United States
Environmental Protection
Agency

Environmental Research
Laboratory
Gulf Breeze FL 32561

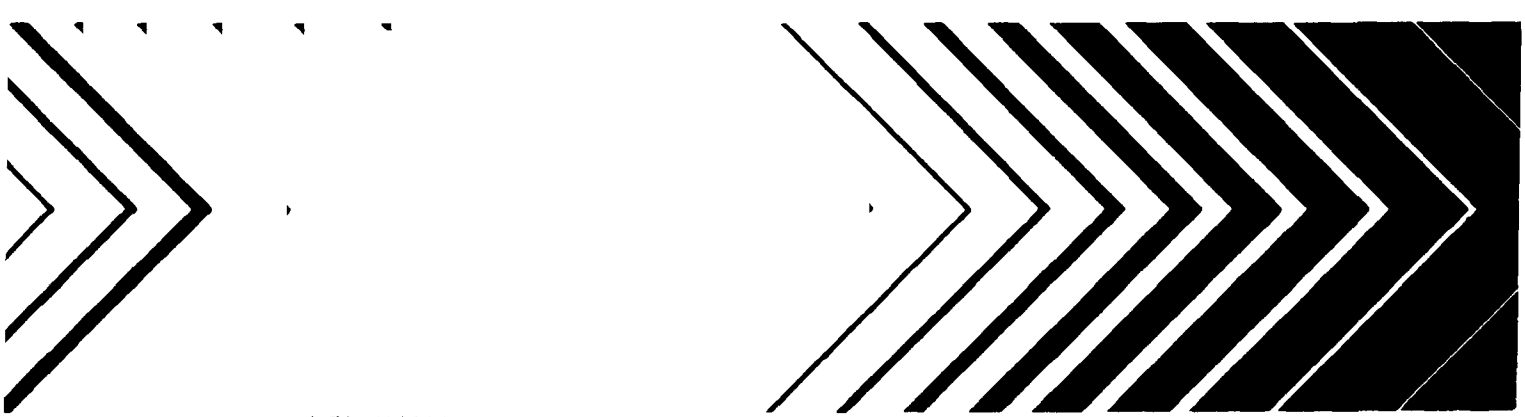
EPA-600/3-79-111
November 1979

Keep Paper Copy

Research and Development

EPA

Atrazine Fate and Effects in a Salt Marsh



RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into nine series. These nine broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The nine series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

This report has been assigned to the ECOLOGICAL RESEARCH series. This series describes research on the effects of pollution on humans, plant and animal species, and materials. Problems are assessed for their long- and short-term influences. Investigations include formation, transport, and pathway studies to determine the fate of pollutants and their effects. This work provides the technical basis for setting standards to minimize undesirable changes in living organisms in the aquatic, terrestrial, and atmospheric environments.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

ATRAZINE FATE AND EFFECTS IN A SALT MARSH

by

Donald E. Davis
J.D. Weete, C.G.P. Pillai, F.G. Plumley
J.T. McEnerney, J.W. Everest, B. Truelove, A.M. Diner
Department of Botany & Microbiology
Alabama Agricultural Experiment Station, Auburn University
Auburn, Alabama 36830

Grant No. R803835

Project Officer

Frank G. Wilkes
Environmental Research Laboratory
U.S. Environmental Protection Agency
Gulf Breeze, Florida 32561

ENVIRONMENTAL RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
GULF BREEZE, FLORIDA 32561

DISCLAIMER

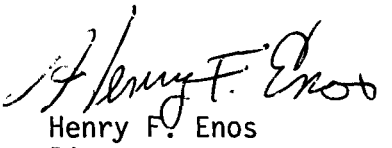
This report has been reviewed by the Environmental Research Laboratory, Gulf Breeze, Florida, U. S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

Atrazine is the herbicide most widely used in the United States. This report describes the fate and effects of this pesticide in salt-marsh ecosystems, both in the field and in laboratory microecosystems. Data such as these are useful in the U.S. Environmental Protection Agency in developing strategies that will minimize the harmful impact of toxic substances on the environment.



Henry F. Enos
Director
Environmental Research Laboratory
Gulf Breeze, FL 32561

ACKNOWLEDGMENTS

We thank the Environmental Protection Agecny for financial support and the project officer, Dr. Frank G. Wilkes, for guidance and understanding of the innumerable problems that invariably arise in an investigation of this magnitude. We also acknowledge the gift of ^{14}C-ring labeled atrazine and N-dealkylated derivates of atrazine by the CIBA-GEIGY Corporation. Some of the atrazine metabolites were identified by Dr. John L. Laseter of the Center for Bioorganic Studies, University of New Orleans, and some of the amino acids were identified by Dr. Paul Melius of the Auburn University Chemistry Department. Ms. Ingrid Kircher and Mr. Craig Weatherby provided technical assistance in the crab studies; and Drs. Ruth Patrick and Francis R. Trainor provided technical advice and training that made the study with the diatoms possible.

CONTENTS

ABSTRACT

A series of experiments were conducted to determine the effect of atrazine on various biological components of a salt marsh ecosystem such as exists along the Georgia coast and the associated offshore islands and the fate of atrazine when introduced into this ecosystem. Investigative procedures involved supplying either formulated atrazine, AAtrex , or ^{14}C-labeled technical atrazine to individual organisms in the laboratory, to groups of organisms in microecosystems of various levels of complexity, and to the salt marsh itself.

Spartina alterniflora Loisel was grown for 2 days with its roots in ^{14}C-labeled atrazine solution (2.6 μ M) followed by 28 days in atrazine-free solution. *S. alterniflora* was moderately resistant to this herbicide. Radioactivity in the chloroform fraction of 80% methanol extract was ca 82, 38, and 24% after 2, 10, and 30 days, respectively. In the aqueous fraction the values were ca 15, 57, and 60%, respectively. The chloroform fraction contained atrazine and three N-dealkylation products. About half of the 14 water-soluble metabolites detected contained fully N-alkylated triazine rings while almost all of the others contained the 4-amino-6-isopropylamino derivative. 2-Hydroxyatrazine and 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine were identified as water-soluble metabolites of atrazine.

Box crabs (*Sesarma cinereum*) were fed for 10 days with leaves from *S. alterniflora* grown for 2 days in ^{14}C-labeled atrazine solution followed by 3 days in atrazine-free solution with no significant effect on crab survival or behavior. At the end of the 10-day feeding period, only 1.2% and 0.5% of the total radioactivity in the crabs and their feces, respectively, was atrazine compared to 24% in the *S. alterniflora* used as a food source.

Leaves from *S. alterniflora* plants grown in solutions containing ^{14}C ring-labeled atrazine was converted to detritus. During the 20-day conversion to detritus, the chloroform fraction of the 80% methanol extract decreased from 55 to 9%. *Uca pugnax* fed this detritus or detritus wetted with ^{14}C-labeled atrazine decreased the percent radioactivity in the chloroform fraction. Thus, atrazine concentration decreased as leaves were converted to detritus and again when the detritus was consumed by *U. pugnax*.

A 10^{-5} M (2.2 ppm) atrazine concentration significantly reduced photosynthesis rate, chlorophyll content, and cell numbers in unialgal cultures of *Nitzschia sigma* Grun. and *Thalassiosira fluviatilis* Hustedt isolated from a salt marsh habitat. Atrazine effects were less in the field than in

microecosystems or cultures. Diatom species diversity was not affected by 10^{-5} M atrazine in microecosystems or in the field, but the number of *Cymatosire belgica* was increased in both situations.

Atrazine was sprayed at 0.0, 0.05, 0.5, and 5.0 g/m^2 on plots on Sapelo Island and on microecosystems. Residue determinations were made 3 months later. Atrazine concentration in the 0 to 1-, 1- to 10-, and 10- to 25-cm layers of soil from plots on Sapelo treated with 5 g/m^2 averaged 1.20, 0.77, and 0.25 ppm, respectively, and together accounted for 3% of that applied. Atrazine concentrations in *S. alterniflora* from plots on Sapelo treated with 5.0 g/m^2 were 21.6 and 12.8 ppm for *S. alterniflora* < 0.5 m and > 0.5 m tall, respectively, and for similarly treated microecosystems were 16.8 and 21.1 ppm, respectively. Periwinkle snails, horse mussels, and fiddler crabs from microecosystems receiving 5.0 g/m^2 contained 7.8, 3.5, and 0.31 ppm atrazine, respectively. Less atrazine was found in animals from Sapelo. Lower rates of atrazine application gave lower residue levels, often too low to be detectable.

This report was submitted in fulfillment of Grant No. R803835 by the Auburn University Agricultural Experiment Station under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period of July 1, 1975, to March 1, 1979.

FIGURES

TABLES

SECTION I

INTRODUCTION

Over the past few years a growing concern has developed regarding the pollution and misuse of our coastal salt marshes. These areas are very important because they provide substantial amounts of food substances and protection for the many organisms that use them as breeding grounds and nurseries (Odum, 1971 a). A variety of energy flow models have been prepared for the *Spartina alterniflora* marshes of Georgia which were the site of these investigations (Odum,1961 and 1971; Teal, 1962). Figure 1 is similar to these proposed models, but it identifies all of the species used in this study. *Spartina alterniflora* is the dominant macrophyte on the 192,508 ha of Georgia coastal salt marshes (Reimold,1977). Net annual primary production of *S. alterniflora* is as high as 3800 g/m^2 (Gallagher et al., 1979) with approximately 90% of the fixed carbon entering the detritus food web (Smalley, 1959). Teal (1962) estimated that only 7% of the *S. alterniflora* productivity was consumed directly by herbivores.

The soils in the *S. alterniflora* marsh are high in organic matter and may be water-saturated for extended periods of time (Cotnoir, 1974); sand, silt, and clay constitute the bulk of the inorganic matter with the percentage of each varying with the position relative to tidal creeks. The pH of the aerobic surface sediments is 7-8 while the anaerobic layers (beneath approximately 2 mm) have pH values as low as 5. The high rate of *S. alterniflora* production is due to lack of competition and adequate nutrient supply from the sediments and flooding tides; only nitrogen limits the growth of *S. alterniflora* in the Georgia salt marshes (Gallagher, 1975). A relatively high rate of mineralization and/or oxidation causes a fast turnover of nutrients (Christian et al., 1978) and some nitrogen is added by nitrogen fixation in the surface sediments (Hanson, 1977).

The second major group of primary producers in these marshes is the surface-inhabiting algae, primarily diatoms, blue greens, and euglenoids (Williams, 1962). The importance of the edaphic algae has been variously estimated (Eaton and Moss, 1966; Gallagher, 1971; Round, 1971; Sullivan and Daiber, 1975; Van Raalte et al., 1974; Williams, 1962). Although the data are incomplete, it now appears that the algae supply a major portion of the utilizable fixed carbon to the estuarine ecosystem (Haines, 1977). The biomass, productivity, and species composition of these algae vary widely between the different zones in the marsh.

Within the marsh, there exist at least four readily distinguishable zones (Teal, 1962) which appear to be delineated by their degree of tidal

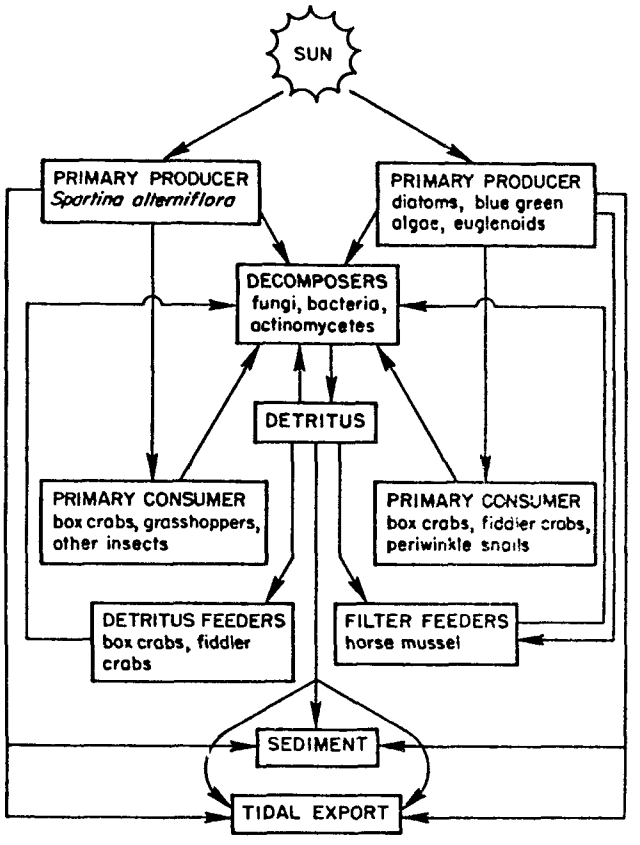


Figure 1. Diagram of energy flow in a Georgia *Spartina alterniflora* salt marsh ecosystem.

exposure. Zone 1, the creek bank zone, with its muddy and/or sandy banks, is devoid of *S. alterniflora* but it is here that the unshaded algae reach their maximum productivity of 245 mg C/m^2/h (Darley et al., 1976). Grazing by the mud snail, *Nassarius obsoletus*, significantly reduces algal productivity in this area (Shimmel, 1979). Immediately adjacent to the creek bank is Zone 2, the 1-3 m wide streamside marsh where *S. alterniflora* reaches its maximum height (> 2m) and productivity (3800 g C/m^2/yr). The shading by *S. alterniflora* reduces algal productivity to approximately 75% of that in Zone 1. The largest individuals of the fiddler crab (*Uca* spp.) population occupy Zones 1 and 2 and graze heavily on the algae and detritus along the creek bank during low tide. Zone 3 consists of the naturally occurring levees bordering the creeks and is occupied by intermediate-height *S. alterniflora*. Due to their higher elevation, the levees are only covered by spring tides and their exposed surface sediments are often dry and cracked. Zone 4, the short *S. alterniflora* marsh, lies directly behind the levees and consists of large flat areas which occupy 40-60% of the total *S. alterniflora* marsh. The productivity of *S. alterniflora* in Zone 4 is relatively low (1300 g C/m^2/yr) (Gallagher et al., 1979). Horse mussels (*Geukensia demissa*) and snails (*Littorina irrorata*) reach their maximum population density in this area and are rare in the others.

All field work for this project was conducted on Cabretta Island, a small strip of land separated from Sapelo Island (31°N Latitude) by Big Hole Creek and Cabretta Creek. The specific zones used for field studies were approximately 50 m WSW of the bridge connecting Cabretta Island with Sapelo Island. Algal studies were conducted in Zone 1 on Cabretta Creek and *S. alterniflora* studies were in Zone 3. Specimens used in laboratory studies were collected from areas adjacent to those used for field studies.

Many pollutants pose potential threats to these salt marshes. Among those of particular concern are oil from oil spills, industrial and domestic sewage, and pesticides. This particular investigation deals with the effect of a very widely used pesticide, atrazine, on various components of the salt marsh ecosystem. Atrazine was selected for use in this study because twice as much atrazine is used as any other single pesticide in the United States. The average amount used yearly is approximately 40 million kg. As much as 2 to 3% of the atrazine may be removed from the soil surface by runoff water (Correll et al., 1978). Atrazine is one member of a very important family of herbicides, the *s*-triazines. It is used at rates of 2 to 4 kg/ha for selective control of broadleaf and some grassy weeds in corn, sugarcane, and sorghum (WSSA Herbicide Handbook Committee, 1979). It is usually applied by ground equipment to the surface of the soil after crop planting and prior to weed emergence. It may sometimes be lightly incorporated into the soil surface and has on occasions been applied from the air (WSSA Herbicide Handbook Committee, 1979).

Atrazine is classified as a photosynthesis inhibitor. More specifically, it inhibits the Hill reaction at the step where a H_2O molecule is split to form O_2 and electrons (Esser et al., 1975). Evidence also suggests that atrazine may affect protein synthesis (Esser et al., 1975). Atrazine is toxic to essentially all broad-leaved plants although some deep-rooted perennial species are unaffected by surface applications because relatively little of the applied herbicide is taken up by their roots (Gunther and Gunther, 1970). (Narrow-leaved annual species such as corn and grain sorghum are able to rapidly convert absorbed atrazine into nontoxic metabolites [Gunther and Gunther, 1970].)

The reported effects of atrazine on animals vary. Rats administered large oral doses of atrazine were relatively unaffected (LD_{50} 3,080 mg/kg) whereas rainbow trout were sensitive to atrazine concentrations of a few ppm (LC_{50} 12.0 ppm) (Pimentell, 1971). Common invertebrates of the salt marsh such as clams, water bugs, mayfly nymphs, common midges, mosquitoes, biting midges, caddice fly larvae, aquatic worms, and brown shrimp have been reported to be reduced in numbers by as much as 50% after applications of atrazine to the soil in concentrations ranging from 0.5 to 2 ppm (Balinke and Bilodub-Pantera, 1964; Pimentell, 1971). However, other invertebrates such as damselfly nymphs and water beetles have doubled their numbers after applications of 1 ppm atrazine (Pimentell, 1971). Since these studies were conducted in the field, it is not known whether atrazine was directly involved or whether the changes in numbers were due to atrazine-induced changes in some of the food sources.

Although considerable information has been gathered about the toxicity and metabolism of atrazine by important crop and some weed species and about its fate in well-drained agricultural soils (Gunther and Gunther, 1970), very little is known about its toxicity to the components of the salt marsh ecosystem or its ultimate fate in the salt marsh.

A series of experiments were performed to expand our knowledge about the fate and effects of atrazine in a salt marsh ecosystem. The major components of this ecosystem are shown in Table 1. As indicated, experiments dealing with the toxicity of atrazine to *S. alterniflora* and the metabolism of atrazine to chloroform-soluble and water-soluble metabolites are reported in Sections 3 and 4 of this paper. Section 5 covers studies of the toxicity of atrazine to the fiddler crab, *Uca pugnax*, and Section 6 the metabolism of atrazine as it passes down the *S. alterniflora*-detritus-*Uca pugnax* food chain. Section 7 reports on studies dealing with the toxicity of atrazine to the

TABLE 1. ATRAZINE EFFECTS INVESTIGATED, WHERE INVESTIGATED, AND WHERE REPORTED

Atrazine effects	Where Investigated	Where Reported
Atrazine toxicity to:		
Smooth cordgrass, *Spartina alterniflora*	Laboratory	Sec. 3
Box crab, *Sesarma cinereum*	Lab. & field	Sec. 7
Fiddler crab, *Uca pugnax*	Lab. & field	Sec. 5
Marine diatoms, *Thallasiosira fluviatilis, Nitzschia sigma, Cymatosira belgica, Melosira sp., Navicula sp.*	Lab. & field	Sec. 8
Atrazine metabolism by:		
Smooth cordgrass, *Spartina alterniflora*	Laboratory	Sec. 3&4
Box crab, *Sesarma cinereum*	Laboratory	Sec. 7
Fiddler crab, *Uca pugnax*	Laboratory	Sec. 6
Detritivores	Laboratory	Sec. 6
Atrazine residues in:		
Smooth cordgrass-detritus-fiddler crab food chain	Laboratory	Sec. 6
Smooth cordgrass; snails, *Littorina irrorata;* horse mussels, *Geukensia demissa;* soil	Field & microecosystems	Sec. 9
Effectiveness of microecosystems	Laboratory	Sec. 10

box crab, *Sesarma cinereum* and metabolism of atrazine by this animal. Section 8 deals with a series of investigations of the toxicity of atrazine to marine diatoms in monocultures, in microecosystems, and in the field and includes an evaluation of the effect of this herbicide on species diversity. The amount of atrazine remaining in various components of the ecosystem after a single application of three different rates of atrazine in the field and to microecosystems in the subject of Section 9. Section 10 summarizes some of the advantages and limitations of the microecosystems used in various aspects of these investigations.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

These studies on the fate and effects of atrazine on the salt marsh have largely dissipated fears of adverse effects on the marsh by atrazine in runoff water from herbicide-treated fields. Concentrations of atrazine present in runoff waters entering estuaries seldom reach parts per billion (ppb). Based on the results of this study, this concentration is far below the concentration needed to adversely affect the conversion of *S. alterniflora* leaves to detritus or to be toxic to box crabs, fiddler crabs, or *S. alterniflora*. The least effect level for diatoms, the most sensitive organisms tested, is approximately 5×10^{-7}M (ca 100 ppb). This is about 50-fold greater than the maximum atrazine concentration expected in runoff water, but much of the atrazine removed from the application site is adsorbed on soil particles. It is conceivable that when such particles are deposited in the marsh that they might adversely affect the growth of diatoms on the soil surface.

Because atrazine was found to be readily metabolized by *S. alterniflora*, box crabs, fiddler crabs, and detritivores, there seems to be little probability that atrazine introduced in the marsh would remain as a problem for more than a very few months unless present in concentrations far in excess of those used for weed control in agricultural crops. This conclusion is substantiated by the finding, that 10 weeks after atrazine was applied to the marsh at rates over 10 times those used for weed control, less than 5% of that applied remained in the marsh. Nearly half of the atrazine that was applied was removed in the tidal water. There was no evidence of bioaccumulation of atrazine by any species tested.

Microecosystems worked well in giving qualitative answers to questions about atrazine toxicity or metabolism. However, the amounts of atrazine residues remaining in various components in the microecosystems were sometimes significantly different from the residues in those same components in the field. Apparently, the major problem of developing a microecosystem that accurately predicts behavior in the field is the difficulty of getting a flux of water onto and off of the microecosystem comparable to that in the marsh. The relatively small areas involved have different flow patterns than occur in a large marsh.

Additional work is needed to develop microecosystems capable of more accurately predicting the amount of a pesticide remaining in various ecosystem components in the field. Research is also needed to determine whether atrazine, in the amounts found adsorbed on soil particles in runoff

SECTION 3

ATRAZINE METABOLISM BY *SPARTINA ALTERNIFLORA:* CHLOROFORM SOLUBLE METABOLITES[1]

OBJECTIVES

Spartina alterniflora, a marsh grass commonly known as smooth cordgrass, is the primary autotroph in the salt marsh under investigation. The objectives of the research presented in this section were: 1) determine the tolerance of *S. alterniflora* to atrazine; 2) describe the metabolism of this herbicide to chloroform-soluble forms, and 3) identify the metabolites of atrazine produced by *S. alterniflora* that may be ingested by consumers of this ecosystem component.

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-liter (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 plant heights recorded. Plants were then transferred to 1-L plastic beakers containing 900 ml of Hoagland's solution with 0, 5 x 10^{-8}, 5 x 10^{-7}, 5 x 10^{-6}, or 5 x 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 10 groups of four plants each. Each group of plants was

---

[1]Most of the material in this section is reprinted from the Journal of Agricultural and Food Chemistry 25:852-855. Copyright 1977, American Chemical Society. Used by permission.

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 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 with atrazine concentrations of 5 x 10^{-5} and 5 x 10^{-6} M when compared with the control (Table 2). There was an average of 37% reduction of the growth parameters measured at the most concentrated atrazine solution used (5 x 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 x 10^{-7} M. Only root dry weight was significantly less than the control at 5 x 10^{-8} M concentration.

TABLE 2. EFFECT OF ATRAZINE ON GROWTH OF *SPARTINA ALTERNIFLORA* 45 DAYS AFTER TREATMENT*

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 a	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

*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.

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 adsorbed 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 2). 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

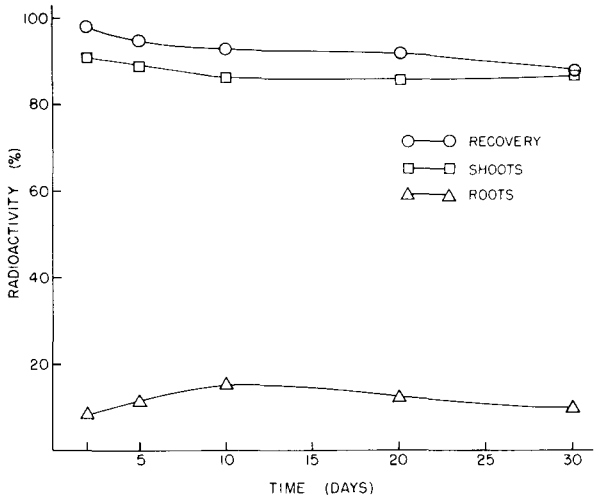


Figure 2.

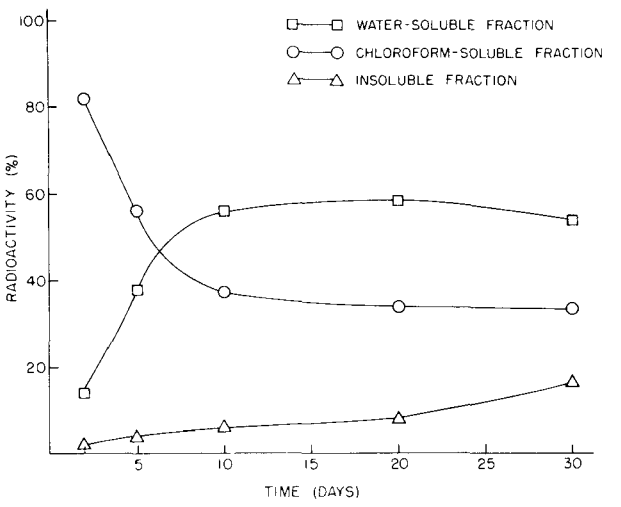


Figure 3.

