Metabolism of Atrazine by *Spartina alterniflora*. 1. Chloroform-Soluble Metabolites

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The tolerance to atrazine and the translocation and metabolism of this herbicide by the marsh grass *Spartina alterniflora* were studied for 28 days in an atrazine-free solution after an initial 2-day root exposure to the radiolabeled compound. No visual symptoms of atrazine toxicity were observed at the concentrations tested and *S. alterniflora* was considered at least moderately resistant to this herbicide. Atrazine was readily absorbed by the roots and translocated to the shoots; after 2 days exposure to [^{14}C]atrazine, 90% of the radioactivity was present in shoots. Atrazine was readily metabolized to chloroform, aqueous, and subsequently to insoluble substances. The chloroform fraction, which contained atrazine, showed an initial rapid decrease and then a steady decrease from 84.8 to 23.9% of radioactivity in the total extract. Along with atrazine, three N-dealkylation products were identified by thin-layer chromatography: 2-chloro-4-amino-6-ethylamino-*s*-triazine, 2-chloro-4-amino-6-isopropylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine.

A four-component model microecosystem is being tested in our laboratories for studying the fate of atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) in a simulated marsh environment. *Spartina alterniflora*, a marsh grass commonly known as smooth cordgrass, has been selected as the primary autotroph for this system. The initial objective of this study is to determine the metabolites of atrazine produced by *S. alterniflora* that will be ingested by feeders on this component of the ecosystem. This paper describes the tolerance of *S. alterniflora* to atrazine and the metabolism of this herbicide to chloroform-soluble forms.

MATERIALS AND METHODS

Ring-labeled [^{14}C]atrazine (24.9 μCi/mg) and the following atrazine metabolites were obtained from the Agricultural Division of Ciba Geigy Corporation, Greensboro, N.C.: 2-chloro-4-amino-6-ethylamino-*s*-triazine, 2-chloro-4-amino-6-isopropylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine.

Plants were collected from the marsh at Sapelo Island, Ga. They were maintained at Auburn University in 2-L beakers containing Hoagland's solution placed in a growth chamber having a 14-h photoperiod with 60% RH, a temperature of 28 °C, and 30 klux of light provided by a mixture of incandescent and fluorescent lamps. The photoperiods were followed by 10-h dark periods at 60% RH and 24 °C.

In a preliminary experiment to determine the tolerance of *S. alterniflora* to atrazine, plants were divided into five groups of 16 plants each. The plants were weighed and the number of leaves per clump of plants and plants heights recorded. Plants were then transferred to 1-L plastic beakers containing 900 mL of Hoagland's solution with 0, 5×10^{-8}, 5×10^{-7}, 5×10^{-6}, or 5×10^{-5} M atrazine. Each beaker contained four plants and each treatment was replicated four times.

In time course experiments, a uniform lot of vigorously growing plants was divided into ten groups of four plants each. Each group of plants was placed in 300 mL of Hoagland's solution containing 2.0 μCi of [^{14}C]atrazine (2.6 μM). After 2 days, the plants were removed from the [^{14}C]atrazine solutions, the roots were rinsed, and all but two groups were placed in atrazine-free Hoagland's solution. The two groups not transferred to the atrazine-free solution and two additional groups collected at 3, 8, 18, and 28 days after transferring them to the atrazine-free nutrient solution were extracted as described below.

The extraction methods used in this study were essentially those described by Shimabukuro et al. (1973). Roots and shoots were separated and extracted with 10 mL of 80% methanol for each gram of tissue. The extracts were concentrated by flash evaporation at 37 °C, diluted with water, and then washed with chloroform. Each phase was brought to volume and the amount of radioactivity in each was determined by liquid scintillation spectrometry (Beckman LS-200B). Radioactivity in the insoluble plant residue was estimated by grinding a portion of this material to a fine powder, suspending it in Aquasol (Beckman) liquid scintillation cocktail containing Cab-O-Sil (Beckman), and counting as before.

