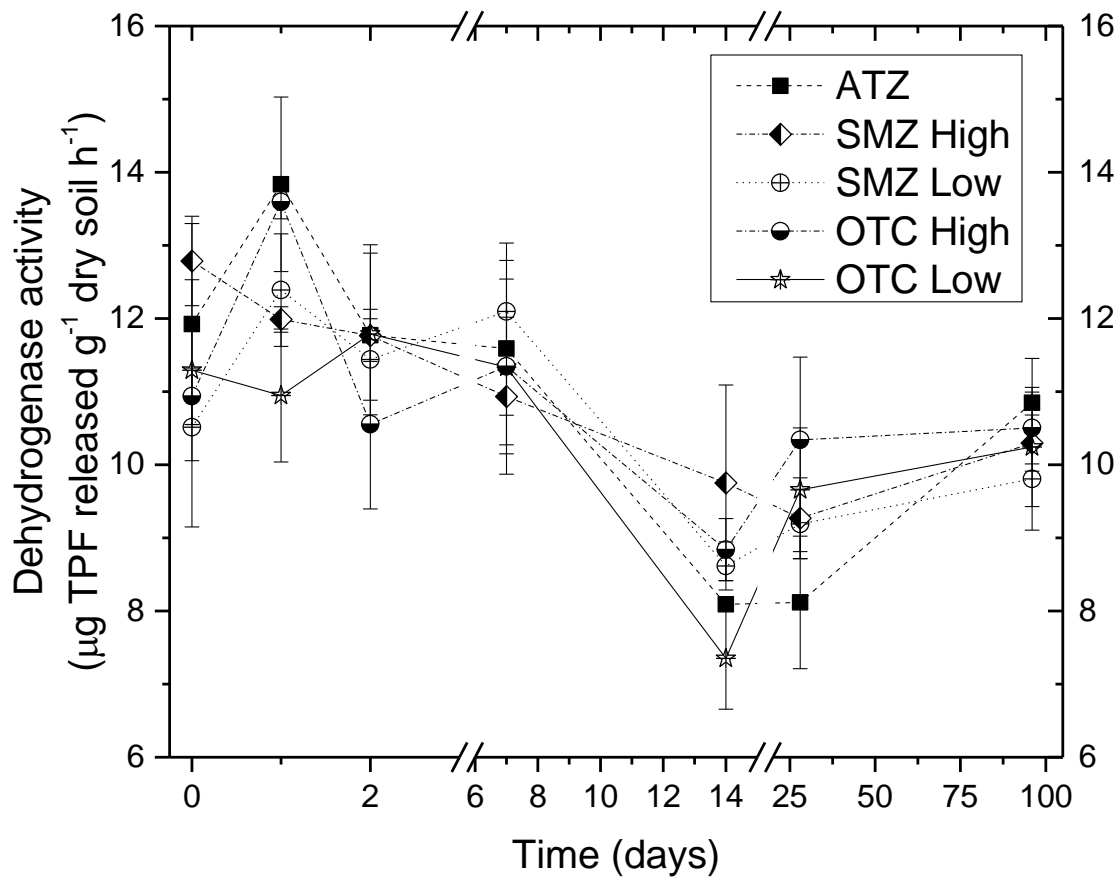
Time had a significant effect on DH activity in the first incubation experiment (Table 2.6). Mean values of DH activities as a function of time are represented in Figure 2.25. An initial increase in DH activity between days 0 and 1 was observed. At day 2 and day 7 the DH activity decreases to the levels initially observed at day 0. Dehydrogenase activity reached a minimum at day 14. A subsequent increase in DH activity was observed on days 28 and 96 of the incubation experiment; however, DH activity never reached the same levels that were observed at the beginning of the incubation experiment. As with the treatment effect, DH activity over time

contrasted  $\beta$ -glu activity; the minimum DH activity observed on day 14 corresponds to the maximum observed  $\beta$ -glu activity. This is most likely due to the differences between the two enzymes.

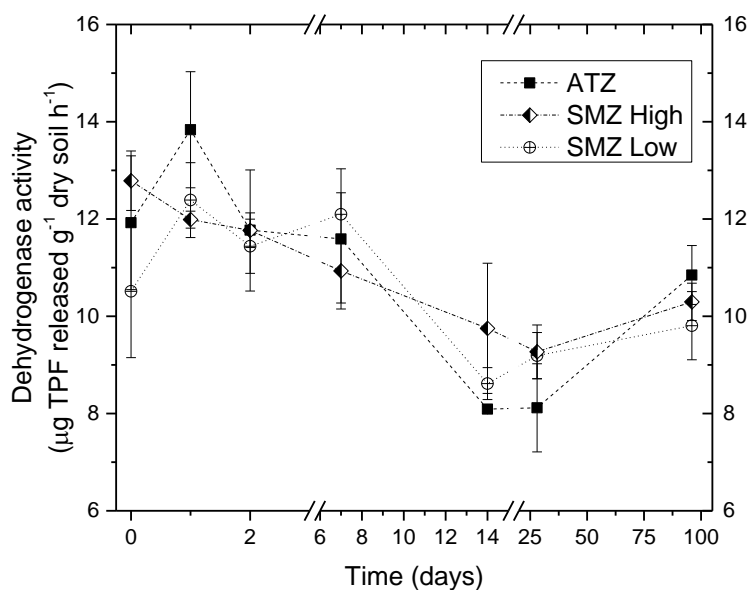
Even though treatment did not significantly affect DH activity, a complex interaction between treatment and time was observed. Figures 2.26-2.28 display DH activity for each treatment over the course of the 96 day incubation period. While a general decrease in DH activity was observed with time, DH activity on different days varied with treatment. None of the treatments consistently exhibited elevated DH activity.



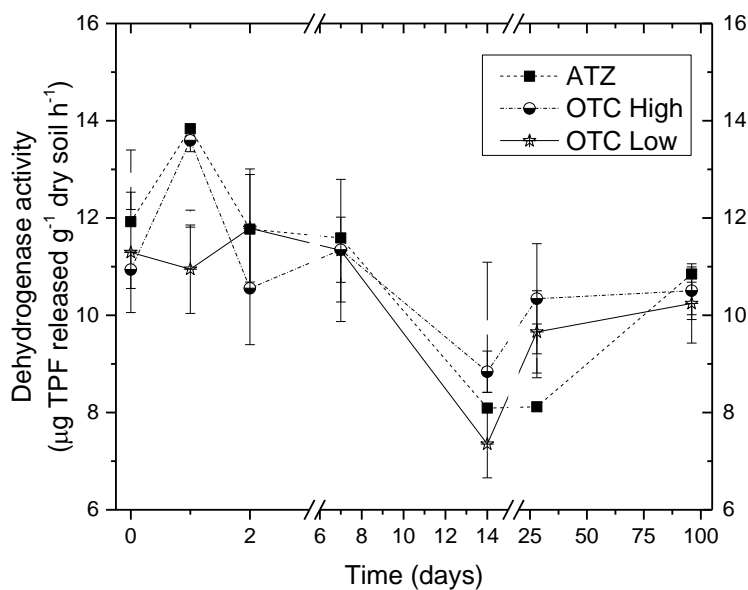
**Figure 2.25** Mean dehydrogenase activity in soils based on time (days). Soils were amended with  $500 \mu\text{g kg}^{-1}$  atrazine (ATZ) and 0, 100, or  $1000 \mu\text{g kg}^{-1}$  of sulfamethazine (SMZ) or oxytetracycline (OTC). Error bars represent one standard deviation.



