Bioremediation of Atrazine-Contaminated Soil by Forage Grasses: Transformation, Uptake, and Detoxification

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A sound multi-species vegetation buffer design should incorporate the species that facilitate rapid degradation and sequestration of deposited herbicides in the buffer. A field lysimeter study with six different ground covers (bare ground, orchardgrass, tall fescue, timothy, smooth bromegrass, and switchgrass) was established to assess the bioremediation capacity of five forage species to enhance atrazine (ATR) dissipation in the environment via plant uptake and degradation and detoxification in the rhizosphere. Results suggested that the majority of the applied ATR remained in the soil and only a relatively small fraction of herbicide leached to leachates (<15%) or was taken up by plants (<4%). Biological degradation or chemical hydroxylation of soil ATR was enhanced by 20 to 45% in forage treatment compared with the control. Of the ATR residues remaining in soil, switchgrass degraded more than 80% to less toxic metabolites, with 47% of these residues converted to the less mobile hydroxylated metabolites 25 d after application. The strong correlation between the degradation of *N*-dealkylated ATR metabolites and the increased microbial biomass carbon in forage treatments suggested that enhanced biological degradation in the rhizosphere was facilitated by the forages. Hydroxylated ATR degradation products were the predominant ATR metabolites in the tissues of switchgrass and tall fescue. In contrast, the ***N***-dealkylated metabolites were the major degradation products found in the other cool-season species. The difference in metabolite patterns between the warm- and cool-season species demonstrated their contrasting detoxification mechanisms, which also related to their tolerance to ATR exposure. Based on this study, switchgrass is recommended for use in riparian buffers designed to reduce ATR toxicity and mobility due to its high tolerance and strong degradation capacity.

Atrazine (ATR) has been used in corn production for more than 30 yr, and an estimated 36.3 million kg of ATR was applied annually to more than 69% of all USA corn acreage (USDA, 2004). Recently, public health and ecological concerns have been raised about contamination of surface- and ground water by ATR and its chlorinated metabolites (Hayes et al., 2002; Swan et al., 2003).

Phytoremediation is an innovative technology that uses the natural properties of plants in engineered systems to mitigate chemical pollutants (Burken and Schnoor, 1997). Among several applications, the implementation of multispecies vegetative buffers has been proven to be one of the most cost-effective mitigation practices to intercept ATR in surface runoff. In a watershed study conducted in central Texas, Hoffman et al. (1995) observed a 44 to 50% reduction in ATR concentrations when a 9-m filter strip was used. Enhanced infiltration rates within vegetative buffers were identified as the major physical mitigation process by which herbicides transported in surface runoff were intercepted (Misra et al., 1996).

After the physical trapping process, several physiochemical and biochemical mechanisms may be involved in the vegetative buffer to degrade and take up the ATR and its metabolites deposited in the rhizosphere. The degradation of ATR can be a physicochemical or a biochemical process, and more than 15 metabolites have been identified (Jensen, 1982). The major ATR degradation products include *N*-dealkylated metabolites deethylatrazine (DEA) and deisopropylatrazine (DIA) and the hydroxylated metabolites hydroxyatrazine (HA), deethylhydroxyatrazine (DEHA), and deisopropylhydroxyatrazine (DIHA) (Fig. 1). Increased microbial activity and the release of exudates in the root zone have been shown to enhance the degradation of many triazine herbicides through various biochemical mechanisms including enzymatic oxidation, dealkylation, and hydrolysis (Mandelbaum et al., 1993; Wenger et al., 2005). *N*-dealkylation, hydrolysis, and dechlorination are the predominant pathways for the microbial degradation of chloro-s-triazines, methoxy-s-triazines, and methylthio-triazines in soil and under pure culture conditions (Skipper et al., 1967; Struthers et al., 1998). The major chlorinated biological

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Abbreviations: ATR, atrazine; DEA, deethylatrazine; DIA, deisopropylatrazine; HA, hydroxyatrazine; DEHA, deethylhydroxyatrazine; DIHA, deisopropylhydroxyatrazine; HADPs, hydroxylated ATR degradation products; LOD, limit of detection; SPE, solid-phase extraction; TTR, total transpiration rate.

degradation products of ATR metabolism, DEA and DIA, are produced by *N*-dealkylation of the ethyl or isopropyl side chains of the parent molecule (Mandelbaum et al., 1993; Mougin et al., 1994). Dechlorination of ATR, DEA, and DIA during the early stages of ATR metabolism can be catalyzed by ATR-chlorohydrolase produced by bacteria *Pseudomonas sp* strain ADP and *Rhodococcus corallinus* (Mulbry, 1994; De Souza Mervyn et al., 1995). The action of this class of enzyme results in the formation of hydroxylated ATR metabolites, including HA, DEHA, and DIHA (Mulbry, 1994; Sadowsky et al., 1998). The positive correlation between microbial population and rate of herbicide degradation has been established in several previous studies. For instance, Struthers et al. (1998) reported that the rate of ATR mineralization and degradation coincided with increased microbial population. However, this correlation is not consistently observed. In a ^{14}C-labeled ATR incubation study, Ghani et al. (1996) found that ATR mineralization was largely independent of the microbial biomass. The mineralization of ATR and its metabolites can be significantly enhanced by high plant residue and organic carbon in the soil (Holford et al., 1989; Horswell et al., 1997). The rich humic acid in riparian buffer soils also has been shown to enhance the process of HA formation through chemical hydrolysis, resulting in a less toxic and mobile metabolite than ATR (Martin-Neto et al., 1994).