Radiolabeled components of the chloroform fractions were separated by thin-layer chromatography (TLC) using glass plates coated with a 250-μm layer of silica gel HF-254 and activated for 1 h at 110 °C. Plates were developed initially in benzene–acetic acid–water (60:40:3, v/v/v) and radioactivity was located using a Berthold TLC-Scanner. Silica gel from radioactive areas was removed from the plates, washed with methanol, and removed from the solvent by centrifugation. Atrazine and its metabolites in these extracts were again spotted on TLC plates and developed in chloroform–ethanol (90:10, v/v) and identified by comparing their R_f values with those of authentic standards. Standards of atrazine and its N-dealkylation products were visualized on the TLC plates with ultraviolet light (254 nm). Radioactivity in each component was determined by liquid scintillation spectrometry after removing the compound from silica gel as before.

RESULTS AND DISCUSSION

Tolerance of *S. alterniflora* to Atrazine. For *S. alterniflora* to be a successful component of the model ecosystem for long-term studies, it must be at least moderately resistant to atrazine. To determine the effects of atrazine on this species, plants were grown for 45 days in the different atrazine solutions and fresh and dry weights of roots and shoots, plant heights, and number of leaves were determined. There were no obvious symptoms of atrazine toxicity such as chlorosis, necrosis, or wilting. However, with the exception of plant height, significant decreases in all growth parameters measured were obtained

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Table I. Effect of Atrazine on the Growth of *Spartina alterniflora* 45 Days after Treatment[a]

Atrazine concn, M	Fresh wt. of roots, g	Fresh wt. of shoots, g	No. of leaves	Total height, cm	Dry wt. of roots, g	Dry wt. of shoots, g
Control	19.7 a	27.3 a	15.6 a	68.3 a	3.3 a	7.2 a
5×10^{-5}	7.5 b	7.9 c	5.1 c	45.9 b	0.7 c	2.6 e
5×10^{-6}	7.9 b	13.8 bc	9.9 be	61.1 a	1.1 bc	4.6 be
5×10^{-7}	9.4 b	16.6 b	11.2 ab	63.1 a	1.4 bc	5.5 ab
5×10^{-8}	13.7 ab	24.1 a	15.0 a	69.7 a	2.0 b	7.9 ab

[a] Each value is the average of four replications with four plants in each replication. Values in a column followed by the same letters are not significantly different at the 5% level using Duncan's multiple range test.

with atrazine concentrations of 5×10^{-5} and 5×10^{-6} M when compared with the control (Table I). There was an average of 37% reduction of the growth parameters measured at the most concentrated atrazine solution used (5×10^{-5} M). Root and shoot fresh weights and root dry weights were significantly less than the control at all atrazine concentrations down to 5×10^{-7} M. Only root dry weight was significantly less than the control at 5×10^{-8} M concentration.

These data are similar to those obtained for resistant species. Corn is considered a resistant plant, but its degradation system can be overloaded. Couch and Davis (1966) reported a 50% decrease in fresh and dry weights when corn was grown in a 5×10^{-5} M atrazine solution and ca. 5×10^{-5} atrazine decreased photosynthesis about 60%. A correlation between net CO_2 exchange (NCE) and atrazine resistance was reported for certain grasses (Jensen et al., 1977). Grasses with NCE recovery rates exceeding 1.2 mg of CO_2 dm^{-2} h^{-1} were considered tolerant to 1.0 kg/ha preemergence and 1.25 kg/ha postemergence atrazine applications. When representatives of the subfamilies Festucoideae, Eragrostoideae, and Panicoideae were screened, only some members of the latter subfamily had NCE recovery rates exceeding 1.2 mg dm^{-2} h^{-1}. Although the NCE recovery rates for *S. alterniflora*, a member of the Festucoideae, are not known, our data suggest that this species is resistant to atrazine.