**Figure 2.26** Dehydrogenase enzyme activity in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ) and low and high (100 and 1000 µg kg<sup>-1</sup>) concentrations of sulfamethazine (SMZ) or oxytetracycline (OTC). Control represents ATZ amendment only. Error bars represent one standard deviation.



**Figure 2.27** Dehydrogenase enzyme activity in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high (100 and 1000  $\mu\text{g kg}^{-1}$ ) concentrations of sulfamethazine (SMZ). Control represents ATZ amendment only. Error bars represent one standard deviation.

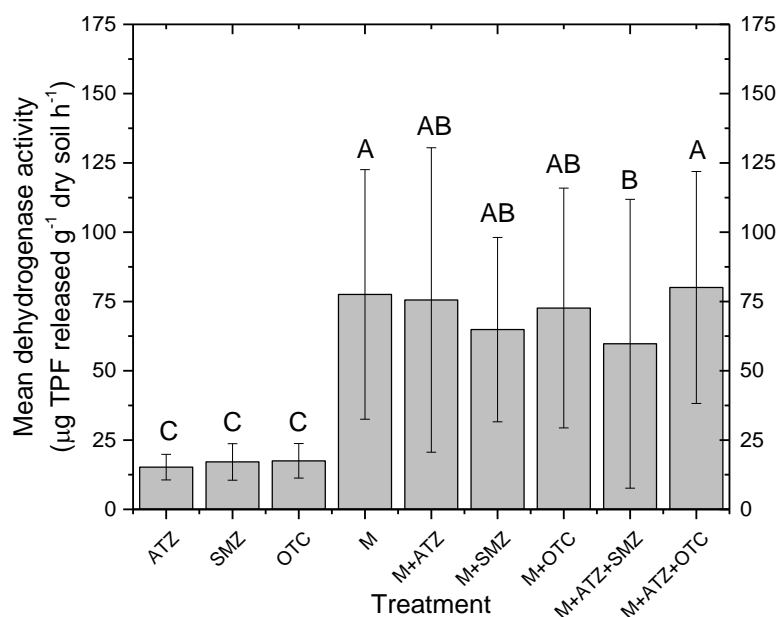


**Figure 2.28** Dehydrogenase enzyme activity in soil amended with 500  $\mu\text{g kg}^{-1}$  atrazine (ATZ) and low and high (100 and 1000  $\mu\text{g kg}^{-1}$ ) concentrations of oxytetracycline (OTC). Control represents ATZ amendment only. Error bars represent one standard deviation.

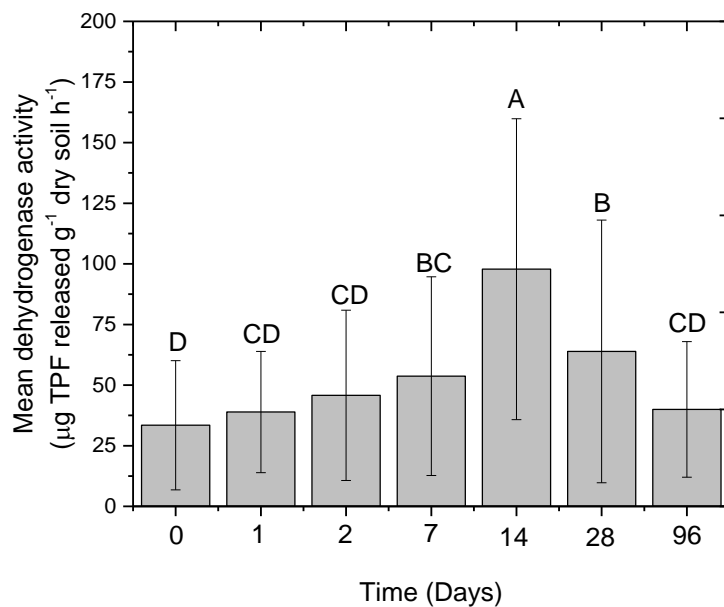
### 2.3.3.2 Manure-Amended Soil Incubation Experiment

Dehydrogenase activity exhibited a significant treatment effect in the second incubation experiment (Table 2.6). The mean values of DH activity for the various treatments are displayed in Figure 2.29. The statistical analysis indicated that treatments amended with manure exhibit substantially greater DH activity. Samples containing manure had DH activity levels nearly three times greater than the soil only samples. There were no significant differences between the soil only treatments (ATZ, SMZ, and OTC), suggesting the input of these agrichemicals by themselves did not promote different responses in DH activity. Slight dissimilarities between treatments containing manure were observed. The M+ATZ+SMZ sample exhibited significantly less DH activity relative to the manure only (M) and M+ATZ+OTC treatments. Amongst the manure-amended soils, M+ATZ+SMZ exhibited the greatest  $\beta$ -glu activity, which continues to suggest the two enzymes respond to the presence of manure and VAs in opposite manners. Even though  $\beta$ -glu activity was reduced in samples containing manure, the DH activity results for manure-amended soils suggest that the input of ATZ and VAs does not systematically reduce enzyme activity of the total microbial biomass. These results agree with the findings from the first incubation study, where the addition of ATZ and VAs did not significantly influence DH activity. Just as with the soil incubation experiment, a significant negative correlation between DH activity and  $^{14}\text{CO}_2$  evolution was observed ( $r=-0.24734$  and  $p=0.0362$ ). However, the addition of manure seems to have weakened the strength of this correlation.

The main effect of time also had a significant influence on DH activity in the second incubation study (Table 2.6). Figure 2.30 depicts the mean values of DH activity for each sampling day. Activity of DH was least at day 0 of the incubation and gradually



**Figure 2.29** Mean dehydrogenase activity in soils based on treatment. Soils were amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