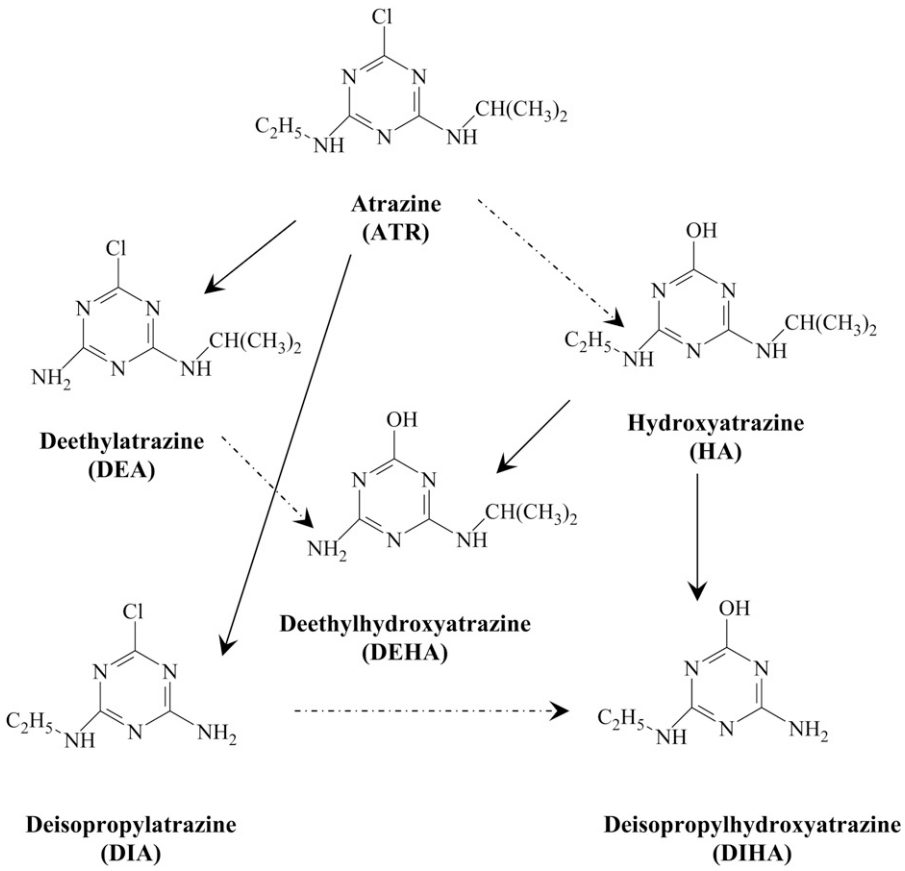

Fig. 1. Degradation pathways of atrazine.

Numerous radiolabeled studies have shown rapid ATR uptake and detoxification by a wide range of woody and grass species (Burnet et al., 1993; Burken and Schnoor, 1996; Schroll and Sabine, 2004). In these studies, high transpiration rates were shown to strongly correlate with ATR accumulation (Burken and Schnoor, 1996; Burken and Schnoor, 1997; Chang et al., 2005). However, the amount of ATR taken up by plants was limited by the concentration and bioavailability of ATR in the rhizosphere. Uptake was found to be significantly greater in hydroponic systems and sandy soils than in agricultural soils (Burken and Schnoor, 1996). The uptake of ATR was expected to be less than 5% of applied ATR under the field conditions (Weber, 1977; Schroll and Sabine, 2004; Henderson et al., 2007).

After plant uptake, ATR could be detoxified through non-enzymatic hydroxylation and enzyme-mediated *N*-dealkylation or glutathione and cysteine conjugation (Ballantine and Simoneaux, 1991; Gronwald, 1994; Hatton et al., 1999). Tolerant species rapidly detoxify ATR through hydrolytic and conjugation processes. The ability of the cell sap in a number of C_4 plants to hydrolyze ATR was closely correlated with herbicide tolerance (Kearney and Kaufman, 1975). Conversions of ATR and its metabolites into nonphytotoxic free (nonconjugated) hydroxylated products can be catalyzed by non-enzymatic agents, such as benzoxazinone, whereas conjugated (hydroxylated) metabolites involve enzyme-mediated reactions (Kearney and Kaufman, 1975; Ballantine and Simoneaux, 1991; Gronwald, 1994). Benzoxazinone, an ATR hydrolytic phytochemical found primarily in the roots of tolerant plants, facilitates ATR hydrolysis when ATR uptake occurs via the root system (Owen, 1989; Gronwald, 1994). The formation of glutathione conjugates, a reaction catalyzed by the enzyme glutathio-S-transferase, can account for the rapid detoxification of ATR in some tolerant plants. However, in several agronomically important plants, this mechanism mainly occurs in seedling leaves and may be less significant for mature plants (Kearney and Kaufman, 1975; Ballantine and Simoneaux, 1991; Hatton et al., 1996). For example, examination of ^{14}C-treated ATR-tolerant corn suggests that glutathione conjugation is not a major contributor to detoxification as plants mature (Ballantine and Simoneaux, 1991). Most ATR degradation products found in mature corn are hydroxylated metabolites, their oxidation products, and corresponding conjugates. However, a recent study reveals that glutathione conjugation remains a major pathway to detoxify atrazine in mature vetiver plants (Marcacci et al., 2006).

The efficacy of vegetative buffer strips in intercepting ATR from surface runoff has been well established. However, a sound buffer design should also consider incorporating plant species that facilitate rapid degradation or sequester herbicides deposited within the buffers before they have a chance to be released to surface and subsurface flow. The objective of this

work was to assess the bioremediation capacity of five forage species to enhance ATR dissipation in the environment via plant uptake and degradation and detoxification in the rhizosphere. The identification of species showing promising bioremediation potential is needed to optimize vegetative buffer designs for mitigating ATR transport from cropped fields.

Materials and Methods

Experimental Design and Sampling Process

A field lysimeter study was established at the University of Missouri Horticulture and Agroforestry Research Center. Details of the field lysimeters and experimental design were previously reported by Lin et al. (2003). Briefly, three replications of six ground cover treatments, including a bare ground (control); the C_3 species orchardgrass (*Dactylis glomerata* L.), smooth bromegrass (*Bromus inermis* Leyss.), tall fescue (*Festuca arundinacea* Schreb.), and timothy (*Phleum pratense* L.); and the C_4 species switchgrass (*Panicum virgatum* L.) were established in 18 0.5-m-deep lysimeters (1 m diameter). Each lysimeter was filled with a sandy loam soil with an average pH of 7.0, organic matter content of 0.72%, and cation exchange capacity of 3.0 cmol kg^{-1}. When all species were vigorous and well established, ATR was applied to the soils in each lysimeter as a 3-L solution containing 500 μg L^{-1} of ATR. The site had no previous history of ATR application. Leachate from each lysimeter was collected and processed as described by Lin et al. (2003). The study was conducted for a long enough period to allow for distribution of ATR throughout the soil-plant-water system, such that reasonable treatment effects were apparent. Additionally, the growth of C3 species was significantly inhibited during hot summer, whereas the growth of C4 species was the best during late summer to early fall. Therefore, to allow high levels of vigorous growth for C3 (cool-season) and C4 (warm-season) species, the 25-d period during summer to fall was the only window of opportunity to conduct the experiment. Six rainfall events, with total precipitation ranging from 5 to 58 mm, occurred within a 25-d period after herbicide application (Lin et al., 2003). Leachate volume was generally proportional to the amount of precipitation, with the greatest volume observed on the third (12 d after application) and sixth (25 d after application) rainfall events. The grass-treated lysimeters generally showed less leachate volume than the control treatment due to their higher evapotranspiration.

Soil and plant samples were collected 25 d after herbicide application. Soil samples were composite samples of five representative 35-cm-deep soil cores collected from each lysimeter. Soil samples were stored at −20°C until analyzed. For the plant samples, all aboveground plant material was collected, rinsed with water to remove soil, and ground with dry ice to pass a 1-mm sieve. Plant samples were stored at −20°C before analysis. The samples were homogenized and extracted followed by the procedure described. The recoveries were 73 to 95% for ATR, 78 to 93% for DEA, and 76 to 105% for DIA (Lerch et al., 1999). For hydroxylated ATR degradation products (HADPs), recoveries were 74 to 81% for HA, 79 to 88% for DEHA, and 64 to 77% for DIHA (Lerch and Li, 2001).