Figure 2. 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 3. 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.

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 3). 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 al., 1971; Lamoureux et al., 1972). Conversion of atrazine to water-soluble metabolites by *S. alterniflora* is slower than in sorghum. After 2 days of 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 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, the changes in radioactivity with time in the three fractions from the roots and shoots were compared, and 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 4). 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 products 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 5). 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

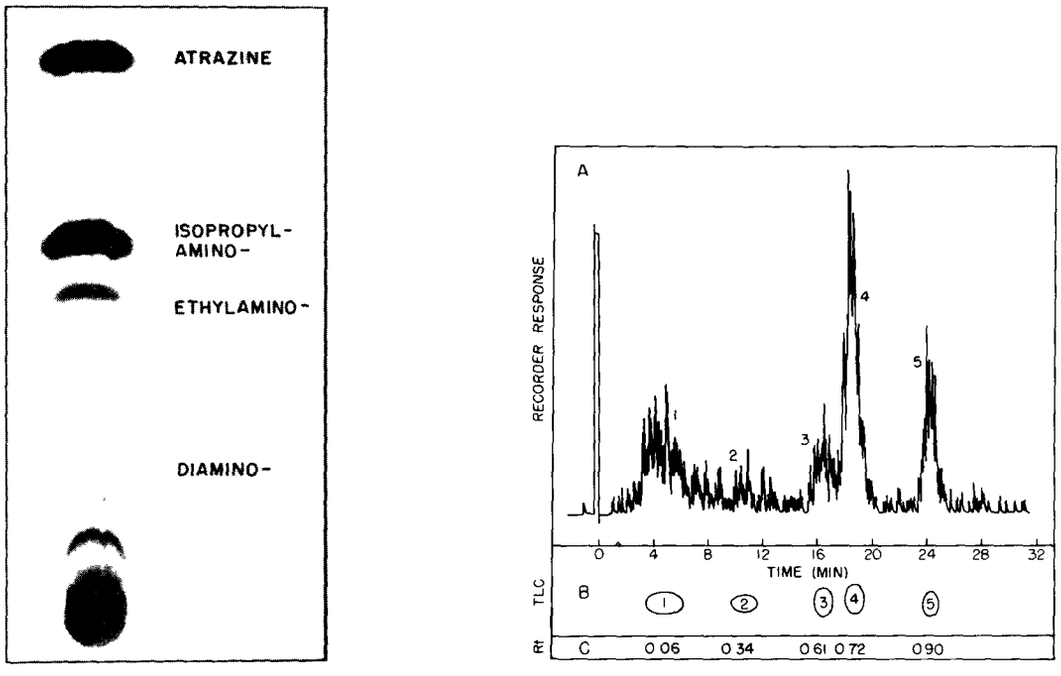


Figure 4. Left, an autoradiograph of a TLC plate showing radioactive components present in the chloroform fraction of an 80% methanol extract of *Spartina alterniflora* treated with [^{14}C]-atrazine for 2 days and then transferred to a herbicide-free nutrient solution. Right, (A) radiochromatogram of the chloroform fraction, (B) diagram of TLC autoradiograph shown on the left, and (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 (atrazine). The TLC plate was developed two times in benzene-acetic acid (50:4, v/v).

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 3). 2-Chloro-4-amino-6-isopropylamino-*s*-triazine was present

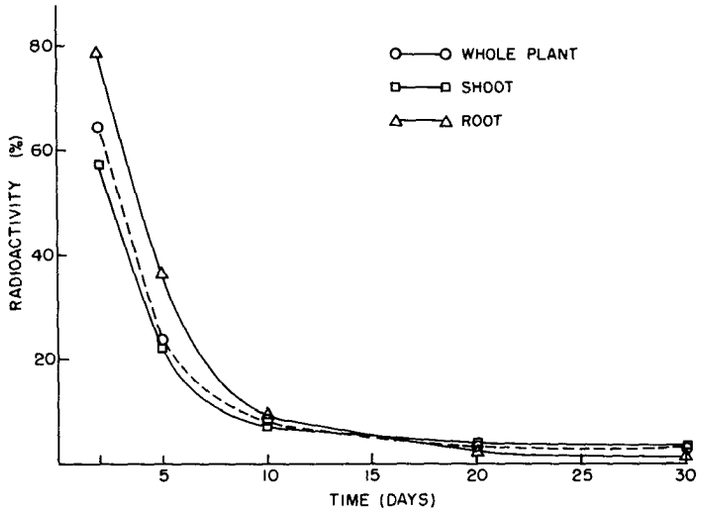


Figure 5. Change in radioactivity with time in atrazine extracted from shoots and roots of *Spartina alterniflora* treated for 2 days with [^{14}C]-atrazine and subsequently transferred to an atrazine-free nutrient solution for 28 days.

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 3).

TABLE 3. RADIOACTIVITY IN N-DEALKYLATED PRODUCTS OF ATRAZINE METABOLISM ISOLATED FROM SHOOTS OF *SPARTINA ALTERNIFLORA* COLLECTED AT INTERVALS OVER A 30-DAY PERIOD

Days after treatment	Radioactivity, % of total		
	I*	II†	III‡
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	5.1	8.7

* I, 2-chloro-4-amino-6-isopropylamino-*s*-triazine.

† II, 2-chloro-4-amino-6-ethylamino-*s*-triazine.

‡ III, 2-chloro-4,6-diamino-*s*-triazine.

SUMMARY

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.

SECTION 4

ATRAZINE METABOLISM BY *SPARTINA ALTERNIFLORA:* WATER-SOLUBLE METABOLITES

OBJECTIVES

Research conducted in Section 3 established that *Spartina Alterniflora* rapidly converted the absorbed atrazine to water soluble metabolites. The objectives of Section 4 were: 1) to identify the metabolites produced and 2) to determine the pathway(s) followed in the metabolism of atrazine by *S. alterniflora*.

MATERIALS AND METHODS

Growth and Treatment of Plants

S. alterniflora plants were collected from the salt marsh at Sapelo Island, Ga., and maintained at Auburn University as described previously (Section 3). In time course experiments, a uniform lot of vigorously growing plants was divided into 10 groups of four plants each. Each group of plants was placed in 300 ml of Hoagland's solution containing a 2.0 µCi of [^{14}C]-atrazine (24.9 µCi/mg). After 2 days, the plants were removed from the [^{14}C]-atrazine solution, the roots 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 transfer to the atrazine-free nutrient solution were extracted as described previously (Section 3). Briefly, the plants were separated into roots and shoots which were ground separately and extracted with 80% methanol. The extract was filtered and the filtrate was concentrated using a rotary flash evaporator. The plant material remaining after filtration is referred to as the 80% methanol-insoluble fraction. The concentrated extract was diluted with distilled water and washed three times with chloroform. The combined chloroform fractions were taken to dryness. The aqueous fraction was also concentrated and the radioactive components isolated as described below.

Isolation of Water-Soluble Atrazine Metabolites

Plants used specifically for the isolation of water-soluble metabolites of atrazine were selected and placed in 1-L plastic cups, five plants per

[2]Most of the material in this section has been submitted to the Journal of Agriculture and Food Chemistry for consideration for publication. Copyright 1979, American Chemical Society. Printed by permission.

cup, with their roots in 400 ml of Hoagland's solution. Each cup had 2.0 μCi [^{14}C]-atrazine and sufficient nonlabeled atrazine to give a 5 x 10^{-5} M atrazine concentration. The plants were maintained in these cups for 2 days after which the roots were rinsed with nonlabeled atrazine solution and placed in a fresh atrazine-free Hoagland's solution for 18 days.

Approximately 1 kg of fresh shoot tissue from these plants was ground, in 10-g portions, 3-min in a Waring Blender containing 80% aqueous methanol (10 ml solvent/1 g tissue). After each grinding step, the extract was filtered and the residue was placed in the extracting solvent for 1-2 days at 4 C until all the material was ground. The grinding, soaking, and filtering steps for all the tissue was repeated two times. The combined extracts were concentrated by flash evaporation at 37 C, dissolved in 400 ml water, and washed with four 500-ml volumes of chloroform. Portions of the aqueous fraction containing about 10^7 dpm were concentrated as before and dried by lyophilization. The dried sample was dissolved in 50 ml of pyridine-acetate buffer (pH 2.15) and applied to 2.5 x 95 cm column of AG 50W-X2 (200-400 mesh) cation-exchange resin. The resin and buffers were prepared as described by Schroeder et al. (1962). The column was maintained at 15 C while being washed with pyridine-acetate buffer gradient at 0.6 ml/min. The first chamber of the gradient device contained 400 ml of 0.2 N buffer (pH 3.1), and the second and third chambers each contained 400 ml of the same buffer solution (2N) at pH 5.0. Five-ml fractions were collected during each column chromatography step. Adjacent fractions containing no radioactivity were combined. Five major radioactive fractions were obtained from the cation-exchange column; each was evaporated to dryness by flash evaporation at 37 C, dissolved in minimum volume of water, and applied to a separate 2.5 x 95-cm column of AG 1x2 (200-400 mesh, acetate form) anion-exchange resin, also maintained at 15 C. The column was eluted with an acetic acid concentration gradient at a flow rate of 0.4 ml/min. The gradient device contained 275-ml of water in each of the first two chambers, 275-ml of 0.5 N acetic acid in chamber 3 and 275-ml of 0.38 N acetic acid in chamber 4. The column was subsequently washed with 150-ml of 0.5 N acetic acid followed by 150-ml/fractions each of 1 to 6 N acetic acid. Radioactive fractions from this column were obtained as before and evaporated to dryness by flash evaporation. The residue was washed from the flask with one 5-ml portion and two 1-ml portions of absolute methanol which was then evaporated to dryness under N_2. The residue was then dissolved in few ml of water and placed on a 1.5 x 95-cm column of Sephadex LH-20 which was washed with water at the rate of 0.5 ml/min at 15 C. Each radioactive fraction from this column was purified by TLC using silica gel G (250 μm thickness) on 20 x 20 cm glass plates. The TLC plates were developed three times in benzene-ethyl acetate-acetic acid-water (25:50:30:3, v/v/v/v) (Solvent System I), and radioactive areas were located with a Berthold TLC Scanner. The silica gel spots containing radioactivity were removed from the plate and washed five times with methanol. The radioactive substances were then applied to silica gel plates which were developed one time in n-butanol-acetic acid-water (60:15:25, v/v/v) (Solvent System II). In most cases, further purification of the radioactive substance was by dialysis followed by TLC, using Solvent System II. A schematic outline of the column and thin-layer chromatographic procedures is given in Figure 6.

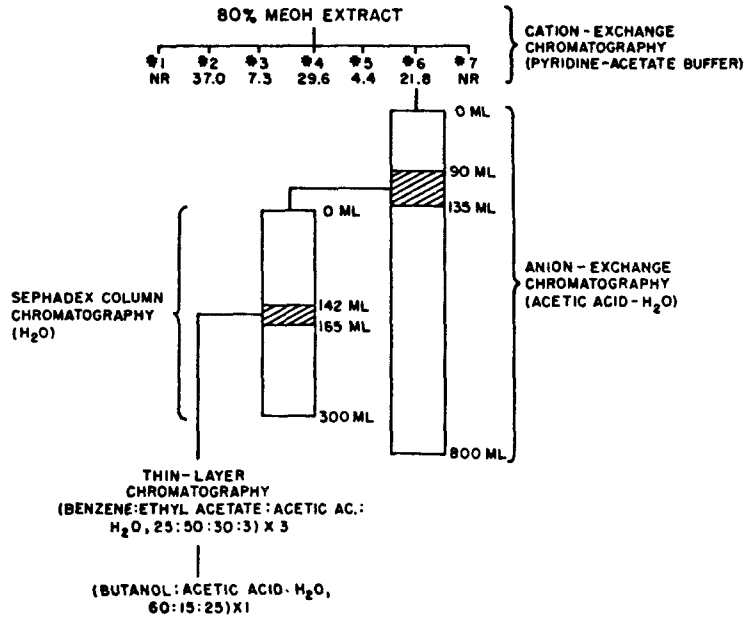


Figure 6. Outline of procedures used in the isolation and purification of water-soluble metabolites of atrazine from *S. alterniflora*. The example is given for fraction #6 from the cation-exchange column, but each fraction was treated in a similar manner.

Characterization of Metabolites

For amino acid analysis, a portion of each purified metabolite was hydrolyzed under nitrogen in 200 µl of 6 N HCl in a sealed ampule under nitrogen at 110 C for 16 h. Each hydrolysate was diluted with distilled water and evaporated to dryness several times under vacuum to remove the HCl and then treated for 2 h at room temperature with a mixture of 200 µl of formic acid and 20 µl of 30% hydrogen peroxide (Lamoureux et al., 1973). Amino acids were analyzed using a Beckman Amino Acid Analyser.

For analysis of the triazine portion of the purified substances from the aqueous fraction of the 80% methanol extract, a portion of each substance was hydrolyzed under nitrogen in 200 µl of 6 N HCl at 50 C for 8 h. Hydroxy derivatives of atrazine and its metabolites were obtained. The hydrolysates were compared by TLC using silica gel HF_{254} (250 µm thickness) with standards of authentic hydroxy-atrazine and hydroxy derivatives of N-dealkylated products of atrazine,using Solvent System II and 2-propanol-28% ammonium hydroxide-water (80:10:10, v/v/v) (Solvent System III).

Derivatization for Mass Spectral Analysis

A portion of each isolated metabolite was suspended in 2.0 ml of absolute

methanol and treated with diazomethane as described by Schlenk and Gellerman (1960). After methylation, the product was dried under nitrogen and purified by TLC (Solvent System II). The major radioactive derivatives were washed from the silica gel with absolute methanol and analyzed by mass spectrometry.

RESULTS AND DISCUSSION

Translocation and Metabolism of Atrazine

Atrazine was readily absorbed and translocated by *S. alterniflora*. After 2 days of continuous exposure to the radiolabeled herbicide, approximately 90% of the absorbed atrazine was present in the shoots (Section 3). The extracts of *S. alterniflora* shoots were separated into chloroform, aqueous, and 80% methanol-insoluble fractions. The chloroform fraction had approximately 80% of the radioactivity after the initial 2-day exposure to radioactive herbicide (Figure 7). Radioactivity in this fraction declined rapidly during the first 5-6 days, followed by a slower decrease. There was a corresponding increase in radioactivity of the aqueous fraction, suggesting a precursor-product relation between components of the chloroform and aqueous fractions.

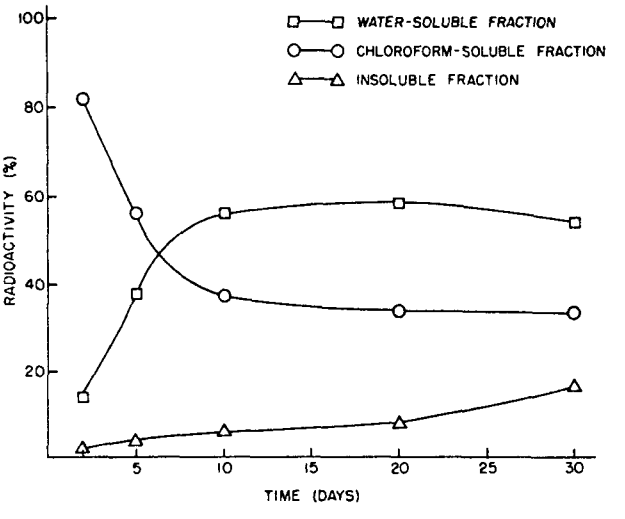


Figure 7. Change in radioactivity with time in the aqueous, chloroform, and insoluble fractions of an 80% methanol extract of shoots from *S. alterniflora* plants grown in [^{14}C]-atrazine for 2 days and then transferred to an atrazine-free nutrient solution for 28 days. (This figure is also shown in Section 3 but is reproduced again here for easier access.)

In smooth cordgrass, atrazine and its N-dealkylation products of the chloroform phase were accompanied by relatively polar metabolites (Section 3). The amount of radioactivity in these polar metabolites increased slowly with time, ranging from approximately 2% in plants treated for 2 days to about 11% in *S. alterniflora* 28 days after transfer to the atrazine-free solution. Similar metabolites were reported in the chloroform fraction from sorghum. The chloroform-soluble atrazine metabolites in both sorghum and *S. alterniflora* appear to be minor metabolites of the herbicide and do not accumulate rapidly with time. Perhaps they are transitory intermediates that are converted to water-soluble atrazine metabolites (Shimabukuro et al., 1973).

Radioactivity in the 80% methanol-insoluble fraction of the *S. alterniflora* extract also increased slowly with time, reaching about 18% of the total after 28 days in the atrazine-free nutrient solution (Figure 7). An increase in radioactivity in the insoluble fraction of sorghum extracts occurred with time but at a higher rate than in *S. alterniflora* (Lamoureux et al., 1973), reaching ca 30% by the end of the experimental period. The chemical form of the 80% methanol-insoluble metabolites of atrazine are unknown.

Water-soluble Metabolites of Atrazine

Five to 8 days after exposure of *Spartina* to [^{14}C]-atrazine, all but about 3% of the herbicide was metabolized to either chloroform or water-soluble substances, which appear to persist for a considerable length of time (Figure 7). For example, after 28 days in atrazine-free nutrient solution, radioactivity in the aqueous extract of [^{14}C]-atrazine-treated *S. alterniflora* represented ca 58% of the total. The resistance of this marsh grass to atrazine is attributed to the rapid metabolism of the herbicide to nontoxic substances (Section 3). Atrazine metabolism follows a similar pattern to that in sorghum, which is also very resistant to the herbicide but it is slower in *S. alterniflora* (Lamoureux et al., 1973). For example, extracts of sorghum plants treated as described for *S. alterniflora* contained over 60% of the radioactivity in the aqueous fraction at the end of the 2-day exposure to the herbicide. Radioactivity increased slightly thereafter but decreased after 20 days in the atrazine-free solution.

After passing the aqueous fraction of the *S. alterniflora* extract through a series of cation- and anion-exchange and Sephadex columns, it was apparent that there are numerous water-soluble metabolites of atrazine most of which appear to have a high turnover rate during the 28 days after exposure to the herbicide. Five radioactive fractions (designated #2 through #6) were obtained by passing the aqueous fraction through a cation-exchange column (Figure 8), and 79% of the radioactivity placed on the column was recovered. There was a shift from polar to less polar substances during the first 8 days after transfer of the plants to the atrazine-free nutrient solution. Fifty-two to 60% of the radioactivity accumulated in fraction 2 (fraction 1 contained no radioactivity) after the first 8 days, and changes in the relative proportions of radioactivity in other fractions occurred during the final 20 days of the experiment.

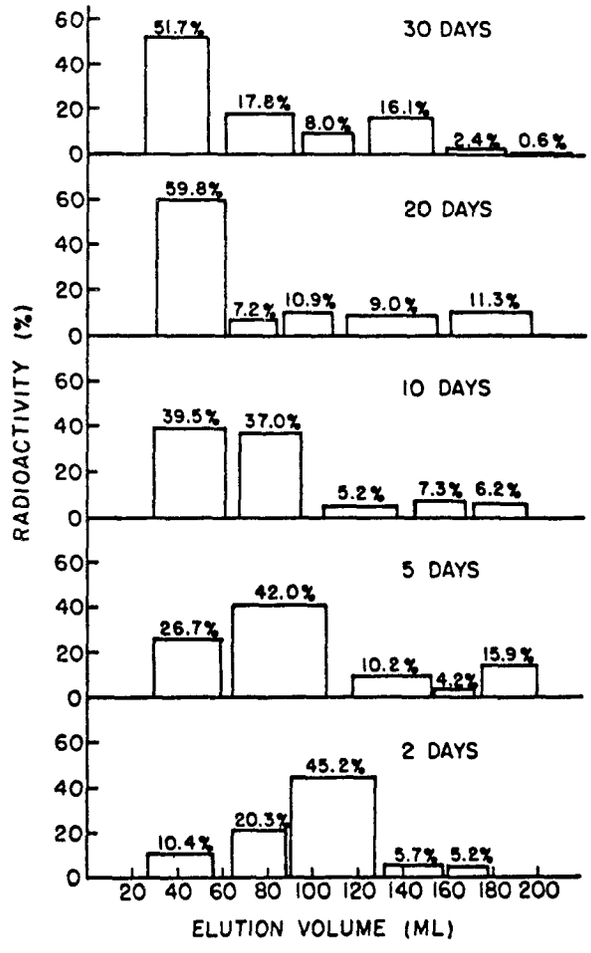


Figure 8. Radioactive fractions of the aqueous fraction of the *S. alterniflora* extract passed through a cation-exchange (50W-X2 Aminex resin, 200-400 mesh) column. Samples were taken at 0, 3, 8, 18, and 28 days after a 2-day exposure to [^{14}C]-atrazine. Values at the top of each bar are the percentages of radioactivity in the particular fraction and are averages of two replications. Each replication consisted of four plants.

Chromatographic treatment of fractions from the cation-exchange column resulted in the isolation of 16 individual water-soluble radioactive substances. To simplify discussion of the isolation and purification of the atrazine metabolites, each radioactive fraction from the cation-exchange column was assigned a number 2 through 6 as indicated above and each radioactive fraction from the anion-exchange column was given a letter. For example, fraction 2C represents the third radioactive fraction from the anion-exchange column when fraction 2 of the cation-exchange column was chromatographed on the former column. Upon isolation, individual atrazine metabolites were arbitrarily given a number (Table 4).

TABLE 4. SUMMARY OF RESULTS OF THE ISOLATION OF SOME WATER-SOLUBLE METABOLITES OF ATRAZINE FROM *S. ALTERNIFLORA* SHOOTS

Number & letter designation*	Radioactivity (%) of		Compound
	Fraction from anion-exchange column†	Total aqueous fraction‡	
2A	30.7	11.9	1
2B§	5.3	2.0	-
2C	24.6	9.5	2,3,3a
2D	8.6	3.4	10
2E	15.1	5.8	4#
2F§	6.8	2.6	-
2G§	8.7	3.4	-
3A	100	7.6	12
4A	28	8.6	13**
4B	72	22.3	14,14a,15,15a,16
5A	100	4.7	17
6A	100	22.8	8,8a

* The numeral indicates the cation-exchange column fraction number. Fraction 1 contained no radioactivity. The letter designates the fraction(s) resulting when the cation-exchange column fraction was chromatographed on the anion-exchange column.

† Values are percentages of the radioactivity in the parent fraction from the cation exchange column.

‡ Values are percentages of the aqueous fraction (ca 3.5×10^6cpm) of the 80% methanol extract.

§ These fractions were not processed further; #2-Hydroxyatrazine; **2-Hydroxy-4-amino-6-isopropylamino-*s*-triazine.

Passage of each cation-exchange column fraction separately through an anion-exchange column achieved further separation of the atrazine metabolites (Figure 9). Each radioactive fraction from the anion-exchange column was subsequently passed through a Sephadex LH-20 column, but little separation of radioactive substances was achieved. This procedure only aided in the purification process by removing some nonradioactive substances. Fractions from the anion-exchange column containing relatively small amounts of radioactivity (2B, 2F, and 2G) were not processed further. Each radioactive component was further purified (or separated) by TLC, using two solvent systems. A summary of the results obtained by these chromatographic procedures is given in Table 4. TLC in two solvent systems indicated that fractions 2A, 2D, 2E, 3A, 4A, and 5A from the anion-exchange columns contained only a

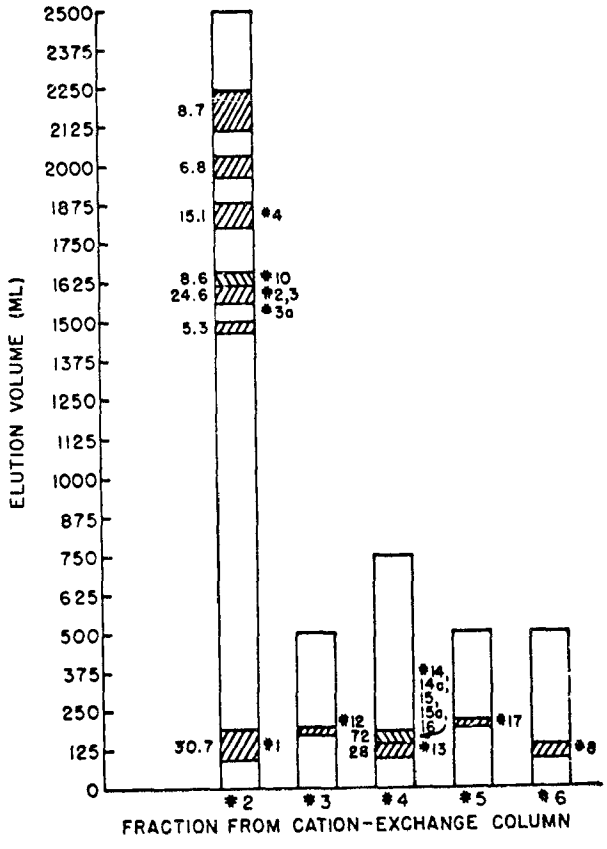


Figure 9. Separation of components in each fraction from the cation-exchange column by anion-exchange chromatography (AG-1 x 2, 200-400 mesh). Values on the left of each bar represent radioactivity in the fractions from the column, and values on the right designate the particular atrazine metabolite isolated from that fraction by TLC.

single radioactive component. 2-Hydroxyatrazine (#4) was identified in fraction 2E and 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine (#13) was identified in fraction 4A (Table 4); together they represented about 14% of radioactivity extracted from the *S. alterniflora* shoots. Hydrolysis appears to be the principal mode of atrazine detoxification in some resistant plants but seems to be limited to benzoxazinone-containing species such as corn (Shimabukuro, 1967). Benzoxazinone is a naturally occurring catalyst of atrazine hydrolysis (Hamilton et al., 1962). Several resistant and intermediately susceptible species do not produce the 2-hydroxy derivatives of atrazine (Shimabukuro et al., 1971). Whether dealkylation occurs before or after the hydrolysis of atrazine in *S. alterniflora* is not known, but apparently both alternatives occur in some plants.