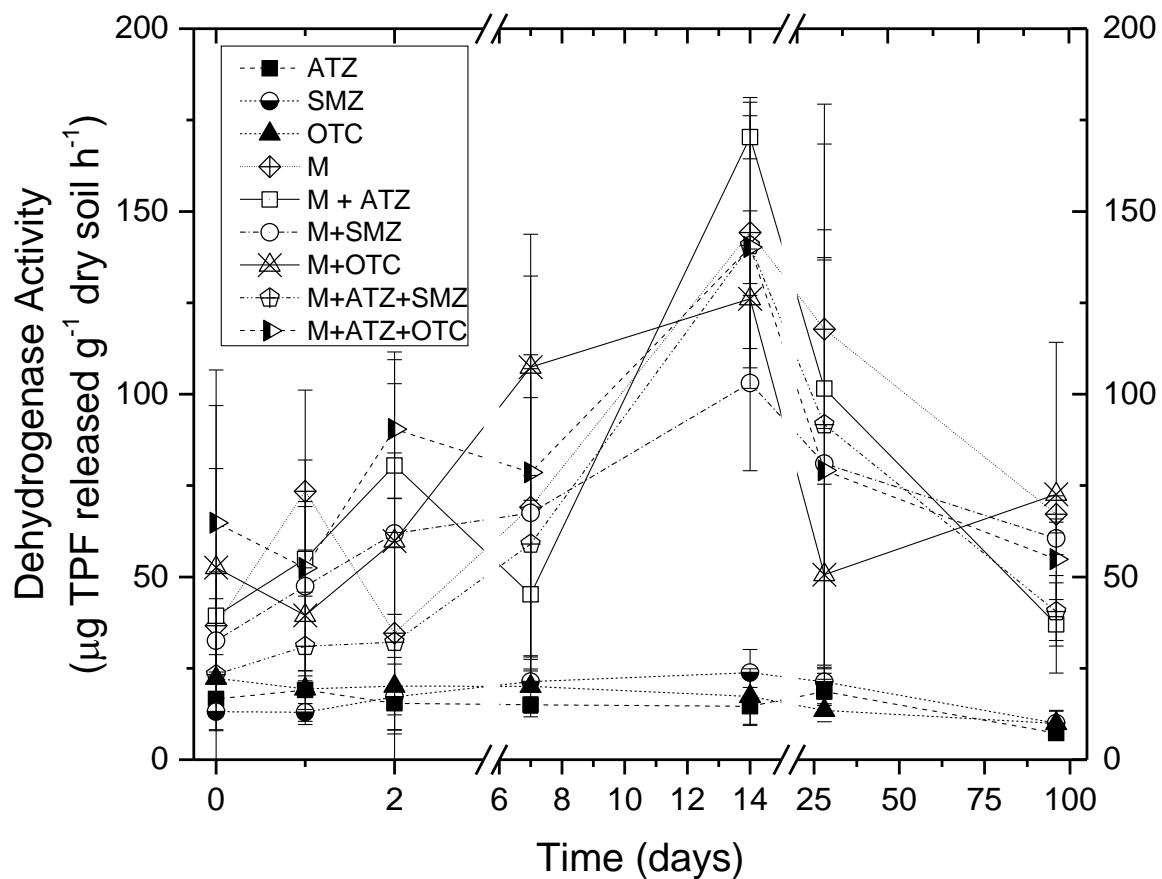


**Figure 2.30** Mean dehydrogenase activity in soils based on time. Soils were amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

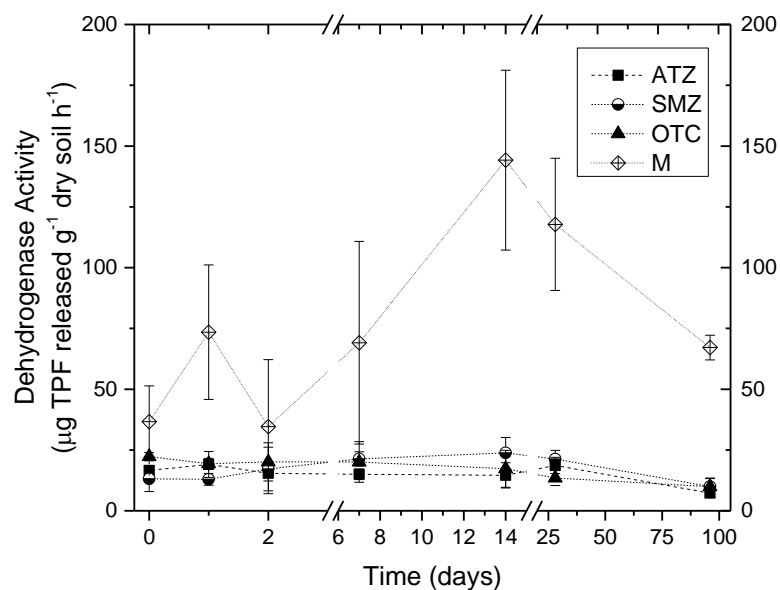
increased at 1, 2, and 7 days. Dehydrogenase activity reached its peak at day 14 and then declined during day 28 and 96. Activity levels after 96 days were equivalent to levels observed during the first week of the incubation. The spike at day 14 and subsequent decline in activity could be a result of manure application, utilization of agrichemicals as a C source, and sensitivity of soil microorganisms to ATZ/VAs. As previously discussed, the addition of manure could provide readily available C and N sources which may stimulate microbial growth (Paul and Beauchamp, 1989). Although their contribution would be minor compared to the manure amendment, the VAs and ATZ are another potential energy source for soil microorganisms (Yanze-Kontchou and Gschwind 1994; Topp et al. 2013). Based on ATZ degradation through time (Figure 2.13), ATZ levels were considerably reduced by day 14. If the bioavailable ATZ was being used as a microbial substrate, it is possible that the observed reduction in ATZ within the soil contributed to decreased DH activity after day 14. The reported half-life for SMZ is 18.6 days; the half-life of OTC is 33 days in manure-amended soil and 55 days in unamended soil (Wang and Yates, 2006, 2008). Consequently, it can be inferred that VAs were degraded somewhat during the incubation period, at which point their bioactivity was neutralized and may have been available as an energy source. It is possible the VAs were available to the microbes as an energy source leading up to day 14, after which they were metabolized or became less available, resulting in decreased DH activity. Comparison of PLFA markers revealed soil microorganisms were sensitive to both ATZ and VAs (data not shown). As a result, it is possible DH activity increased once the agrichemicals had been partially degraded within the soil.

The interaction between treatment and time was significant for DH activity in the second incubation experiment. For this reason, it is difficult to separate the two variables and determine their individual significance. The interaction between the two variables is complex, with manure-amended treatments showing substantial variation throughout the incubation. However, two trends are visually apparent for DH activity over time (Figures 2.31 - 2.33). Dehydrogenase activity for samples without manure remains relatively constant throughout the incubation, and the ATZ and VA treatments do not cause significant changes in DH activity. The manure-amended treatments appear to have a stronger response to time, as illustrated by an increase in DH activity up to day 14 followed by a decline in activity. This pattern is consistent with the classical response of the “zymogenous” soil microbial community, which acts quickly on readily available substrates resulting in an activity (growth) spike and subsequently diminishes as substrates are depleted (Killhan and Prosser, 2007). The manure apparently provided abundant labile C and N substrates to the soil thereby stimulating metabolic activity of the zymogenous bacterial community. Even though this overall pattern is visually apparent, the treatments respond differently through time. There is no one treatment that consistently demonstrates elevated DH activity relative to the others, and the resulting interaction effect is difficult to interpret. Nonetheless, it appears that overall DH activity through time was predominantly influenced by the presence or absence of manure and not the VA/ATZ treatment.

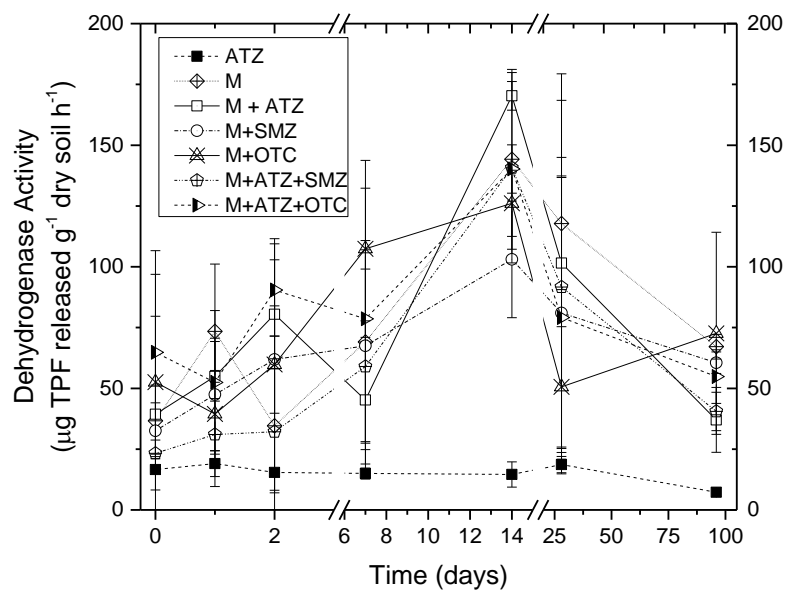




**Figure 2.31** Dehydrogenase enzyme activity in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.



**Figure 2.32** Dehydrogenase enzyme activity in soil amended with either 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), or low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.