Uptake and Translocation of [^{14}C]Atrazine. [^{14}C]Atrazine was readily absorbed and translocated by *S. alterniflora*. After 2 days of continuous exposure to the radiolabeled herbicide, approximately 90% of absorbed atrazine was present in the shoots. Methanol extractable radioactivity in the roots and shoots remained relatively constant throughout the 28-day period in the atrazine-free nutrient solution (Figure 1). This is consistent with the concept that absorption and translocation limitations are not the primary factors that determine susceptibility to atrazine (Davis et al., 1959).

Extracts of *S. alterniflora* roots and shoots were separated into chloroform, aqueous, and insoluble fractions, and the radioactivity in each was determined. Radioactivity in the insoluble fraction represented atrazine or its metabolites in the plant residue that remained after exhaustive washing with 80% methanol. The chloroform fraction, which contained atrazine, had approximately 80% of the radioactivity after the initial 2-day exposure to the radiolabeled herbicide (Figure 2). Radioactivity in this fraction declined rapidly during the first 5 to 6 days after transferring the plants to an atrazine-free solution. This was followed by a slower decrease. There was a corresponding increase in radioactivity of the aqueous fraction, which is consistent with the expected precursor-product relation between components of the chloroform and aqueous fractions. Similar results were reported for the distribution of radioactivity between the chloroform and aqueous fractions of extracts of sorghum treated with [^{14}C]atrazine (Lamoureux et al., 1973). It is well established for atrazine-resistant corn and sorghum that the chloroform fraction contains atrazine, its N-dealkylated products, and some conjugated metabolites, whereas the aqueous fraction contains 2-hydroxyatrazine and most of the conjugated metabolites (Shimabukuro, 1967b; Shimabukuro et al., 1970; Shimabukuro et at., 1971; Lamoureux et al., 1972). Conversion of atrazine to water-soluble metabolites by *S. alterniflora* is slower than in sorghum. After 2-days continuous exposure to [^{14}C]-atrazine, radioactivity in the aqueous fraction from sorghum was near maximum (Lamoureux et al., 1973) whereas in our studies the increase in radioactivity in the same fraction from the smooth cordgrass did not level off

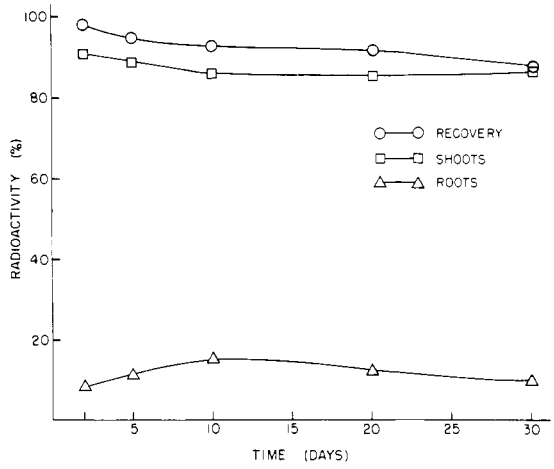

Figure 1. Distribution of methanol extractable radioactivity in roots and shoots of *Spartina alterniflora* treated for 2 days with [^{14}C]atrazine and then transferred to an atrazine-free nutrient solution for 28 days.

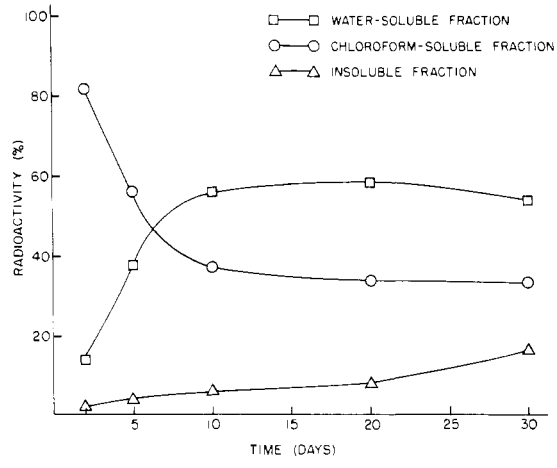

Figure 2. Change in radioactivity with time in the water, chloroform, and insoluble fractions of an 80% methanol extract of shoots of *Spartina alterniflora* plants grown in [^{14}C]atrazine for 2 days and then transferred to an atrazine-free nutrient solution.