The remaining fractions from the anion-exchange column (2C, 4B, and 6A) contained 2 to 5 individual radioactive substances, none of which cochromatographed with the remaining 2-hydroxy derivatives of atrazine,

2-hydroxy-4-amino- 6-ethylamino-*s*-triazine and 2-hydroxy-4,6-diamino-*s*-triazine (Table 4).

Based on the previous research on atrazine metabolism in sorghum (Shimabukuro et al., 1971), it was suspected that the remaining water-soluble metabolites were intermediates in the glutathione conjugation pathway. With the exception of compounds #4 and #13, each radioactive water-soluble substance isolated was subjected to acid hydrolysis so that the chemical nature of the *s*-triazine ring and possibly the conjugate groups might be determined. The results of hydrolysis showed that the conjugated water-soluble metabolites were nearly equally divided between derivatives of atrazine and 2-chloro-4-amino-6-isopropylamino-*s*-triazine (Table 5). 2-Hydroxy-4-amino-6 -ethylamino -*s*-triazine was obtained on hydrolysis of only compound #15a, and no 2-hydroxy-4,6-diamino-*s*-triazine was detected in any of the hydrolysates.

TABLE 5. CHEMICAL NATURE OF THE *S*-TRIAZINE RING IN THE INDIVIDUAL RADIOACTIVE COMPONENTS FROM THE AQUEOUS FRACTION OF THE 80% METHANOL EXTRACT OF *S. ALTERNIFLORA* GROWN IN ^{14}C-LABELED ATRAZINE SOLUTION

S-Triazine	Water-soluble compounds isolated
2-hydroxy-atrazine	1, 2, 4, 8a, 10, 16
2-hydroxy-4-amino-6-isopropylamino-*s*-triazine	3, 3a, 8, 14, 14a, 15, 17
2-hydroxy-4-amino-6-ethylamino-*s*-triazine	15a
2-hydroxy-4,6-diamino-*s*-triazine	--

Amino acid analysis of the hydrolysates did not reveal much about the chemical nature of the conjugate portion of the water-soluble atrazine metabolites. In each case, the usual protein amino acids were detected in very low concentrations (< 5 nmoles), suggesting that the samples may have contained small amounts of protein. However, in some cases, a particular amino acid was present in higher relative abundances than the others and was possibly part of the conjugate material. The hydrolysate of compound #2, for example, contained high relative proportions of glutamic acid, glycine, and an unidentified substance. Since glutathione is composed of these two amino acids and cysteine, the results suggest that glutathione conjugation occurs in *Spartina* as in other resistant plants (Shimabukuro

et al., 1971). Compounds #8, #8a, #14, #16, and possibly #3a contained glycine in higher relative proportions than "background" amino acids, suggesting that they may also be intermediates in glutathione conjugation as in sorghum. A conjugated intermediate containing glycine would be expected to occur early in the pathway (Lamoureux et al., 1973). Compound #14a contained valine, the significance of which is not clear. Amino acids at levels above background were not detected in the other samples.

Fraction 6A appeared to be a single radioactive substance through each of the column and first thin-layer chromatographic steps (Figure 6). However, TLC of fraction 6A using solvent system II showed that it was an equal mixture of two substances (Figure 10). Based on the hydrolysis data, they differ by the chemical nature of the *s*-triazine, #8 being a derivative of atrazine and #8a a derivative of 2-chloro-4-amino-6-isopropylamino-*s*-triazine. Compounds #3, #14, and #15 appeared chromatographically pure according to Solvent System I. As before, compounds #15 and #15a differed according to the *s*-triazines, being derivatives of 2-chloro-4-amino-6-isopropylamino and 2-chloro-4-amino-6-ethylamino-*s*-triazine, respectively. The other two pairs of radioactive substances did not differ according to the triazine portion of the molecule, but did differ in the amino acid portion of the module obtained on hydrolysis. For example, compounds #14 and #14a differed by glycine and valine, respectively. The basis on which compounds #3 and #3a were separated by TLC is not clear, (Table 5).

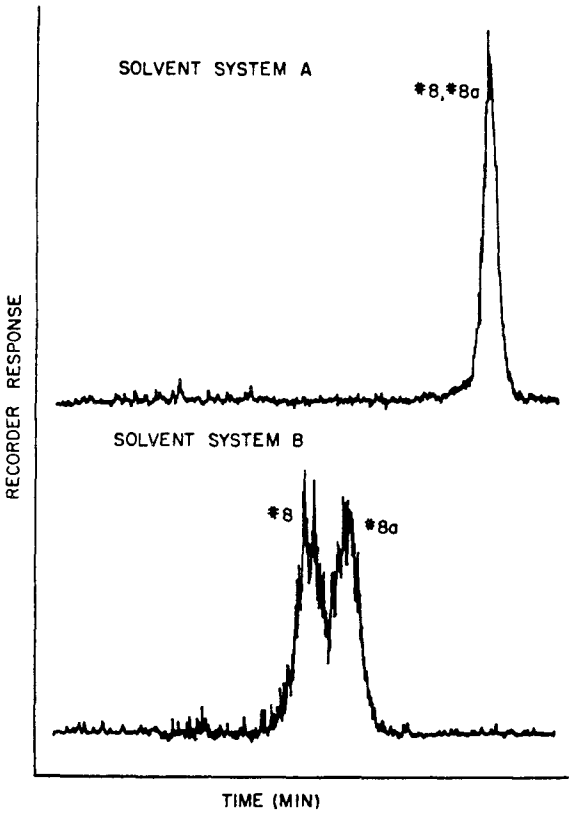


Figure 10. TLC of fraction 6A from the anion-exchange column.

As in sorghum (Shimabukuro et al., 1971; Lamoureux et al., 1973), atrazine is metabolized to a large number of substances in *S. alterniflora*. The atrazine molecule may be simply hydrolyzed; it may be degraded through dealkylation or both. However, the process leading to the largest number of atrazine metabolites appears to be initiated by conjugation followed by extensive chemical modification of the non-*s*-triazine portion of the molecule. The *s*-triazine portion of atrazine shuttled through the conjugation pathway was present in numerous compounds, 16 of which were isolated and partially characterized. The predominant chemical forms of the *s*-triazine ring among these metabolites were the fully alkylated and 4-amino-6-isopropylamino forms (Table 5). Although collectively the water-soluble substances (conjugation plus 2-hydroxy) represent the greatest number of atrazine metabolites in *Spartina*, nonconjugated chloroform-soluble metabolites represent the quantitatively most important chemical species. For example, after 20 days exposure to atrazine, ca 25% of the radioactivity from [^{14}C] atrazine in *Spartina* shoots was represented by only 3 compounds: 2-chloro-4-amino-6-isopropylamino-*s*-triazine, 2-chloro-4-amino-6-ethylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine (Section 3). This combined with the ca 14% of the total radioactivity represented by 2-hydroxy-atrazine and 2-hydroxy-4-amino-6-iospropylamino-*s*-triazine amounts to ca 39% of the atrazine metabolites represented by only 5 substances. The extent to which these substances are conjugated after 30 days in the plant is not known. The final form of a large portion of the *s*-triazine ring appears to be 80% methanol-insoluble as suggested by extrapolation in Figure 7. The material appears to be tightly linked to the structural components of the cells. Accumulation of atrazine metabolites in this fraction of *S. alterniflora* is considerably slower than in sorghum (Lamoureux et al., 1973). How incorporation of the atrazine metabolites into this insoluble form relates to persistence in the environment is not known.

SUMMARY

Spartina alterniflora plants were incubated 2 days in half-strength Hoagland's solution containing 2.0 µCi ring-labeled atrazine and then transferred to an atrazine-free nutrient solution. Samples were taken 3, 8, 18, and 28 days after transfer and the change in radioactivity in the water-soluble atrazine metabolites as a function of time was determined. After 2 days, ca 90% of the radioactivity was present in the shoots. Radioactivity in the aqueous fraction of *S. alterniflora* shoot extracts increased for ca 8 days, after which the radioactivity remained relatively constant representing ca 60% of the total radioactivity taken up by the plants.

The aqueous extract was fractionated using cation-and anion-exchange and sephadex column chromatography followed by thin-layer chromatography using two solvent systems. Some of the water-soluble metabolites of atrazine were isolated and partially characterized. About half the water-soluble metabolites contain fully N-alkylated triazine rings while the other half contained the 4-amino-6-isopropylamino derivative. Only one contained the 4-amino-6-ethylamino derivative and no 4,6-diamino forms were detected. 2-Hydroxyatrazine and 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine were identified as water-soluble metabolites of atrazine. Acid hydrolysates of

the isolated metabolites contained low amounts of amino acids such as glutamic acid, glycine, and valine, suggesting the glutathione conjugation pathway of atrazine detoxification may be operative in *S. alterniflora*.

Atrazine is metabolized to a large number of substances by *S. alterniflora* some of which tend to slowly accumulate in the insoluble plant residue with time.

SECTION 5

EFFECT OF ATRAZINE ON *UCA PUGNAX*[3]

OBJECTIVES

The most conspicuous detritivore in the salt marsh is the fiddler crab, *Uca pugnax*. The objectives of the research in Section 5 were to determine: 1) whether AAtrex was toxic to fiddler crabs and whether the toxicity was due only to the active ingredient, atrazine; 2) the effect of size and sex of the crab on atrazine toxicity; 3) whether the presence of marsh soil affected toxicity; and 4) whether there were seasonal variations in sensitivity of the crabs to atrazine.

MATERIALS AND METHODS

Atrazine Studies in the Field and in Microecosystems

In these studies the effect of atrazine on fiddler crabs in a natural salt marsh (field studies) were compared with those in microecosystems (microecosystem studies). Both experiments were started in the middle of June 1977 and terminated 10 weeks later.

Field Studies--

Twelve 1.8-m diameter 0.75-m tall metal cylinders were sunk 15 cm deep in a uniform stand of *Spartina alterniflora* in a salt marsh near Sapelo Island, Ga. The cylinders minimized the movement of fiddler crabs into and out of these enclosures. Holes were cut in each cylinder at soil level and then the holes covered with 0.6-cm mesh hardware cloth. The holes permitted tide water to flow into and out of the enclosures, and the hardware cloth prevented movement through the holes of all except the very small crabs. The areas enclosed by cylinders were selected for uniformity of *S. alterniflora* stands and depth of flooding by the tides. At a time appropriate for comparison with the microecosystem studies, three randomly selected enclosures were sprayed with 0, 100, 1,000, or 10,000 ppm atrazine by applying the appropriate concentrations of the 80% wettable powder, AAtrex. The amount of spray solution applied was sufficient to give 0, 0.05, 0.5, and 5 g atrazine/m^2. The herbicide was applied at a period of low tides and when no flooding would be expected for at least 6 h after herbicide applications.

[3]Most of the material in this section is reprinted from a paper accepted for publication in Estuaries and is reproduced here with their permission.

Daily records were made on the appearance and general well being of plants and animals in each of the treated areas and outside the enclosures. Daily records were also made of the depths and durations of flooding and the amounts of rainfall. Ten weeks after treatment, crab populations were estimated using the method of Wolf et al. (1975).

Microecosystem Studies--

Each microecosystem used in these studies was constructed from 75-L molded plastic, laundry tubs (described and illustrated in Section 10). Each resultant container was 51 cm long by 51 cm wide by 97 cm deep and had a drain hole in the bottom. A 45-cm long 2.5-cm-diam plastic pipe, perforated with 3-mm diam holes every 2 or 3 cm, was inserted through the drain hole upward into the container. It made possible movement of water into and out of the system but prevented any larger animals from escaping through this port. Twelve of these containers were taken to the salt marsh for filling. Approximately 25- by 25- by 20-cm-deep sections of soil on which a dense stand of *S. alterniflora* was growing were removed and fitted into the bottom of the ecosystem containers. The spaces between the sections were filled with soil also taken from the marsh. In securing these *S. alterniflora*-bearing soil sections, the associated algae, soil microorganisms, and blood worms were also automatically transferred into the container. The larger animal components, fiddler crabs *(Uca pugnax)* horse mussels *(Geukensia demissa)*, and periwinkle snails *(Littorina irrorata)* were collected separately. The ecosystem containers and animals were then transported back to the greenhouse at Auburn, AL, for final assembly. Each ecosystem was attached to a 75-L seawater reservoir that was raised and lowered by a "tide machine" as described by Everest and Davis (1977). Diagrams and additional details about these systems are presented in Section 10.

As soon as the 12 systems were coupled with their reservoirs and tides initiated, ca 15 randomly selected fiddler crabs were added to each system giving a fiddler crab density comparable to that in the field. Additional small crabs, both *Uca pugnax* and *Sesarma cinereum*, may have been present in the soil placed in the ecosystems. After all components had been added, the systems had stabilized, and the *S. alterniflora* was growing well, word was sent to Sapelo Island to spray atrazine at the prescribed rates on the 12 plots on the Island. One week after the herbicide was applied at Sapelo Island, equal rates were applied to randomly selected microecosystems at Auburn. Tidal frequency and depth and rainfall amounts were duplicated at Auburn 1 week after they occurred at Sapelo Island. Ten weeks after herbicide application, all fiddler crabs in each microecosystem were collected and the number found was recorded.

Laboratory Studies.

Fiddler crabs, *Uca pugnax*, were collected several times during the year in the pristine marshes of Sapelo Island and Cabretta Island, GA. Crabs were transported to Auburn, AL, and acclimated for 2 weeks to the new environment before testing began. Laboratory temperature was 27 C and a regime of 12 h light and 12 h darkness was maintained. Oven-dried leaves

of *S. alterniflora* were provided as a food source.

To determine if age (as determined by size), sex, or the presence or absence of marsh soil were important factors affecting atrazine toxicity, the following experiments were performed in August 1977. Crabs were placed singly in 144 plastic boxes (18 x 13 x 5 cm) with covers. Half of the 144 boxes contained approximately 1 inch of marsh soil. One hundred ml of atrazine at 0, 1, 100, or 1000 ppm of atrazine suspension was added to each box. This was sufficient to cover the ventral surface but not the dorsal one. AAtrex 80 (80% atrazine) was the formulation of atrazine employed, therefore, the concentration of the formulated material used was actually 0, 1.25, 125, and 1250 ppm. All solutions were prepared in Instant Ocean with a salinity of 20%. Four different crab classes were exposed to each of the atrazine concentrations with or without marsh soil. These were large males (carapace width > 1.5 cm), large females (> 1.5 cm), small males (< 1.5 cm), or small females (< 1.5 cm). The experiment was replicated four times. Crabs were observed 6, 12, and 24 h after atrazine addition and then every 24 h for 8 days. Toxicity to crabs was assessed in terms of percentage of death-free days (DFD), percentage surviving, percentage stress-free days (SFD), and percentage that passed the escape-response test (ER test). The percentage death-free days was calculated by the formula: $DFD(\%) = \frac{(C_1 + C_2 + C_3 \ldots)}{N \times D} \times 100$ where: C_1, C_2, C_3, etc. = the number of days each crab lived: N = number of crabs tested; and D = duration of the experiment in days. The percentage of stress-free days was calculated by the formula $SFD(\%) = \frac{(S_1 + S_2 + S_3 \ldots)}{N \times D} \times 100$ where: S_1, S_2, S_3, etc. = the number of days when each crab was neither dead nor showing obvious signs of stress; and N and D are used as described above.

Locomotor response was determined for all live crabs at the end of 8 days by the methods of Ward and Busch (1976). For this locomotor test, a 40- by 25-cm shallow aluminum pan was used. Two lines were drawn across the pan 5 cm from each end (30 cm between lines), and the pan lined with paper towelling was moistened with sea water. The pan was placed inside a large cardboard box having viewing ports which permitted observation of the crabs without frightening them. One crab was placed in the end of each pan and allowed to "habituate" before frightening the crab by waving an object through an opening in the large box near the crab. The crab either "escaped" (crossed far line in 15 sec) or had "no response" (did not cross far line within 15 sec).

The above test was supplemented by another to ascertain whether the additive in the formulated atrazine (AAtrex) or the atrazine itself was the toxicant. In this study small males were exposed in the absence of soil to: 1) 1000 ppm pure atrazine, 2) 250 ppm of material added to atrazine to make AAtrex, 3) 1250 ppm AAtrex, and 4) no chemicals.

All subsequent tests used only small males, no soil, and AAtrex as the source of atrazine. Rates used are expressed in terms of the amount of atrazine present. These tests were otherwise conducted as described above and were repeated in late August, September, and November 1977 and in March and

August 1978. Atrazine concentrations were varied during these experiments as attempts were made to ascertain the least effect level and the LD_{50} concentration.

Feeding Studies

Fiddler crabs were collected in early spring of 1977 in the marshes near Sapelo Island, GA, and transported in marsh soil on ice to Auburn, AL. In Auburn the fiddler crabs were divided into 84 groups of two or three crabs each in such a manner that the weight of crabs per group was approximately the same. One such group was placed in each of 84, 18- by 13- by 5-cm clear plastic boxes. Each box contained one-half of an 8-cm diameter petri dish, which served as a feeding station for the crabs. The floor of each box was wetted with seawater initially and kept moist by rewetting as needed throughout the 20-day experiment. The boxes were cleaned every 3 days. The lids were kept on the boxes except when the crabs were being tended.

The 84 boxes were further divided into 3 groups which corresponded to treatments. Each group (each group contained ca 60 crabs) was fed detritus wetted with either 0, 10^{-6} M,or 10^{-4} M atrazine suspensions. Throughout the 20-day feeding period, daily observations were made of crab behavior and mortality. All treatments were completely randomized, and a randomized analysis of variance was performed on the data from the experiments. However, before the analysis of variance was performed on the crab mortality, percentage data were converted by arcsin transformation (Steel and Torrie, 1970).

RESULTS AND DISCUSSION

Atrazine Effects in the Field and in Microecosystems.

Studies in the field involved the effect of various atrazine concentrations on the survival of crabs that happened to be trapped in the areas when the 1.8-m diam cylinders were put in place. Similar studies in the microecosystems included 15 *Uca pugnax* deliberately added to each system plus any small crabs that were inside the soil mass when it was placed in the ecosystem containers.

Field Studies--

At harvest time the average number of fiddler crabs (*Uca pugnax*)/m^2 for the 10,000, 1000, 100, and 0 ppm treatments were 4, 67, 40, and 65, respectively. Only the 10,000 ppm rate had a significant effect. Other crabs (mostly *Sesarma cinereum* and a few *Eurytium* and *Panopeus* spp) averaged 38, 21, 28, and 21/m^2, respectively. Numbers of these crabs were not significantly affected by the treatments.

Microecosystem Studies--

At harvest time average numbers of fiddler crabs/m^2 for the 10,000, 1000, 100, and 0 ppm treatments were 8, 40, 36, and 24, respectively. *Sesarma cinereum* numbers/m^2 were 0, 4, 12, and 8, respectively. The

variability between replications was too great for any of the differences in averages to be significant. However, 2 weeks after atrazine application, dead fiddler crabs were found in microecosystems treated with 10,000 ppm atrazine but not in microecosystems receiving any other rate of treatment. The observed kill by 10,000 ppm led to the other studies on atrazine toxicity.

Laboratory Studies

In the first experiment, August 1977, 1000 ppm atrazine (1250 ppm AAtrex) was injurious or lethal to some crabs in both sexes and size classes whether applied with or without marsh soil being present (Table 6). The adverse effects of this concentration were shown by all paramenters used to measure the crab response, but the percentages of stress-free days (SFD) and percentages that survived and passed the escape response test (S & pass ER test) seemed to be the most sensitive indicators of atrazine toxicity. The effects of 100 ppm were less severe, and 1 ppm had no effect.

In the supplementary study, it was established that 1250 ppm AAtrex did not differ in toxicity from 1000 ppm of atrazine alone and that 250 ppm of the additive did not differ in toxicity from the untreated control.

The presence of marsh soil in the system decreased the toxicity, perhaps because atrazine adsorption on the soil decreased the atrazine concentration in water. The decreased toxicity was probably not apparent at the 1000 ppm rate because the decrease in atrazine concentration was too small to have an effect. Small males seemed to be more sensitive to atrazine than other classes tested. Vernberg et al. (1974), working with a different species of fiddler crab, *U. pugilator*, found that mercury-treated males died sooner than females, and the rate of oxygen uptake by males was significantly lower than by females. Since the uptake of mercury was the same in both sexes, it was concluded that differences in metabolic rates and thus mercury toxicity may reflect neuroendocrine differences. Unfortunately, their studies did not involve different size classes of crabs. It is not certain whether different rates of respiration have any effect on atrazine toxicity.

When the work was repeated in late August and again in September using small males in a soil free environment, atrazine did not adversely affect the crabs. The maximum atrazine concentration used, however, was 100 ppm (data not included). In November the work was once again repeated, and there was no adverse effect of atrazine even with 1000 ppm of atrazine (Table 7).

The last experiments, conducted in March and August, 1978, gave results that confirmed the seasonal variations in sensitivity observed in 1977 (Tables 8,9). In the March experiment, only percentage stress-free days (SFD) was affected by atrazine; whereas in August an increase in mortality was present at higher atrazine concentrations, and adverse effects were again apparent with as little as 100 ppm atrazine. Thus it is apparent that the toxicity of atrazine to fiddler crabs is strongly influenced by the time of year when the test is performed. Newell (1975) found that, in general, there was a reduction in metabolism from February to July. Vernberg and Vernberg (1975), working with the zoeae of *U. pugnax* and *U. pugilator*, found seasonal

TABLE 6. EFFECTS OF ATRAZINE ON FOUR CLASSES OF FIDDLER CRABS IN SYSTEMS WITH AND WITHOUT MARSH SOIL, AUG. 6, 1977 TEST*

Atr. concn. (ppm)	Sex† size class	DFD,%‡ Soil +	DFD,%‡ Soil -	Survive,%§ Soil +	Survive,%§ Soil -	S & PT,%# Soil +	S & PT,%# Soil -	SFD,%** Soil +	SFD,%** Soil -
1,000	LM	69	78	50	75	0	25	44	25
1,000	LF	12	34	0	0	0	0	12	6
1,000	SM	34	41	0	0	0	0	31	0
1,000	SF	53	81	25	25	0	25	47	25
100	LM	100	84	100	50	100	25	97	72
100	LF	100	69	100	0	100	0	97	66
100	SM	100	50	100	25	100	0	100	44
100	SF	100	69	100	50	100	25	100	59
1	LM	100	100	100	100	100	50	100	100
1	LF	100	100	100	100	100	100	100	100
1	SM	100	100	100	100	100	100	100	100
1	SF	100	100	100	100	100	75	100	100
0	LM	100	100	100	100	100	100	100	100
0	LF	100	100	100	100	100	100	100	100
0	SM	100	100	100	100	100	100	100	94
0	SF		100	100	100	100	100	100	97

*Test continued for 8 days and was replicated four times.

†LM = large male, LF = large female, SM = small male, SF = small female.

‡DFD = death-free days expressed as a percentage of that possible.

§Survive = percentage that lived 8 days.

#S & PT = percentage that had survived and passed the escape-response test on day 8.

**SFD = stress-free days expressed as a percentage of that possible.

differences in survival at certain salinity-temperature combinations. In all cases tested, zoeae of *U. pugnax* were more resistant to changes in temperature and salinity in summer as compared to spring. Results with *U. pugilator* were more variable. The significance of these seasonal cycles was not readily apparent, and it was concluded that field acclimation phenomena influence the response of early-stage larvae. Teal (1959) found that a 2-week acclimation period was adequate to acclimate *U. pugnax* to a new thermal regime. Thus, it is concluded that seasonal differences in atrazine sensitivity that we observed cannot be attributed to lack of acclimation or to temperature differences since all crabs were acclimated for at least 2 weeks, and all testing was conducted at the same temperature.

TABLE 7. EFFECT OF ATRAZINE ON SMALL MALE CRABS IN SYSTEMS WITHOUT SOIL, Nov. 7, 1977 TEST*

Atr. concn. (ppm)	DFD† (%)	Survived‡ (%)	SFD§ (%)
1,000	99	90	100
560	91	70	100
320	90	70	100
180	90	60	100
100	95	80	100
0	97	80	100

*Test continued for 30 days and was replicated 10 times.

†DFD = death-free days expressed as a percentage of that possible.

‡Survived = percentage that lived 30 days.

§SFD = stress-free days expressed as a percentage of that possible.