**Figure 2.33** Dehydrogenase enzyme activity in soil amended with 500 µg kg<sup>-1</sup> atrazine (ATZ), 5% manure (M), and low concentrations (100 µg VA kg<sup>-1</sup>) of sulfamethazine (SMZ) or oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

## 2.4 Conclusions

This study demonstrated that the presence of the VAs SMZ and OTC in sandy loam soil did not significantly reduce ATZ degradation at the investigated concentrations (100 or 1000  $\mu\text{g VA kg}^{-1}$ ). Degradation of ATZ and the formation of the chlorinated metabolites DDA, DEA, and DIA were not significantly different between any treatments in the soil only incubation study. Mineralization of  $^{14}\text{C}$ -ATZ was significantly reduced in soil samples amended with 100  $\mu\text{g SMZ kg}^{-1}$  relative to the ATZ only control, suggesting low concentrations of SMZ might inhibit ATZ mineralizing organisms. No significant differences were observed between VA treatments in manure-amended soil. However, the addition of manure significantly increased the half-life of ATZ and may influence the composition of ATZ metabolites formed relative to unamended soil. For manure-amended soils, the parent compound accounted for nearly 50% more of the remaining ATZ at the end of 96 days, and the formation of DDA was reduced by almost 50% and DEA was completely absent. The type of VA, VA concentration, and presence of manure all significantly affected  $\beta$ -glu activity in soil, as  $\beta$ -glu activity was significantly different between treatments in manure-amended and unamended soils, but the pattern was not easily explained. Manure amendment and the 100  $\mu\text{g kg}^{-1}$  SMZ treatment in unamended soil seemed to inhibit  $\beta$ -glu activity relative to soil spiked with only ATZ. However, DH activity showed that soil microbial communities were not negatively affected by the presence of VAs in soil, and DH activity was stimulated by manure amendment. These results suggest co-application of manure containing VAs and ATZ to agricultural soils will not inhibit ATZ degradation because of the VA's antimicrobial properties. It does not appear the investigated concentrations of VAs were great enough to significantly

inhibit ATZ degrading microorganisms in soil and manure amended soil. However, the impact of manure addition to soil had the greatest overall effect, leading to significantly decreased ATZ degradation in soil. The behavior of these agrichemicals is likely to change depending on soil, manure, and VA properties. As a result, VAs may influence ATZ degradation for some soils. Further research investigating ATZ adapted soils, different VA types and concentrations, and additional manure sources is warranted.

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## CHAPTER 3: SUMMARY AND FUTURE WORK

### 3.1 Summary

Recognizing the relationship between VAs and herbicides is crucial, yet information on the subject is very limited. This research sought to enhance our understanding of herbicide/VA interactions by studying the influence of two VAs [sulfamethazine (SMZ) and oxytetracycline (OTC)] on degradation of the commonly used herbicide atrazine (ATZ). Objectives of this study were to (1) compare ATZ degradation rates in soil amended or not amended with manure in the presence of SMZ or OTC and (2) investigate changes in soil microbial enzymatic activity, specifically the activity of  $\beta$ -glucosidase ( $\beta$ -glu) and dehydrogenase (DH), as a function of time following application of SMZ and OTC to soils amended with ATZ.

Veterinary antibiotics did not significantly influence ATZ degradation or the formation of its chlorinated metabolites in the soil only incubation experiment. This suggests VA treatment did not significantly impact ATZ degradation or the degradation pathway. A kinetic model was used to show that ATZ degradation followed a first-order rate law in the soil incubation experiment. The calculated half-lives ranged from 10.6 to 13.5 days, and no significant differences in ATZ half-lives was observed between the treatments. Similarity of the calculated half-lives supports the conclusion that VA treatment at these concentrations does not inhibit ATZ degradation in soil.

Unlike in the soil incubation experiment, there was a significant effect of treatment, time, and the interaction of treatment and time for ATZ degradation in manure-amended soil samples. However, it appears that the significance of the treatment on ATZ and its metabolites was limited to the presence or absence of manure and not

influenced by VAs. The kinetic model used to fit ATZ degradation followed a first-order rate law for each of the treatments in the second incubation experiment. The calculated half-life of ATZ for the soil only treatment was 21.1 days. The calculated half-lives for the manure-amended soils were approximately double those of the unamended samples ranging from 40.4 to 45.9 days. The ATZ half-life in unamended soil was significantly less than for manure-amended soils and the M+ATZ+OTC had a half-life significantly less than the M+ATZ treatment. These results showed that the input of manure will significantly decrease the rate of ATZ degradation in soil over time.

### **3.1.3.2 $\beta$ -glucosidase Activity**

$\beta$ -glucosidase activity exhibited a significant treatment effect in the soil incubation study but the reason for these differences is not readily apparent.  $\beta$ -glu activity was also significantly affected by time during the primary incubation study, experiencing maximums at days 0, 1, and 14 and a minimum at day 7 of the incubation experiment. It is difficult to determine the relative significance of the treatment and time effects because a complex interaction effect between treatment and time was observed. Since  $\beta$ -glu is indicative of C cycling, inferences about soil microbial activity can be made based on  $\beta$ -glu activity. Changes in  $\beta$ -glu activity with time could be a result of microbial turnover, utilization of VAs/ATZ as C or N sources, and varying sensitivity to the agrichemicals.

There was a significant treatment effect for  $\beta$ -glu activity in the manure-amended incubation experiment. Differences in mean values of  $\beta$ -glu activity among the treatments indicate that the addition of ATZ and VAs may have induced dissimilar effects on soil microorganisms in the presence and absence of manure. Decreased  $\beta$ -glu activity for manure-amended samples may be attributable to  $\beta$ -glu repression resulting from readily

available C in the manure; thus, the decrease in  $\beta$ -glu activity may not be reflective of an overall decrease in microbial activity.  $\beta$ -glu activity varied significantly during the second incubation experiment. Unlike the sharp declines and recoveries observed in the primary incubation experiment, the activity of  $\beta$ -glu generally declined over the course of the second incubation study. Once again, it is difficult to determine the relative significance of the treatment and time because there was an interaction effect between treatment and time.

### **3.1.3.3 Dehydrogenase Activity**

Dehydrogenase activity was not statistically significant in the soil incubation experiment. This demonstrated that VA treatment did affect the overall microbial biomass, despite the large variations in  $\beta$ -glu activity. Dehydrogenase activity reached a maximum at day 1 and a minimum at day 14. Even though treatment was not significant for DH activity, a complex interaction between treatment and time was observed.

Dehydrogenase activity exhibited a significant treatment effect in the second incubation experiment. Manure-amended samples had DH activity levels nearly three times larger than the soil only samples. Time and the interaction of treatment and time also significantly influenced DH activity. Dehydrogenase activity for samples without manure remained relatively constant, and ATZ and VA treatments do not cause significant changes in DH activity. The manure-amended treatments exhibit an increase in DH activity up to day 14 followed by a decline in activity. It appears DH activity through time is influenced by manure amendment and not the VA/ATZ treatment.