One-way ANOVA was performed to test the effects of the treatments (i.e., forage species). Fisher's LSD test was performed to compare the differences between treatments. Due to the variation in total soil weight among the 18 lysimeters (275–320 kg), the soil data were normalized and expressed as proportion of total soil ATR residue, so the differences in soil ATR degradation profile between treatments could be compared. The normalized soil data reflect the distribution of the measured ATR and metabolites remaining in the soil. For plant and leachate data, the results were calculated and expressed as percentage of applied ATR because the weight of plant tissues and volume of leachates can be accurately measured during the sampling processes.

Determination of Atrazine and Its Chlorinated Metabolites in Soil

The liquid–liquid extraction procedure used for analyzing ATR, DEA, and DIA in soils was previously described by Lerch et al. (1999). Briefly, soil samples of 50 g dry weight equivalent were sequentially extracted twice with 100 mL of 80% aqueous CH_3OH (4:1 CH_3OH:deionizeds water [v/v]) in 250-mL Teflon screw cap centrifuge tubes. Extraction was conducted on an end-to-end shaker at about 200 oscillations min^{-1} for 1 h at ambient temperature. Samples were centrifuged, the supernatants were combined, and the CH_3OH was evaporated. The remaining water was extracted three times with 50 mL of chloroform ($CHCl_3$). Chloroform was evaporated to about 5 mL. A small drop of toluene was added as a keeper, and the remaining $CHCl_3$ solution was brought to dryness under a stream of ultra-pure N_2. Samples were reconstituted in 1 mL of 40% CH_3OH and filtered through Anotop 0.2-μm syringe filters (Whitman International, Maidstone, UK) into chromatography vials. High-performance liquid chromatography (HPLC)–ultraviolet analyses were performed with a Beckman Model 338 system (Beckman Instruments, Inc., San Ramon, CA) using a C_8 reverse-phase silica-based column (LC-8-DB, 250 mm × 4.6 mm, 5 μm; Supleco, Inc., Bellafonte, PA) to separate ATR, DEA, and DIA. The HPLC conditions were as follows: injection volume, 40 μL; mobile phase A, DI water; mobile phase B, 100% CH_3CN; mobile phase gradient ramp from 15% B to 30% B in 15 min, hold at 30% B for 13 min, ramp to 85% in 9 min; mobile phase flow rate, 1.45 mL min^{-1} chloroform; and column temperature, 35°C. Detection wavelength was 220 nm. Retention times of DIA, DEA, and ATR were 6.7, 13.3, and 28.6 min, respectively. The limit of detection (LOD) for ATR, DEA, and DIA was 0.04 μg kg^{-1}. The LOD in this work was defined as the concentration that was three times the signal-to-noise ratio.

Determination of Hydroxylated Metabolites in Soil

Extraction of HADPs was based on a method using a mixed-mode extractant developed by Lerch and Li (2001). The mixed-mode extractant was a 3:1 ratio of 0.5 *M* KH_2PO_4 (pH 7.5) to ACN (v/v). The extractant was heated to 70°C before addition to the samples. Soil samples of 25.0 g dry weight equivalent were extracted three times with 50 mL of mixed-mode extractant in 250-mL Teflon centrifuge tubes at 70°C using an orbital shaker at 400 rpm. The sequence of shaking times was 1, 2, and 0.5 h.

After each extraction, the samples were centrifuged, supernatants were combined, and the CH_3CN was evaporated. Sample clean-up and concentration were achieved with a two-step solid-phase extraction (SPE) procedure, using anion exchange (SAX) SPE to remove dissolved organic matter from the extracts followed by acidification and isolation by cation exchange (SCX) SPE. The HADPs were eluted from the SCX SPE with 10 mL of 8:1:1 CH_3OH:NH_4OH:DI water at a flow rate of approximately 1 mL min^{-1}. The eluant was evaporated to dryness under a stream of ultra-pure N_2 at 45°C. Samples were reconstituted with 1.0 mL of 40% CH_3OH, sonicated for 5 min, vortex mixed for about 30 s, and filtered through 0.2-µm Anotop syringe filters.

The analyses of HA, DEHA, and DIHA were performed using a PerkinElmer Sciex API 365 tandem mass spectrometer (Norwalk, CT) with a TurboIonspray atmosphere pressure ionization interface. The compounds were separated with a Shimadzu LC-10AT HPLC system using a Zobax 300 propylbenzenesulfonic acid SCX analytical column (150 × 4.6 mm i.d.). Injection volume was 20 µL, and mobile phase flow rate was 0.3 mL min^{-1}. Mobile phases were as follows: A, 90% DI water:10% CH_3OH containing 1% formic acid (HCOOH) and 5 m*M* ammonium acetate (CH_3COONH_4); and B, 90% CH_3OH:10% DI water containing 0.1% HCOOH and 25 m*M* CH_3COONH_4. The detailed MS/MS and HPLC gradient conditions were as described by Lerch and Li (2001). The TurboIonspray atmosphere pressure ionization interface was operated in the positive ion mode. The MS/MS system was operated in the multireaction monitoring scan mode, and precursor and product ions used for quantification were the same as described by Lerch and Li (2001). Retention times of HA, DEHA, and DIHA were 11.4, 10.1, and 10.5 min, respectively. The LOD for HA, DEHA, and DIHA in the soil was about 0.002 µg kg^{-1}.

Determination of Atrazine and Its Chlorinated Metabolites in Plant Tissue

Due to organically rich plant samples, highly selective detection using gas chromatography–ion trap tandem mass spectrometry (GC–MS/MS) was used to identify the ATR, DEA, and DIA in the plant samples. Grass subsamples of 10 g dry weight equivalent were transferred into 250-mL high-density polypropylene centrifuge tubes. Samples were homogenized with 150 mL of 80% CH_3OH (4:1 CH_3OH:DI water [v/v]) for 1 min using a Brinkmann Polytron Homogenizer at 10,000 rpm. They were then extracted for 24 h at ambient temperature on an end-to-end shaker at 200 oscillation min^{-1} followed by sonication for 20 min. Samples were centrifuged for 20 min at 12,000 *g* (7000 rpm) at 0°C, and the supernatant was decanted. The same extraction procedure was repeated for 1 h on the residue remaining in the centrifuge tubes using 100 mL of 80% CH_3OH. All supernatants were combined and evaporated to remove the CH_3OH. The remaining sample solution was extracted four times with 50 mL of $CHCl_3$. The $CHCl_3$ fraction was evaporated to about 5 mL; this volume was transferred to a 12-mL test tube, and 5 mL of CH_3OH was added. The sample was brought to about 0.5 mL under a stream of ultra-pure N_2 until the $CHCl_3$ was evaporated. The final residue was extracted twice with 0.5 mL of CH_3OH and diluted to 60 mL with DI water. The analytes were isolated from the aqueous solution using 1 g C_{18} silica-based SPE cartridges (Varian, Inc., Harbor City, CA). Cartridges were preconditioned with 10 mL of CH_3OH followed by 10 mL of DI water at a flow rate of 2 mL min^{-1}. Sample solutions were passed through the cartridges at a flow rate of 3 mL min^{-1}, and cartridges were purged with air for 1 h to remove excess water. Samples were eluted with 12 mL of CH_3OH at a flow rate of 2 mL min^{-1}. The eluate was concentrated to approximately 2 mL under a stream of ultra-pure N_2 at 30°C. Terbutylazine (200 µg per sample) was added as an internal standard for final volume correction and recovery calculation. Concentrated samples were filtered through an Anotop 0.2-µm syringe filter into chromatography vials. A 250-µL subsample of the extract solution was diluted and vortexed with 250 µL of CH_3OH.