TABLE 8. EFFECT OF ATRAZINE ON SMALL MALE CRABS IN SYSTEMS WITHOUT SOIL March 20, 1978 TEST*

Atr. concn. (ppm)	DFD† (%)	Survived‡ (%)	S & PT§ (%)	SFD# (%)
1,000	100	100	100	0
560	100	100	100	0
320	100	100	100	3
180	100	100	100	17
100	100	100	90	28
0	100	100	100	99

*Test continued for 9 days and was replicated nine times.

†DFD = death-free days expressed as a percentage of that possible.

‡Survived = percentage that lived 9 days.

§S & PT = percentage that survived and passed the escape-response test on day 9.

#SFD = stress free-days expressed as a percentage of that possible.

TABLE 9. EFFECT OF ATRAZINE ON SMALL MALE CRABS IN SYSTEMS WITHOUT SOIL, AUG. 1, 1978 TEST*

Atr. concn. (ppm)	DFD† (%)	Survived ‡ (%)	S & PT§ (%)	SFD# (%)
10,000	23	0	0	0
1,000	54	10	0	0
560	37	10	0	0
320	53	20	0	0
180	42	10	0	0
100	54	40	0	0
0	100	100	0	0

*Test continued for 9 days and was replicated 10 times.

†DFD = death-free days expressed as a percentage of that possible.

‡Survived = percentage that lived 9 days.

§S & PT = percentage that survived and passed the escape-response test on day 9.

#SFD = stress-free days expressed as a percentage of that possible.

Feeding Studies.

The average percentage survival for four replications of crabs fed detritus wetted with 0, 10^{-6}, or 10^{-4} M concentrations of atrazine solution were 85, 88, and 90, respectively. These values were not significantly different according to Duncan's new multiple range test.

Although the detritus fed to the crabs was wetted with either 0, 10^{-6} M, or 10^{-4} M atrazine solutions, the actual concentration in the detritus may have been slightly higher or lower because both adsorption and absorption of the atrazine were involved; some atrazine probably was metabolized by the microflora during the course of the experiment.

Several investigators have reported adverse effects on behavior and locomotion of fiddler crabs exposed to or fed pesticides (Odum et al., 1969; Ward and Busch, 1976). *Uca pugnax* fed detritus containing DDT and its metabolites in concentrations ranging from 1.44 to 51.93 ppm developed impaired coordination and a sluggish behavior (Odum et al., 1969). An organophosphorus insecticide (Temefos), at low concentrations, has also been shown to adversely affect the ability of *Uca pugnax* to respond to stimuli (Ward and Busch, 1976). In contrast to those studies, *Uca pugnax* fed detritus wetted with a 10^{-4} atrazine solution (22 ppm) did not exhibit any noticeable differences in behavior or impairment in movement during the 20-day feeding trial.

The highest concentration of atrazine reported in waters of coastal estuaries is 2.5 ppb (Anonymous, 1977). The lowest concentration at which atrazine appears to have a detectable effect on the fiddler crab, *Uca pugnax*, from those areas is about 100 ppm and then only in late spring and summer. Atrazine use is greatest during this period, and its maximum concentration in coastal waters would be expected to coincide with the period at which *U. pugnax* is most sensitive. From our data, we conclude that atrazine, even if present at concentrations over 1000 times greater than expected as run off from agricultural land, would have no significant direct effect on *U. pugnax* of the size classes tested; however, effect of atrazine on the larval stage was not investigated.

SUMMARY

Atrazine concentrations of 1000 ppm either killed or eliminated the escape response ability (considered to be analagous to death) of *Uca pugnax* in laboratory experiments in August 1977. Adverse effects were observed at concentrations as low as 100 ppm and the severity was dependent on size and sex of the crab. However, in subsequent experiments, each with a new group of crabs, the effects became smaller and smaller until a November experiment when no deleterious effects were observed even at the 1000 ppm concentration. Experiments in August 1978 confirmed the data obtained 1 yr earlier. Ecological and physiological considerations of this seasonal variation in response are discussed. Crabs fed for 20 days with detritus wetted with 10^{-4} M atrazine were not adversely affected. Crabs exposed to a single application of 0, 100, 1000, or 10,000 ppm atrazine in the field and in microecosystems were adversely affected only by the 10,000 ppm rate. Toxicity of atrazine to the larval stage of the crab was not investigated.

SECTION 6

METABOLISM OF ATRAZINE IN *SPARTINA ALTERNIFLORA*-DETRITUS-*UCA PUGNAX* FOOD CHAIN[4]

OBJECTIVES

One of the important food chains in the salt marsh starts with *Spartina alterniflora* which, upon death, is decomposed to detritus, which in turn is consumed by fiddler crabs, *Uca pugnax*. Any toxicant absorbed by *S. alterniflora* may also pass along this food chain. The objective of this study was to determine what happened to atrazine absorbed by *S. alterniflora* as it passed along this food chain.

MATERIALS AND METHODS

Atrazine Metabolism During Conversion of Spartina to Detritus

This study used a model ecosystem that simulated the formation of detritus from *S. alterniflora* in the salt marsh (Figure 11). Each system consisted of a 12-cm diam buchner funnel containing marsh soil and connected by Tygon tubing to a 1-L bottle which held 750 ml of seawater prepared from Instant Ocean.[5] The bottom of each funnel was covered by a nylon mesh filter on which was placed first a 1.5-cm layer of washed sand and then a 1.5-cm layer of soil collected in the marsh at Sapelo Island. The nylon mesh filter and layer of washed sand prevented the movement of soil out of the funnel when the reservoir bottles were raised and lowered to flood and drain the soil surface. This system allowed for the quantification of atrazine metabolites that were degraded, absorbed, and leached during the decomposition of *S. alterniflora*.

S. alterniflora was collected from the marsh on Sapelo Island, GA, transferred to Auburn, AL, and cultured in Hoagland's nutrient solution, and transferred into six 1-L beakers containing ^{14}C-labeled atrazine (24.9 µCi/mg), i.e., 0.26 ppm or 1.2×10^{-6}M. After 2 days the plants were removed from the ^{14}C-atrazine solution, the roots rinsed, and the plants placed in atrazine-

---

[4]Most of the material in this section is reprinted from the Journal of Environmental Quality with their permission. The paper has not yet been published.

[5]Instant Ocean. Ward Natural Science Establishment Inc., Rochester, NY 14601.

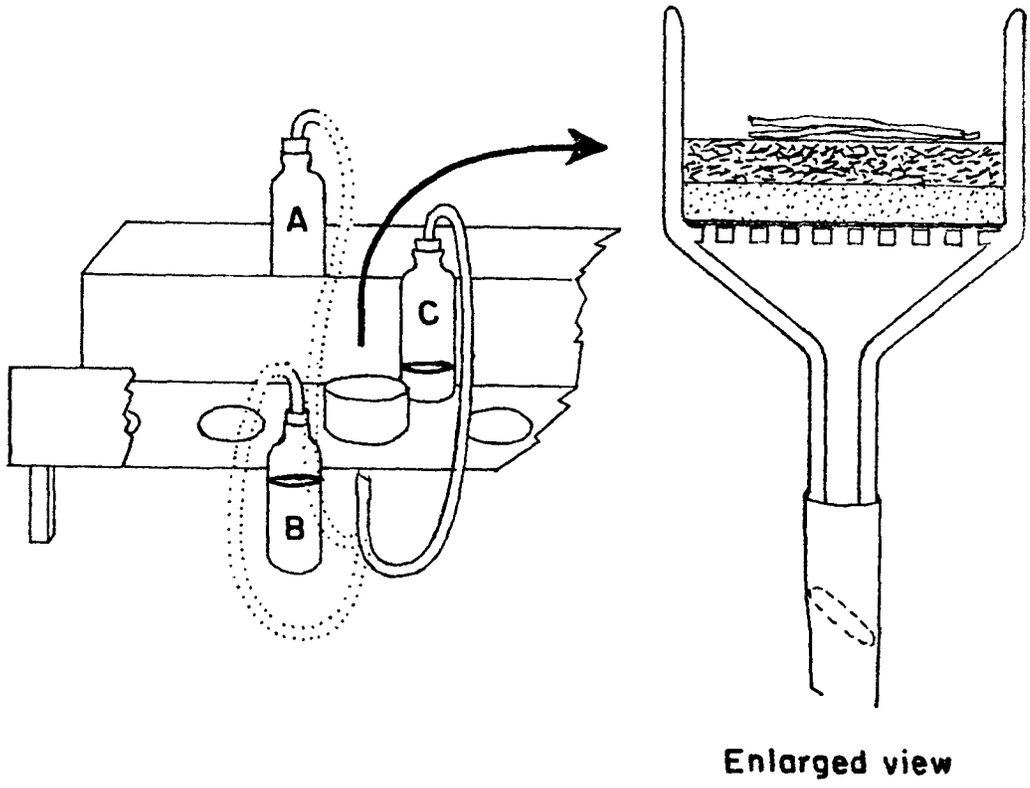


Figure 11. Diagrammatic representation of experimental set-up used to make detritus. Positions A, B, and C are the ones used to flood, drain, and maintain a moist soil surface, respectively.

free Hoagland's solution. Three days later the leaves of the plants were harvested, cut into 1-cm-long sections, and dried at 70 C to a constant weight (34% of fresh weight).

The conversion of the dried *S. alterniflora* to detritus was studied in six model ecosystems. About 5 g of the dried material was placed in each of seven nylon mesh bags. One bag was placed on the soil surface in each system and one was kept to determine the total radioactivity initially present in the *S. alterniflora* leaves. The reservoirs were raised and lowered twice daily during the 20-day study. The time between raising and lowering of the reservoirs was adjusted so that the soil surface was flooded for 30 min twice each day. At other times the height of the reservoirs was adjusted so that the soil surface was kept moist but not flooded. The twice-daily complete wetting of the detritus and constant contact with moist soil surface created conditions commonly found in the *S. alterniflora* marshes. After 10 days the seawater was emptied from all reservoirs, saved for herbicide and metabolite assay, and replaced with fresh seawater. After 20 days the bags of partially decomposed spartina, hereinafter referred to as

detritus residue, were removed from the funnels, washed thoroughly with deionized water to remove any material small enough to pass through the 0.01 mm holes in the nylon bags, dried to constant weight, and stored in a freezer for later chemical and radiochemical analyses. The marsh soil and sand from each buchner funnel, the seawater removed after 10 days, the seawater removed at the end of the experiment, the water from the final wash of the detritus residue, and the nylon bags and filters were also stored under refrigeration for later analysis.

The procedures used to extract atrazine and atrazine metabolites from the dried *S. alterniflora* and the detritus residue were essentially those described in Section 3. The material was homogenized in a Waring blender in 80% (v/v) methanol and suction-filtered through a buchner funnel. The insoluble material was resuspended in 400 ml of 80% methanol, stirred overnight, and filtered the next morning. The filtrates were combined, concentrated by flash evaporation at 37 C, diluted with water, and washed with three 25-ml portions of chloroform. Each fraction was brought to volume, and the amount of radioactivity in each was determined by liquid scintillation spectrometry. The insoluble fraction was dried at 70 C to constant weight, pulverized, and 50-mg portions radioassayed by suspending with Cabosil. Observed counts were corrected for quenching, and the radioactivity was expressed as dpm per mg of dry weight of the original *S. alterniflora* as percentages of the total radioactivity in all samples.

The seawater removed from the reservoirs 10 and 20 days after the start of the experiment was concentrated by flash evaporation, centrifuged at low speed to remove insoluble material, and washed with chloroform. The water and chloroform fractions were brought to volume and assayed for radioactivity. The material removed by centrifugation was dried, and a 50-mg portion suspended in a scintillation vial with Cabosil and radioassayed. Counts were corrected and radioactivity expressed as described above. The water from the final wash of the detritus was treated as described above except that it was not centrifuged.

Any atrazine or atrazine metabolites which had been adsorbed to the nylon bags or filters were removed by a 48-h extraction with 80% methanol. The extracted material was concentrated, washed with chloroform, and radioassayed.

The soil was air dried and extracted by gently refluxing with 600 ml of 50% (v/v) methanol for 24 h. The methanol was filtered to remove suspended soil, concentrated to near dryness by flash evaporation, and washed with chloroform; and aliquots of the chloroform and water extracts were radioassayed.

Atrazine Metabolism by Fiddler Crabs Fed Detritus

The crabs used in these experiments were collected in early spring of 1977 in the marshes near Sapelo Island, GA, and transported in marsh soil on ice to Auburn, AL. In Auburn the crabs were divided into 56 groups of two or three crabs each in such a manner that the weight of crabs per group was

approximately the same. One such group was placed in each of 56 clear plastic boxes (18 x 13 x 15 cm). Each box contained one-half of an 8-cm-diameter petri dish which served as a feeding station for the crabs. The floor of each box was wetted with seawater initially and kept moist by re-wetting as needed throughout the 20-day experiment. The boxes were cleaned every 3 days. The lids were kept on the boxes except when the crabs were being tended. This experimental set up was used in all of the crab feeding experiments.

Two experiments, each using the same general procedure, were performed to study atrazine metabolism in the mud fiddler crab. In both experiments the atrazine concentrations used were not toxic to the crabs. In the first experiment, crabs were fed detritus residue (0.1 g/crab) made from the leaves of spartina plants grown in ^{14}C-labeled atrazine. The detritus residue used in the latter experiment was made by harvesting spartina leaves from the marsh and converting them to detritus in a system similar to the one described earlier. At the end of the 20-day feeding period, the crabs in each group were sacrificed and stored under refrigeration. The crabs in each group were analyzed by first grinding the crabs in a mortar with a pestle in 80% methanol to pulverize their exoskeltons and were then homogenized in a Waring blender. The extract was filtered, and the filtrate was concentrated to near dryness and extracted with chloroform. The chloroform and water-soluble fractions were brought to volume and assayed for radioactivity. The insoluble portion was dried and a 50-mg portion suspended with Cabosil and radioassayed. Observed counts were corrected for quenching, and the percentage of the total radioactivity in each fraction was determined.

RESULTS AND DISCUSSION

Atrazine Metabolism During Conversion of Spartina to Detritus

Table 10 summarizes the distribution of atrazine and atrazine metabolites in the various components of the model ecosystem. The chloroform-soluble extracts from plants, animals, and microorganisms exposed to atrazine have been shown to contain predominantly unchanged atrazine and N-dealkylated atrazine metabolites (Esser et al., 1975; and Shimabukuro et al., 1973), and the water-soluble extracts to contain primarily hydroxy atrazine and glutathione-conjugated metabolites of atrazine (Esser et al., 1975). The most conspicuous feature of Table 10 is the relatively large amount of water-soluble atrazine metabolites (78%) and small amount of chloroform extractable material (9%) recovered. In the *S. alterniflora* leaves from which the detritus was formed, about 38% of the total radioactivity was in the water-soluble fraction and about 55% was in the chloroform-soluble fraction (Section 3). Thus during the 20-day period, there was a decrease in the amount of chloroform-soluble material (which contained any atrazine present) and an increase in the amount of water-soluble atrazine metabolites. This is probably attributable to the metabolism of atrazine by the microorganisms living in association with the decomposing *S. alterniflora* leaves.

During the 20-day experiment, the decomposing *S. alterniflora* was inundated twice daily with seawater thereby leaching atrazine and atrazine

TABLE 10. AMOUNTS OF RADIOACTIVITY IN THE CHLOROFORM-SOLUBLE, WATER-SOLUBLE, AND INSOLUBLE FRACTIONS IN PHYSICAL COMPONENTS OF THE SYSTEM

Physical Component	Chloro-soluble		Water-soluble		Insoluble		Total*	
	(dpm/mg)†	(%)	(dpm/mg)†	(%)	(dpm/mg)†	(%)	(dpm/mg)†	(%)
Nylon bag	1.02	52	.95	48	0.0	0	1.97	0.5
Nylon filter	.34	30	.81	70	0.0	0	1.15	0.3
Soil & sand	6.59	16	33.36	84	0.0	0	39.95	11.0
Water 10 day	11.75	9	117.80	91	0.0	0	129.55	36.0
Water 20 day	8.86	8	97.25	92	0.0	0	106.11	30.0
Final wash	.28	16	4.85	84	0.0	0	5.13	1.5
Detritus	4.72	6	23.90	33	45.30	61	73.90	20.5
Total	33.60	9	278.90	78	45.30	13	357.80	100.0

*Total radioactivity recovered was about 90% of that initially supplied.

†Expressed as dpm/mg of dried spartina leaves supplied to the system.

metabolites out of the decomposing leaves. About 2/3 of the initial total radioactivity in the leaves was recovered in the two water samples (Water 10 day, Water 20 day) (the largest portion of this radioactive material was soluble in water). A somewhat larger fraction of the total radioactivity was recovered from the first 10-day than the second 10-day period (36.0% vs 30.0%), and only a small amount was recovered in the final wash. This suggests that at the end of the 20-day experiment, most of the readily soluble radioactive material in the detritus had leached out. The amount of radioactivity extracted from the soil and sand layers was approximately 11% of the total radioactivity recovered.

Weber et al. (1969) have shown that soils high in clay content and organic matter are efficient in the adsorption of atrazine which reduces the herbicidal toxicity of atrazine in such soils. Thus, in a marsh soil with substantial amounts of organic matter and clay, a significant amount of atrazine introduced into the marsh would be adsorbed on clay and organic colloids, making it unavailable. In our study, we found that much of the atrazine present in *S. alterniflora* was metabolized as the plant material was converted to detritus. This metabolism plus adsorption of atrazine by clays and organic matter would thus be expected to quickly detoxify atrazine introduced into the food chain through *S. alterniflora*.

Atrazine Metabolism by Fiddler Crabs Fed Detritus

The percentage of [^{14}C]-atrazine-derived radioactivity in the various fractions extracted from the crabs and the detritus fed the crabs are given in Tables 11 and 12.

TABLE 11. AMOUNTS AND PERCENTAGES OF TOTAL RADIOACTIVITY IN THE VARIOUS FRACTIONS EXTRACTED FROM RADIOLABELED DETRITUS RESIDUE* FED TO FIDDLER CRABS AND IN THE FIDDLER CRAB THEMSELVES

Item	Chloroform-soluble		Water-soluble		Insoluble residue	
	(dpm)	(% total)	(dpm)	(% total)	(dpm)	(% total)
Detritus residue	51,000	5.0	318,000	34.0	566,000	61.0
Crabs	4,500	5.0	27,000	41.0	50,700	54.0

*The detritus residue was derived from *S. alterniflora* which was grown in a solution containing ^{14}C ring-labeled atrazine. The amount of radioactivity in the detritus residue was determined at the end of the 20-day feeding period.

TABLE 12. AMOUNTS AND PERCENTAGES OF THE TOTAL RADIOACTIVITY IN THE VARIOUS FRACTIONS EXTRACTED FROM DETRITUS RESIDUES* WETTED WITH RADIOLABELED ATRAZINE AND FED TO FIDDLER CRABS AND IN THE FIDDLER CRABS THEMSELVES

Item	Chloroform-soluble		Water-soluble		Insoluble residue	
	(dpm)	(% total)	(dpm)	(% total)	(dpm)	(% total)
Detritus residue	380,000	8.5	795,000	18.0	3,200,000	73.0
Crabs	25,500	17.0	99,000	65.5	36,500	17.5

*The detritus residue was derived from *S. alterniflora* leaves wetted with ^{14}C ring-labeled atrazine. The amount of radioactivity in the detritus residue was determined at the end of the 20-day feeding period.

The common result from the two metabolism studies is the increased percent of the total radioactivity in the water-soluble fraction extracted from the crabs compared to the detritus which they were fed. This increase is due either to a selective absorption of water-soluble atrazine metabolites by the crabs' intestinal mucosa or metabolism of chloroform-soluble atrazine or atrazine metabolites to water-soluble metabolites by the crab or by the flora inhabiting the crab's gut. Metabolism by the crabs seems probable since *Sesarma cinereum* has been shown to metabolize atrazine (Section 7) and because rats given atrazine orally have been shown to convert it to hydroxyatrazine (Bakke et al., 1972). Since fiddler crabs are filter feeders, no

suitable method of introducing pure atrazine into the gut of the crab has been developed. Furthermore, this would not eliminate the possibility of atrazine metabolism by the microflora of the gut. Until a method of introducing pure atrazine into the crab's gut and the control of microfloral activity is developed, the ability and extent to which fiddler crabs can metabolize atrazine will remain undetermined. However, regardless of the mechanism, it is apparent that the level of atrazine in the tissues of the fiddler crab is lower than in the material on which it fed.

Concentrations of Atrazine and Atrazine Metabolites in the Detritus Food Chain

Table 13 summarizes the amount and concentration of chloroform and water-soluble radioactive material extracted from *S. alterniflora* leaves, detritus residue derived from spartina leaves, and fiddler crabs fed such detritus residue. As can be seen, there is a continuing decrease in the concentration of chloroform-soluble atrazine or atrazine metabolites from the leaves, to the detritus residue, and finally to the fiddler crab. Also, there is a decrease in the concentration of water soluble metabolites; however, this decrease is not progressive as in the chloroform-soluble metabolites. Thus,as atrazine is absorbed by the plant, decomposed to detritus and detritus residue which is eaten by the fiddler crabs, there is a marked decrease in the concentration of atrazine and atrazine metabolites present. Extrapolation of these results suggests that there would be a progressive decrease in atrazine concentration from lower to higher trophic levels in salt marshes exposed to atrazine.

TABLE 13. THE AMOUNTS AND CONCENTRATIONS OF WATER-SOLUBLE AND CHLOROFORM-SOLUBLE ATRAZINE OR ATRAZINE METABOLITES IN SPARTINA LEAVES, IN DETRITUS DERIVED FROM THE LEAVES, AND IN FIDDLER CRABS FED DETRITUS DERIVED FROM THE LEAVES

Item	Water-soluble Concentration (ppm)	Chloroform-soluble Concentration (ppm)
Spartina leaves*	2.14	3.10
Detritus residue	0.48	.085
Fiddler crabs	0.70	.080

*The values for *S. alterniflora* are from Section 3.

SUMMARY

Leaves from *Spartina alterniflora* plants grown with their roots in solutions containing ^{14}C ring-labeled atrazine were converted to detritus in model ecosystems that simulated the salt marsh environment. Percentages

of radioactivity in chloroform-soluble, water-soluble, and insoluble materials in the leaves were 55, 38, and 7, respectively. Twenty days later these values for detritus were 9, 78, and 13, respectively. Thus, there was a decline in the percentage of radioactivity in the chloroform fraction which contains atrazine and nontoxic metabolites and a concurrent increase in the water-soluble fraction that contains only nontoxic metabolites. The fiddler crab, when fed detritus labeled with atrazine, further decreased the percentage of the chloroform-soluble atrazine or atrazine metabolites. Radioactivity originally present in either atrazine or atrazine metabolites fed to fiddler crabs was concentrated in the water-soluble extract from the crabs, suggesting either selective absorption through the gut or metabolism of the chloroform-soluble form(s) to water-soluble material by the fiddler crabs or the crabs' enteric flora. Atrazine and atrazine metabolite concentrations were reduced from lower (detritus) to higher trophic levels (fiddler crabs) in salt-marsh microecosystem.

SECTION 7

METABOLISM OF ATRAZINE BY *SESARMA CINEREUM*[6]

OBJECTIVES

Box crabs *Sesarma cinereum*, feed directly on the leaves of cordgrass, *Spartina alterniflora*, and by so doing ingest toxicants taken up by *S. alterniflora*. The objectives of this investigation were: 1) to determine whether altrazine present in *S. alterniflora* leaves was toxic to box crabs, and 2) to compare the concentrations of atrazine and atrazine metabolites present in leaves fed box crabs with those in the crabs and in crab feces.

MATERIALS AND METHODS

Box crabs and cordgrass, *Spartina alterniflora*, were collected from the marsh on Sapelo Island, GA, and transported in marsh soil to Auburn, AL. Each crab was washed in seawater and placed in an individual plastic container approximately 20 cm x 20 cm x 18 c. Cordgrass plants were grown and maintained in a controlled environment chamber as described in Section 3. Uniform lots of four plants were transferred to six 1-L beakers containing 300 ml of Hoagland's nutrient solution, 0.2 ppm (1.2×10^{-6}M),and 2 µCi of ring-labeled [^{14}C] atrazine (24.9 µCi/mg) (Ciba-Geigy, Greensboro, NC). After 2 days, the plants were removed from the atrazine solution, the roots rinsed free of radioactive material, and placed in an atrazine-free Hoagland's solution. After 3 days in the atrazine-free solution, leaves were harvested and cut into 5 mm x 2 mm sections; and 100 mg of these sections were placed in each of the plastic containers with the crabs (Daiber and Crichton, 1967). Each container was covered with a transparent lid to prevent loss of water by evaporation. The crabs were maintained in a growth chamber with 14 h of diffused light (1.8 klux) at 22 C and a 10-h dark period at 20 C. Crabs were removed from the containers daily and placed in the seawater (1/2 strength Instant Ocean, Carolina Biological Supply House) to allow them to refill their gill chambers. Feces were collected from the containers with a rubber policeman. *S. alterniflora* leaf fragments were removed, rinsed with deionized water, combined, and kept frozen until extraction and analysis. The crabs were then returned to their containers with fresh *S. alterniflora* leaf sections. Two experiments were conducted, the first with 50 crabs and the second with 68.

---

[6]Most of the material in this section is reprinted from the Journal of Environmental Quality with the permission of the Journal of Environmental Quality. Volume and page numbers have not been established at this time.

