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Abstract
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Keywords
atrazine, phytoremediation, switchgrass, metabolites

Disciplines
Entomology | Other Ecology and Evolutionary Biology | Plant Biology

Comments
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DISPOSITION OF ATRAZINE METABOLITES FOLLOWING UPTAKE AND DEGRADATION OF ATRAZINE IN SWITCHGRASS

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ABSTRACT

Extensive use of the agricultural herbicide atrazine has led to contamination of numerous ground and surface water bodies. Research has shown that it can have a variety of negative impacts on numerous non-target organisms in the environment. Phytoremediation is one strategy that has been studied to remove atrazine contamination. This paper investigates the hypothesis that switchgrass (*Panicum virgatum*) can exude metabolites of atrazine after uptake and degradation, which has been suggested by prior research. Pots planted with switchgrass were treated with a 4 ppm solution of atrazine spiked with [^{14}C]atrazine. After 4 days, switchgrass plants were transplanted to new pots with fresh sand. Four days later, the pots were sacrificed, and sand and plant samples were extracted. Plant and sand samples were analyzed for the presence of atrazine and its major metabolites. The percentage of radiotracer remaining as the parent atrazine was observed to decrease over the course of the study while the percentages of the metabolites were observed to increase. The presence of the metabolite cyanuric acid in a switchgrass phytoremediation system is reported for the first time.

Keywords – Atrazine Phytoremediation Switchgrass Metabolites

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INTRODUCTION

The triazine herbicide atrazine has been one of the most widely used herbicides in agriculture for control broadleaf weeds, mainly in corn, sorghum, and sugarcane crops by a reversible inhibition of photosynthesis (Kruger *et al*. 1993; Henderson, Belden, and Coats 2007; Shimabukuro and Swanson 1969). In 2010, 51 million pounds of atrazine were applied across 18 states, with 6.8 million pounds applied in Iowa alone (NASS 2011). As a result of widespread usage, atrazine and its metabolites are common contaminants of both ground water and surface water sources. The United States Environmental Protection Agency (USEPA) estimated that atrazine is present in 1,570 community water source wells and 70,800 private wells, but these numbers may be as high as 2,700 and 214,000 wells respectively (USEPA 1990). Battaglin *et al*. (2000) found atrazine was present in 100% of 129 samples from 75 Midwestern rivers and streams in 1998. The median concentration in these samples was 3.97 µg/L and the highest concentration detected was 224 µg/L (Battaglin *et al.* 2000). Thus, more than half of the samples exceeded the maximum contaminant level (MCL) of 3 µg/L for drinking water set by the USEPA (USEPA 2009). Another study found that 22% of atrazine detections were above the MCL in surface waters of Louisiana (Southwick *et al.* 2002).

The presence of atrazine in water has been shown to have many detrimental effects on aquatic organisms. Inhibition of photosynthesis in algae, phytoplankton, and macrophytes as a result of exposure to atrazine has been observed (Graymore, Stagnitti, and Allinson 2001). Numerous detrimental effects have been observed in amphibians including decreases in fat body size and liver weights in *Xenopus laevis* tadpoles, decreased spermatogenesis and fertility in male *X. laevis*, and reduced successful egg hatching in spotted salamander embryos, *Ambystoma maculatum* (Zaya *et al*. 2011; Hayes *et al*. 2010; Olivier and Moon 2010). In humans, atrazine

has been linked to various cancers including breast, brain, ovarian, stomach, and testicular cancer (Patlak 1996; Powell *et al*. 2011).

One major source of atrazine contamination is non-point source runoff from agricultural fields. It has been reported that 2-5% of the atrazine applied to a field may be lost to water runoff; this number may be even higher if a storm event occurs immediately after application (Mersie *et al*. 2006). As a result, several strategies have been developed in an effort to mitigate the amount of atrazine that reaches surface bodies of water. One promising method is phytoremediation, the use of plants to degrade, sequester, or otherwise mitigate organic and inorganic contaminants in soil and water. A wide variety of plants have been shown capable of metabolizing atrazine. Sorghum, soybeans, and pea plants are capable of performing a N-dealkylation on either of the two alkyl groups (Shimbukuro 1967). Maize and wheat can also perform these N-dealkylation reactions in addition to removing the chlorine atom through a hydroxylation reaction (Shimbukuro 1967). Wheat is also capable of detoxifying atrazine by conjugating it with a glutathione molecule (Mauch and Dudler 1993). Poplar trees have been shown to take up and degrade atrazine from contaminated soils through a dealkylation reaction (Burken and Schnoor 1997). Others have hypothesized that some atrazine resistant plants may confer resistance by exclusion of the herbicide from the site of action by a mutation in a protein that can affect binding affinity (De Prado, Lopez-Martinez, and Gonzalez-Gutierrez 2000). Other research has considered the use of several native prairie grasses in phytoremediation strategies as they have an extensive fibrous root system that produces a root surface area greater than any other vegetation and can penetrate as much as ten feet below the surface (Aprill and Sims 1990). Belden and Coats (2004) found that a mix of three different prairie grasses reduced atrazine in leachate by 43% and that it was degraded more quickly in vegetated soils than unvegetated soil.

Disposition of atrazine metabolites in switchgrass

Leaf and root tissue have been shown to be equally capable of taking up and degrading atrazine (Henderson *et al*. 2007).

The purpose of the current research was to examine movement of atrazine and its metabolites in a vegetative system. Previous research by Murphy and Coats (2011) indicated that switchgrass may exude or diffuse some of the chlorinated metabolites of atrazine into the soil after degradation. To test this hypothesis, radiolabeled [^{14}C]atrazine was utilized to track uptake and degradation of atrazine and possible exudation of the metabolites deethylatrazine (DEA), deisopropylatrazine (DIA) and didealkylatrazine (DDA) into virgin soil (Figure 1).

METHODS AND MATERIALS

Experimental setup

Approximately 22.7 kg of commercial sand (Lowe's) was obtained, was sifted (sieve size: 0.841 mm) to remove particulate matter, and was washed with water to remove dust (pH 8.15, 0.3% organic matter; soil composition: sand 98.27%, clay 1.65%, silt 0.08%). The sand was allowed to dry in a greenhouse (16 h light: 8 h dark schedule at 27°C/day and 22°C/night) for five days. Seven days prior to the start of the study, 18 pots (8.5 cm x 8.5 cm x 10 cm), each with a hollow propylene tube (9.5 cm x 2.7 cm) placed in the center, were filled with 400 g of sand. All 18 pots were autoclaved for one hour at 121°C once a day for three consecutive days.

Switchgrass preparation

Switchgrass plants (*Panicum virgatum*, Cave-in-rock variety) utilized in this experiment were planted in Sunshine® Professional Growing Mix potting soil (Sun Gro Horticulture, Bellevue, WA, USA) in March of 2010 and grown throughout 2010 in a greenhouse on a 16 h light: 8 h dark schedule at 27°C/day and 22°C/night. In the fall of 2010, the greenhouse temperature was slowly lowered to 4.5°C day and night over eight weeks at a rate of 2.8°C per

week. At the end of the ramp down, the day: night cycle was changed to 12 h light: 12 h dark. Switchgrass plants were allowed to senesce over winter. The temperature was slowly ramped back up and the day: night cycle changed back to 16 h light: 8 h dark in the spring of 2011, and the plants were allowed to grow for four months before being utilized in this study. Plants were watered 3 times a week during the growing season and once every two weeks during the winter.

Experimental conditions and time frame

Four days prior to the start of the study (Day -4), switchgrass plants were removed from their growing medium, and their roots were stored in Hoagland's solution for 30 minutes (Hoagland and Arnon 1938). The plants then had their roots dipped in 10% hydrogen peroxide for 10 seconds to minimize microbial growth on the roots and then placed in each of the 18 pots with one plant per pot. After a four-day acclimation period, four pots were sacrificed, and the sand as well as the plant material (including roots) was extracted to show that no background radioactivity was present. The remaining 14 pots were treated with 50 mL of a 4 μg/ml (parts per million) solution of atrazine spiked with approximately 3 x 10^6 disintegrations per minute (dpm) of [^{14}C]atrazine (specific activity = 28.9 μCi/mg) that was applied to the surface of each pot. This represents Day 0 of the study.

One day after the start of the study, an additional 10 sand pots were prepared as previously described and autoclaved for one hour at 121°C once a day for three consecutive days. Four days after the start of the study (Day 4), four pots were sacrificed, and the sand, as well as the plant material, including roots, was extracted to show how much atrazine has been taken up into the plant. The 10 remaining pots had their plants and roots extracted and placed in a Hoagland's solution to rinse off any adhered sand. The switchgrass plants then had their roots dipped in 10 % hydrogen peroxide and then were placed in each of the 10 recently autoclaved

pots. Four days after transplantation (Day 8), the switchgrass was removed from the pots. Sand from each of the pots was extracted and analyzed for the presence of atrazine and/or metabolites. Each pot was watered with 50 mL autoclaved Hoagland's solution on Days -4, -2, 2, 4, and 6. All treatment groups were kept in a greenhouse for a total of 12 days on a 16h light: 8h dark schedule at 27°C/day and 22°C/night.

Extraction of sand

Sand was homogenized by placing it in a 470 ml glass Mason jar and shaken for 5 minutes. Twenty grams of sand were weighed and placed in a 200 ml glass French square bottle with 40 mL of ethyl acetate and mechanically shaken horizontally at 300 rpm's for 20 minutes. The ethyl acetate was decanted off into a paper filter with 15 g of anhydrous granular sodium sulfate. This procedure was repeated for a total of three times for each sand sample.

Extraction of switchgrass material

Roots were teased out of the sand. Roots of each sample of switchgrass were rinsed with water to remove any adhered sand. Entire plants were weighed and then cut into pieces that were approximately one-half inch in length. Each sample was then homogenized with a mortar and pestle using 30 mL of ethyl acetate. The solvent was decanted off through a filter containing 15 g of anhydrous granular sodium sulfate to absorb any water contained in the sample. This procedure was repeated for a total of three times for each plant sample.

Concentration of switchgrass and sand samples

All solvent extractions were placed in a N-evaporator. Samples were dried down with nitrogen gas to approximately 1 mL. The extracts were quantitatively transferred into a syringe with a 0.45-µm micropore filter attached. The tube originally containing the solvent extract was subsequently rinsed with ethyl acetate, and the rinse was also placed into the syringe. The extract

was passed through the filter into a vial to a total volume of approximately 10 mL. The vials were then blown down with a N-evaporator and reconstituted with methanol to 1ml and placed in an HPLC vial. No futher clean-up of the plant samples was needed as the plant pigments did not interfere with the detection of the radioactivity in the samples. Recovery efficiencies for the sand extraction method and the switchgrass extraction method were determined to be 99% and 97.5% respectively.

Analysis of samples

A 100-µl sample of both plant and sand extracts were analyzed using high-performance liquid chromatography (HPLC). A Hewlett-Packard 1100 series HPLC equipped with an autosampler was used to separate the metabolites and parent compound. An Atlantis dC18 5µm, 4.6x250 mm column (Waters Corporation, Milford, MA, USA) was used with a mobile phase as described in Table 1. Flow rate was 1 ml/min; column temperature was maintained at 30°C. A Model 2B Beta-Ram detector with Laura Lite 3 radiochromatography software (IN/US Systems, Inc., Tampa, FL, USA) connected to the HPLC was used in identification and quantification of the metabolites (Figure 2). After entering the Beta-Ram detector, the elutant from the HPLC was mixed with Flow Logic ES liquid scintillation cocktail (LabLogic, Brandon, FL, USA) at a ratio of 3:1. Non-radioactive analytical standards and radioactive standards were used to determine retention times of the compounds on the HPLC and the Beta-Ram. All radioactive standards and non-radioactive atrazine, deethylatrazine, deisopropylatrazine, and didealkylatrazine standards were obtained from CIBA-GEIGY (Syngenta), Greensboro, North Carolina, USA. Unlabeled hydroxyatrazine was obtained from Sigma-Aldrich (St. Louis, MO, USA), and unlabeled cyanuric acid was obtained from Fluka (St. Louis, MO, USA).

Statistical analysis

A T-test ($p = 0.05$) was used to test the null hypothesis that switchgrass does not detoxify atrazine into the metabolites DEA, DIA, DDA, and cyanuric acid by comparing their concentrations across days. Due to the unequal variance between days for DIA, unequal variance was assumed for all tests. Only the mean and standard error of the mean were calculated for the sand samples. No comparisons were made between Day 4 and Day 8 sand samples. Statistical calculations were performed using SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

Sand Samples

No radioactivity was detected in any of the Day 0 sand samples (data not shown). After four days, approximately 73% of the radioactivity extracted from the sand samples remained in the form of the parent compound atrazine (Table 2). Deethylatrazine (DEA) accounted for approximately 19% of the extractable radioactivity, and deisopropylatrazine (DIA) accounted for approximately 8%. No radioactivity was detected in the Day 8 sand samples.

Plant Samples

Plant material was extracted after it was determined there was no radioactivity in the sand samples. No radioactivity was detected in any of the Day 0 plant samples. In the Day 4 samples, atrazine comprised 43.7% of the extractable radioactivity and was present at significantly higher concentrations than the four metabolites (Table 3). The metabolites DEA, DIA, DDA, and cyanuric acid comprised 13.6%, 13.9%, 20.0%, and 8.8% of the extractable radioactivity, respectively. In the Day 8 plant samples, 52.8% of the extractable radioactivity that remained was in the form of didealkylatrazine (DDA). Cyanuric acid, atrazine, and DEA accounted for

22.7%, 16.5%, and 8.0% of the extractable radioactivity, respectively. DIA was not detected in the Day 8 plant samples.

DISCUSSION

The main objective of this research was to study the potential of atrazine metabolites to diffuse or be exuded from roots after uptake and degradation of atrazine by switchgrass. This was suggested in preliminary research by Murphy and Coats (2011). In that study, the authors compared degradation of atrazine in pots of autoclaved sand without switchgrass against degradation of atrazine in pots of autoclaved sand planted with switchgrass. Atrazine, DIA, and DDA were found in both sand samples, while DEA was found only in pots planted with switchgrass. This led the authors to propose two possible scenarios: (1) plant enzymes were released into the sand and removed the ethyl group of atrazine or (2) atrazine was taken up by the plant, degraded to DEA, and then released back into the sand.

The current study shows that no metabolites were detected in the second set of sand samples four days after being planted with switchgrass that were exposed to atrazine. In the original extractions, only 20 grams of sand was extracted. After running the samples on the HPLC and observing no metabolites, the decision was made to extract more sand in an effort to concentrate any metabolites that may be present. The remaining sand in three pots was split into two portions and extracted by mechanical shaker three times with 300 ml of ethyl acetate for 30 min at 300 rpm; sand weights in these extractions ranged from 186 g to 190 g. The new samples were then analyzed on the HPLC, and no metabolites of atrazine were detected. This leads us to conclude that after uptake and degradation of atrazine by switchgrass plants, no metabolites were diffused or exuded.

Disposition of atrazine metabolites in switchgrass

The use of sterile sand as the growth medium allowed us to definitively separate detoxification occurring in the plant from detoxification occuring in the sand in the rhizosphere of switchgrass plants. This method did not account for degradation due to endophytes within the plant tissues. Exudation of metabolites after degradation was the primary focus and no effort was made to identify degradation due to plant processes from degradation due to endophytes. The use of radiolabeled [^{14}C]atrazine in this study also allowed better identification and tracking of atrazine metabolites and degradation pathways. It also allowed the detection and tracking of specific atrazine metabolites, such as hydroxyatrazine and cyanuric acid, which were not detectable with gas chromatography methods. Uptake and rapid degradation of atrazine was observed as evidenced by the large decrease in atrazine from the Day 4 to Day 8 plant samples, from 516 parts per billion (ppb) to 93.2 ppb (Table 3). The decreasing concentrations of the metabolites DEA and DIA and the increasing concentrations of DDA and cyanuric acid from Day 4 to Day indicate that the plant continues to degrade atrazine and its metabolites over time (Table 3). The total concentration of atrazine and metabolites also decreased from Day 4 to Day 8. (1194 ppb to 564 ppb), suggesting that the plant continues to degrade atrazine beyond the metabolite cyanuric acid. These results are consistent with a mass balance study by Henderson *et al.* (2007). In that study, the authors also used 14C atrazine to track degradation and observed production of [^{14}C]CO_2, signifying complete mineralization of atrazine. While it is unclear if the mineralization is the result of plant degradation or soil microbial degradation, the research presented here is illustrates that the former is at least plausible due to the overall decrease in atrazine and metabolite concentrations.

The present study found no hydroxyatrazine present in either the sand or the plant tissues. This is in contrast to Lin *et al.* (2008) who detected hydroxyatrazine present in switchgrass

Disposition of atrazine metabolites in switchgrass tissues. The major difference between that study and the present study was the growing media utilized in the research. In Lin *et al.* (2008), the switchgrass plants were grown in sandy loam soil, and no attempt was made to sterilize or otherwise reduce the microbe population present in the soil. In the present study, sand was autoclaved for 1 hour at 121°C on three consecutive days, which has been shown to significantly reduce the number of microbes present (Murphy and Coats 2011). Therefore, it is possible that the presence of hydroxyatrazine observed by Lin *et al.* (2008) in switchgrass tissues was a result of atrazine degradation to hydroxyatrazine by microbes in the rhizosphere followed by uptake by the switchgrass plants.

The presence of ammeline (Figure 1) also was not detected in switchgrass tissues. Although ammeline could be separated out from the other metabolites during method development, it did not appear in any of the chromatographs. Thus, it is possible that once ammeline is formed, it is rapidly converted to cyanuric acid. Detecting the presence of the metabolites deethylhydroxyatrazine (DEHA) and deisopropylhydroxyatrazine (DIHA) (Figure 1) was not focus of this study. However, it is possible small amounts of these metabolites were formed through dechlorination of DEA and DIA respectively. Since no unidentified peaks were observed in the analysis, the contributions from these metabolites is either very small or they were no capable of being sufficiently separated from the DEA and DIA peaks with the solvent system that was utilized.

The current study also reports the presence of cyanuric acid in switchgrass tissues. This compound was previously undetectable in using gas chromatography methods. The use of radiolabeled [^{14}C]atrazine allowed cyanuric acid to be detected and quantified, since the ring structure, which contains the radiolabel, was not compromised in the degradation process. Cyanuric acid made up 9% of the extractable radioactivity in the Day 4 samples. This was higher

in the Day 8 samples where cyanuric acid accounted for 23% of the extractable radioactivity. To our knowledge, this is the first time this compound has been reported to be detected in switchgrass tissues.

Formation and detection of the metabolites of atrazine are important, as they are generally considered to be less toxic than the parent compound. DEA and DIA have been shown to be significantly less toxic than atrazine to some amphipods and algae, as well as to some bacteria and plants (Ralston-Hooper *et al.* 2009; Kross, Vergara, and Raue 1992; Winklemann and Klaine 1991). However, the chlorinated metabolites have still been included in regulatory limits for atrazine in drinking water in the past. Hydroxylated metabolites, such as hydroxyatrazine and cyanuric acid, may be even less toxic as they do not contain the chlorine atom. In fact, hydroxyatrazine has been shown to be nontoxic to green algae and cyanobacteria that have their growth and photosynthetic processes inhibited by atrazine (Stratton 1984). Thus, by degrading atrazine to its metabolites, the risks to organisms in aquatic ecosystems can be reduced.

The final objective of this course of research will be to integrate switchgrass, alone or in combination with other native prairie grasses, into buffer strips between agricultural fields and waterways, and on terraces around existing tile line intakes. The findings reported here are very significant in advancing knowledge toward that goal. By showing that metabolites of atrazine are not exuded, we show that atrazine can safely remove atrazine and its metabolites from surface water runoff. The metabolites DEA and DIA have been shown to be mildy toxic to plants, but much less so than atrazine (Winklemann and Klaine 1991). Exudation of atrazine metabolites did not occur, and these compounds remained in the plant, where they were further detoxified to cyanuric acid, which is of minimal concern in the environment.

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TABLES

Table 1. High-performance liquid chromatography (HPLC) conditions for compound separation and determination.

Compound	Retention Time	Column Conditions
Blank		Isocratic 75:25 water:acetonitrile (0-3 min)
Cyanuric acid	6.6	Linear gradient from 75:25 water:acetonitrile to
DDA	7.7	25:75 water:acetonitrile (3-11 min)
DIA	8.4	
DEA	9.9	
Hydroxyatrazine	11.5	Linear gradient from 25:75 water:acetonitrile to
Atrazine	12.6	75:25 water:acetonitrile (11-16 min)
Blank		Isocratic 75:25 water:acetonitrile (16-20 min)

Table 2. Percentages and concentrations of atrazine and metabolite radioactivity extracted from sand samples.

Day 4 Sand Compound	% of extractable radioactivity	SEM[1]	Concentration (ppb)
Atrazine	73.23	± 1.10	65.9
Deethylatrazine	18.51	± 0.99	16.7
Deisopropylatrazine	8.26	± 0.62	7.44

[1]Standard error of the mean

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Table 3. Percentages and concentrations of atrazine and metabolite radioactivity extracted from plant samples.

	Day 4 Plant			Day 8 Plant			
Compound	% of extractable radioactivity	SEM[1]	Concentration (ppb)	% of extractable radioactivity	SEM[1]	Concentration (ppb)	P-value[2]
Atrazine	43.71[A3]	± 4.46	516	16.47[B]	± 1.73	93.2	0.0053
Deethylatrazine	13.57[A]	± 4.57	170	8.03[A]	± 1.12	45.6	0.3160
Deisopropylatrazine	13.85[A]	± 2.53	172	0.00[B]	± -	0.00	0.0120
Didealkylatrazine	20.03[A]	± 4.99	233	52.77[B]	± 1.91	297	0.0042
Cyanuric Acid	8.84[A]	± 4.05	103	22.72[B]	± 0.87	128	0.0388

[1]Standard error of the mean.

[2]Significance at p = 0.05.

[3] Letters indicate significance within compound across days.

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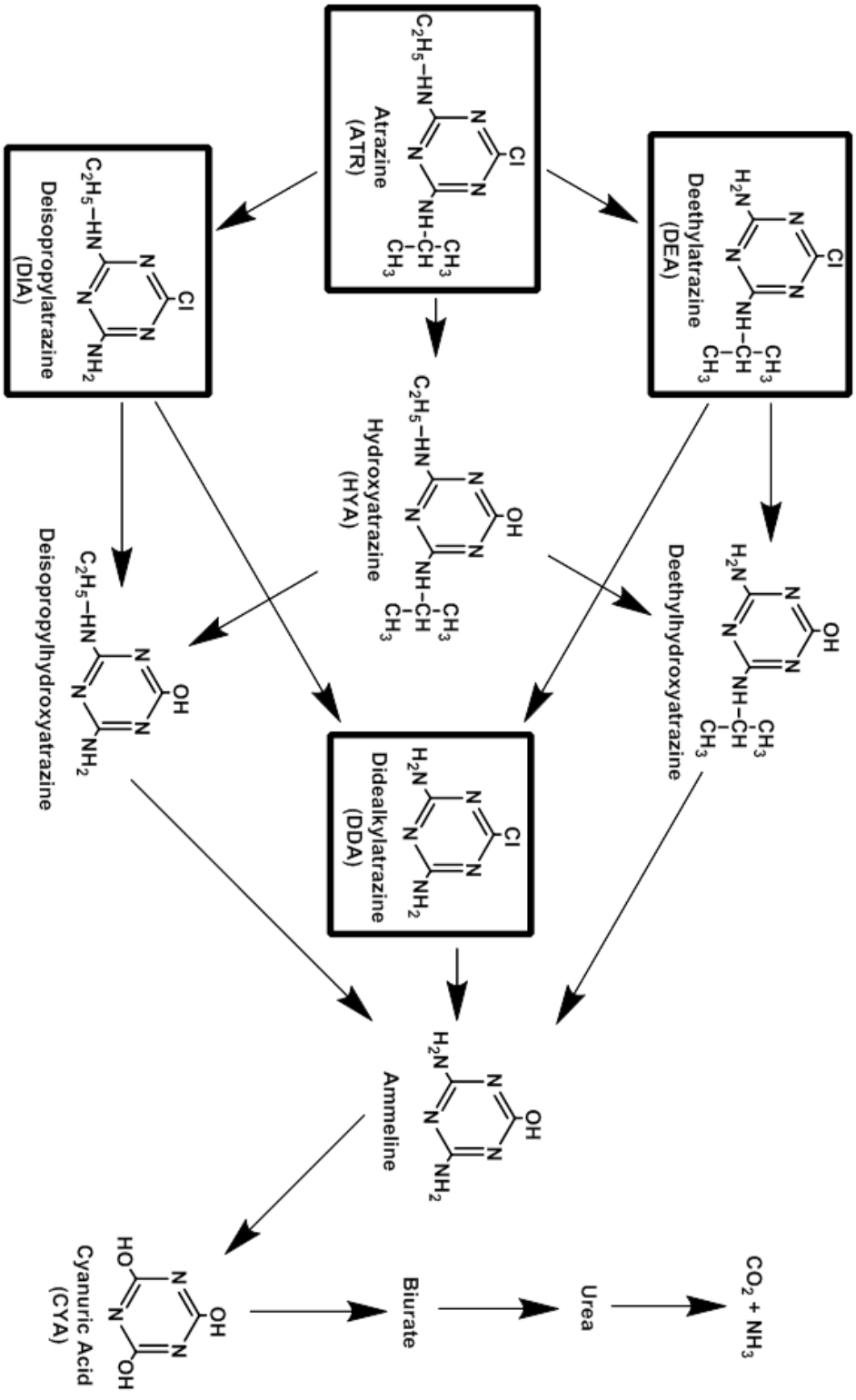
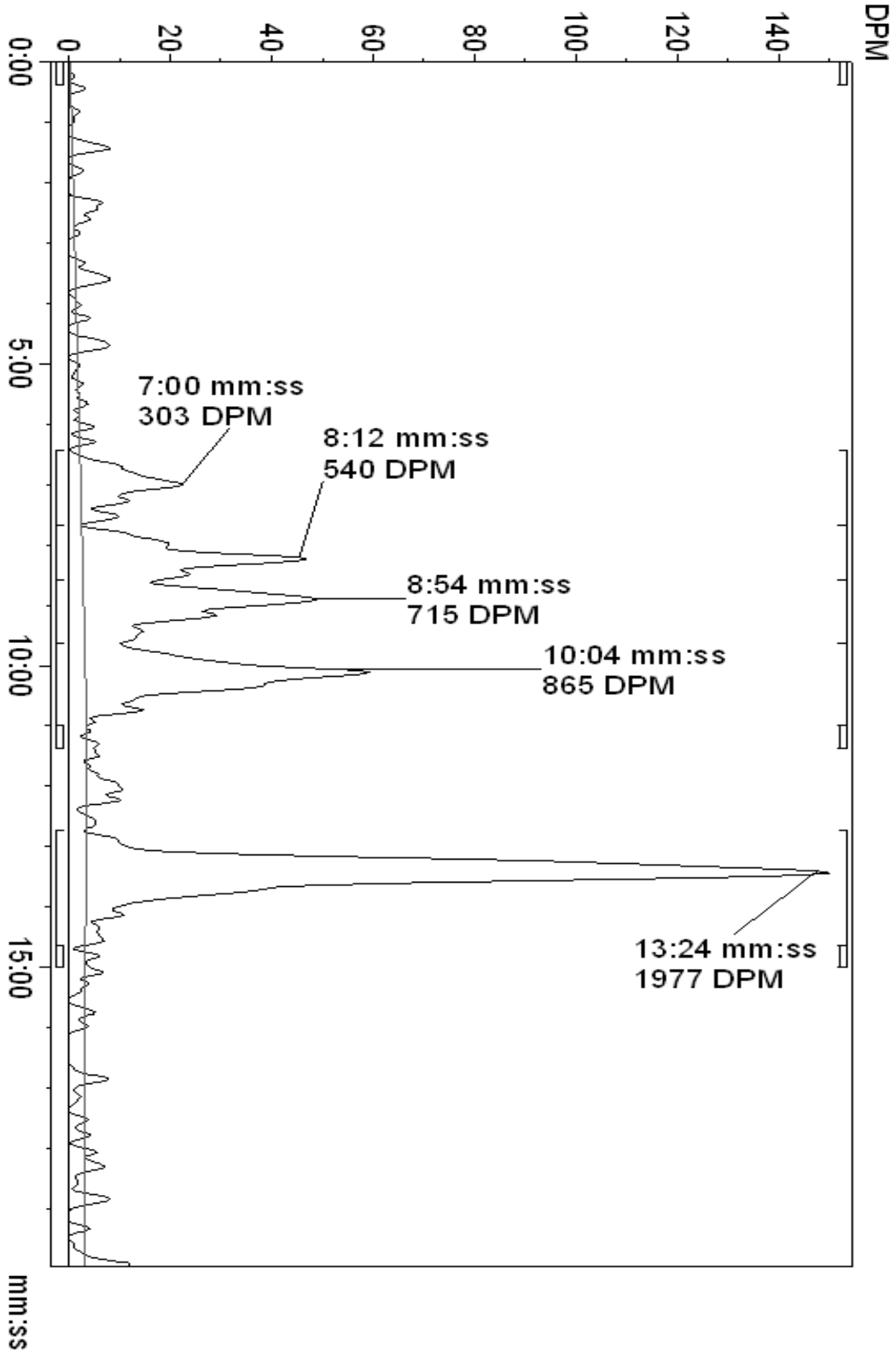
FIGURES