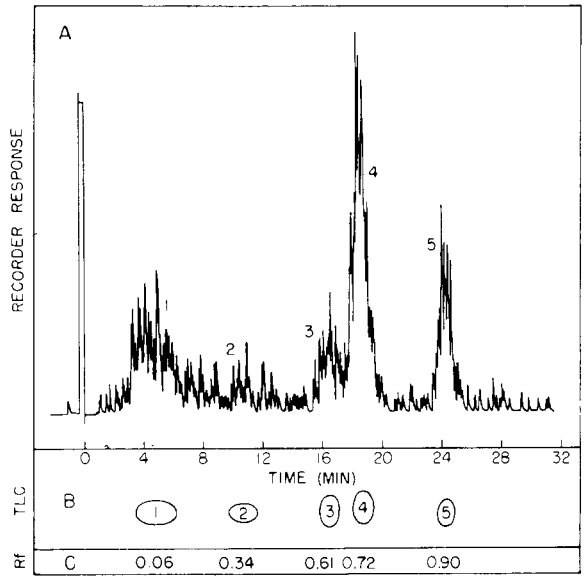

Figure 3. (A) TLC radiochromatogram showing radioactive components present in the chloroform fraction of an 80% methanol extract of *Spartina alterniflora* treated with [^{14}C]-atrazine for 2 days and then transferred to a herbicide-free nutrient solution. (B) Diagram of TLC chromatogram of atrazine and its metabolites. (C) R_f values of standards of atrazine and its metabolites. (1) Polar metabolites, (2) 2-chloro-4,6-diamino-*s*-triazine, (3) 2-chloro-4-amino-6-ethylamino-*s*-triazine, (4) 2-chloro-4-amino-6-isopropylamino-*s*-triazine, and (5) 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine (atriazine). The TLC plate was developed two times in benzene–acetic acid (50:4, v/v).

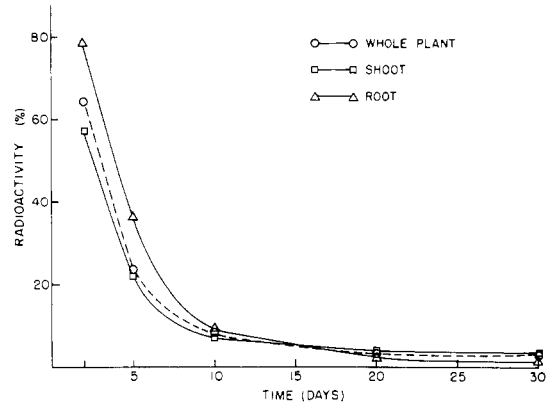

Figure 4. Change in radioactivity with time in atrazine extracted from shoots and roots of *Spartina alterniflora* treated for 2 days with [^{14}C]atrazine and subsequently transferred to an atrazine-free nutrient solution for 28 days.

Table II. Radioactivity in N-Dealkylated Products of Atrazine Metabolism Isolated from Shoots of *Spartina alterniflora* Collected at Intervals over a 28-Day Period

Days after treatment	Radioactivity, % of total		
	I[a]	II[b]	III[c]
2	14.4	2.5	0.8
5	20.4	4.2	2.2
10	18.2	4.2	2.8
20	9.4	12.1	3.0
30	5.8	5.9	8.7

[a] I, 2-chloro-4-amino-6-isopropylamino-*s*-triazine. [b] II, 2-chloro-4-amino-6-ethylamino-*s*-triazine. [c] III, 2-chloro-4,6-diamino-*s*-triazine.

until 10 days after exposure to the herbicide. Radioactivity in the 80% methanol-insoluble plant residue increased slowly with time, reaching approximately 20% of the total radioactivity after 30 days.