In each case, half the crabs were fed *S. alterniflora* leaf sections from plants grown in atrazine-free solutions (controls) and half with leaves from plants grown in solutions containing [^{14}C]-labeled atrazine.

At the end of the 10-day feeding period, the box crabs were frozen and homogenized in 80% aqueous methanol (10 ml/g of fresh weight of crab). The homogenate was filtered, and the filtrate was concentrated by flash evaporation at 37 C, diluted to 50 ml with water, and partitioned with chloroform. The chloroform and aqueous fractions were concentrated separately, brought to volume, and the radioactivity in each fraction was determined by liquid scintillation spectrometry. The insoluble residue was refluxed for 2 h with 25 ml of 0.5 N HCl at 70 C, cooled, and filtered. The filtrate was partitioned three times with 50 ml of ether, and combined washes were dried over sodium sulfate. The aqueous phase of the hydrolysate was dried by flash evaporation to remove the acid and then radioassayed. Radioactivity in the insoluble residue remaining after refluxing was estimated by grinding a portion of the material to a fine powder, suspending it in Aquasol (Beckman) scintillation cocktail containing Cab-O-Sil (Beckman), and radioassayed as before. Extraction and fractionation of various components of the *S. alterniflora* leaf sections and crab feces were conducted essentially as described for box crab except that the 80% methanol-insoluble residue was not hydrolyzed. All radioactive determinations were corrected for quenching.

Radiolabeled components of the chloroform fractions were separated by thin-layer chromatography (TLC) using 20 cm x 20 cm glass plates coated with a 250 µm-layer of silica gel HF-254 and activated for 1 h at 100 C. The plates were developed twice in benzene-acetic acid (50:4, v/v), and the metabolites were identified by comparing their RF values with those of authentic standards (Ciba-Geigy Corporation, Greensboro, NC, U.S.A.). Atrazine and its N-dealkylation products were visualized on the TLC plates with UV light (254 nm). Radioactivity in each component was determined by scraping the radioactive areas from the plates and radioassaying.

Radioactive components of the aqueous fractions were separated by ion-exchange chromatography. Portions of these fractions containing approximately 150,000 dpm were concentrated to dryness by flash evaporation, dissolved in 2 ml of 0.2 N (pH 2.1) pyridine-acetate buffer, and applied to a 1 x 80 cm water-jacketed column of AG 50 W-X2 Aminex resin (200-325 mesh) (Bio-Rad Laboratories) at 15 C. The column was washed at 0.3 ml/min with a pyridine-acetate buffer gradient developed from three chambers and the fractions collected. The radioactivity in each fraction was determined. Preparation of the buffers and regeneration of the ion-exchange resin was described by Schroeder et al. (1962). The first chamber of the gradient device contained 300 ml of 0.2 N buffer at pH 3.1, and the second and third chambers contained 300 ml of 2.0 N buffer at pH 5.0.

RESULTS AND DISCUSSION

Response of Box Crabs to Dietary Atrazine

To determine whether atrazine is toxic to box crabs, male and female

box crabs of different sizes and stages of maturity were fed fresh *S. alterniflora* leaf strips either from plants grown in nutrient solution containing atrazine or in atrazine-free solution. Forty-five crabs were used in preliminary study, and in two subsequent experiments, 50 and 68 crabs were tested. Preliminary work indicated that the crabs consumed about 20 mg of cordgrass leaves per day per g body weight containing about 0.015 ppm atrazine and 0.046 ppm of its metabolites. At the end of the 10-day feeding period, about 0.4 ppm atrazine and its metabolites accumulated in the body of the crab and about 0.21 ppm was excreted with feces. During the feeding period, 11.7% and 12.4% of the crabs died in the control and the treatment groups, respectively. This was probably due to the stress conditions imposed on the crabs during the test. No dissimilar behavior was observed between crabs fed atrazine-treated leaves and crabs in the control group. Thus, it appears that atrazine, at the level present in the *S. alterniflora* diet, was not toxic to the crabs.

Atrazine and Atrazine Metabolites in *S. alterniflora*

The plants used in this study were grown as described in Section 3 except that the plant material was collected 3 days after transferring the plants from solutions containing [^{14}C]-atrazine to atrazine-free solution. Leaf material used in the feeding experiments contained 56%, 39.5%, and 4.5% radioactivity in the chloroform, aqueous, and 80% methanol-insoluble fractions, respectively (Table 14). Although the *S. alterniflora* used for feeding was stored at 4 C prior to use and a new supply of previously stored leaves were given to the crabs daily over the 10-day feeding period, it was necessary to know the changes that occurred in the relative proportions of radioactivity in the plant material over a 10-day period (equivalent to 13 days after transfer of the plants to an atrazine-free nutrient solution). Data in Section 3 indicate that after 10 days the leaves contained 36%, 57%, and 7% radioactivity in the chloroform, aqueous, and 80% methanol-insoluble fractions, respectively. This shows a slower shift in radioactivity from the chloroform to aqueous fractions in the *S. alterniflora* compared to crab (Table 14).

Metabolism of Atrazine and its Metabolites in the Box Crab

Chloroform Fraction--

The relative proportions of radioactivity in the various fractions of the box crabs and its feces were quite different from those of the plant material fed to the crabs initially and after 10 days incubation (Table 14). The chloroform fraction of the crab and feces extracts contained 6.6% and 25% of the total radioactivity, respectively. The *S. alterniflora* leaves fed to the crabs contained 24% of the radioactivity as atrazine and after 10 days only about 13% (Section 3). Atrazine was 1.2% and 0.5% of the radioactivity in the crab tissue and feces, respectively, at the end of the feeding experiment (Table 15). It appears that atrazine is metabolized more rapidly by the crab than by *S. alterniflora* since it represents only 1.7% in the crab and feces after an equivalent period of time. It cannot be determined unequivocally from these data whether atrazine metabolism occurs

TABLE 14. RADIOACTIVITY IN CHLOROFORM, AQUEOUS, AND INSOLUBLE FRACTIONS OF *S. ALTERNIFLORA*, CRABS, AND FECES*

Fractions	*S. alterniflora*†		Crabs†	Feces†
	3 days‡	13 days‡		
Chloroform	56.0	36.0	6.6	25.0
Aqueous	39.5	57.0	86.2	51.0
Insoluble	4.5	7.0	7.2	24.0

*Leaves from the *S. alterniflora* were fed to the crabs and feces collected from the crabs.

†Each value is the mean of two experiments and is expressed as the percentage of the total radioactivity in the 80% methanol extract.

‡*S. alterniflora* leaves were collected 3 days after transferring the plants from atrazine-containing solution to an atrazine-free nutrient solution. Leaves were analyzed at harvest and after 10 days, the length of time that feeding experiment lasted. The 13-day data are from Section 3.

mainly by enzymatic, chemical, or microbial action in the gut or in the crab tissue after absorption.

Radioactive components other than atrazine in the chloroform fraction of crab and feces extracts included the N-dealkylation products of atrazine and relatively polar unidentified substances (Table 15). Both the box crab and its feces contained the three expected N-dealkylation products; 2-chloro-4-amino-6-isopropylamino-*s*-triazine, 2-chloro-4-amino-6-ethylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine (Figure 12). 2-Chloro-4-amino-6-isopropylamino-*s*-triazine was the principal N-dealkylation product found in the crab and its feces after 10 days of feeding. Whether this is due to selective absorption of this metabolite relative to others or preferential removal of the ethyl group cannot be determined from these data. However, 2-chloro-4-amino-6-isopropylamino-*s*-triazine is the principal dealkylation product of most systems including *S. alterniflora* (Section 3). N-dealkylation appears to be the principal way in which atrazine is degraded and partially detoxified by soil fungi (Kaufman and Kearney, 1970), cotton, pea, and soybean (Shimabukuro et al., 1966; Shimabukuro, 1967a) and seems to be a universal reaction in higher plants, animals, and microorganisms (Shimabukuro et al., 1970). N-dealkylation products represented a quantitatively minor amount of the atrazine metabolites in the crab and feces after the 10-day feeding period (4 and 11% of the total radioactivity, respectively), and it would appear that N-dealkylation may represent a relatively important pathway in the metabolism of atrazine in box crabs. However, interpretation of the importance of this pathway in crabs is complicated by the fact that in addition

TABLE 15. RADIOACTIVITY IN ATRAZINE, N-DEALKYLATION PRODUCTS, AND UNINDENTIFIED POLAR COMPONENTS OF THE CHLOROFORM FRACTION OF EXTRACTS FROM *S. ALTERNIFLORA* LEAVES*

Atrazine or metabolite	Percentage of total†		
	S. alterniflora	Crabs	Feces
Atrazine	23.7	1.2	0.5
2-chloro-4-amino-6-isopropylamino-*s*-triazine	20.4	2.2	7.2
2-chloro-4-amino-6-ethylamino-*s*-trizaines	4.2	1.4	2.0
2-chloro-4,6-diamino-*s*-triazine	2.8	0.4	1.6
unidentified	4.8	1.4	13.8

*The *S. alterniflora* was grown in [^{14}C]-atrazine solution. These leaves were fed the crabs and feces collected from the crabs.

†Each value is the mean of two experiments with three replications each and is expressed as a percentage of the total radioactivity in the 80% methanol extract.

to atrazine, polar metabolites (see below) of the aqueous fraction can also originate from the N-dealkylation products. That is, N-dealkylation may proceed at a higher rate than is apparent because the products can be rapidly converted to water-soluble substances which do not accumulate in the chloroform fraction.

In addition to atrazine and its N-dealkylation products, the chloroform fraction of crab and feces extracts contained unidentified, relatively polar radiolabeled substances. N,N-bis(4-ethylamino-6-isopropylamino-*s*-triazine-2)-cystine was identified in the chloroform fraction of extracts of atrazine-treated sorghum (Shimabukuro et al., 1973), and an unidentified substance(s) with similar TLC migration properties was detected in *S. alterniflora* (Section 3). Similar chloroform-soluble substances comprised 20% of the radioactivity in the chloroform fraction from crab and 55% of those in feces (Table 15). Since the *S. alterniflora* leaves fed to the crabs contained only 8.6% of these substances, it appears that reactions leading to the production of polar metabolites from atrazine or its N-dealkylation products were occurring in the gut of the box crab. It cannot be determined if these substances in the crab were formed and absorbed from the gut or were also produced in the crab tissue.

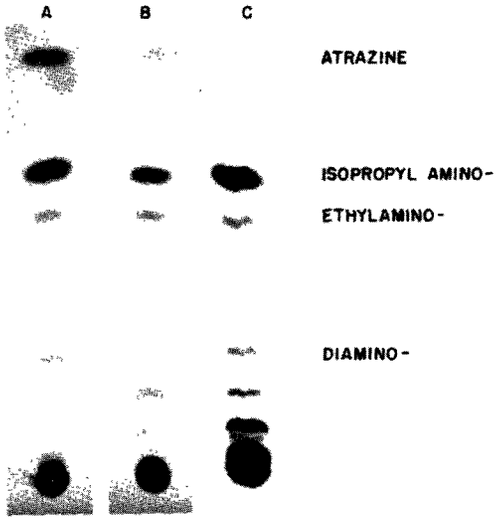


Figure 12

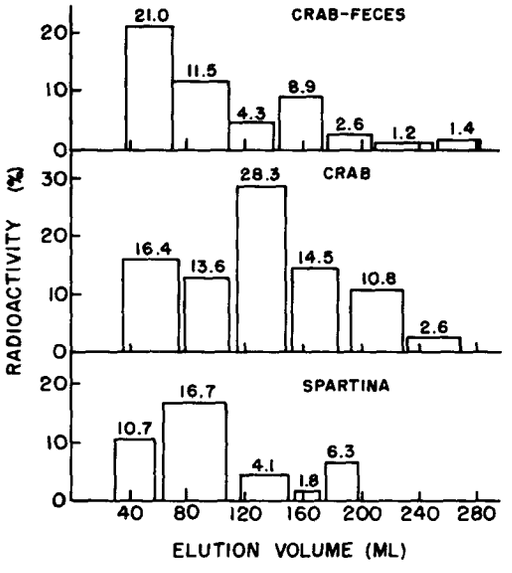


Figure 13

Figure 12. Autoradiogram showing the ^{14}C separation of radioactive components of the chloroform extracts from A) *S. alterniflora* leaves, B) crabs, and C) crab feces.

Figure 13. Radioactivity in subfractions of aqueous fractions from leaves of *S. alterniflora* grown in ^{14}C-labeled atrazine solution, crabs fed leaves from these plants, and feces of these crabs.

Water-Soluble Metabolites--

Two of the reactions leading to the detoxification of atrazine are 2-hydroxylation and conjugation with glutathione at the 2-position of the triazine ring. The latter reaction is particularly important in sorghum (Shimabukuro et al., 1971) and appears to occur in *S. alterniflora* also (Section 4). Most of the compounds resulting from these reactions are relatively water-soluble and would be present in the aqueous fraction of extracts prepared as described above (Shimabukuro et al., 1970; Shimabukuro et al., 1971; Lamoureux et al., 1972). Relatively little change in the amount of radioactivity in the aqueous fraction of extracts of *S. alterniflora* was observed over a period equivalent to that of the feeding experiment (Table 14). The aqueous fraction of box crab extracts contained 86% of the total radioactivity (Table 14). There was little difference in the relative amounts of radioactivity in this fraction of the feces and the *S. alterniflora* extracts. The amount of radioactivity in the chloroform fraction of crab was considerably less than in the feces, suggesting that atrazine and its N-dealkylation products ingested with the *S. alterniflora* were absorbed by

the box crab and converted to water-soluble substances, possibly via the hydroxylation or conjugation reactions. However, the dechlorination reaction is not considered important in detoxification of atrazine in those animals tested (Shimabukuro et al., 1971).

Although the relative amounts of radioactivity in the aqueous fraction of cordgrass remained relatively constant between 10 and 20 days after transferring the plants from a nutrient solution containing ^{14}C-labeled atrazine to an atrazine-free solution (Section 3), considerable changes in the radiolabeled components within this fraction occurred during this time (Section 4). Passing portions of the aqueous fractions from crab, feces, and the *S. alterniflora* fed to the crabs through a cation-exchange column showed that the relative proportions of the components differed among samples (Figure 13). From previous studies (Section 4), it was anticipated that approximately 42% of the radioactivity would accumulate in the first two column fractions after 10 days incubation under the feeding experimental conditions. While this appeared to be true for the feces, radioactivity was more evenly distributed between the 6 column fractions for the crab extract, with about 28% in fraction 3. This further suggests that reactions unique to the crab involving atrazine, N-dealkylation products, and possibly intermediates in the conjugation pathways may have occurred. Studies of column fractions of cordgrass extracts showed that each fraction is a mixture of two to several individual atrazine metabolites (Section 4).

Of the metabolites isolated from *S. alterniflora* leaves, about half yielded amino acids such as glycine and glutamic acid on hydrolysis, suggesting a possible link to glutathione conjugation (Section 4). In excised sorghum leaves, the glutathione conjugation pathway operates almost exclusively (Lamoureux et al., 1972), and the contribution of this pathway to the detoxification of atrazine by sorghum leaves may approach 87% (Lamoureux et al., 1973). Glutathione conjugation is the initial reaction leading to mercapturic acid biosynthesis, a pathway recognized as a means for detoxification and excretion of foreign compounds in mammals (Boyland and Chasseaud, 1969), birds (Wilt and Leeuwaugh, 1969), and insects (Cohen et al., 1960). Almost complete detoxification of atrazine and the abundance of the water-soluble metabolites (86%) suggests that, as in other animals, the metabolism of this herbicide in crabs may occur via the glutathione conjugation pathway.

SUMMARY

The metabolism of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] in box crabs (*Sesarma cinereum*) was determined. Leaves of smooth cordgrass (*Spartina alterniflora* Loisel) collected from plants grown for 2 days in nutrient solution containing [^{14}C]-atrazine, followed by 3 days in an atrazine-free nutrient solution, were fed to box crabs for 10 days. No significant effects of atrazine on the behavior or survival of crabs were found. At the end of the 10-day feeding period, box crabs and their feces were extracted with 80% methanol, and the extracts were concentrated and partitioned with chloroform. Radioactivities in the chloroform, aqueous, and 80% methanol-insoluble fractions (remaining crab material) of the extract were 7%, 86%, and 7% for crabs and 25%, 51%, and 24% for feces, respectively.

Only 1.2% and 0.5% of the total radioactivity in the crab and feces, respectively, was atrazine, compared to 24% in the *S. alterniflora* used as a food source. This indicates that atrazine is metabolized in the crab. The accumulation of water-soluble metabolites in the crab suggests that, as in other animals, glutathione conjugation or a comparable pathway is responsible for the almost complete degradation and detoxification of atrazine in these organisms.

SECTION 8

EFFECT OF ATRAZINE ON MARINE DIATOMS[7]

OBJECTIVES

Since algae, in particular the marine diatoms, make a major contribution to the food supply in the *S. alterniflora* salt marsh ecosystem, it is important to know the effect of atrazine on this group of plants. The objectives of this investigation were to determine the effect of atrazine on: 1) cell numbers, 2) chlorophyll synthesis, 3) carbon fixation, and 4) species diversity of marine diatoms common in the *S. alterniflora* salt marsh when grown in monocultures, microecosystems, and in the field.

MATERIALS AND METHODS

Culture Studies

Guillard's (1962) f/2 nutrient solution with 26.5 ppm NH_4Cl adjusted to a salinity of 20 % with Instant Ocean and buffered with 500 ppm of tris buffer-HCl at pH 7.4 was used. Culture conditions were 14-h days with 4 klux of light at 25 C and 10-h nights at 22 C. Cultures were constantly shaken at 160 cycles/min. Cultures of *Thalassiosira fluviatilis* Hustedt and *Nitzschia sigma* Grun. were isolated from marsh soil samples from the bank of Cabretta Creek, Cabretta Island, GA, by the methods of Pringsheim (1946) as modified by Trainor (1978).

Effects of a 7-day exposure to 0, 10^{-7}, 10^{-6}, and 10^{-5} M atrazine on a mixed culture of *T. fluviatilis* and *N. sigma* were investigated. There were 14 replications for each concentration, and cultures were maintained in 1.5- x 12.5-cm culture tubes. Cultures were initiated by taking 1 ml from an actively dividing culture and adding it to 4 ml of nutrient solution containing sufficient atrazine to give the desired final concentration. After the 7-day exposure period, chlorophyll content was determined for three randomly selected cultures from each atrazine concentration as described by Yentsch and Menzel (1963). Another three randomly selected tubes from each concentration were used to determine cell numbers with a hemocytometer using the methods of Guillard (1973). Each of the remaining tubes had 2 ml of the appropriate atrazine solution containing a constant amount of ^{14}C (supplied as $^{14}CO_2$) added. The 8 remaining tubes of each concentration

---

[7]Most of this material is from a paper accepted for publication in Estuaries and is reproduced here with their permission.

were divided into two sets of four each, and one set was held in the dark for 2 h and the other in the light for 2 h. After the 2-h incubation period, the cells were separated by filtration, washed, and the fixed ^{14}C measured with a liquid scintillation spectrometer.

Microecosystems

In February 1977 and again in April 1977, atrazine effects on edaphic algae were monitored in model ecosystems at Auburn, AL. A 10-cm layer of soil obtained from the creekbank zone of Cabretta Creek was placed in 35-cm diam plastic tubs. Tidal action was simulated by the raising (high tide) or lowering (low tide) of 9-L buckets containing 7.6 L of water adjusted to a salinity of 20% with Instant Ocean. The plastic tubs were connected to the moveable buckets by rubber tubing. Water in the buckets was continually aerated by bubbling air. The "tide machine" employed for raising and lowering the water reservoirs was designed by Everest and Davis (1977) and was capable of simulating tidal flow with a high degree of accuracy. Ten fiddler crabs, *Uca pugnax*, were added to each system to prevent blue-green algal mats from becoming the dominant vegetation.

Atrazine was introduced into four of the eight water reservoirs at a concentration of 10^{-5} M (2.2 ppm or 0.16 g/m^2). Systems were flooded with herbicide-containing seawater "twice" daily for 5 consecutive days. Water in the reservoirs was changed following the last herbicide application, and the system was allowed to cycle for several days during which time the edaphic algae were sampled. The motile algal population, primarily diatoms, was sampled by placing 10 sections of double layer lens paper, 2.5 by 2.5 cm, on the soil surface of each system at low tide (Eaton and Moss, 1966). Following removal of the lens paper, the nonmotile and/or nonsurface edaphic algae were sampled for chlorophyll analysis with 2.3 cm diameter PVC coring tubes. All algal samples were held in icebox coolers until initiation of the various assay procedures. Core samples were frozen until the chlorophyll was to be extracted (Gallagher, 1971).

Chlorophyll was measured fluorometrically in 3 of the 10 lens paper samples by the methods of Yentsch and Menzel (1963). Core samples were sectioned horizontally with a thin spatula into two segments, 0 to 2 mm and 2 to 5 mm, and chlorophyll was determined similarly.

Diatom cell number was assayed by boiling three of the lens paper samples from each microecosystem in nitric acid and then identifying the cleaned diatoms (Patrick and Reimer, 1966). Species diversity (H') and the structure of diatom communities (SIMI) were computed with methods described by Sullivan (1975). In the February experiment, 100 values from each of the four replicates for each of the two treatments were enumerated. Species diversity (H') was calculated and the results pooled for presentation. Diatom community structure (SIMI) was calculated with the pooled data for the 400 enumerated values. In the April experiment, one system was selected at random from each of the two treatments and 100 values enumerated. Results of species diversity determinations were tested for statistical differences

using the Student T test. No statistical tests were applied to the SIMI values. Photosynthesis rates were determined by placing four of the 10 lens paper samples from each microecosystem on 3- by 3-cm glass slides. The lens paper was spread as evenly as possible, and a few drops of distilled water added to prevent desiccation. The lens paper samples were placed in an air tight chamber (Darley et al., 1976) and incubated for 30 min in the light (3 reps) or dark (1 rep) with a known amount of $^{14}CO_2$. Light intensity was 4 klux from cool white fluorescent lamps, and the temperature was 25 C. At the end of the exposure period, samples were exposed to HCl fumes to remove any unfixed ^{14}C, placed in scintillation vials with cocktail, and vortexed prior to determining ^{14}C incorporation by liquid scintillation spectrometry. Carbon fixation estimates were obtained by the formula of Darley et al. (1976).

Field Studies (Tubs)

Plastic tubs 36 cm in diam and 29 cm deep were filled to a depth of 15 cm with surface soil obtained from the area previously identified. The tubs were partially buried in the soil so that both the inside and outside soil surfaces were at the same level. A 3-cm diameter hole in the side of the tubs at ground level provided tidal water movement into and out of the systems. Tubs were positioned on a level area of creekbank marsh in Southend Creek, Sapelo Island, GA, in June 1977. Edaphic algal populations were allowed to habituate to their new surroundings for 7 days prior to herbicide application. For herbicide application, the tubs were removed from the creekbank and placed on high ground. The systems were then flooded twice daily for 5 consecutive days with either 0 or 10^{-5} M atrazine solution in 7.6 L on nonfiltered water collected from the nearby creek. Each treatment was replicated four times. Flooding periods were from 1.5 h before to 1.5 h after high tide as given in the tide tables for the Savannah River entrance to the marsh. After the 5-day treatment period, the tubs were returned to their original positions along the creekbank, and 10 *U. pugnax* were added to each system.

Edaphic algal productivity was measured as described for the microecosystems. Samples were taken both inside and outside of the tubs to determine not only the effects of atrazine but also any effect induced by the tubs. Cell number, species diversity, and SIMI values were determined as previously described. A total of 900 values were enumerated: 300 from each of the three collection zones. Chlorophyll levels were not determined in this experiment.

Field Studies (Metal Cylinders)

To help insure the continued presence of fiddler crabs in test plots, 12 aluminum cylinders 1.8 m in diam and 80 cm high were pressed 15 cm into the soil surface, leaving 75 cm above ground. A single 5-cm diameter hole, covered with 2 thicknesses of hardware cloth, at ground level permitted normal exchange of tidal water and prevented the movement into and out of the system of all but the smallest fiddler crabs. Three of the 12 plots were chosen at random for each atrazine concentration.

Atrazine was applied as a spray in 500 ml of water at concentrations of 0, 100, 1000, and 10,000 ppm (0.0, 0.05, 0.5, and 5.0 g/m^2), respectively. The 0.5 g/m^2-rate is similar to the 0.22-0.45 g/m^2 used for weed control in corn. The methods of Van Raalte et al. (1974) were modified for determining ^{14}C-uptake by edaphic algae following addition of atrazine. Cores of surface soil were taken with PVC coring tubes as previously described. Effects of the sides of the cylinders were minimized by taking cores at least 25 cm from them. The top 0.5 cm of each core was placed in an air-tight 75-ml light or dark jar and inoculated with 10 ml of distilled water containing a known amount of $NaH^{14}CO_3$. Salinity was adjusted to 20%, with NaCl and the pH adjusted to 8 with NaOH. Samples were shaken vigorously to expose the maximum surface area of each core to incident radiation and incubated in afternoon sunlight for 2 to 4 h in a shallow-water bath. Samples were killed with 0.5 ml of concentrated HCl. The samples were frozen to aid in cell rupture prior to lyophilization; 10 ml of concentrated nitric acid were added to each dried sample, and the samples were allowed to digest overnight. A 0.1-ml aliquot was pipeted into scintillation vials containing 15 ml of Aquasol and radioassayed for 10 min or 10,000 cpm with a liquid scintillation spectrometer.