### **3.1.4 Conclusions**

These results indicated that co-application of manure containing VAs and ATZ to agricultural soils will not inhibit ATZ degradation because of the VA's antimicrobial properties. The concentrations of VAs were apparently not high enough to significantly inhibit ATZ degrading microorganisms in soil or manure-amended soil. Overall, the results showed that manure application had a much greater effect on ATZ degradation than the VAs, with manure amendment doubling the degradation rate of ATZ. Given the common use of manure application to corn, this finding indicated that ATZ persistence in soil will increase in manured fields. This will have important implications to ATZ weed control efficacy and its hydrologic transport. However, environmental risks related to ATZ and VAs individually cannot be ignored. Veterinary antibiotics in the environment could impact food webs, water quality, soil microorganisms, and induce antimicrobial resistant bacteria. Transport of ATZ to water resources could have detrimental impacts on wildlife and human health. The behavior of these agrichemicals is likely to change depending on soil, manure, and VA properties. As a result, VAs may influence ATZ degradation under different circumstances. Further research investigating different VA types and concentrations, ATZ adapted soils, and additional manure sources is warranted.

### **3.2 Future Work**

Since only a few studies have investigated the interaction of herbicides and VAs in the environment, and the results of previously published studies do not agree, further research in the area is necessary. Laboratory experiments are a good starting point, but conditions are much more stable in the lab compared to the actual environment. A more realistic interaction between ATZ and VAs could be established by utilizing a field study.



The behavior and degradation of ATZ may also differ based on soil properties, especially between adapted and nonadapted soils. The soil investigated in this study had not received ATZ application for decades, but behaved like an adapted soil. Examining the same chemical interactions within soils that receive annual ATZ applications, have different crop management systems, and different physical properties may yield different results which would be very relevant to agriculture in the U.S. Since the addition of manure drastically changed the composition of metabolites in samples, forthcoming work should include the hydroxylated ATZ metabolites in addition to the chlorinated metabolites so a complete mass balance can be included. Enzyme activity did not prove to be a useful indicator in this study. The enzymes of interest were not specifically sensitive to the VA treatments and the results were not useful in distinguishing treatment differences for VAs or ATZ. The enzyme activity is, however, a good indicator of major changes in soil such as the addition of C, N, and P with the manure amendment. In the future, more sensitive indicators of microbial activity should be utilized to investigate changes resulting from VA and ATZ treatment.

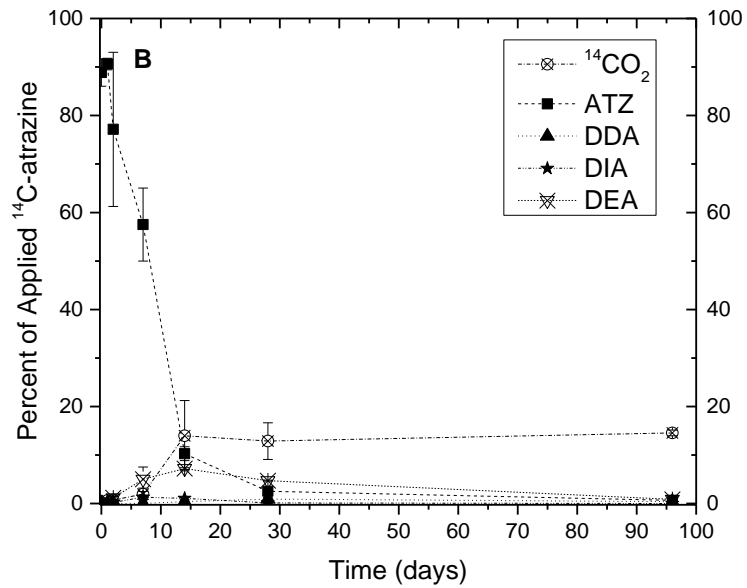
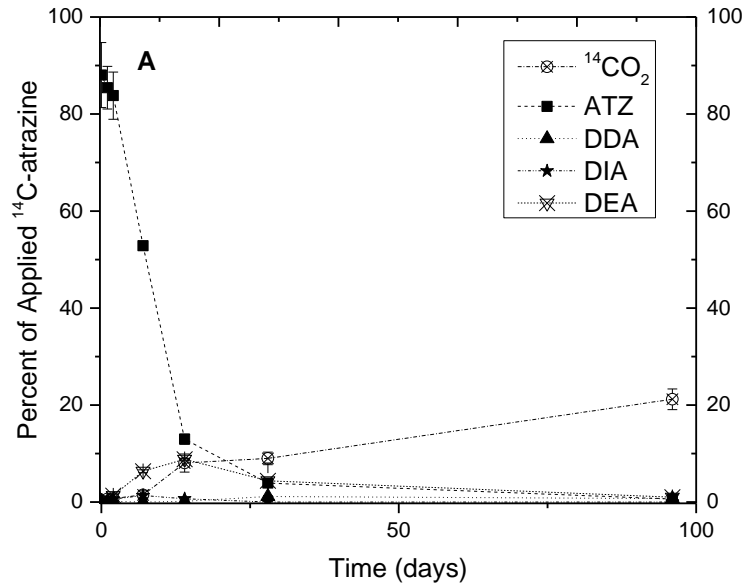
Another aspect that should be examined relating to VA/herbicide interaction is the influence of different manure amendments. Manure from different sources (beef, dairy, swine, poultry, etc.) that has been handled in different manners (composted vs. fresh) could influence the interaction between VAs and herbicides. Nutrient composition, pH, and moisture content of manure is highly variable. These differences could influence VA behavior, microbial activity, and ATZ degradation in soils. In this study ATZ degradation decreased with the addition of manure but several studies have found the opposite to be true. Herbicide bioavailability in soil may vary with different manure amendments