It appears that in *S. alterniflora* the root plays a relatively minor role in the metabolism of atrazine, since about 90% of the radioactivity was present in the shoots when the plants were transferred to the atrazine-free solution. However, when the changes in radioactivity with time in the three fractions from the roots and shoots were compared, a similar relationship between the fractions was found, suggesting that at least some of the same reactions occur in both tissues (data not given).

Chloroform-Soluble Metabolites of Atrazine. The basis of atrazine resistance in higher plants is due primarily to conversion of the herbicide to nontoxic metabolites (Shimabukuro et al., 1970). The primary types of reactions in the atrazine degradation are well known and include 2-hydroxylation, N-dealkylation, and conjugation. The three possible N-dealkylation products of atrazine are 2-chloro-4-amino-6-isopropylamino-*s*-triazine, 2-chloro-4-amino-6-ethylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine. Along with unchanged atrazine, each of these N-dealkylation products was detected in the chloroform fraction of extracts of *S. alterniflora* by TLC and cochromatography with authentic standards (Figure 3). N-Dealkylation appears to be the principal reaction in the degradation of atrazine by soil fungi (Kaufman and Kearney, 1970) and pea plants (Shimabukuro et al., 1966; Shimabukuro, 1967a) and seems to be a universal reaction in higher plants, animals, and microorganisms (Shimabukuro et al., 1970). The two monodealkylated products of atrazine degradation seem to be most common in higher plants, but the diamino product has been identified in sorghum (Shimabukuro et al., 1973). In smooth cordgrass, atrazine and its N-dealkylation products were accompanied by a polar metabolite(s). The identity of the polar metabolite(s) in the chloroform fraction was not determined, but they may be similar to those reported in the chloroform fraction from sorghum which are intermediates in the conjugation pathway of atrazine metabolism (Shimabukuro et al., 1973).

Change in Atrazine and Its Metabolites with Time. Davis et al. (1959) showed that there was a correlation between the amount of atrazine in exposed plants and susceptibility. After 2-days of continuous exposure to [^{14}C]atrazine, 77.9 and 57.1% of the total radioactivity in the cordgrass roots and shoots, respectively, was present as atrazine (Figure 4). Atrazine in each tissue declined rapidly as shown by the decrease in radioactivity between 2 and 10 days after the initial exposure. After 10 days from the initial exposure to [^{14}C]atrazine, the rate of decline of absorbed radioactive atrazine was similar in the roots and shoots.

As noted above, *S. alterniflora* can be classified as resistant to atrazine. Although differences in absorption and translocation are possible factors that determine the degree of resistance or susceptibility of a plant to atrazine, it is well established that resistance and selectivity are due primarily to the plant's ability to degrade the herbicide to nontoxic substances (Shimabukuro, 1967a; Lamoureux et al., 1970; Robinson and Greene, 1977; Lamoureux et al., 1973). Eight days after transferring the plants to an atrazine-free solution, radioactivity in the chloroform fraction remained nearly constant for 20 days at about 34.5 to 37.3% of the total. The chloroform fraction contains primarily atrazine and its N-dealkylation products which at least partially contribute to detoxification of the herbicide (Shimabukuro, 1967a; Shimabukuro, 1967b). 2-Chloro-4-amino-6-isopropylamino-*s*-triazine is the primary product of N-dealkylation in *S. alterniflora*. Radioactivity in this metabolite extracted from shoot tissue ranged from