RESULTS

Culture Studies

Atrazine, at the highest concentration tested, 10^{-5} M, significantly reduced the chlorophyll level, rate of photosynthesis, and cell number of *N. sigma* and *T. fluviatilis* following a 7-day exposure (Figure 14), while the lowest concentration, 10^{-7} M, had no significant effect on any paramanter assayed. The results with the intermediate concentration, 10^{-6} M, were variable. There was no effect on chlorophyll content for either species, a reduction in cell numbers only in T. fluviatilis, and a reduction in photosynthesis rate in both cultures.

Microecosystems

In the first experiment (February 1977), primary productivity was reduced from 191.4 to 29.0 mg $C/m^2/h$ in microecosystems flooded for 5 days with 10^{-5} M atrazine (Table 16). Chlorophyll content was also reduced in the 0-2 and 2-5 mm soil layers. Cell numbers and chlorophyll content of the surface algae (lens paper harvested) were not affected. In the April experiment, primary productivity was again reduced. Chlorophyll content was reduced in the surface algae but not in the soil segments (Table 16). There was no clearly identifiable effect on diatom species diveristy in either study (Table 17), but atrazine increased the numbers of *Cymatosira belgica* in both experiments (Figure 15). Atrazine had little effect on community structure in either experiment as evidenced by SIMI values (Table 17).

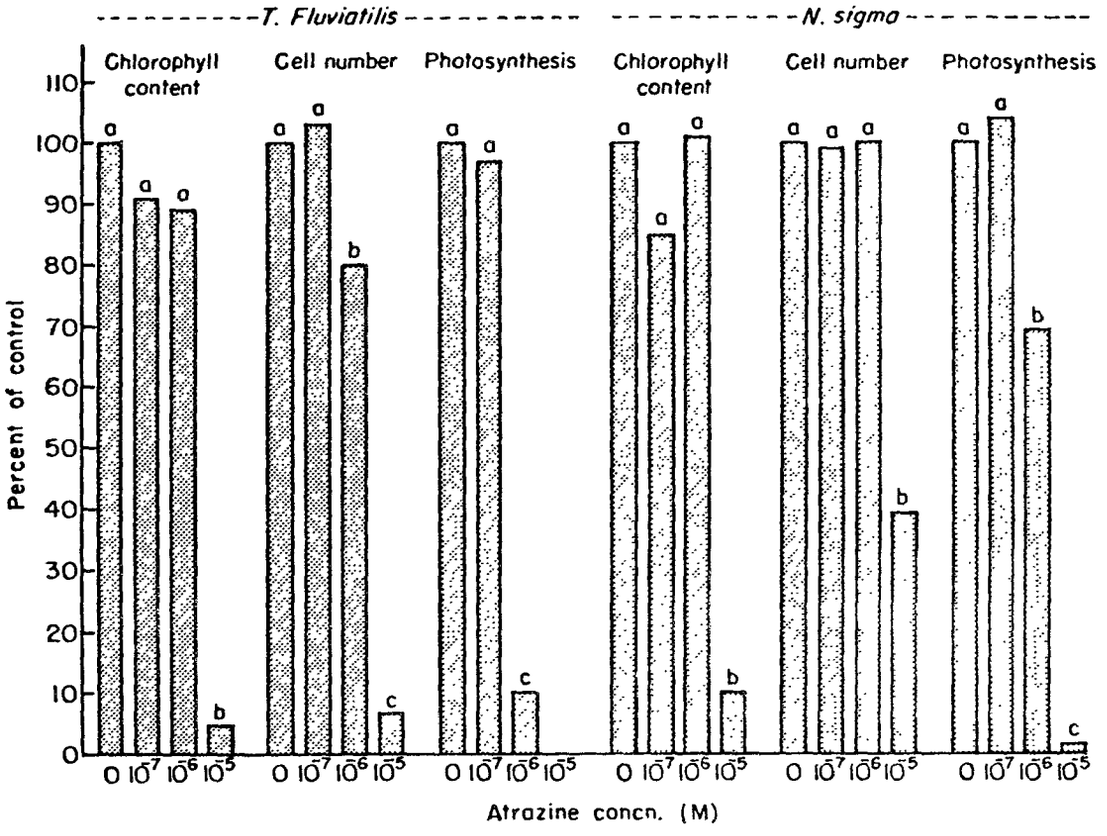


Figure 14. Effect of 7 days of exposure to various atrazine concentrations on the chlorophyll content, cell number, and rate of photosynthesis of laboratory cultures of *Thallasiosira fluviatilis* and *Nitzschia sigma*. Within a given measurement, columns having the same letter (a, b, or c) are not significantly different at the 5% level according to Duncan's new multiple range test.

Field Studies (Tubs)

Five days of treatment by flooding with 10^{-5} M atrazine solutions did not significantly reduce the number of cells counted 1 or 7 days after completion of treatment; the actual counts for the controls and treated areas averaged 2.81 x 10^8 and 2.85 x $10^8/m^2$, respectively, on day 1 and 13.5 x 10^8 and 7.1 x 10^8, respectively, on day 7. Cell numbers inside the enclosures did not vary significantly from outside the enclosures. Rates of carbon fixation (mg $C/m^2/h$) were measured 7 and 18 days after treatment. Carbon fixation was significantly decreased by the treatment at both of these sampling times. The average fixation rates for the controls versus the treated areas were 577 and 155, respectively, on day 7 and 334 and 161, respectively, on day 18. Average rates of photosynthesis were six times lower outside the containers than for the controls inside the containers 7 days after initiation of the experiment.

TABLE 16. EFFECTS OF ATRAZINE ON PHOTOSYNTHESIS, CELL NUMBERS, AND CHLOROPHYLL CONTENT OF DIATOMS IN MICROECOSYSTEMS

	Herb. Concn (M)	Photo. mg C per m^2/h	Cells per $m^2 x 10^8$	Chlorophyll Surface (mg/m^2)	Chlorophyll 0-2 mm (mg/m^2)	Chlorophyll 2-5 mm (mg/m^2)
Feb.	0	191.4 a*	12.2 a	2.5 a	77 a	54 a
	10^{-5}	29.0 b	11.2 a	2.1 a	43 b	35 b
April	0	283.4 a	28.6 a	4.8 a	87 a	61 a
	10^{-5}	30.3 b	6.5 a	1.6 b	62 a	69 a

*Means in the same column for the same date followed by a common letter are not statistically different at the 5% level as determined by Duncan's new multiple range test.

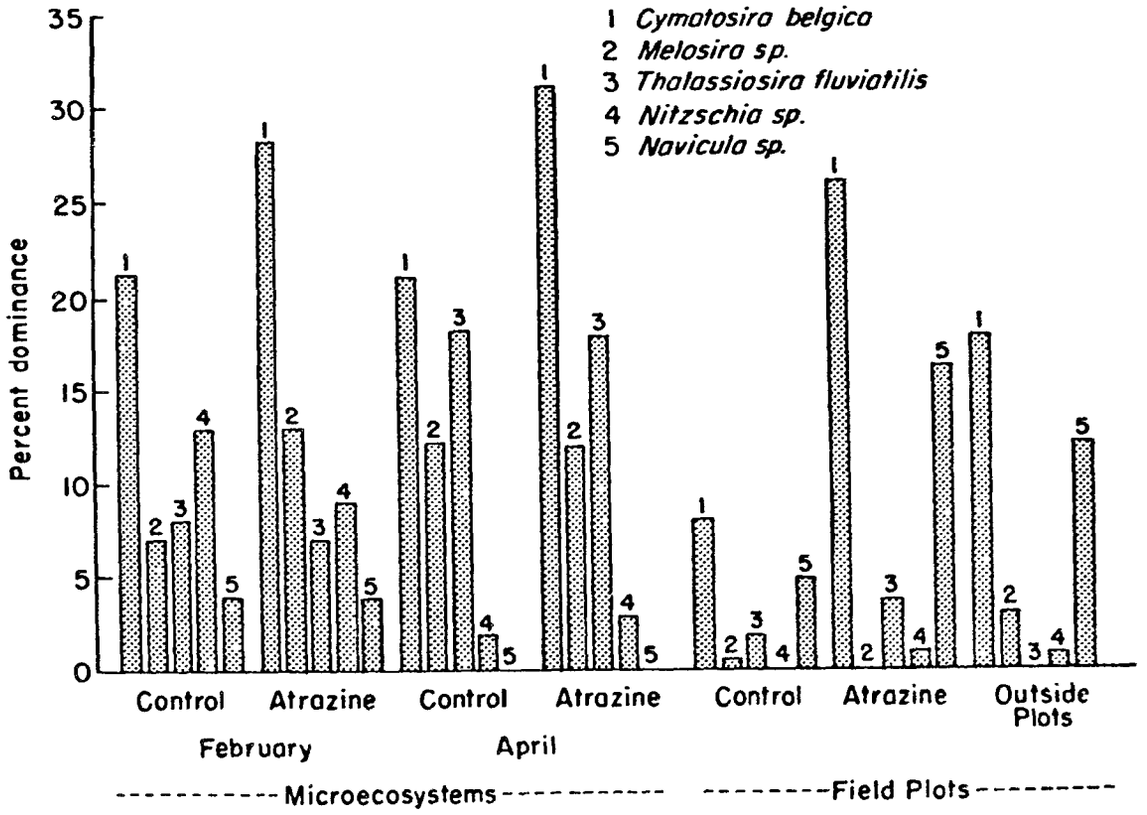


Figure 15. Percentage dominance for each of the five most common diatom species in atrazine-treated and control areas in microecosystems and in the field.

Atrazine did not significantly affect species diversity (H') in the microecosystems or in the field (Table 17). However, atrazine did consistently increase the number of *C. belgica* (Figure 15). Atrazine appeared to cause an increase in *Navicula* sp. in the field but not in the microecosystems. There were larger numbers of *C. belgica* and *Navicula* sp. in the untreated controls inside than outside the tubs. In the microecosystems, the matrix of similarity values (SIMI) between atrazine-treated and untreated systems was 0.838 for the February experiments and 0.906 for the March experiments. A value of 1 results when two communities support the same species in the same relative abundance and 0 when the communities are completely different. In the field, when atrazine-treated areas inside the tubs were compared with untreated areas outside the tubs, the SIMI-value was 0.786 again, indicating that atrazine did not greatly alter community structure. However, when untreated areas inside the tub were compared with untreated areas outside the tub, the SIMI-value was 0.506. This relatively low value indicates that the presence of the tub had a significant effect on community structure which in turn means that some other means should have been devised to determine the effect of atrazine in the salt marsh. The use of larger containers and a longer delay time after transfer of the soil into the container might alleviate this problem.

TABLE 17. DIATOM SPECIES DIVERSITY (H'=BITS/INDIVIDUAL) AND NUMBER OF SPECIES (S) FOR ATRAZINE-TREATED AND UNTREATED MICROECOSYSTEMS AND IN THE FIELD*

Value	Herb.	Microecosystems		Field (tubs)	
		February	April	Inside	Outside
H'	No	2.99 ± 0.42	3.80	3.85 ± 0.53	3.66 ± 0.17
H'	Yes	3.51 ± 0.44	3.43	3.42 ± 0.32	----------
S	No	16.20 ± 1.50	25.00	24.70 ± 7.50	24.70 ± 2.50
S	Yes	21.00 ± 6.30	23.00	22.36 ± 3.50	----------

Values for the microecosystems in the February experiments are averages of four replications (100 values each) ± 1 standard deviation (SD), and for the April experiments are for one replication with 100 values used. Values for the field plots are averages of three replications (100 values each) ± 1 SD. None of the H' values for the atrazine-treated plots vary significantly from the companion control plots (p=0.05).

Field Studies (Metal Cylinders)

The studies in the metal cylinders involved much higher rates of atrazine than were used in other studies. Carbon fixation was significantly reduced by 0.05 and 0.5 g/m^2 16 days after treatment but not 26 days after treatment. The 5.0-g/m^2 rate inhibited carbon fixation through the 42-day assay but not on the 67-day assay. Assays were continued on all plots through the 108th day. Even the lowest rate used, 0.05 g/m^2 (100 ppm), is several hundred times greater than would be expected in the runoff from treated fields (Anonymous, 1977).

The degree of inhibition of photosynthesis, chlorophyll production, and cell numbers in cultures on *N. sigma* and *T. fluviatilis* by atrazine are in close agreement with values obtained for other algal types. Davis et al. (1976) found that prometryn, another s-triazine, reduced O_2 production in *Chorella pyrenoidosa* by 94% after a 40-min exposure at 5×10^{-5} M. The ability to maintain chlorophyll synthesis in *T. fluviatilis* and cell division and chlorophyll production in *N. sigma* with significantly reduced photosynthesis rates (atrazine at 10^{-6} M) poses interesting questions. It would appear that these two benthic alga species may be able to maintain themselves with reduced light (if atrazine addition is considered to be analagous to a reduction in light intensity). Research by others partially supports our findings.

Admiraal (1977) reported a reduction in doublings/day for *N. sigma* when grown under 16-h days as compared to 8-h days; photosynthesis rates and chlorophyll production were not determined. In our experiments with *N. sigma*, cell number was decreased about 50% when photosynthesis was reduced about 33%. Similarly, *Cyclotella meneghiniana*, a freshwater diatom, has the same chlorophyll level and photosynthesis rate when grown under 3 or 30 klux (Jorgensen, 1964). If *T. fluviatilis* and *N. sigma* can maintain chlorophyll synthesis and cell division in the equivalent of reduced light, their optimal dominance should be in the winter. Williams (1962) has previously reported a winter maximum for *N. sigma* in a Georgia salt marsh. Williams also found maximum photosynthesis rates at approximately 50% of full sunlight, in winter and summer, further suggesting that at least some members of the community may be saturated at less than 100% of full sunlight.

Carbon fixation by untreated controls in the microecosystems (Table 16) were similar to previously reported values; e.g., Pomeroy (1959) estimated that winter production in a Georgia salt marsh was 150 mg $C/m^2/h$ at low tide, and Darley et al. (1976) reported a summer value as high as 244.7 mg $C/m^2/h$ for creekbank samples from the same area. Estimates obtained in the April microecosystem experiment were somewhat higher (283.4 mg $C/m^2/h$) than expected. Possible explanations for these high values include increased productivity due to greenhouse effects, an alteration of light quality and intensity inside the plastic tubs used as microecosystems, or addition of algal cells to the soil surface from the water reservoirs. Estimates of cell numbers in microecosystems were in close agreement with those for the field. Thus these microecosystems appeared to give reasonable values for cell numbers and primary productivity even though species diversity was

adversely affected. The value for cell numbers in April nontreated microecosystems (Table 16) was higher than expected. This was due to a bloom in one system of *T. fluviatilis*, a small diatom which could be present in large numbers and still not significantly alter chlorophyll level or rate of photosynthesis.

Chlorophyll estimates in microecosystems were also similar to reported values of Sullivan and Daiber (1975 b), who estimated a yearly average of 100 mg Chl/m^2 in the top 1 cm of a Delaware marsh. The nonatrazine-treated systems in this study contained ca 135 mg Chl/m^2 in the top 0.5 cm of soil.

The presence of high rates of photosynthesis in the lens paper samples which contained only a small percentage of total chlorophyll indicates that there may be a large population of photosynthetically inactive algae living just beneath the surface. Examination of this layer with a light microscope verified the presence of numerous living cells. Heterotrophy would be the only means of survival for this community, but Darley et al. (1979) have found that the surface community could only obtain up to 1.0% of its carbon by this means; no work has been undertaken to ascertain heterotrophic potential of subsurface algae.

The effects of atrazine in the field were less severe than in the culture work or microecosystems. This could have been due to the higher summer temperatures with correspondingly higher rates of microbial degradation of atrazine and increased volatilization. However, the dilution of the applied atrazine by tidal flux may have been more important. The nearly six-fold increase in primary productivity observed inside the test enclosures as compared to adjacent nonenclosed controls is possibly explainable on the basis of decreased sunlight intensity and/or duration inside the containers. Pomeroy (1959) has reported an inhibition of photosynthesis by the summer sun, but this has not always been confirmed in other studies (Gallagher, 1971; Van Raalte et al., 1976). It is possible that the shade-adapted diatoms produced more chlorophyll and had a correspondingly greater ability to assimilate CO_2.

In the field as in the microecosystems, *C. belgica* was the most dominant diatom species, and the addition of atrazine appeared to increase its numbers. Although only 100 values were enumerated per sample in determining species diversity, the low coefficient of variation (always < 15%) between replicate samples would indicate that sufficient values were enumerated. The species diversity values, both for the field plots and the microecosystems, agree well with the data of Sullivan (1975) who reported H'-values of 4034 to 4688 in a Delaware salt marsh. The degree of similarity between the atrazine-treated microecosystems and the nontreated systems indicates either (1) that only a few species can survive in this artificial environment and that the resultant population is stable even when exposed to atrazine or (2) (less likely) the severity of atrazine effects on species diversity is less in the winter than the summer. Similarity values were not computed for microecosystem diatom populations versus those in the field because of known differences in taxa throughout the year (Williams, 1962).

The primary objective of this study was to evaluate the effects of atrazine on the salt marsh edaphic algae and, based on these findings, estimate safe levels of atrazine for this ecosystem. Several methods have been proposed for designating acceptable levels in the aquatic environment; only two will be discussed here.

The American Society for Testing and Materials Standards (1964) has devised a system whereby the I_{50} level obtained for diatoms in laboratory cultures exposed to the pollutant for 7 days is multiplied by a safety factor of 0.3. For this study, the I_{50} for *N. sigma* and *T. fluviatilis* was computed to be 4.36×10^{-6} M atrazine; 4.36×10^{-6} M x 0.3 = 1.3×10^{-6} M (0.28 ppm).

EPA has sometimes used a general "rule of thumb" of a 10-fold safety factor below the least effect level when this level is well-known. The least effect level (I_1) for algae grown in culture was computed to be about 5×10^{-7} M atrazine in these studies. With a 10-fold safety factor, acceptable levels would be 5×10^{-8} M (10 ppb). The authors consider this to be a more acceptable value than the 0.28 ppm calculated by the first method. One study (Anonymous, 1977) has reported atrazine concentrations in the range from 0.0 to a maximum of 2 ppb in water in tributaries of the Chesapeake Bay. It has been postulated that atrazine might be responsible for declines in aquatic vegetation in the Chesapeake Bay. Results from this study do not support this hypothesis.

SUMMARY

A 10^{-5} M (2.2 ppm) concentration of atrazine significantly reduced the rate of photosynthesis, chlorophyll content, and cell numbers in unialgal cultures of *Nitzchia sigma* Grun. and *Thalassiosira fluviatilis* Hustedt isolated from a salt marsh habitat. Results with lower atrazine concentrations indicated an ability to maintain chlorophyll production and cell division with reduced photosynthesis. The effects of a 10^{-5} M concentration of atrazine in unialgal cultures were also evident in microecosystems and in the field at the same concentration. Severity of atrazine effects was less in the field than in microecosystems or cultures. Cell number and productivity of the diatoms from nonatrazine treated microecosystems agreed well with field data and previously published data. Diatom species diversity was not affected by 10^{-5} M atrazine in microecosystems or in the field but the number of *Cymatosira belgica* was increased. Diatom populations in atrazine-treated versus nontreated microecosystems were very similar (SIMI value = 0.906). Results were less conclusive in the field but the trend was toward a lower level of similarity (0.838). Based on the least effect level of atrazine to diatoms, the maximum safe level for atrazine in the salt marsh is estimated to be 10 ppb.

SECTION 9

ATRAZINE RESIDUES IN SALT-MARSH ECOSYSTEM COMPONENTS: A COMPARISON OF FIELD AND MICROECOSYSTEM RESULTS

OBJECTIVES

The ultimate test of how well an investigator has succeeded in building a microecosystem that faithfully reproduces conditions in the field is to conduct an experiment in the field and in the microecosystem and compare results. The objectives of this investigation were: 1) to measure persistence of atrazine in various compartments in the salt-marsh ecosystem and 2) to compare the values obtained in the field with those in the microecosystem.

MATERIALS AND METHODS

Herbicide Application

Plots on the salt marsh on Sapelo Island, GA, and microecosystems at Auburn, AL, were sprayed with atrazine suspensions in amounts and concentrations sufficient to give atrazine application rates of 0.0, 0.05, 0.50, and 5.00 g/m^2. The atrazine concentrations used were 0.0, 100, 1000, and 10,000 ppm, respectively. Rates of application were replicated three times and were completely randomized. Atrazine residues in various ecosystem components were determined 10 weeks after herbicide application as described below. In addition to determining the amounts of atrazine remaining, the experiment was designed to test how well the results in the microecosystems matched those in the field. In order to enhance the parallelism between the microecosystems and the field plots, herbicide application were made in microecosystems 1 week after they were made in the field. This made it possible to duplicate variables observed in the field such as rains or height and periodicity of tides 1 week later in the microecosystems. Samples were taken for atrazine residue determination in the microecosystems 1 week after they were taken in the field.

Establishing Field Plots

Twelve 1.8-m diam cylinders 105 cm tall were sunk 25 cm deep in soil along a tidal creek in the salt marsh on Sapelo Island. Each cylinder had a 5-cm diam hole cut in the side at soil level. The hole was covered with 8-mm mesh hardware cloth. This port allowed free movement of tidal water into and out of the enclosures but prevented movement of all of the large fiddler crabs into and out of the area.

Establishing the Microecosystems

Each microecosystem was constructed from two 75-L molded plastic laundry tubs which were taken to Sapelo Island for filling with soil and biological components as described in Section 5. Additional details about these microecosystems are presented in Section 10 and by Everest and Davis (1977).

The ecosystem containers were returned to Auburn, placed on greenhouse benches, and the drainage pipes for the containers were connected to water reservoirs by means of 1.8-cm diameter rubber hoses and standard plumbing fittings. The water reservoirs (also 75-L laundry tubs) were filled with one part natural seawater (enough to supply an inoculum of associated microorganisms) and nine parts synthetic seawater. Each water reservoir contained 37.9 L of seawater (20 ppt). At least 2 weeks were allowed to lapse before any experiments were initiated to allow the systems to stabilize. Sufficient demineralized water was added every 3 days to compensate for water lost by evaporation and not added by simulated rainfall.

A "tide machine" was designed that mimicked tidal frequency, duration on the marsh, and rate of flow onto and off the marsh. The "tide machine" had three main components: (1) a supporting frame, (2) a mechanical drive system, and (3) an electrical timing system. The maximum depth of seawater above the soil level in each microecosystem was 17.5 cm; and during each 24 h and 50 min period, two tidal cycles were completed, each consisting of 2 h of flooding and 10 h and 25 min of no flooding. Additional details concerning the construction and function of this system are given in Section 10 and by Everest and Davis (1977).

Three days after the tides were initiated and soil had settled in place, mud fiddler crabs *(Uca pugnax)*, periwinkle snails *(Littorina irrorata)*, and ribbed mussels *(Geukensia demissa)* were placed in the systems at densities comparable to those observed on the plots on Sapelo Island. A 2-week delay was imposed between the introduction of the animals into the systems and herbicide treatment to insure that the animals had survived and had established normal movement and feeding behavior. At this time personnel on Sapelo Island were told to apply the herbicide when conditions become suitable (no rain and no tides that would flood the area within 6 h after application). The herbicide was applied the third week in July on Sapelo and 1 week later at Auburn.

The water in the microecosystem reservoirs was emptied and replaced by fresh synthetic seawater 6, 20, 37, 70, and 73 days after the herbicide was applied.

Sampling Procedure for Atrazine Residue Determination

Sampling was done 10 weeks after herbicide application. Each sample from each field plot or microecosystem was kept separate through the final atrazine residue determination. *Spartina alterniflora* was cut off at the

soil surface and divided into stems longer than or shorter than 0.5 m. Fresh and dry weights were determined, and then 500- to 1000-g subsamples were drawn, ground, and stored in a deep freeze until assayed. Fiddler crabs samples consisted of all crabs collected. The collected crabs were counted, thoroughly washed, weighed, and stored frozen in a deep freeze. Periwinkle snail samples consisted of all snails found on the *S. alterniflora* or on the soil surface. They were prepared as described for the fiddler crabs. Horse mussels were collected, counted, washed thoroughly, and weighed; their flesh was removed and stored frozen in a deep freeze.

Soil samples were taken from the 0-1, 1-10, and 10-25-cm layers from four randomly selected areas in each plot or microecosystem. Soil samples were taken before the soil was disturbed by any other sampling procedure. A soil surface sample was made by carefully removing a 1-cm thick layer from each of four ca 100 cm^2 areas. Polyvinyl coring tubes were inserted into these areas to a depth of 25 cm (24 cm + 1 cm removed initially). The coring tubes were then removed, and the contents were divided into the soil present in the 1-10 cm layer and the 10-25 cm layer. Sufficient tubes were used to give composite samples of 200-300 g for each soil layer. The soil was then freeze-dried, ground, and stored in a deep freeze until analyzed.

Water samples (500 ml) were taken from the microecosystems that were treated at the rate of 0.5 g/m^2 when the water was changed in the reservoirs (6, 20, 37, 70, and 73 days after herbicide application).