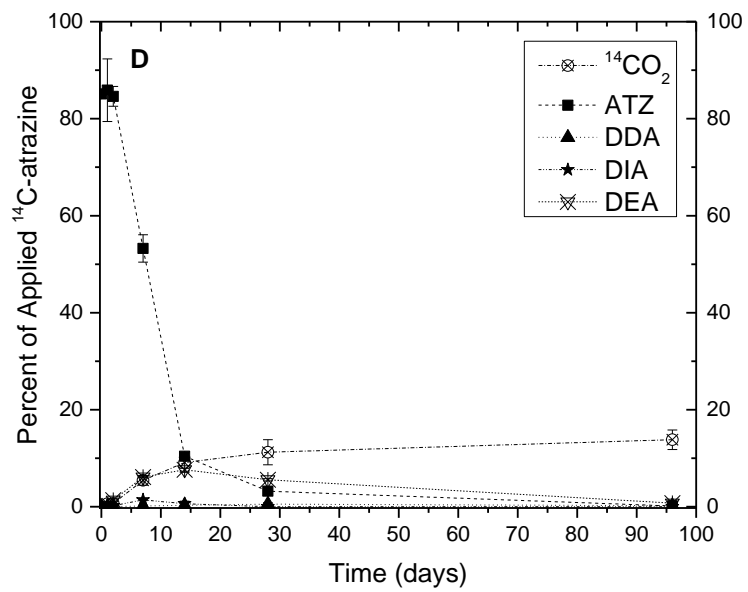
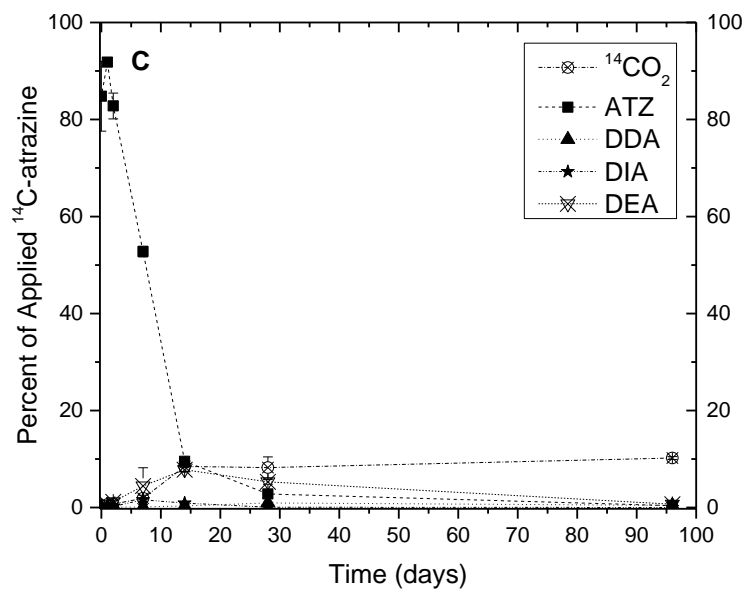
because of changes in sorption potential. For these reasons, future work should elucidate how different manure amendments influence VA/herbicide interaction.

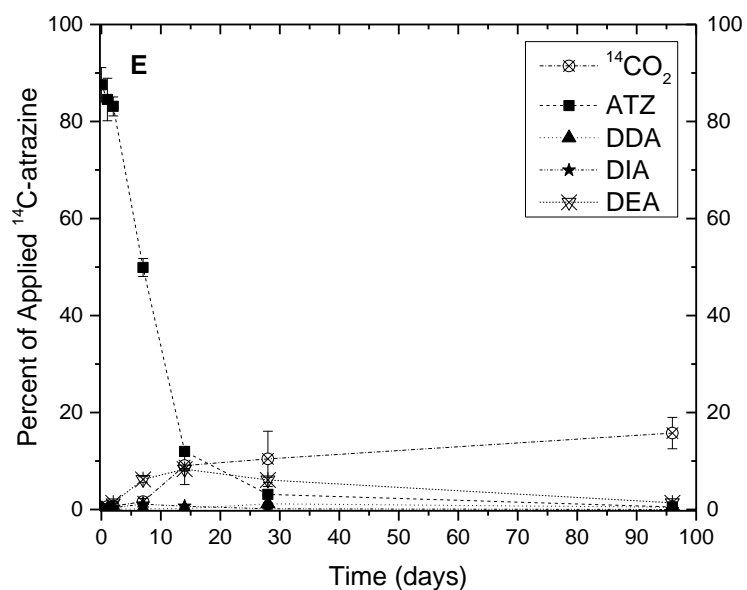
The concentration and class of VAs selected for this study aimed to represent realistic concentrations of two commonly used VAs. However, there are a number of additional components that should be addressed in the future. Due to vast differences in behavior within the soil, additional research involving different classes of VAs at various concentrations is warranted. It is not uncommon for manure to contain more than one VA. As a result, further investigations are necessary to determine whether the input of multiple VAs influence ATZ degradation in manure amended soils. Atrazine was the only herbicide of interest in this study, but future research could evaluate the influence of VAs on different pesticides; just like VAs, individual classes of herbicides may behave differently within the environment.

# APPENDICES

## Appendix 1: Atrazine degradation and metabolite formation through time for the soil incubation study

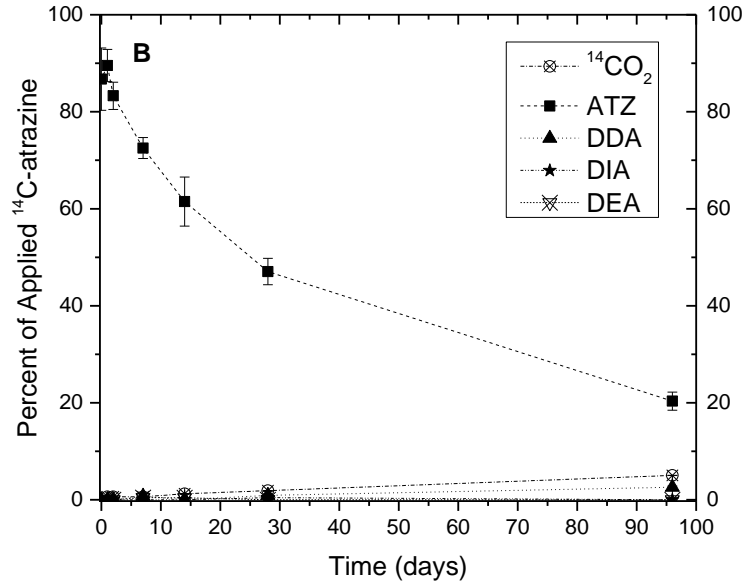
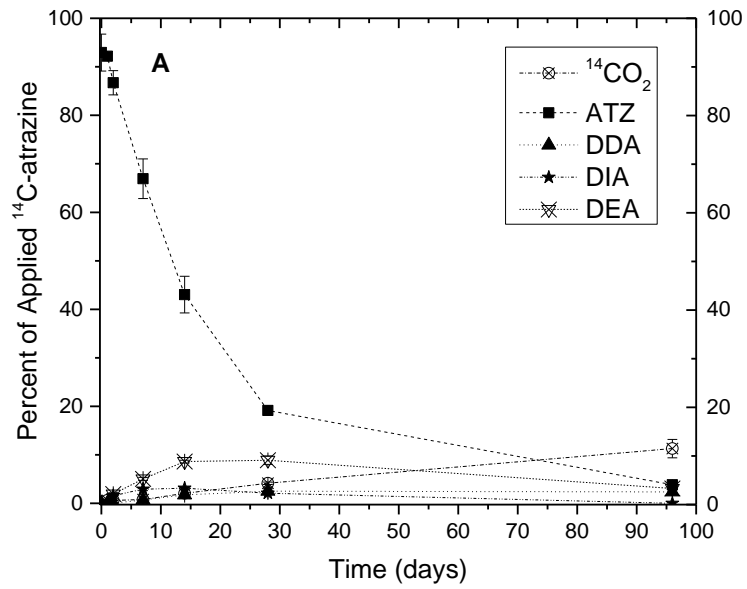


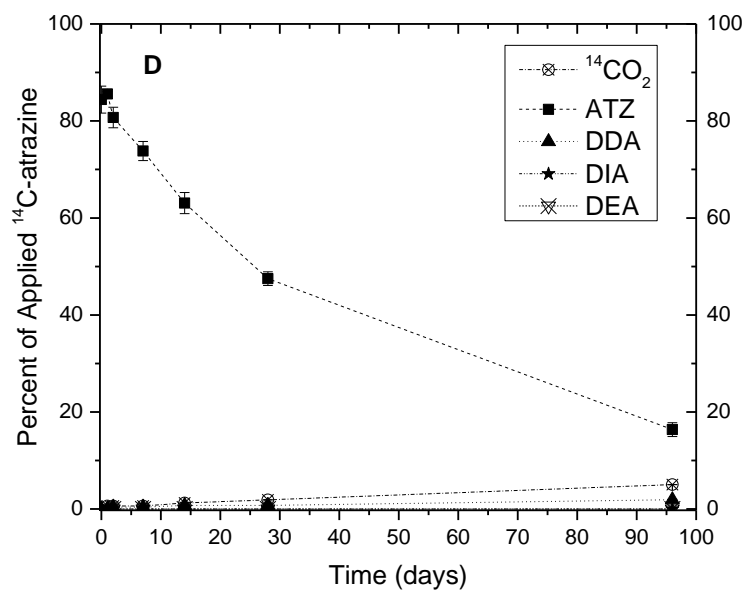
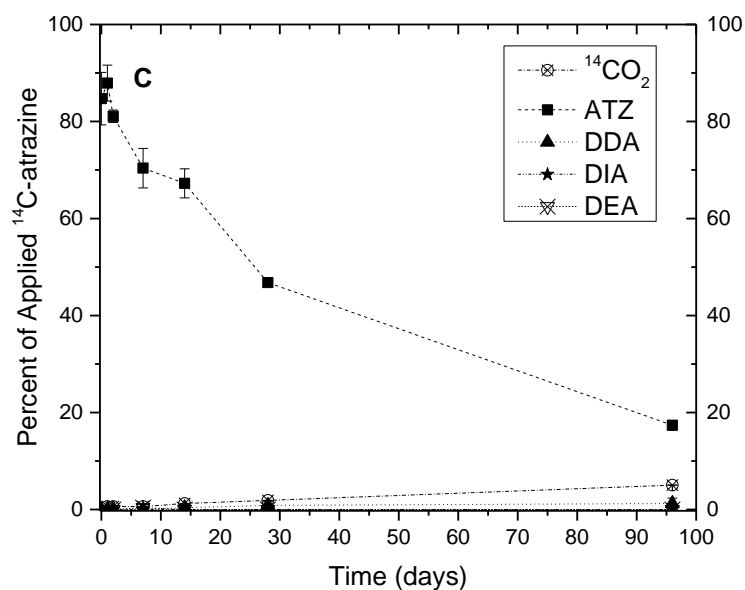




**Figure A.1** Presence of Atrazine (ATZ),  $^{14}\text{CO}_2$ -atrazine, dehyatrazine (DEA) deisopropylatrazine (DIA), and didealkyatrazine (DDA) in soil amended with  $500 \mu\text{g kg}^{-1}$  and (A)  $0 \mu\text{g kg}^{-1}$  VA (B)  $1000 \mu\text{g kg}^{-1}$  sulfamethazine (SMZ) (C)  $100 \mu\text{g kg}^{-1}$  SMZ (D)  $1000 \mu\text{g kg}^{-1}$  oxytetracycline (OTC) (E)  $100 \mu\text{g kg}^{-1}$  OTC during a 96 d incubation period. Error bars represent one standard deviation.

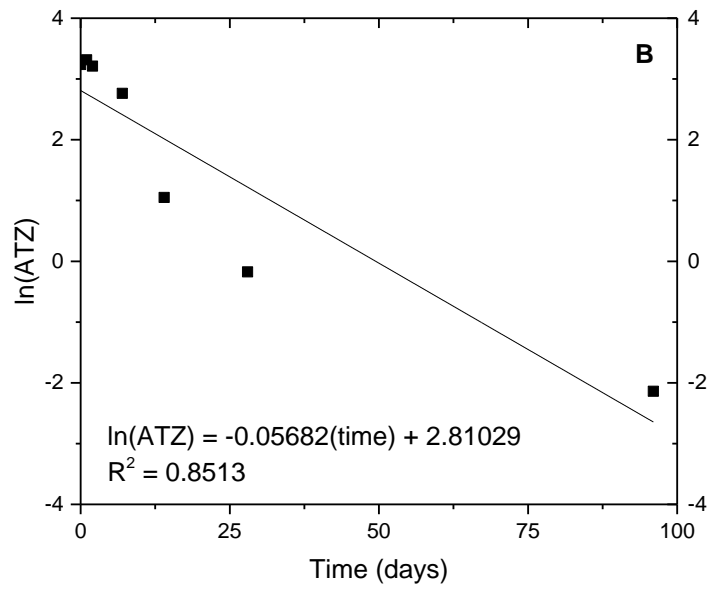
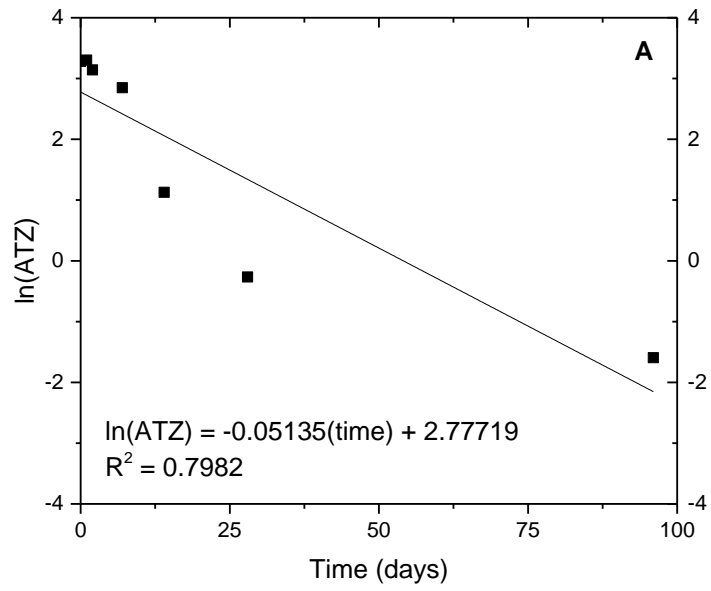
## Appendix 2: Atrazine degradation and metabolite formation through time for the manure-amended soil incubation study



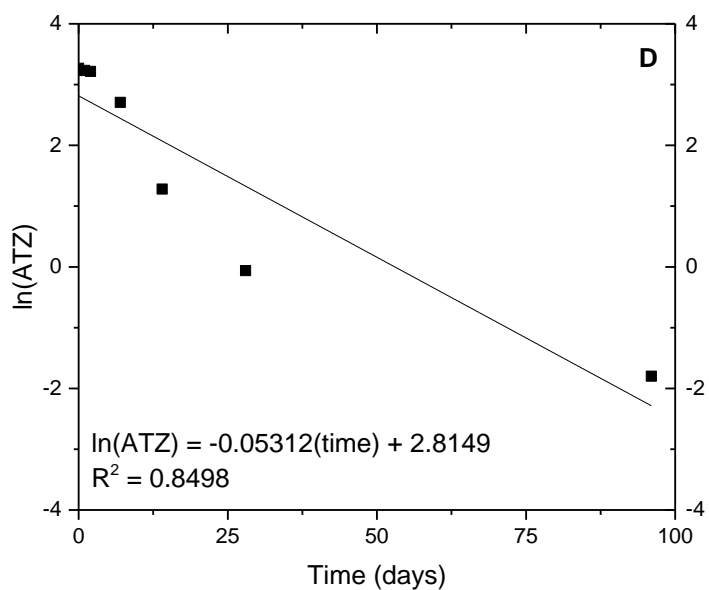
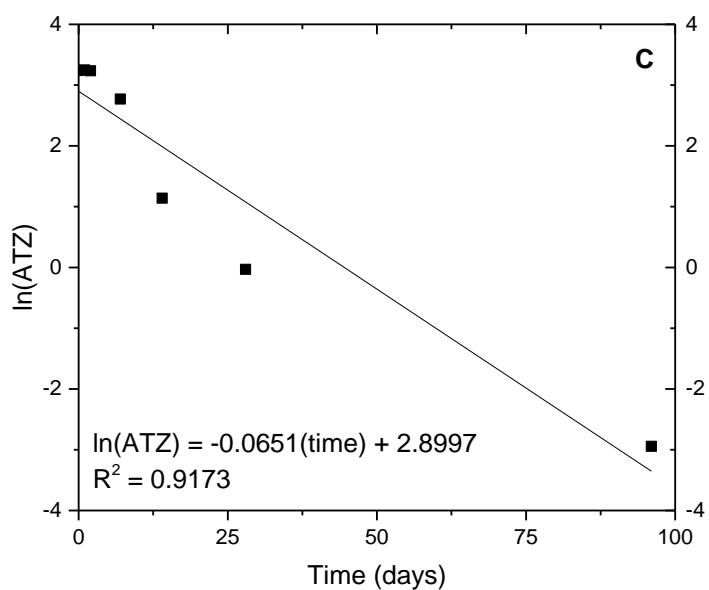


**Figure A.2** Presence of Atrazine (ATZ), <sup>14</sup>CO<sub>2</sub>-atrazine, dehyatrazine (DEA) deisopropylatrazine (DIA), and didealkyatrazine (DDA) in soil amended with (A) 500 μg kg<sup>-1</sup> ATZ (B) 500 μg kg<sup>-1</sup> ATZ and 5% swine manure (C) 500 μg kg<sup>-1</sup> ATZ, 5% swine manure, and 100 μg kg<sup>-1</sup> sulfamethazine (SMZ) (D) 500 μg kg<sup>-1</sup> ATZ, 5% swine manure, and 100 μg kg<sup>-1</sup> oxytetracycline (OTC) during a 96 d incubation period. Error bars represent one standard deviation.

### Appendix 3: Atrazine degradation kinetics for the soil incubation study

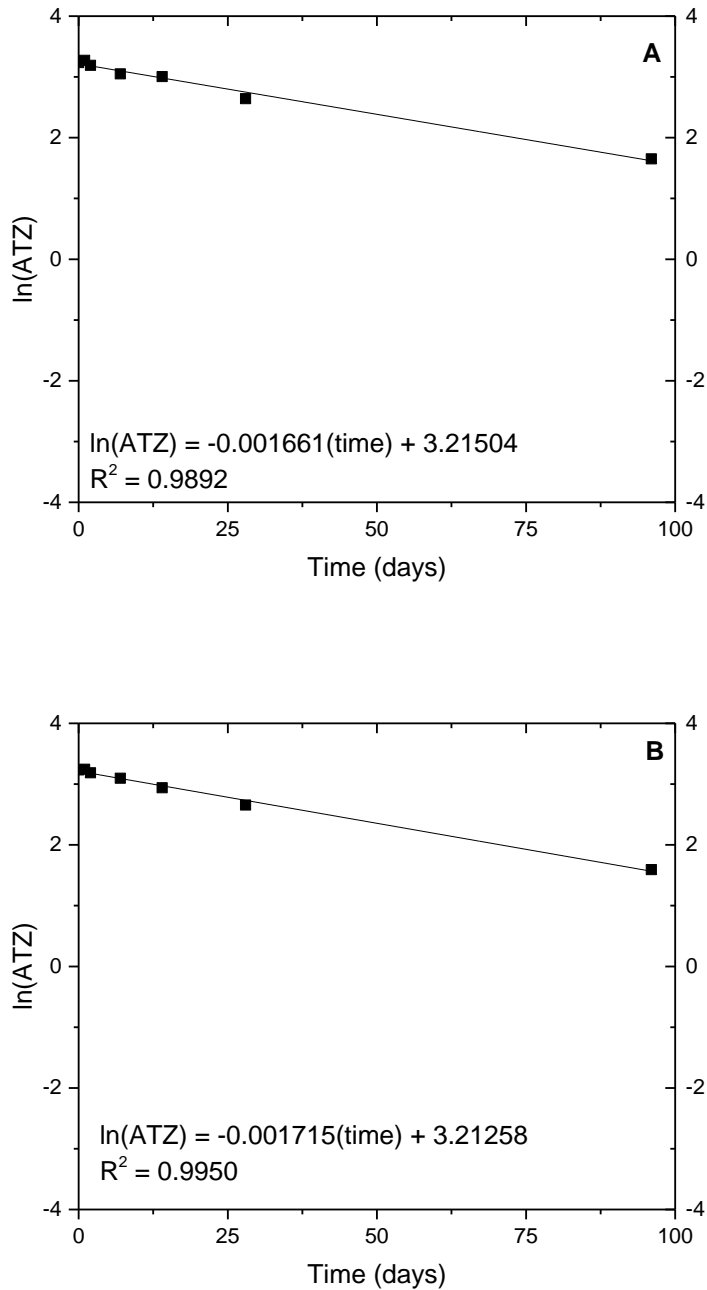






**Figure A.3** Relationship between time (days) and the natural log of atrazine (ATZ) concentration in µg in soil amended with 500 µg kg<sup>-1</sup> ATZ and (A) 1000 µg kg<sup>-1</sup> sulfamethazine (SMZ) (B) 100 µg kg<sup>-1</sup> SMZ (C) 1000 µg kg<sup>-1</sup> oxytetracycline (OTC) (D) 100 µg kg<sup>-1</sup> OTC during a 96 d incubation period.

#### Appendix 4: Atrazine degradation kinetics for the manure-amended soil incubation study



**Figure A.4** Relationship between time (days) and the natural log of atrazine (ATZ) concentration in  $\mu\text{g}$  in soil amended with  $500 \mu\text{g kg}^{-1}$  ATZ, 5% swine manure, and (A)  $100 \mu\text{g kg}^{-1}$  sulfamethazine (SMZ) (B)  $100 \mu\text{g kg}^{-1}$  oxytetracycline (OTC) during a 96 d incubation period.

## Appendix 5: Statistical Analysis Software (SAS) Code

**A.5.1** Code used to conduct an analysis of variance to evaluate treatment and time effects in SAS

```
proc mixed; class trt day;  
model act =trt day trt*day;  
lsmeans trt day trt*day /diff;  
ods output diffs=ppp lsmeans=mmm;  
run;  
%include 'C:\Users\rmnvz2\Desktop\pdmix800.sas';  
%pdmix800(ppp,mmm,alpha=0.05,sort=yes);  
run;
```

**A.5.2** Code used to check normality of residuals in SAS

```
ods graphics on;  
proc mixed; class trt day;  
model act =trt day trt*day/residual;  
lsmeans trt day trt*day;  
run;  
ods graphics off;
```

**A.5.3** Code used to conduct Pearson Correlation

```
proc corr data=one;  
  
**the following names (NaOH, BG, DH etc.) are my variable names;  
**just substitute the names you gave the variables in your sheet;  
**you can have as many or as few as you want;  
var NaOH BG DH;  
run;
```