5.8 to 20.4% of the total, while the corresponding mono-N-dealkylated product ranged between 2.5 and 12.5% over a 30-day period (Table II). 2-Chloro-4-amino-6-isopropylamino-*s*-triazine was present at the highest levels between 5 and 10 days after the initial herbicide treatment, while the corresponding mono-N-dealkylated product was detected at the highest levels at 20 days. Although the level of each mono-N-dealkylated atrazine product decreased between 20 and 30 days after the initial exposure, it seems that 2-chloro-4-amino-6-isopropylamino-*s*-triazine is the favored substrate for the second N-dealkylated or possibly the conjugation reaction. The diamino atrazine metabolite resulting from the second dealkylation reaction represented a relatively minor component of the chloroform fraction, but did tend to increase with time through the 30-day experiment (Table II).

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Cometabolism of Products of 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) by *Pseudomonas putida*

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Pseudomonas putida, an organism capable of utilizing diphenylmethane and benzhydrol as sole sources of carbon, cometabolized bis(*p*-chlorophenyl)methane (DDM) to *p,p'*-dichlorobenzhydrol (DBH), *p,p'*-dichlorobenzophenone (DBP), benzhydrol, benzophenone, *p*-chlorophenylacetic acid, and *p*-chlorophenylglycolaldehyde. DBH was converted by the resting cells to DBP, benzhydrol, and benzophenone. No products were detected when the bacterium was incubated with DBP. Bis(*p*-chlorophenyl)acetic acid was cometabolized to DDM, DBH, and DBP. The addition of diphenylmethane to the resting cells did not stimulate the cometabolism of DDM, and no new chlorinated products were detected when the bacteria were provided with both substrates. 1,1,1',1'-Tetra(*p*-chlorophenyl)dimethyl ether was not formed microbiologically from either DDM or DBH.

Cometabolism refers to the metabolism of a substance by a microorganism which is unable to use that compound for energy or as a source of any of the elements required for growth (Horvath and Alexander, 1970). Cometabolism has been employed as a technique for isolating the degradation products of many chlorinated molecules, which are otherwise resistant to biodegradation. With the exception of a few reports, extensive degradation of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) has not been reported. Focht and Alexander (1970) demonstrated the extensive transformation of a DDT metabolite in a sequence that involved ring cleavage of bis(*p*-chlorophenyl)methane (DDM), a metabolite generated from DDT. The ring cleavage product was identified as *p*-chlorophenylacetic acid (PCPA). PCPA was also found to be formed by cell-free extracts of *Pseudomonas* sp. (originally classified as *Hydrogenomonas* sp.) incubated first with DDT anaerobically for 4 days and then in the presence of O_2 (Pfaender and Alexander, 1972). Anderson et al. (1970) noted that unidentified water-soluble products were formed from DDT by *Mucor alternans*.

To define more completely the pathways of DDT metabolism, attempts were made to isolate the products formed during the cometabolism of bis(*p*-chlorophenyl)acetic acid (DDA), DDM, *p,p'*-dichlorobenzhydrol (DBH), and *p,p'*-dichlorobenzophenone (DBP) by resting cells of *Pseudomonas putida*. A study was also made of the influence of nonchlorinated analogues on the cometabolism of DDM by *P. putida*.

MATERIALS AND METHODS

Bacterium. The strain of *P. putida* used is capable of utilizing diphenylmethane (DPM) and benzhydrol (BH) as sole sources of carbon. The mineral salts solution used in preparing the growth medium for *P. putida* and for testing the degradability of DDT metabolites was described by Pfaender and Alexander (1973).

Chemicals. The DDT metabolites were obtained from Aldrich Chemical Co., Milwaukee, Wis., except that DDM was purchased from Eastman Organic Chemicals, Rochester, N.Y. 1,1,1',1'-Tetra(*p*-chlorophenyl)dimethyl ether (DCBHE) was synthesized according to the method used for the preparation of its nonchlorinated analogue (Pratt and Draper, 1949).

Respirometry. To obtain resting cells, *P. putida* was

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