Atrazine Residue Determination

Atrazine residues were determined by gas-liquid chromatography. Water samples were washed with CH_2Cl_2, taken to dryness, and the extracts analyzed directly. The other materials were dried, ground to a powder, extracted by refluxing for 1 h with 90% acetonitrile, made to volume; an amount equivalent to 10 g of the sample was removed and a known amount of ^{14}C-label added to the extract. The acetonitrile solution was concentrated by flash evaporation, washed several times with CH_2Cl_2 and hexane, and the extract passed through an aluminum oxide column to isolate the atrazine. Recovery efficiency was measured by liquid scintillation radioassay of the column eluate. Atrazine concentration in the eluate was determined by a Hewlett-Packard (HP) gas chromatograph (model 5710 A) equipped with a 1.22 m x 2 mm glass column packed with 2% OV-101, a HP Nitrogen-Phosphorus detector, and an HP 3380 A data system.

RESULTS AND DISCUSSION

Residues in Soils

Atrazine residues found in soils 10 weeks after herbicide application are given in Table 18. The variability between replications was so large and the residue levels so low that there is no certain evidence that either soil from the field or microecosystems treated at the 0.05 or the 0.50 g/m^2 rates had a measurable level of atrazine remaining. It is apparent that the amount, if any, must be no more than 0.1 ppm. Field plots treated with

TABLE 18. AVERAGE CONCENTRATIONS OF ATRAZINE IN VARIOUS SOIL LAYERS IN THE FIELD AND IN MICROECOSYSTEMS 10 WEEKS AFTER HERBICIDE APPLICATION

Atrazine rate g/m^2	Location	Soil layer samples (cm) 0-1 (ppm)	1-10 (ppm)	10-25 (ppm)	0-25 (ppm)
0.00	Field	0.03 ± 0.07*	0.05 ± 0.08	0.00 ± 0.03	0.02†
0.00	Microeco	0.06 ± 0.15	0.09 ± 0.19	0.00 ± 0.00	0.03
0.05	Field	0.05 ± 0.03	0.03 ± 0.05	0.04 ± 0.11	0.04
0.05	Microeco	0.11 ± 0.18	0.00 ± 0.02	0.00 ± 0.07	0.00
0.50	Field	0.06 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.02
0.50	Microeco	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.02	0.05
5.00	Field	1.20 ± 0.39	0.77 ± 0.33	0.25 ± 0.27	0.48
5.00	Microeco	0.00 ± 0.03	0.01 ± 0.03	0.01 ± 0.03	0.01

*Values are given ± N-1 standard deviation. All samples were spiked with known amounts of radiolabeled atrazine. When the amount added was subtracted from the value determined for the sample, the result was sometimes negative. Occasional averages were slightly negative. Negative values were used in calculating the standard deviation, but any apparently negative averages are presented as 0.

†The value for the 0-25 cm layer is calculated from the values for the three layers (0-1 cm, 1-15 cm, 15-25 cm) involved.

5.0 g/m^2 had 1.20, 0.77, and 0.25 ppm in the 0 to 1-, 1- to 10-, and 10- to 25-cm layers, respectively. The average was 0.48 ppm in the top 25 cm. If one assumes a specific gravity of 1.2 for the soil, then this 25-cm layer contained 144 mg of atrazine or 2.88% of that applied. The microecosystems averaged only 0.01 ppm which is not significantly different from the control. It is not known why the microecosystems lost more atrazine than the field plots. It is possible that it was easier for the tidal flux to remove the atrazine from the small surface area (0.26 m^2) of the microecosystems than from the relatively larger area (2.5 m^2) in the field plots. No doubt areas of very slow movement occurred in the field plots since all of the water had to go into or out of one comparatively small hole.

Residues in *S. alterniflora*

Atrazine residues in *S. alterniflora* are presented in Table 19. As for the soil, atrazine residues in plants grown in the field or in microecosystems treated with 0.05 or 0.50 g/m^2 of atrazine were too low and too variable to be significantly greater than the controls. The value of 0.50 ppm for *S. alterniflora* > 0.5-m-tall harvested in the field is higher than one would expect in view of the other data. We have no basis for explaining this one relatively large value.

TABLE 19. AVERAGE CONCENTRATIONS OF ATRAZINE IN TWO SIZE CLASSES OF *S. ALTERNIFLORA* (< 0.5 M AND > 0.5 M TALL) IN THE FIELD AND IN MICROECOSYSTEMS 10 WEEKS AFTER HERBICIDE APPLICATION

Atrazine Rate (g/m^2)	Location	Size of Plant Sampled	
		< 0.5 m (ppm)*	> 0.5 m (ppm)*
0.00	Field	0.00 ±	0.04 ± 0.06
0.00	Microeco	0.04 ± 0.13	0.00 ± 0.03
0.05	Field	0.11 ± 0.23	0.50 ± 0.23
0.05	Microeco	0.02	0.07 ± 0.07
0.50	Field	0.16 ± 0.20	0.09 ± 0.11
0.50	Microeco	0.04 ± 0.05	0.51 ± 0.46
5.00	Field	21.58 ± 9.85	12.83 ± 2.46
5.00	Microeco	16.80 ± 6.59	21.07 ± 7.73

*Values given are averages ± the N-1 standard deviation. When no standard deviation is given, the material from all three plots were combined in order to get one sample large enough to assay.

The amounts of *S. alterniflora* harvested from these plots did not vary significantly either between rates of atrazine applied or between the ecosystems and the field. Average dry weight yields were 45.5 g/m^2 for *S. alterniflora* < 0.5 m tall and 231.2 g/m^2 for plants > 0.5 m tall. Using these values for *S. alterniflora* yields and the concentration of atrazine found in subsamples from *S. alterniflora* < or > 0.5 , the total amount of atrazine in *S. alterniflora* harvested from field plots treated with 5.0 g/m^2 of atrazine was 3.9 mg/m^2 or ca 0.08% of that applied. For the microecosystems the average of the atrazine residues was ca 5.6 mg/m^2 or ca 0.11% of that applied. The higher concentration of atrazine in the *S. alterniflora* from the microecosystems than from the field is probably because in the field each tidal change brought in essentially atrazine-free water whereas new water was not placed in the microecosystem reservoirs until 6 days after herbicide application.

Residues in Animals

Atrazine residues in periwinkle snails, mussels, and fiddler crabs from plots treated with 5.0 g/m^2 of atrazine are given in Table 20. None of the animals from plots receiving lower rates of treatment contained a significant amount of atrazine except for snails in the plots treated with 0.50 g/m^2 atrazine which contained 0.45 and 0.34 ppm from the field and the microecosystems, respectively. Atrazine concentration was 15-20 times higher for snails from the microecosystems receiving 5.0 g/m^2 atrazine than for snails from plots in the field treated at the same rate. It is possible that part of this difference is due to snail migration between plots in the

TABLE 20. AVERAGE CONCENTRATIONS OF ATRAZINE IN PERIWINKLE SNAILS, HORSE MUSSELS, AND FIDDLER CRABS IN THE FIELD AND IN MICROECOSYSTEMS 10 WEEKS AFTER HERBICIDE APPLICATION

Atrazine rate (g/m^2)	Location	Organism		
		Snails (ppm)*	Mussels (ppm)*	Crabs (ppm)*
5.00	Field	0.45	0.00	-----
5.00	Microeco	7.76	3.49	0.31

*Values are for one composite sample. There was insufficient material to any single plot to make a reliable assay of the atrazine present.

field whereas this was only remotely possible between microecosystems. However, the atrazine concentration in mussels was also much greater in the microecosystems than in the field, and these organisms did not migrate out of their plots. It seems possible that the greater concentration of atrazine in the microecosystem than in the field is (as was mentioned above) because atrazine-containing water flushed back and forth into and out of the microecosystems whereas the tide brought in essentially atrazine-free water in the field. Perhaps some food organisms or organic material containing atrazine also moved back and forth into and out of the microecosystems. There were not enough crabs in the field plots receiving the highest atrazine concentration to analyze the atrazine in their bodies. Therefore, it is not known whether the crabs from the microecosystems also contained more atrazine than those from the field.

Residue in Water

In order to estimate the amount of atrazine removed in tidal water, periodic samples were taken from the three reservoirs attached to ecosystems treated with atrazine at the rate of 0.50 g/m^2. The samples were taken just prior to replacement of the water with fresh seawater. The concentrations of atrazine present in these samples are given in Table 21. After 6, 20, 37, and 70 days, the accumulated atrazine discarded when the water was changed amounted to 42, 49, 50, and 50% of that applied, respectively. After harvesting was completed, an additional assay was made. During this 3-day period, an additional 0.1% of that applied had moved into the water. It is difficult to apply these results to what might be expected in the field. If atrazine is accidentally spread on a small area of the marsh, it would seem reasonable to guess that about half of it would be washed away by the tide in 1 week. The rate of disappearance would be expected to be higher than that found for the microecosystems; each tidal invasion in the field would bring essentially atrazine-free water, whereas fresh water was introduced in the microecosystems only after 6 days. However, if the source of the atrazine contamination to the marsh is the tide itself, it

TABLE 21. AVERAGE AMOUNTS OF ATRAZINE COLLECTED IN WATER RESERVOIRS ATTACHED TO MICROECOSYSTEMS TREATED WITH 0.50 g/m^2 (0.13 mg/system) ATRAZINE

Collection period (days)	Concn (ppm)	mg in 37.9 L	% of applied
0 - 6	1.440 ± 0.114*	54.57	41.98
6 - 20	0.235 ± 0.026	8.91	6.85
20 - 37	0.035 ± 0.003	1.33	1.02
37 - 70	0.007 ± 0.002	0.265	0.11
70 - 73	0.003 ± 0.008	0.114	0.09

*Values given are average ± the N-1 standard deviation. The average value for the 0- to 6-day sample from the untreated controls was 0.00165 ± 0.0008.

would be expected to diminish in the marsh as atrazine concentration in the tidal water decreased.

Care must also be exercised in applying the data accrued for atrazine concentrations in soil, plants, and animals to situations found in the field. The relatively high residues found in these components from areas treated with 5.00 g/m^2 have no relevance to any ordinary happening in the field. The 5.00 g/m^2-rate is 10-20 times the rate of application that is used for weed control in corn (WSSA Herbicide Handbook Committee [1979]) and was included only to insure that measurable atrazine concentrations could be found in some of the ecosystem components after 10 weeks. To achieve the lowest atrazine treatment level used, the areas were sprayed with suspensions containing 100 ppm atrazine. Tidal water contamination would not be expected to be as much as 0.1 ppm (Chesapeake Bay Research Consortium, 1977). However, two conclusions are possible. First, if a small section of the marsh is accidentally contaminated with atrazine, atrazine would be rapidly exported in the tide, and unless the contamination rate was extremely high, residue levels should approach 0 within 3 months. Second, ordinary levels of atrazine contamination would not result in any atrazine carryover from one growing season to the next.

Atrazine levels found in the microecosystems were similar to those in the enclosed plots in the field. Many of the differences observed are explainable in terms of the fact that each tidal flush in the field involved essentially atrazine-free water; whereas in the microecosystems, the water running off the systems was replaced by atrazine-free water only at irregular intervals. The first change was 6 days after atrazine application.

SUMMARY

Within 3 months after atrazine application the total amount of atrazine remaining in the soil, *S. alterniflora*, snails, fiddler crabs, and horse mussels was less than 3% of that applied. At the lower rates of application

(0.05 and 0.50 g/m^2) the amounts of herbicide remaining were often below the sensitivity of the assay system. Although both the tests with microecosystems and in the field confirmed the rapid disappearance of atrazine from the treated areas, there were significant differences in results between the two systems. Considerably more atrazine remained in the soil of systems in the field than in the microecosystems. This was perhaps because the enclosures around the field plots permitted tidal movement out only through one relatively small hole. This may have resulted in areas of stagnation such that atrazine was not swept away nearly as vigorously as in the microecosystems. Contrariwise, more atrazine was found in the *S. alterniflora*, snails, and mussels in the microecosystems than in the fields. This was probably because each new tide in the field introduced new water,whereas the water in the reservoirs was not changed until after 6 days.

SECTION 10

MICROECOSYSTEMS

LITERATURE REVIEW

Over the last two decades, there has been a growing interest in the ecosystem concept in biology. Natural ecosystems are often quite large and variable. The most difficult problem in working with ecosystems is to establish boundaries between them. In nature, ecosystems tend to blend or grade into one another, making any boundary which is established purely artificial.

Researchers have developed a new technique to aid in the study of ecosystems. The technique involves enclosing a small portion of an ecosystem to isolate this functional unit from the rest of the biosphere. This deliberately isolated ecosystem is known as a microcosm or a microecosystem (Booth, 1977). There are several advantages of using microecosystems in ecosystem studies. Their small size and isolation from the surrounding environment make possible the study of the effects of very expensive or highly toxic materials and the more precise measurement of responses. Furthermore, precise control of environmental conditions such as temperature, gas composition, or light intensity is more feasible. The microecosystem approach also makes possible replicated studies using very similar ecosystems, which is seldom feasible in nature.

Ideally, each microecosystem should include: one or more primary producers which serve as a food source for one or more primary consumers, one or more trophic levels of carnivores, and decomposers. Furthermore, population densities and soil, air, and water amounts and composition should be comparable to those in nature. The systems should be self-sustaining for long periods of time without the need to supply more organisms, feed, or fertilizer.

Most of the microecosystems which have been used in research have been aquatic rather than terrestrial and they have rarely approached the ideal microecosystem described above. They have usually been quite simple in design, containing only two trophic levels and a very limited number of kinds of macroscopic organisms.

---

[9]Several portions of this section are reprinted from Pages 167-171 of the 2nd ed of Research Methods in Weed Science. Copyright Sout. Weed Sci. Soc. Used by permission.

Cooke (1969) gave an excellent review of the literature dealing with aquatic microecosystem usage. He pointed out the parameters that could be measured or controlled, and the benefits and disadvantages of using microecosystems in a wide variety of investigations. Beyers (1963) subjected microbial populations in glass containers to different environmental regimes and determined the effect that these modifications had on diurnal metabolism. Abbott (1966, 1967, 1969) used similar systems to determine the effects of nitrate and phosphate enrichment. Taub (1976) investigated the effect of some algicides, insecticides, and heavy metals in aquatic microecosystems containing bacteria, algae, and grazing microorganisms. He showed that shifts in species composition occurred with increased populations of resistant forms.

Other aquatic and aquatic-terrestrial microecosystems have been designed and used to study the accumulation and effects of various pesticides in the environment (Isensee et al., 1973; Metcalf et al., 1971; Sanborn, 1974; Yu et al., 1975). These studies demonstrated that some pesticides tended to accumulate in organisms in the higher trophic levels, while others had a low potential for bioaccumulation. Results from these studies have shown the value of microecosystems for predicting the fate of pesticides in the environment.

MICROECOSYSTEMS USED

During the course of our investigations, several different types of microecosystem were designed and used to determine the fate and effects of atrazine in a salt marsh. The microecosystems employed varied in size, complexity, and mode of operation.

The microecosystem used to study the metabolism of atrazine in *Spartina alterniflora*-detritus-*Uca pugnax* food chain (Section 6) was the smallest and simplest in design. This system consisted of a Buchner funnel containing a nylon mesh liner covered with a 1.5-cm layer of sand on top of which was a 1.5-cm layer of marsh soil containing the associated microflora. Each funnel was connected to a small water reservoir by plastic tubing. The tidal flux was obtained by the raising and lowering of the water reservoirs (Figure 11, Section 6). This microecosystem made it possible to follow the conversion of dried *S. alterniflora* to detritus, and the fate of ^{14}C-atrazine in the *S. alterniflora* during this conversion. Quantitative assessment of the various metabolites of atrazine produced would not be feasible in the field. There were several ways in which these systems did not match conditions in the salt marsh. Since the microecosystems did not contain grazing organisms, the microflora populations present in each system were not reduced by normal feeding activity. Probably the most important variation from the field was the closed seawater system. The same seawater, except for one change after a 10-day period, was used to flush the systems during each tidal cycle. This did not bring about dilution comparable to the tides and may have resulted in the gradual accumulation of toxic materials. Daily changes of the seawater or the design of a more complex chemostat system could have been used to overcome this weakness. The small size of the systems limited the amount of detritus that could be produced.

The microecosystems used in the greenhouse to study the effect of atrazine (Section 8) on marine diatoms were intermediate in size and complexity. Each system consisted of a plastic tub containing a 10-cm layer of creek bank soil, associated diatoms, and other microorganisms. These tubs were connected to individual water reservoirs (plastic buckets) by rubber tubing fastened to glass tubes inserted into rubber stoppers fitted into holes in the tubs and buckets. There were several benefits in using these microecosystems. The tubs and buckets used were inexpensive and relatively heat and sunlight stable. The soil-containing tubs were yellow-orange in color so that they did not reflect or absorb significant amounts of heat or light. Also, the tubs and buckets were small and relatively lightweight (even when full) so filling in the field and transporting them to the greenhouse was easy. As with any design, there were weaknesses or limitations discovered while using these systems. Since the systems contained no fiddler crabs to graze on the algae or disturb the soil surface and thus produce turbid water, the systems developed unnatural blooms of blue-green algae. Fiddler crabs had to be added to the systems to prevent such blooms. The seawater reservoirs were not large enough to give adequate tidal dilution. This was especially apparent after the herbicide treatments when the systems were recovering from the atrazine effects. This problem might have been overcome by changing the seawater in the reservoirs daily after the herbicide treatments to simulate natural tidal dilution. The single most annoying problem was the splitting of the tubs and buckets where the rubber stoppers were inserted into them. The use of silicone caulking material or cement to hold the stoppers in place or some alternative connectors might have alleviated this problem.

The microecosystems used in the field to study atrazine effects on marine diatoms were much simpler (Section 8). Each microecosystem consisted of a 36-cm-diam plastic tub filled to a depth of 15 cm with soil collected in the immediate vicinity. The tubs were partially buried in the soil so that both the inside and outside soil surfaces were at the same level. A 3-cm-diam hole in the side of the tub at the soil surface could be closed during atrazine treatment or left open to allow normal tidal movement onto or off of the soil in the tub. The ecosystems were placed on the creek bank in the field and thus apparently varied from the natural situation only by the presence of the container walls. The systems were allowed to stabilize for 7 days prior to atrazine treatment. Effects of atrazine on photosynthesis, cell numbers,and cell kinds were compared within the tubs and also between the untreated areas inside and outside the tubs. Algal numbers did not vary significantly from the outside. However, the kinds of algae did,and the rate of photosynthesis inside the tubs was six times greater than that outside. At high tide fiddler crabs could easily crawl out of the tubs. The loss of these grazers, disturbance of the soil when filling the tubs, and shading by the walls of the tubs may have caused the observed variations. The use of larger containers and a longer delay time after transfer of the soil into the containers might decrease the observed differences.

The largest and most complex microecosystem used in these investigations was partially described in Sections 7 and 9. The microecosystem containers and water reservoirs were constructed from 75-L molded plastic tubs. The

microecosystem containers were connected to their individual reservoirs by means of rubber tubing. The microecosystems contained marsh soil and associated microorganisms, *S. alterniflora*, fiddler crabs, ribbed mussels, and periwinkle snails. The water reservoirs containing seawater (salinity-20ppt) were connected to a device called a tide machine which was designed to raise and lower the reservoirs, thus causing water to flow onto and off of the soil in the microecosystems.

The tide machine had three main components: (1) a supporting frame (Figure 16), (2) a mechanical drive system (Figure 17) and (3) an electrical timing system (Figure 18). The supporting frame was 4.8 m long by 1.2 m wide and supported on eight 2.1-m legs. The frame was constructed from 5.1-cm galvanized pipe. A 5.1-cm solid steel rotating shaft supported by pillow block bearings ran lengthwise through the center. A large, 80-tooth cogwheel was attached to the end of the steel shaft. Twelve, 3-mm-diam aircraft cables were attached to the shaft, and the free ends were attached to the water reservoirs.

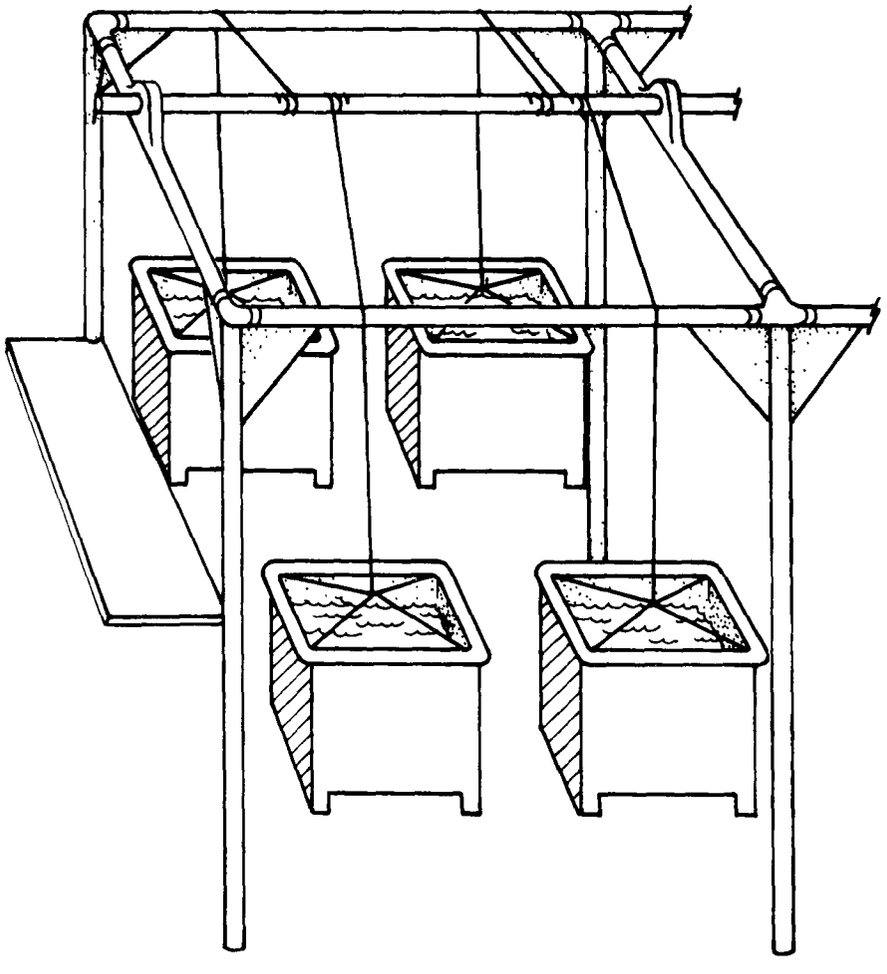
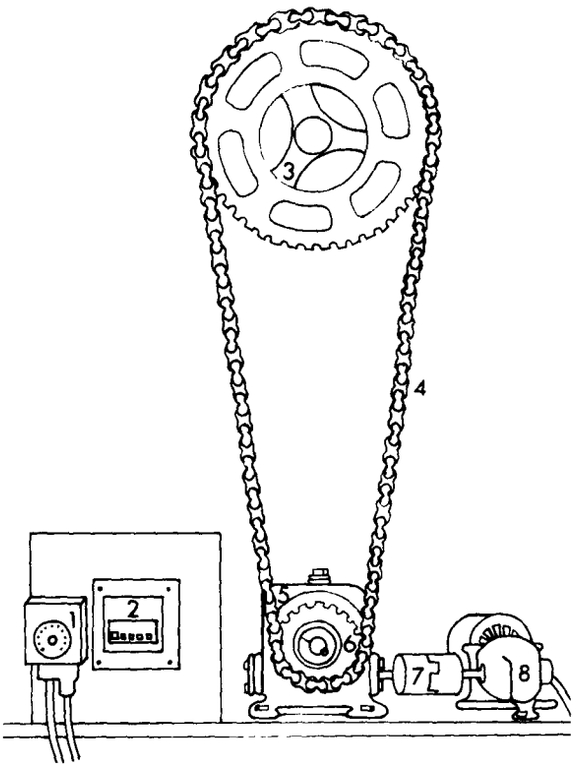


Figure 16. Arrangement of the water reservoirs on the tide machine.



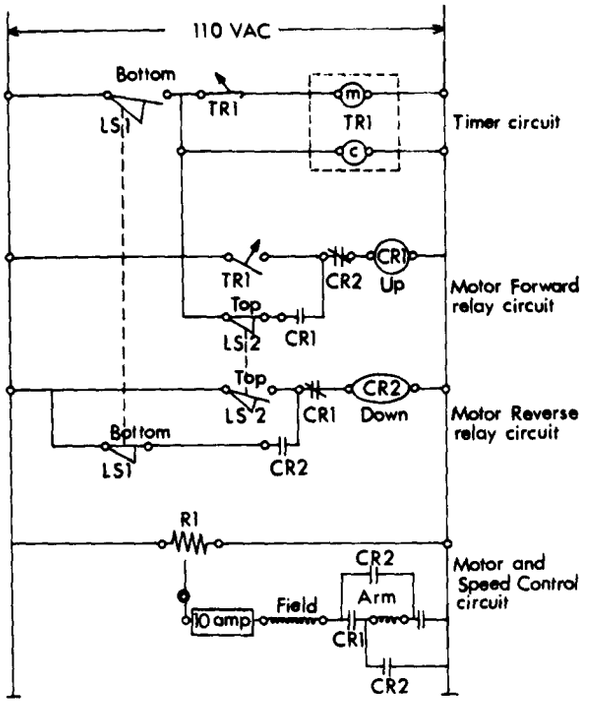


Figure 17

Figure 18

Figure 17. Arrangement of mechanical drive and electrical timing system of the tide machine: 1. variable speed control; 2. digital interval timer; 3. 80-tooth cogwheel; 4. chain; 5. 30:1 right angle speed reducer; 6. 28-tooth cogwheel; 7. coupling; 8. 1/15 HP AC/DC gear motor.