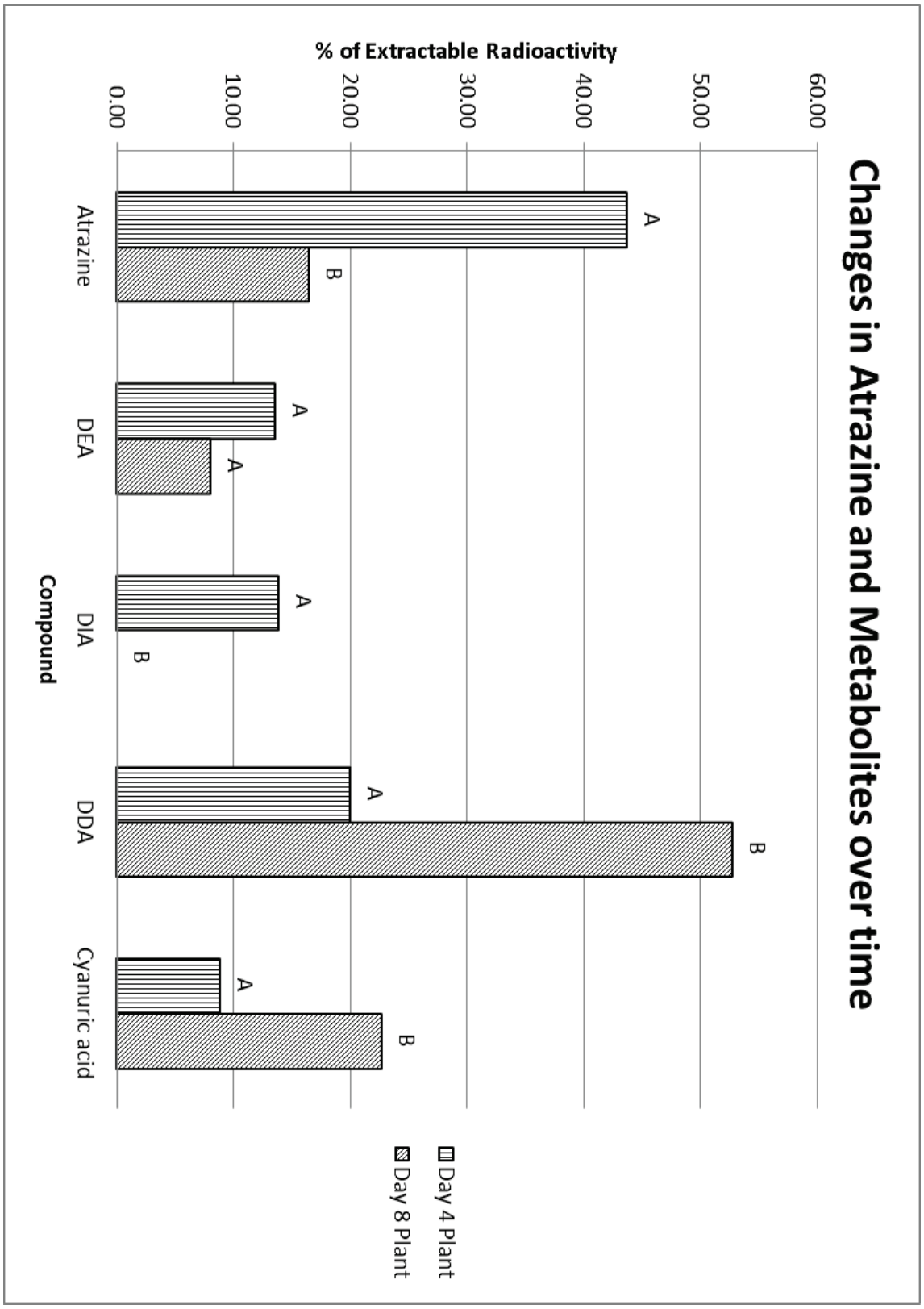
Albright Figure 1.

Disposition of atrazine metabolites in switchgrass



Albright Figure 2.

Disposition of atrazine metabolites in switchgrass



Albright Figure 3.

FIGURE LEGENDS

Albright Figure 1. Atrazine pathway of degradation. Chlorinated metabolites are highlighted. Adapted from Kruger *et al.* 1993.

Albright Figure 2. Representative chromatograph from the Beta-Ram. Peaks for atrazine and metabolites are: Cyanuric acid – 7:00 min; DDA – 8:12 min; DIA – 8:45 min; DEA - 10:04; atrazine 13:24 min.

Albright Figure 3. Changes in the amount of atrazine and metabolites extracted over time. Letters indicate significance within compound across days at $p = 0.05$.