The analyses of ATR, DEA, and DIA were performed using a Varian 3400cx GC coupled with a Varian Saturn 2000 ion trap MS system (Varian, Walnut Creek, CA). A Hewlett-Packard cross-linked methylsiloxane capillary column (12.5 m × 0.20 mm i.d.) was used for separation. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The GC oven temperature was programmed as follows: hold at 70°C for 1 min, ramp to 120°C at 50°C min^{-1}, ramp to 155°C at 3°C min^{-1}, ramp to 290°C at 50° min^{-1}, and hold at 290°C for 12 min. A Varian Universal Capillary Injector with 0.5-mm i.d. insert was used. Splitless injection was used. Injector temperature was held at 250°C for 5 min. The transfer line between the GC and the ion trap MS was held at 280°C, and the ion trap manifold was set to 250°C. Injection volume was 0.4 µL. Selected precursor ions for MS/MS were m/z 200 for ATR and m/z 173 for DEA and DIA. Four product ions (m/z 68, 69, 94, and 104) were summed for quantification of ATR and DEA, and two product ions (m/z 68 and 110) were summed for quantification of DIA. The nonresonant excitation mode was chosen for each analyte. Maximum ionization time was programmed at 65,000 µs, total ion count was set at 5000, and the pre-scan ionization time was 1500 µs. Retention times for GC–MS/MS were 7.4 min for DIA, 7.7 min for DEA, and 9.8 min for ATR. The LODs for ATR, DEA, and DIA were 0.6, 1.3, and 0.3 µg kg^{-1}, respectively. The recovery rates for ATR, DEA, and DIA ranged from 94 to 106%.

Determination of Hydroxylated Metabolites in Plant Tissue

Chemical analysis of hydroxylated ATR degradation products (HADPs: HA, DEHA, and DIHA) in plant tissues was accomplished using methanol extraction, SPE clean-up, and high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). The extraction procedure was the same as that for the chlorinated compounds with the exception that the samples were extracted at 70°C and shaken at 400 oscillations min^{-1}. After evaporation of the methanol, the sample extracts were diluted to 100 mL with DI water. The hydroxylated ATR metabolites were isolated and concentrated using the same anion (SAX) and cation (SCX) exchange SPE as described for the soil extraction procedure. All other HPLC-MS/MS parameters for quantification of HA, DEHA, and DIHA were as described previously for the soil analyses procedure with the exception that injection volume was 70 µL

and the flow rate of the mobile phase was 1.5 mL min^{-1}. An injection split ratio of 10:1 was used. The LODs were 0.4 μg kg^{-1} for HA, 0.8 μg kg^{-1} for DEHA, and 3 μg kg^{-1} for DIHA.

Determination of Total Transpiration Rate

To determine the total transpiration rate (TTR, mmol $H_2O \cdot m^{-2} s^{-1}$) of each vegetated lysimeter, transpiration flux density was multiplied by the estimated total leaf area (m^2). Transpiration flux density (mmol $H_2O \cdot m^{-2} \cdot s^{-1}$) of each forage was measured in the field on a clear day using a Li-COR 6400 Portable Photosynthesis System (Lincoln, NE). Light intensity was 1715 μmol photons $m^{-2} s^{-1}$ of photosynthetically active photon flux density, relative humidity was 18%, and air temperature was 21°C. Ten readings were recorded during each measurement. Total leaf area was calculated by multiplying total aboveground dry weight by the specific leaf area (i.e., cm^2 leaf area/leaf dry weight). Total dry weight of harvested biomass from each lysimeter was determined after drying at 43°C until a constant weight was achieved (approximately 4 d). Specific leaf area was determined by the measurement of leaf area, and the dry weight of 20 leaves was subsampled from each lysimeter. The leaf area was measured using a Li-COR 3000 leaf area meter.

Determination of Sensitivity to Atrazine

Ten seedlings of each species were grown in 15-cm pots for a bioassay study in a greenhouse located on the University of Missouri campus. Herbicide treatments consisted of concentration levels of 0, 500, and 1000 μg L^{-1} for ATR. The applied herbicide concentrations are representative of those expected in surface runoff from cornfields in northern Missouri (Ghidey et al., 2005). Atrazine treatments began 3 mo after seedling establishment. Herbicide solutions were applied in 200-mL volumes every other day until levels reached 0, 500, and 1000 μg kg^{-1} dry soil. The treatments were arranged as a complete randomized design with six replications. Grasses were harvested about 8 wk after herbicide application, which was approximately 4 wk after the first symptoms of herbicide damage were observed. Damage from ATR exposure appeared as necrotic areas on leaf tips. After harvesting, total aboveground dry weight was determined by placing tissues in an oven at 70°C until the dry weight reached a constant value.

Determination of Soil Microbial Biomass Carbon

The soil microbial population of each lysimeter was evaluated by measuring soil microbial biomass carbon. Soil microbial biomass carbon was determined by a modified $CHCl_3$ fumigation and direct extraction method (Jordan and Beare, 1991). A 20-g moist soil sample was collected from each lysimeter. Samples were adjusted to a moisture content of 0.10 g H_2O g^{-1} dry soil. Soil dry weight was determined in an oven at 105°C. Samples were fumigated with $CHCl_3$ at room temperature (approximately 25°C) under vacuum for 24 h or not fumigated. Fumigation allows for the subsequent extraction of microbial carbon. Unfumigated samples were stored at 4°C in a standard refrigerator in a closed specimen cup. Biomass carbon from fumigated and unfumigated samples was extracted by adding 80 mL 0.5 *M* potassium sulfate and shaking for 30 min on a rotary shaker at 320 rpm. The liquid fraction was filtered through Whatman #1 filter paper into a specimen cup. Biomass carbon was evolved as CO_2 by potassium persulfate ($K_2S_2O_8$) digestion. Carbon dioxide was trapped into 1 mL 0.1 *M* NaOH. A volume of 100 μL 1 N barium chloride ($BaCl_2$) was added after CO_2 trapping. This solution was then titrated with 0.01 *M* HCl containing 1% phenolphthalein as an indicator (pink) until a colorless endpoint was reached. The amount of trapped carbon was determined from a standard curve constructed by $K_2S_2O_8$ digestion of glucose standards (0, 100, 200, 300, 400, and 500 μg carbon). Soil microbial biomass carbon was calculated as the difference between duplicate fumigated and unfumigated samples using a mineralization conversion factor of 0.41 (Voroney and Paul, 1984) for the lysimeter soil.

Results and Discussion

Atrazine Fate in Soil and Leachate

Although this study was conducted during a relatively short (25-d) experimental period, it allows us to compare the rates of the atrazine bioremediation between the systems with studied species. A mass balance approach was used for the lysimeter study in which the distribution of ATR in plants, soil, and water were determined. At the end of the collection period 25 d after application, total loss of applied ATR to lysimeter leachate ranged from 8.1 to 12.6% (Fig. 2). Total plant uptake ranged from 0.35 to 3.2% (Fig. 3). Thus, the bulk of the ATR remained in the soil, with 84.3 to 91.6% of the applied ATR existing as the parent or one of five metabolites measured in soil. The mineralization of ATR was not significant in this study.

The *N*-dealkylated ATR metabolites, especially DEA, were the major degradation products detected in leachates (Fig. 2). This finding was consistent with the known high water solubility and low sorption intensity of the *N*-dealkylated metabolites. Total ATR residues (i.e., ATR + metabolites) in leachates were not reduced by the presence of forages. However, the proportion of ATR metabolites detected in the leachates was significantly higher for the grass treatments compared with the control (bare ground, 37.5%; orchardgrass, 64.2%; tall fescue, 51.1%; timothy, 58.7%; smooth bromegrass, 53.3%, and switchgrass, 50.3%). In addition, the mass of DEA was approximately an order of magnitude higher than that of the other metabolites, and HA accounted for only a small fraction (~3.4%) of the metabolites present in the leachates. Detailed results regarding the fate of ATR in leachates were described in Lin et al. (2003).

In contrast to the leachate data, the proportion of HA was consistently higher than other metabolites in all soil treatments (Table 1). Hydroxylated ATR degradation products (HADPs) (HA+DEHA+DIHA) accounted for 34.0 to 47.4% of the total ATR residues in soil, with HA and DIHA making up the majority of HADPs. The *N*-dealkylated ATR metabolites in soil, DEA and DIA, accounted for 21.6 to 33.3% of total ATR residues, and DIA accounted for a greater proportion of the metabolites than DEA in five of six treatments. Concentrations of metabolites in soils were in the following order: HA > DIA > DIHA > DEA > DEHA. This finding was quite different than that reported by Lerch and

Li (2001) for three agricultural soils with a long history of ATR usage. They found that HA was the major constituent, and HADPs comprised an average of 91% of the total ATR residues in these soils. Given the short duration of this experiment, the results reported here suggest that the hydrolysis of DEA and DIA was incomplete and that the HADPs would eventually predominate in soil as observed by Lerch and Li (2001).

The results of the leachate and soil ATR residue data showed that there was preferential transport of DEA compared with DIA. This resulted in the lower relative DEA concentrations in soil compared with DIA and led to greater transformation of DIA to DIHA and limited transformation of DEA to DEHA. These results showed that compared with DEA, DIA was more strongly retained in soil and more susceptible to hydrolysis. Kruger et al. (1993) reported that a high proportion of DIA was retained in surface and subsurface soil, with 24 to 70% of the added DIA forming bound residues in 120-d incubations. Although the methods used by Kruger et al. (1993) were not sufficient to quantitatively extract DIHA, Lerch et al. (1997) showed that HADPs represent an important mechanism for bound ATR residue formation in soil. Thus, a significant proportion of the bound DIA residues reported by Kruger et al. (1993) likely represented DIHA. Their results support those observed in this study in which DIA was largely retained in soil, leading to significant formation of DIHA.

Among the ground treatments, significantly higher levels of ATR degradation products were detected in the soils of grass treated lysimeters than in the bare ground (Table 1). Atrazine degradation was enhanced 20 to 45% by the grass treatments. This may be associated with the increased population or activity of ATR microbial degraders in the rhizosphere or the release of oxidative/hydrolytic agents in exudates from the forage root systems. Switchgrass exhibited the greatest ability to facilitate ATR degradation in soil. Switchgrass treatments resulted in degradation of more than 80% of soil ATR into less toxic degradation products. Tall fescue, orchardgrass, smooth bromegrass, and timothy also showed promising bioremediation ability, degrading 66.5 to 74.7% of soil ATR as compared with 55.5% in the control treatment. All grass treatments had a significantly greater capacity to dealkylate ATR (31.4–34.0%) compared with bare ground (21.6%). However, the extent of dealkylation was similar between grass treatments. The highest capacity for ATR hydroxylation was found in switchgrass lysimeters, with 47.4% of soil ATR converted to HADPs. This was about 40% greater than the hydrolysis capacity of the control lysimeter. These results clearly demonstrated that forage grasses directly or indirectly facilitated ATR degradation in soil compared with the nonvegetated control.

Microbial biomass carbon was significantly higher in every grass treatment than in control. Detailed results of microbial biomass carbon were described in Lin et al. (2003). Enhanced soil ATR degradation in the forage treatments was strongly correlated with

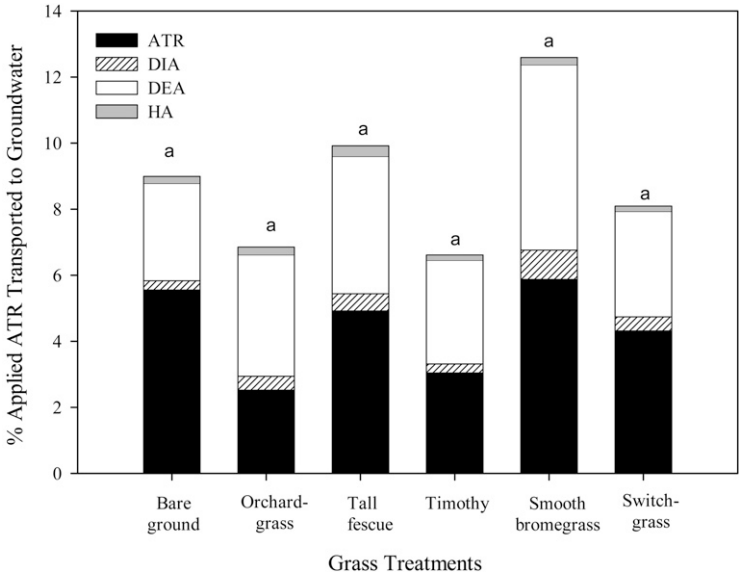

Fig. 2. Loss of atrazine (ATR) and its metabolites deisopropylatrazine (DIA), deethylatrazine (DEA), and hydroxyatrazine (HA) to lysimeter leachates during the 25-d experimental period. Means followed by the same letter do not differ significantly at a 90% confidence level using LSD test (α = 0.1).

increasing microbial biomass carbon in the rhizosphere (Table 2). Further correlation analyses revealed that DEA, DIA, and total dealkylated products (DEA+DIA) were strongly and positively correlated with the microbial population (Table 2). This suggested that dealkylation, in particular deethylation, was closely associated with microbial activity in the lysimeter soil. In contrast, HA, DEHA, DIHA, and total HADPs (HA+DEHA+DIHA) were poorly correlated with microbial biomass carbon (Table 2), suggesting that abiotic hydrolysis was the predominant mechanism for HADP formation.

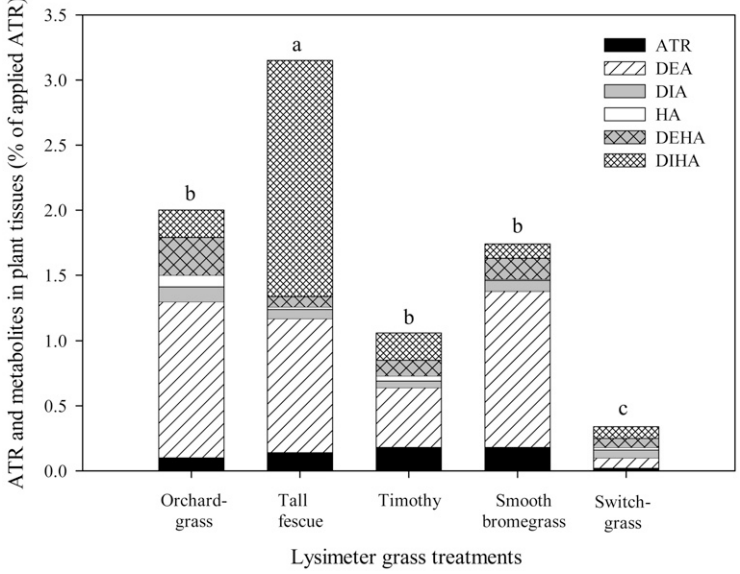

Fig. 3. Total atrazine uptake and the distribution of the degradation products in forage grasses after 25 d. Means followed by the same letter do not differ significantly at a 90% confidence level using LSD test (α = 0.1). ATR, atrazine; DEA, deethylatrazine; DEHA, deethylhydroxyatrazine; DIA, deisopropylatrazine; DIHA, deisopropylhydroxyatrazine; HA, hydroxyatrazine.

Table 1. Proportion (%) of atrazine (ATR) and its metabolites deethylatrazine (DEA), deisopropylatrazine (DIA), hydroxyatrazine (HA), deethylhydroxyatrazine (DEHA), and deisopropylhydroxyatrazine (DIHA) in soil after 25 d.

Grass treatments	ATR†	DEA	DIA	HA	DEHA	DIHA	Dealkylated metabolites	HADPs‡	All metabolites§
Bare ground	44.7a¶	6.3b	14.6b	18.7b	2.5b	13.2b	21.6 (7.1)b	34.0 (4.1)b	55.5 (3.1)c
Orchardgrass	30.9b	19.0a	15.9b	20.4b	3.7b	11.5b	34.0 (5.5)a	35.6 (8.7)b	69.6 (3.2)b
Tall fescue	33.8b	15.4a	17.8b	18.6b	2.3b	12.2b	32.0 (10.0)a	34.5 (10.2)b	66.5 (2.1)b
Timothy	28.1bc	12.2a	20.0b	20.5b	2.6b	16.7b	33.0 (6.0)a	41.7 (9.1)ab	74.7 (13.4)ab
Smooth bromegrass	29.2bc	13.1a	18.7b	20.3b	3.1b	15.5b	31.4 (10.5)a	40.8 (4.6)ab	72.2 (10.8)ab
Switchgrass	19.4c	14.1a	19.2b	24.9a	2.7b	19.7b	33.3 (2.4)a	47.4 (4.0)a	80.7 (1.9)a

† Normalized means of three lysimeter samples. Standard deviation in the parentheses.

‡ Hydroxylated ATR degradation products (HA+DEHA+DIHA).

§ Means derived from ANOVA using normalized values.

¶ Means followed by the same letter within the column do not differ significantly at a 90% confidence level using LSD test.

The significantly enhanced ATR biodegradation in forage treatments, especially switchgrass, may be explained by stimulated degrader populations or enzymatic activities. In addition, many classes of root exudates and plant residues, including carbohydrates, proteins, aliphatic and aromatic organic compounds, and amino acids, provide the carbon and nutrient sources needed to stimulate microbial growth (Burken and Schnoor, 1996). Compared with C_3 species, C_4 species tend to partition more of their total fresh weight to roots (Jiang et al., 2002; Dhawan and Goyal, 2004). Greater partitioning of carbohydrates to the root system enhances root-derived, water soluble, and bioavailable organic carbon in soils and stimulates microbial populations and activities in C_4 plantations (Corre et al., 1999; Pu et al., 2001). As a result, C_4 plants tend to harbor higher numbers of bacteria and actinomycetes in the rhizosphere than C_3 plants (Mahmood and Renuka, 1990). Preliminary results from a growth chamber study also indicated significantly higher soil enzymatic activities (dehydrogenase, β-glucosidase, and fluorescein diacetate hydrolysis) and ATR mineralization rates in the rhizosphere of C_4 species compared with that of C_3 species (Lin et al., 2005).

Many oxidative and hydrolytic compounds are contained in root exudates of C_4 species that are capable of directly hydrolyzing ATR and its metabolites (Wenger et al., 2005). For instance, DIMBOA (3,4-dihydro-2,4-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one), a major benzoxazinone found in corn root exudates, as well as many other hydroxamic acids, are strong ATR hydrolytic agents reported to be abundant in the roots of many C_4 ATR-tolerant plants (Raveton et al., 1997; Wenger et al., 2005). The releases of these phytochemicals can result in rapid hydrolysis of ATR, DEA, and DIA in the rhizosphere (Wenger et al., 2005). Wenger et al. (2005) reported complete conversion of ATR to HADPs within 48 h at a ratio of DIMBOA to ATR of 100:1. In this study, up to 30% of the degraded ATR and DEA and 10% of degraded DIA in a solution medium could be explained by DIMBOA-mediated hydrolysis. Biochemical agents such as this can facilitate the hydrolysis of ATR and its metabolites, especially when ATR is taken up by the roots (Owen, 1989; Gronwald, 1994). This may explain why the highest levels of hydroxylated metabolites were measured in the switchgrass (a C_4 grass) treatments. Atrazine degradation in the switchgrass treatments was consistent with the likelihood that a portion of the HADP formation in soil and within the plant was facilitated by root-exuded DIMBOA, its analogs, or other hydroxamic acids.

Formation of hydroxylated ATR in soil occurs by chemical, biological, or photolytic hydrolysis (Lerch et al. 1995 and 1999). Chemical hydrolysis occurs in association with sorption to soil colloids and pH extremes. Faster rates of hydrolysis have been found in systems containing sterilized soil in comparison with soil-free systems (Armstrong and Konrad, 1974). These authors suggested that sorption-catalyzed hydrolysis of ATR is due to the reaction between protonated carboxyl groups on soil organic matter and ATR ring nitrogens. Soil microbial hydrolysis of ATR and its *N*-dealkylated metabolites catalyzed by hydrolase also results in the formation of HADPs (Mulbry, 1994; Sadowsky et al., 1998). There are at least two advantages resulting from ATR hydrolysis in agronomic or natural soil environments. First, HADPs have not been shown to be carcinogenic, nor do they possess herbicidal activity; thus, they are less toxic to mammals, plants, and aquatic organisms than ATR or its dealkylated metabolites (Lerch et al., 1995). However, the potential of HADPs to behave as endocrine disruptors has not been evaluated. Second, HADPs have greater soil sorption intensity than ATR and the dealkylated metabolites (Brouwer et al., 1990; Moreau-Kervevan and Mouvet, 1998). The greater extent of ATR hydrolysis observed in the switchgrass treatments combined with the low leaching potential of the HADPs suggests that switchgrass buffers more effectively reduce total ATR transport from cropped fields. Therefore, of the grasses tested,

Table 2. Correlation between percentage of atrazine metabolites† and microbial biomass in soil.

	DEA	DIA	HA	DEHA	DIHA	Dealkylated metabolites	HADPs	All metabolites
r	0.85	0.31	0.04	0.36	0.27	0.57	0.23	0.58
p Value	0.00**	0.22	0.88	0.16	0.29	0.02*	0.38	0.015*

* Statistically significant at the 0.05 probability level.

** Statistically significant at the 0.01 probability level.

† DEA, deethylatrazine; DEHA, deethylhydroxyatrazine; DIA, deisopropylatrazine; DIHA, deisopropylhydroxyatrazine; HA, hydroxyatrazine; HADP, hydroxylated atrazine degradation product (HADP = HA+DEHA+DIHA).

switchgrass is recommended for use in grass or tree-grass buffers established for reducing ATR toxicity and mobility.

Plant Uptake and Detoxification

The magnitude of total ATR residues measured in aboveground forage tissues varied considerably between species (Fig. 3). Total ATR residues taken up by the grasses ranged from 0.35 to 3.2% of the applied ATR. Tall fescue had the highest ATR uptake, and switchgrass had the lowest. Orchardgrass, smooth bromegrass, and timothy showed lower uptake rates than tall fescue. The lower uptake by switchgrass may be a result of its significantly lower transpiration flux density and TTR relative to the other forage treatments (Fig. 4). Total transpiration rate is the transpiration flux density multiplied by estimated lysimeter leaf area. Several researchers have reported that the rate of absorption and translocation of triazine herbicides from roots is directly proportional to the transpiration flux density (Ashton and Crafts, 1981; Jensen, 1982; Burken and Schnoor, 1996). Transpiration flux density of C_4 plants is often lower than C_3 plants (Taiz and Zeiger, 1991). The lower transpiration flux density of C_4 species is mainly attributable to the biochemical and anatomic features of their photosynthetic pathway. This pathway allows C_4 plants to eliminate photorespiration and fix CO_2 even under conditions of partial stomatal closure. It is well documented that C_4 species have transpiration ratios on the order of one half to one third of those measured in C_3 species (Salisbury and Ross, 1991). Transpiration ratios (i.e., unit of water used for per unit of carbon fixed) measured in lysimeter forage treatments displayed this characteristic result (Table 3). Greater water use efficiency in C_4 species results in a potentially slower herbicide uptake and translocation than in C_3 species. However, when all transpiration data were pooled, the amount of total ATR uptake did not significantly correlate with TTR (r = 0.371; p = 0.235). This result suggested that there were different ATR translocation mechanisms functioning in different forages (Jensen, 1982).

Atrazine metabolites accounted for 80.4 to 95.6% of the total residue detected in the aboveground forage tissues (Fig. 3). The fraction of total ATR residue as metabolites was highest in tall fescue (95.6%), orchardgrass (94.7%), and switchgrass (94.3%). Timothy and smooth bromegrass showed significantly lower ratios, with average values of 80.4 and 89.6%, respectively. This study was not designed to distinguish whether the metabolites present in the forage tissues were a result of direct uptake from soil or from uptake of ATR and subsequent biochemical detoxification within the plant. However, the much greater proportion of metabolites (80.4–95.6%) in the plants than in the soil (55.5–80.7%) strongly suggested that significant biochemical detoxification occurred within all forages. This was particularly the case for the HADPs. Because these metabolites are strongly bound to soil colloids (Moreau-Kervevan and Mouvet, 1998; Lerch et al., 1999), plant uptake from soil was apparently low. Many biochemical agents found in plants have been reported to be responsible for the *N*-dealkylation, conjugation, or hydroxylation of ATR (Jensen, 1982; Burnet et al., 1993; Gronwald, 1994).

The variation in metabolite composition measured between forages suggested that different species have alternate phytodegradation pathways for ATR, and these pathways may be strongly associated with species tolerance. For switchgrass and tall fescue, biological hydrolysis is the main detoxification mechanism (Fig. 3). For the other C_3 species (orchardgrass, timothy, and smooth

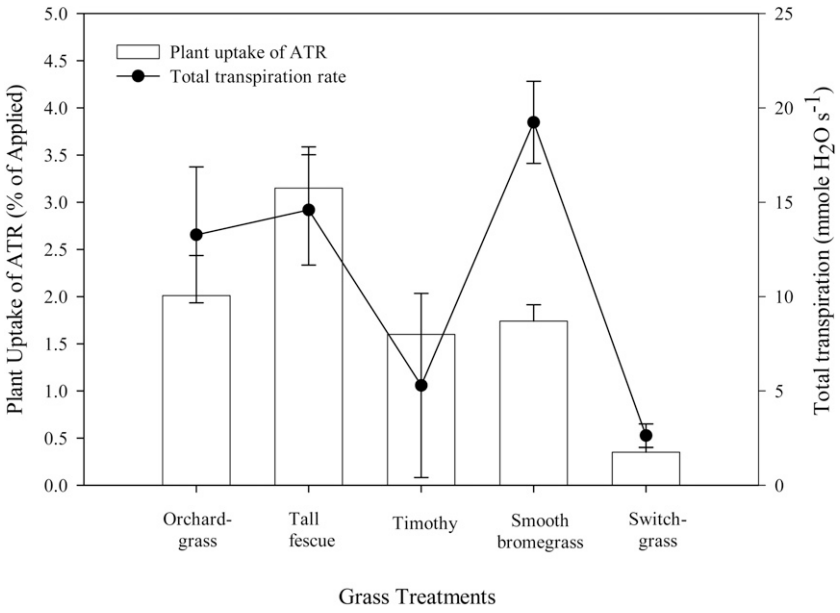

Fig. 4. Plant uptake of atrazine (ATR) and total transpiration rate of forages.

Table 3. Total transpiration rates, transpiration flux densities, photosynthetic flux densities, and transpiration ratios of atrazine-treated lysimeters.

Grass treatment	Total transpiration rate†	Transpiration flux density	Photosynthesis rate	Transpiration ratio
	mmol H_2O s^{-1}	mmol H_2O m^{-2} s^{-1}	μmoleCO_2 m^{-2} s^{-1}‡	μmol H_2O $\mu mole^{-1}$ CO_2
Orchardgrass	13.3 (6.2)a§¶	3.1 (1.7)a	13.1 (4.3)a	222.8 (83.7)ab
Tall fescue	14.6 (5.1)a	3.8 (1.5)a	15.5 (4.1)a	238.9 (31.3)a
Timothy	5.3 (6.9)ab	2.9 (2.1)a	13.8 (6.6)a	193.5 (58.4)b
Smooth bromegrass	19.2 (3.8)a	3.8 (1.3)a	12.8 (2.7)a	290.1 (46.6)a
Switchgrass	2.6 (0.9)b	1.3 (0.7)b	9.6 (1.8)a	133.5 (49.9)b

† Total transpiration rate was determined by multiplying transpiration flux density by the estimated total leaf area (m^2) of each vegetated lysimeter.

‡ Photosynthesis rate was determined by Li-COR 6400 Portable Photosynthesis System and expressed as CO_2 assimilation rate per unit of leaf area.

§ Means of the measurements made with SD in parentheses.

¶ Means followed by the same letter within the column do not differ significantly at a 90% confidence level using LSD test.

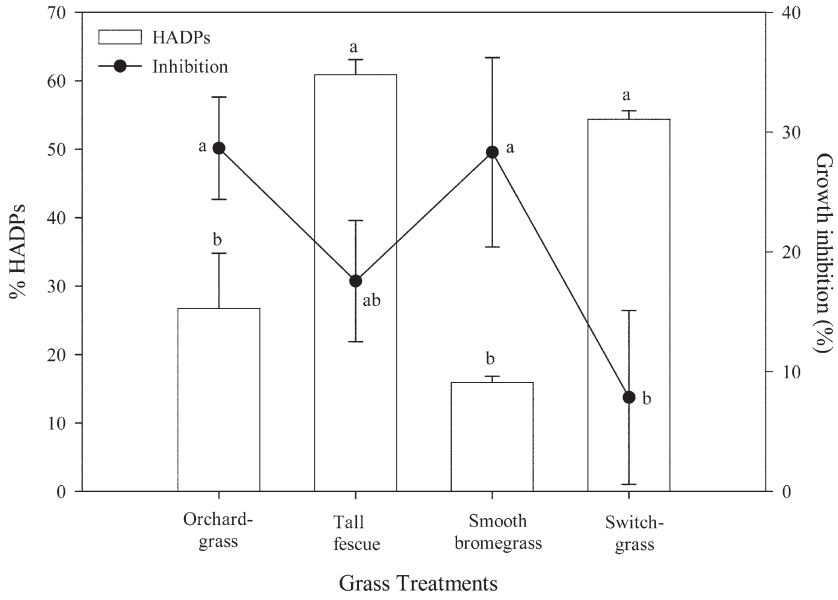

Fig. 5. Proportion of total atrazine (ATR) in forage tissue as hydroxylated ATR degradation products (HADPs) and the susceptibility of forages to ATR at 1000 µg L^{-1}. Means followed by the same letter do not differ significantly at a 90% confidence level using LSD test (α = 0.1).

bromegrass), the *N*-dealkylated products, especially DEA, were the major metabolites (Fig. 3). The hydroxylated forms of ATR are much less phytotoxic than the parent molecule or the dealkylated metabolites (Gronwald, 1994). Thus, hydroxylation of ATR is considered a more effective detoxification mechanism than *N*-dealkylation. The degree of hydroxylation of ATR has been suggested to account for ATR tolerance in mature plants of several species (Kearney and Kaufman, 1975; Ballantine and Simoneaux, 1991). Results of a bioassay on 3-mo-old plants suggested that the proportion of hydroxylated residue observed in lysimeter treatments coincided with forage tolerance to ATR (Fig. 5).

N-dealkylation, especially *N*-deethylation, has been identified as the major detoxification pathway for triazine herbicides in a wide range of C_3 species, such as annual ryegrass (*Lolium rigidum*) (Burnet et al., 1993; Burken and Schnoor, 1997) and hybrid poplar (*Populus deltoides x nigra* DN34) (Burken and Schnoor, 1997). Although hydroxylation is a more effective detoxification pathway, *N*-dealkylation can significantly enhance tolerance to ATR in partially susceptible plants, such as pea and cotton (Shimabukuo and Swanson, 1970). Burnet et al. (1993) postulated that an accelerated *N*-dealkylation rate might be associated with an increased cytochrome P450 activity in an annual ryegrass biotype that displayed ATR resistance (Burnet et al., 1993). This mechanism is considered a minor contributor to ATR-tolerant C_4 species (Gronwald, 1994). Early studies indicated that hydroxylation was an essential detoxification mechanism in many ATR-tolerant C_4 species, especially when ATR was taken up through the roots (Hamilton, 1964; Kern et al., 1975). Hydroxylation was shown to be catalyzed by the presence of DIMBOA in roots of tolerant C_4 species (Jensen, 1982; Raveton et al., 1997).

In many ATR-tolerant C_4 seedlings, including big bluestem, corn, Indian grass, and switchgrass, glutathione conjugation is the predominant ATR detoxification pathway (Weimer et al., 1988). This mechanism becomes less important for ATR detoxification as C_4 plants mature (Ballantine and Simoneaux, 1991; Hatton et al., 1996). Atrazine was applied to the switchgrass treatments approximately 1 yr after establishment. In the work reported here, no analysis for conjugated forms of ATR was conducted. Therefore, the mechanism of ATR detoxification in switchgrass could not be distinguished between glutathione conjugation and DIMBOA-catalyzed hydrolysis. However, based on previous reports (Ballantine and Simoneaux, 1991; Hatton et al., 1996) and the age of the switchgrass in this study, ATR hydrolysis was considered to be the likely detoxification mechanism. A similar finding was reported for a radiolabeled study of ATR detoxification products in corn (Mathew et al., 1996). On the other hand, Jensen (1982) has documented that metabolism rather than uptake accounts for differences in tolerance in many agronomic crops. The low ATR uptake resulting from low transpirational flux densities of switchgrass may also contribute to its greater tolerance to ATR.

Conclusion

The work reported here is the first using a nonradiolabeled field study to extensively examine and contrast the bioremediation capacity of various forages for ATR degradation, uptake, and detoxification under field conditions. Rhizodegradation seems to be the major remediation pathway, accounting for 55 to 81% of the ATR degradation. Compared with rhizodegradation or transport to leachates, a much smaller portion of applied ATR was taken up and detoxified by the selected forage plants.

All grass treatments significantly facilitated soil degradation of ATR to *N*-dealkylated metabolites compared with the control. In addition, the formation of HADPs in soils was significantly facilitated by the presence of switchgrass. The strong positive relationship between microbial biomass C and stimulated *N*-dealkylation of ATR in forage treatments further suggested that forage treatments enhance rhizosphere microbial populations and activity of ATR degraders. As a result of stimulated ATR degradation, an increased proportion of metabolites was observed in ground water collected from forages treatments.

Among the tested grasses, switchgrass could provide significant benefits to reduce ATR toxicity and mobility when incorporated as a groundcover in riparian buffer designs due to its high tolerance and ability to facilitate HADP formation. However, switchgrass can be difficult to establish and is not well suited for growth in colder climates. Of the C_3 species tested, smooth bromegrass and timothy resulted in slightly lower amounts of degradation products formed in soil compared with switchgrass. Thus, these C_3 species may be effective alternatives for grass buffers when ATR exposure is low to moderate and when the use of a C_4 species is impractical. This study has provided baseline information on the bioremediation capacity of the studied forage

grasses for removing ATR from the systems. Future studies are needed to further examine the long-term effects of forage plantation on transport and transformation of ATR.

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