Figure 18. Schematic diagram of electrical system of the tide machine.

The mechanical system consisted of a variable speed, reversible, 1/15 H.P., right angle gearmotor connected directly to a 30:1 right angle speed reducer bearing a 28-tooth cogwheel. Drive was transmitted from this small cogwheel to the larger cogwheel on the steel shaft by a chain (Figure 17).

The electrical system consisted of a synchronous, motor driven, automatic reset timer, and a series of adjustable limit switches and reversal relays. When the system was activated, the turning of the gearmotor resulted in rotation of the shaft which wound the aircraft cable around the shaft and raised the water reservoirs. As they continued to rise, water flowed from the reservoirs into the microecosystems and over the soil surfaces. When the tidal seawater reached the desired height, a limit switch and relay reversed the motor and permitted the unwinding of the cable and the lowering of the water reservoirs. When the reservoirs descended to the desired point (low tide), a limit switch and relay reversed the motor and,simultaneously, the the system was shut off by the timer. After a predetermined time interval,

the timer started the motor and a new cycle was initiated. By controlling the rate and distance of rise and fall, the tide machine permitted the simulation of tidal water movement onto and off of the marsh at the rate and with the duration of a typical tidal cycle.

These more complex microecosystems with the associated tide machine permitted a close simulation of the natural situation. Mortality of plants and animals in the microecosystem was low, and normal feeding and animal movements occurred in these systems after a short acclimatization period (usually 2 weeks). These systems were used in several studies over a period of 3 years and, once the initial design and sampling problems were resolved, the systems functioned well with routine maintenance. However, some difficulties were encountered.

Animal death or escape from the microecosystems was a problem in the early stages of development of these systems. Initially, fiddler crabs tended to move to the drain opening at the bottom of each system and suffocate in the trapped water between tidal cycles. A perforated plastic pipe was introduced into each drainage port and this pipe prevented entrapment. In some preliminary experiments some of the periwinkle snails escaped by moving up and over the sides of the microecosystem containers. This problem appeared to be due to excessive concentrations of snails in the systems. When the snail numbers were reduced to naturally occurring levels, snails seldom left the systems.

Insect infestations of *S. alterniflora* are rarely a problem in a salt marsh apparently due to the presence of some control agent or agents. Apparently the control agent or agents was missing from the microecosystems, as some cordgrass plants were lost due to a heavy scale infestation. Two different scale insects, *Greenisca palustris* Dodds and *Haliaspis spartinae* (Cmst.), fed heavily on the stems and leaves of the infested plants. Careful washing of the plants, hand removal of the scale insects, and good sanitation procedures controlled this problem. Daily observation of the systems was necessary to control the insects.

A problem was encountered in recovering fiddler crabs from the systems for analysis. Most of the components of the microecosystems were readily sampled. However, the burrowing and evasive behavior of the crabs made sampling them extremely difficult without disturbing the soil surface. It was found that if a small trench was dug along one side of the container, water remained in these depressions during low tide and the crabs hid in these pools. This facilitated their capture.

Electrical and mechanical malfunctions did present problems at times. Despite routine maintenance, mechanical and electrical components deteriorated rather rapidly because of the use of salt water. It was necessary to continually watch for corrosion and rust and replace components periodically. Since the tide machine had to function throughout an experiment, replacement parts for every electrical and mechanical component were purchased. The design was such that a faulty part could be replaced within a few minutes.

Probably the most serious limitation of this design was that it did not include a mechanism to simulate rain. The *S. alterniflora* leaves were washed twice each week with deionized water to remove accumulated salt and to simulate the frequent rains common in the salt marsh,and deionized water was added to the reservoirs at periodic intervals to maintain relatively constant levels of salinity. Approximately monthly replacement of the sea water in the reservoirs helped to maintain healthy systems, perhaps by removing accumulating toxic materials.

SUMMARY

The use of microecosystems made a major contribution to these investigations. Many would have been impossible without them. However, it must always be remembered that in the final analysis many of the findings resulting from studies using microecosystems need to be confirmed in the field. Furthermore, the novice proposing to use the microecosystem approach should realize that development of a microecosystem that closely simulates a natural ecosystem requires a thorough knowledge of the relationships among the components of the system, and a good understanding of the relative numbers and biomasses of the living components. He also must be aware of the many cycles (seasonal, diurnal, tidal, etc.) which may affect activities of the organisms under study. Once developed, the systems make possible better replicated and more accurately monitored experiments with a higher level of environmental control than can be achieved in the natural setting.

REFERENCES

Abbot, W. Microcosm studies on estuarine water. I. The replicability of microcosm. J. Water Pollut. Control Fed., 38:258-273, 1966.

Abbot, W. Microcosm studies on estuarine water. II. The effect of single doses of nitrate and phosphate. J. Water Pollut. Control Fed., 39:113-122, 1967.

Abbot, W. High levels of nitrate and phosphate in carboy microcosm studies. J. Water Pollut. Control Fed., 41:1748-1751, 1969.

Admiraal, W. Influence of light and temperature on the growth of estuarine benthic diatoms in culture. Mar. Biol., 39:1-9, 1977.

American Society for Testing and Materials Standards, Part 23. In: Tentative method of test for evaluating inhibitory toxicity of industrial waste waters. Am. Soc. Test. Mater., Philadelphia, PA., pp. 517-526, 1964.

Anonymous. Assessing nonpoint source pollution: A detailed study of a rural watershed in the coastal plain of Maryland. Chesapeake Bay Center for Environmental Studies. Smithsonian Institution, Washington, DC, 59 pp., 1977.

Bakke, J.E., J.D. Larson, and C.E. Price. Metabolism of atrazine and 2-hydroxyatrazine by the rat. J. Agric. Food Chem., 20:602-607, 1972.

Balicka, N., and H. Bilodub-Pantera. The influence of atrazine on some soil bacteria. Acta. Microbiol. Pol., 13:149, 1964.

Beyers, R.J. The metabolism of twelve aquatic laboratory microecosystems. Ecol. Monogr., 38:199-122, 1963.

Booth, G.M. Using model ecosystems to predict the environmental behavior of pesticides. Am. Biol. Teach., 39:275-278, 1977.

Boyland, E., and L.F. Chasseaud. The role of glutathione and glutathione s-transerases in mercapturic acid biosynthesis. Adv. Enzymol. Relat. Areas Mol. Biol., 32:173-219, 1969.

Chesapeake Bay Center for Environmental STudies. Assessing nonpoint source pollution: A detailed study of a rural watershed in the coastal plain of Maryland. Chesapeake Bay Center for Environmental STudies, Smithsonian Institution, 39 pp., 1977.

Christian, R.R., K. Bancroft, and W.W. Wiebe. Resistance of the microbial community within salt marsh soils to selected perturbations. Ecology, 59: 1200-1210, 1978.

Cohen, A.J., J.N. Smith, and H. Turbert. Comparative detoxification. 10. The enzymatic conjugation of chloro compounds with glutathione in locusts and other insects. Biochem. J., 90:457, 1964.

Cooke, G.D. Aquatic laboratory microecosystems and communities. In: The structure and function of freshwater microbial communities. John Cairn, Jr., ed. Virginia Polytechnic Institute and State University, Research Division, Blacksburg, VA, pp. 47-85, 1969.

Correll, D.L., J.W. Pierce, and T.L. Wu. Studies of the transport of atrazine and Arochlor from minimum till corn fields in Chesapeake Bay tidal waters. Proc. Northeast. Weed Sci. Soc. 32:21-32, 1978.

Cotnoir, L.J. Marsh soils of the Atlantic coast. In: Ecology of halophytes. R.J. Reimold and W.H. Queens, eds. Academic Press, NY, pp. 441-447, 1974.

Couch, R.W., and D.E. Davis. Effect of atrazine bomacil, and diquat on $C^{14}O2$-fixation in corn, cotton, and soybeans. Weeds, 14:251-255, 1966.

Daiber, F.C., and C. Crichton. Tide marsh ecology and wildlife. Annual Pittman-Robertson Report on Delaware Board of Game and Fish Commissioners. Project W-22-R-2. Job #4, 1967.7.

Davis, D.E., H.H. Funderburk, Jr., and N.G. Sansing. The absorption and translocation of C^{14}-labeled simazine by corn, cotton, and cucumber. Weeds, 7:300-309, 1959.

Davis, D.E., C.G.P. Pillai, and B. Truelove. Effects of prometryn, diuron, fluometuron, and MSMA on *Chlorella* and two fungi. Weed Sci., 24:587-593, 1976.

Darley, W.M., E.L. Dunn, K.S. Holmes, and H.G. Larew, III. A ^{14}C method for measuring epibenthic microalgal productivity in air. J. Exp. Mar. Biol. Ecol., 25:207-217, 1976.

Darley, W.M., C.T. Ohlman, and B. Wimpee. Utilization of dissolved organic carbon by natural populations of epibenthic salt marsh diatoms. J. Phycol. 15:1-5, 1979.

Eaton, J.W., and B. Moss. The estimation of numbers and pigment content in epipelic algal populations. Limnol. Oceanogr., 11:584-595, 1966.

Edwards, A.C., and D.E. Davis. Effects of herbicides on the *Spartina* salt marsh. In: Ecology of halophytes, R.J. Reimold and W.H. Queen, eds. Academic Press, NY, pp. 531-545, 1974.

Eno, C.F. The effects of simazine and atrazine on certain of the soil microflora and their metabolic processes. Soil Crop Sci. Soc. Fla. Proc., 22:49-56, 1962.

Esser, H.O., G. Dupuis, E. Ebert, C. Vogel, and G. Marco. *S*.-triazines. In: Chemistry, degradation, and mode of action of herbicides. P.C. Kearney, and D. D. Kaufman, eds. Marcel Debber, Inc., NY, pp. 129-308, 1975.

Everest, J.W. Predicting phosphorus movement and pesticide action in salt marshes with microecosystems. Ph.D. Dissertation. Auburn University, Auburn, AL, 88 pp., 1978.

Everest, J.W., and D.E. Davis. Microecosystems for herbicides research and other studies. In: Research methods in weed science, 2nd Ed., B. Truelove, ed. South. Weed Sci. Soc., Auburn, AL, pp. 167-171, 1977.

Gallagher, J.L. Algal productivity and some aspects of the ecological physiology of the edaphic communities of Canary Creek tidal marsh. Ph.D. Dissertation, University of Delaware, Newark, DE, 120 pp., 1971.

Gallagher, J.L. Effect of an ammonium nitrate pulse on the growth and elemental composition of natural stands of *Spartina alterniflora* and *Juncus roemerianus*. Am. J. Bot. 62:644-648, 1975.

Gallagher, J.L., R.J. Reimold, R.A. Linthurst, and W.J. Pfeiffer. Aerial productivity, mortality, and mineral accumulation--export dynamics in *Spartina alterniflora* and *Juncus roemerianus* plant stands in a Georgia salt marsh. Ecology (In press), 1979.

Guillard, R.R.L. Division rates. In: Handbook of phycological methods: Culture methods and growth measurements. J.R. Stein, ed. Cambridge Univ. Press, NY, pp. 289-311, 1973.

Guillard, R.R.L., and J.H. Ryther. Studies of marine planktonic diatoms. I. *Cyclotella nana*. Hustedt and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol., 8:229-239, 1962.

Gunther, F.K., ed., and J.D. Gunther, asst. ed. The triazine herbicides. Residue Rev. 32. Springer-Verlag, NY, 413 pp., 1970.

Haines, E.B. Relation between the stable carbon isotope composition of fiddler crabs, plants, and soils in a salt marsh. Limnol. Oceanogr., 21:880-883, 1976.

Haines, E.B. The origins of detritus in Georgia salt marsh estuaries. Oikos, 29:254-260, 1977.

Hall, J.K., M. Pawlus, and E.R. Higgins. Losses of atrazine in runoff water and soil sediment. J. Environ. Qual., 1:172-176, 1972.

Hamilton, R.H., R.S. Bandurski, and W.H. Reusch. Isolation and characterization of cyclic hydroxamate from *Zea mays*. Cereal Chem., 39:107-113, 1962.

Hanson, R.B. Nitrogen fixation (acetylene reduction) in a salt marsh amended with sewage sludge and organic carbon and nitrogen compounds. Applied Environ. Microbiol., 33:846-852, 1977.

Isensee, A.T., P.C. Kearney, E.A. Woolson, G.E. Jones, and V.P. Williams. Distribution of alkyl arsenicals in model ecosystems. Environ. Sci. Technol., 7:841-845, 1973.

Jensen, K.I.N., G.R. Stephenson, and L.A. Hunt. Detoxification of atrazine in three gramineae subfamilies. Weed Sci., 25:212-220, 1977.

Jorgensen, E.G. Adaptation to different light intensities in the diatom *Cyclotella meneghiniana* Kutz. Physiol. Plant., 17:136-145, 1964.

Kaufman, D.D., and P.C. Kearney. Microbial degradation of triazine herbicides. In: The triazine herbicides, Gunther, F.K. ed., and J.D. Gunther asst. ed. Springer-Verlag, NY, pp. 235-266, 1970.

Lamoureux, G.L., R.H. Shimabukuro, R.H. Swanson, and H.R. Frear. Metabolism of 2-chloro-4-ethylamino-6-isopropyl-*s*-triazine (atrazine) in excised sorghum leaf sections. J. Agric. Food Chem., 18:81-86, 1970.

Lamoureux, G.L., L. E. Stafford, and R.H. Shimabukuro. Conjugation of 2-chloro-4,6 bis (alkylamino)-*s*-triazines in higher plants. J. Agric. Food Chem., 20:1004-1010, 1972.

Lamoureux, G.L., L.E. Stafford, R.H. Shimabukuro, and R.C. Zaylskie. Atrazine metabolism in sorghum: Catabolism of the glutathione conjugate of atrazine. J. Agric. Food Chem., 21:1020-1030, 1973.

Metcalf, R.L., G.K. Sangha, and I.P. Kapoor. Model ecosystems for the evaluation of pesticide biodegradability and ecological magnification. Environ. Sci. Technol., 5:709-713, 1971.

Newell, R.C. Factors controlling metabolic capacity adaption in marine invertebrates. In: Physiological ecology of estuarine organisms, F.J. Vernberg, ed. University of South Carolina Press, Columbia, SC, pp. 112-127, 1975.

Odum, E.P. The role of tidal marshes in estuarine production. The conservationists, New York State Conserv., Albany, NY, 15:12-15, 1961.

Odum, E.P. Fundamentals of ecology. W. B. Saunders Co., Philadelphia, PA, 574 pp., 1974.

Odum, E.P., and A.A. de la Cruz. Particulate organic detritus in a Georgia salt marsh-estuarine ecosystem. In: Estuaries, G.H. Lauff, ed. Am. Assoc. Adv. Sci. Publ. 83, Washington, DC, pp. 383-388, 1967.

Odum, W.E., G.M. Woodwell, and C.F. Wurster. DDT residues absorbed from organic detritus by fiddler crabs. Science, 164:576-577, 1969.

Patrick, R., and C.W. Reimer. The diatoms of the United States. Vol. 1. Acad. Nat. Sci., Philadelphia, PA, Monograph No. 13, 688 pp., 1966.

Pimentel, D. Ecological effects of pesticides on nontarget species. U.S. Govt. Printing Office, Washington, DC, 220 pp., 1971.

Pomeroy, L.R. Algal productivity in salt marshes of Georgia. Limnol. Oceanogr., 4:386-397, 1959.

Pomeroy, L.R., L.R. Shenton, R.D.H. Jones, and R.J. Reimold. Nutrient flux in estuaries. In: Nutrients and eutrophication: The limiting nutrient controversy. Proc. Symp. on nutrients and eutrophication, G.E. Lichens, ed., Vol. 1., Am. Soc. Limnol. Oceanogr. Lawrence, KS, pp. 274-291, 1972.

Pringsheim, E. Pure cultures of algae. Cambridge Univ. Press, 119 pp., 1946.

Reimold, R.J. Mangals and salt marshes of eastern United States. In: Ecosystems of the world: Wet coastal ecosystems, V.J. Chapman, ed. Elsevier Scientific Publishing Co., Amsterdam, The Netherlands, pp. 157-166, 1977.

Robinson, D.E., and D.W. Greene. Metabolism and differential susceptibility of crab grass and witch grass to simazine and atrazine. Weed Sci., 24:500-504, 1976.

Round, R.E. Benthic marine diatoms. Oceanogr. Mar. Biol. Annu. Rev.,9:83-139, 1971.

Sanborn, J.R. The fate of selected pesticides in the aquatic environment. EPA 660/3-74-025, National Environmental Research Center, U.S. Environmental Protection Agency, Corvallis, OR, 81 pp., 1974.

Schroeder, W.A., R.T. Jones, J. Cormick, and K. McCalla. Chromatographic separation of peptides on ion-exchange resins. Separation of peptides from enzymatic hydrolysates of the α, β, γ, chains of human hemoglobins. Anal. Chem., 34:1575, 1962.

Shimmel, S.M. The effect of grazing on epibenthic algae in a Georgia salt marsh. MS Thesis., University of Georgia, Athens, GA, 44 pp., 1979.

Shimabukuro, R.H. Significance of atrazine dealkylation in root and shoots of pea plants. J. Agric. Food Chem., 15:557-562, 1967a.

Shimabukuro, R.H. Atrazine metabolism and herbicidal selectivity. Plant Physiol., 42:1269-1276, 1967b.

Shimabukuro, R.H., D.S. Frear, H.R. Swanson, and W.W. Walsh. Glutathione conjugation: An enzymatic basis for atrazine resistance in corn. Plant Physiol., 47:10-14, 1971.

Shimabukuro, R.H., R.E. Kadunce, and D.S. Frear. Dealkylation of atrazine in mature pea plants. J. Agric. Food Chem., 14:392-395, 1966.

Shimabukuro, R.H., G.L. Lamoureux, D.S. Frear, and J.E. Bakke. Pesticide terminal residues. Supplement to Middle East Tech. Univ. J. Pure Appl. Chem., pp. 323-342, 1971.

Shimabukuro, R.H., H.R. Swanson, and W.C. Walsh. Glutathione conjugation: Atrazine detoxification mechanism in corn. Plant Physiol., 46:103-107, 1970.

Shimabukuro, R.H., W.C. Walsh, G.L. Lamoureux, and L.E. Stafford. Atrazine metabolism in sorghum: Chloroform-soluble intermediates in the N-dealkylation and glutathione conjugation pathways. J. Agric. Food Chem., 21:1031-1036, 1973.

Smalley, R.J. The role of two invertebrate populations, *Littorina irrorata* and *Orchelimum fidicinium*, in the energy flow of a salt marsh ecosystem. Ph.D. Dissertation, University of Goergia, Athens, GA, 126 pp., 1959.

Steel, R.G.D., and J.H. Torrie. Principles and procedures of statistics. McGraw Hill Book Co., Inc., NY, 481 pp., 1970.

Sullivan, M.J. Diatom communities from a Delaware salt marsh. J. Phycol., 11:384-390, 1975.

Sullivan, M.J., and F.C. Daiber. Light, nitrogen, and phosphorus limitation of edaphic algae in a Delaware salt marsh. J. Exp. Mar. Biol. Ecol., 18:79-88, 1975.

Taub, F.B. Demonstration of pollution effects in aquatic microcosms. Int. J. Environ. Stud., 10:23-33, 1976.

Teal, J.M. Distribution of fiddler crabs in Georgia salt marshes. Ecology, 39:185-193, 1958.

Teal, J.M. Respiration of crabs in Georgia salt marshes and its relation to their ecology. Physiol. Zool., 32:1-14, 1959.

Teal, J.M. Energy flow in the salt marsh ecosystem of Georgia. Ecology, 43:614-624, 1962.

Thompson, O.C., B. Truelove, and D.E. Davis. Effects of triazines on energy relations of mitochondria and chloroplasts. Weed Sci., 22:164-166, 1974.

Trainor, F.R. Introductory phycology. John Wiley & Sons, Inc., NY, 525 pp., 1978.

Van Raalte, C.D., W.C. Stewart, I. Valiela, and E.J. Carpenter. A ^{14}C technique for measuring algal productivity in salt marsh muds. Bot. Mar. 17:186-194, 1974.

Van Raalte, C.D., I. Valiela, and J.M. Teal. Production of epibenthic salt marsh algae. Light and nutrient limitation. Limnol. Oceanogr., 21:862-872, 1976.

Vernberg, F.J., and W.B. Vernberg. Adaptations to extreme environments. In: Physiological ecology of estuarine organisms. F.J. Vernberg, ed. Univ. of South Carolina Press, Columbia, SC, pp. 165-180, 1975.

Vernberg, W.B., P.J. DeCoursey, and J. O'Hara. Multiple environmental factor effects on physiology and behavior of the fiddler crab, *U. pugilator*. In: Pollution and physiology of marine organisms. F.J. Vernberg, ed. Academic Press, NY, pp. 381-425, 1974.

Ward, D.V., and D.A. Busch. Effects of Temefos, an organophosphorus insecticide, on survival and escape behavior of the marsh fiddler crab *Uca pugnax*. Oikos, 27:331-335, 1976.

Weber, J.B., S.B. Weed, and T.M. Ward. Adsorption of *s*-triazines by soil organic matter. Weed Sci., 17:417-421, 1969.

Williams, R.B. The ecology of diatom populations in a Georgia salt marsh. Ph.D. Dissertation. Harvard University, Cambridge, MA, 146 pp., 1962.

Wilt, J.G., P. Leeuwaugh. Mercapturic acid formation and enzyme-catalyzed conjugation with glutathione in pigeons. Biochim. Biophys. Acta., 177:329, 1969.

Wolf, P.L., S.F. Shanholtzer, and R.J. Reimold. Population estimates for *Uca pugnax* (Smith, 1870) on the Duplin estuary marsh, Georgia, USA, Crustaceana (Leiden), 29:79-91, 1975.

WSSA Herb. Handbook Comm. Herbicide handbook of the Weed Science Society of America, 4th Ed. Weed Science Society of America, Champaign, IL, 479 pp., 1979.

Yentsch, C.S., and D.W. Menzel. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res., 10:221-231, 1963.

Yu, Ching-Chieh, G.M. Booth, and J.R. Larson. Fate of the triazine herbicide cyanazine in a model ecosystem. J. Agric. Food Chem., 23:1014-1015, 1975.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/3-79-111		

4. TITLE AND SUBTITLE
Atrazine Fate and Effects in a Salt Marsh

5. REPORT DATE
November 1979

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
D.E. Davis, J.D. Weete, C.G. Pillai, F.G. Plumley, J.W. Everest, B. Truelove, and A.M. Diner

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Department of Botany and Microbiology
Auburn University Agricultural Experiment Station

10 PROGRAM ELEMENT NO.
IEA615

11 CONTRACT/GRANT NO.
R803835

12. SPONSORING AGENCY NAME AND ADDRESS
Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, FL 32561

13. TYPE OF REPORT AND PERIOD COVERED
Final July 9,1979-March 1,1979

14. SPONSORING AGENCY CODE
EPA/600/4

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Components of the Spartina alterniflora salt marsh were exposed to atrazine individually, in microecosystems, and in the field, to determine its effects on salt marsh components and its fate in the salt marsh. Components studied were S. alterniflora; horse mussel, Geukensia demissa; periwinkle snail, Littorina irrorata; box crab, Sesarma cinereum; fiddler crab, Uca pugnax; diatom spp, including Nitzschia sigma and Thallassiosira fluviatilis; detritivores, soil, and tidal water. Only algae were affected by possible contaminant concentrations (0.01 ppm) in seawater in the marsh. S. alterniflora was fairly tolerant but 0.1 ppm decreased growth slightly. Adult U. pugnax at their most sensitive stage may have been slightly affected by 100 ppm. S. cinereum was unaffected when fed leaves from S. alterniflora grown in nutrient solution containing 0.6 ppm atrazine. Conversion to detritus was unaffected when S. alterniflora leaves were wetted with 0.26 ppm atrazine solution. No effects on snails or mussels were detected when the marsh was sprayed with 5 g/m^2 atrazine. Atrazine was metabolized by S. alterniflora, S. cinereum, U. pugnax, and detritivores. Three months after atrazine application to the marsh the total herbicide remaining in the soil, S. alterniflora, L. irrorata, U. pugnax, and G. demissa was less than 3% of that applied. Seventy days after atrazine application to microecosystems, 50% had removed in tidal water; 42% within the first 6 days.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Herbicide Marshes Aquatic animals Aquatic biology Atrazine Food chains	Spartinia alterniflora Littorina irrorata Sesarma cinereum, Uca pugnax, Nitzschi sigma, Thallassiosira fluviatilis diatoms, detritivores, S-triazines, non-target organisms, microecosystems	06/C 06/T 08/A

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to public	Unclassified	84
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE