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Title <u>MICROBIAL DEGRADATION OF ATRAZINE IN SOILS</u>

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Atrazine is an asymmetrical <u>s</u>-triazine herbicide used pre- and post-emergence for the control of weeds in many crops. Under conditions considered unfavorable for microbial activity, atrazine may persist in soils for extended periods of time. However, the significance of chemical versus microbial degradation is not known.

This study was conducted to determine the significance of microbial degradation of atrazine by pure cultures and the native soil population in non-sterile soils. Isolated bacterial cultures were used to inoculate seeds in an attempt to provide protection against atrazine residues. Atrazine-treated soil was incubated at 30°C for varying periods and the subsequent loss of activity was correlated to evolution of $^{14}CO_2$ from labeled-atrazine in a radiorespirometric system.

Microorganisms, mostly bacteria, were isolated from a soil solution; pour plates of atrazine-treated and non-treated soil; and the rhizosphere of corn, oats, tomatoes, and soybeans. Viable cell counts were used as an index to test for the utilization of atrazine as the sole source of carbon. Eight bacterial isolates did

not show an appreciable difference in cell counts with or without atrazine as the sole source of carbon. Seed inoculation with a mixture of three bacterial isolates did not increase the growth of oats grown in atrazine-treated soil as an indication of crop protection.

In synthetic media bacterial cultures evolved a small amount of $^{14}CO_2$ from chain-labeled atrazine during the first 24 hours and none thereafter. In sterile soil the same isolates evolved 0.4-0.7 percent of the input activity in two weeks. A mold respired 4.0 percent. No ring breakage was observed.

In non-sterile soils, 1.4-1.6 percent of chain and 0.6-1.0 percent of ring-labeled atrazine was evolved in two weeks and 1.1-1.6 percent of ring-labeled hydroxyatrazine. The latter rate was 2-3 fold greater than from ring-labeled atrazine and indicated the formation of hydroxyatrazine as the rate limiting step in the dissipation of atrazine from soils.

Data from the incubation experiment showed a 73 percent loss of the initial atrazine after 3-4 weeks. In a similar time period, only 2.2-2.6 percent of chain and 1.0-1.2 percent of ring-labeled atrazine was respired. Thus, the $^{14}CO_2$ data did not account for the loss of atrazine and further supports the formation of hydroxyatrazine as the rate limiting step. Extraction of the soils containing labeled-atrazine showed the presence of hydroxyatrazine in non-sterile and sterile soils after two weeks.

The radiorespirometric system designed for these studies is proposed as a means to obtain a relative index of the residual life

of herbicides or pesticides. The $^{14}CO_2$ data may be extrapolated to give an index based on microbial participation. Extraction of the soils would provide a test for possible non-toxic metabolites that may be formed via chemical reactions. Such data would be most beneficial in selecting and recommending new herbicides.

MICROBIAL DEGRADATION OF ATRAZINE IN SOILS

by

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Typed by Gloria M. Foster

To my Parents: for the inspiration to set goals

and then apply my best to achieve

them.

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MICROBIAL DEGRADATION OF ATRAZINE IN SOILS

INTRODUCTION

Herbicides are valuable tools for the selective control of undesirable plants in crop production. However, various herbicides at recommended rates, whether applied to the foliage or soil, often persist in the soil for extended periods of time. These residues may cause serious damage to sensitive plant species grown the season(s) following application of the herbicides.

The climatic and edaphic factors, e.g., temperature, moisture, pH, soil composition, and cation exchange capacity which affect the residual life of herbicides, are numerous and complex. Several factors have or are being studied with others yet to be investigated.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a widely used herbicide for pre- and post-emergence control of grasses and small broadleaf weeds in a variety of crops. It may also be used as a soil sterilant for industrial weed control.

Although atrazine is a popular herbicide, its use may, in some instances, result in a residue that restricts the growth of subsequent sensitive crops. Field, greenhouse, and laboratory studies have shown that atrazine residues vary considerably in duration in the soil and may persist for more than one growing season. These studies indicate that conditions favorable for microbial activity are of major importance in reducing these residues. A number of microorganisms have been reported to use atrazine as the sole source of carbon and/or nitrogen. However, the significance of pure culture

degradation under field conditions is unknown and detailed studies of microbial versus chemical degradation are lacking.

The overall objective of this investigation was to study the effects of native and pure populations of soil microorganisms on atrazine degradation. Ring- or chain-labeled atrazine was used to follow $^{14}CO_2$ evolution in laboratory experiments. Radiochromatograms of extractions were made following periods of exposure to isolated microorganisms, native populations, and sterile conditions to detect metabolites. A bioassay was employed to test for atrazine disappearance in greenhouse studies following periods of incubation. Cultures, demonstrating resistance and possibly degradation of atrazine, were used to inoculate seeds in atrazine-treated soil. Possible degradation and thus protection of the inoculated seeds was detected via bioassay.

LITERATURE REVIEW

Physical, Chemical, and Herbicidal Properties of Atrazine

The physical and chemical properties of atrazine are reported
in a technical bulletin from Geigy Chemical Corporation (15).
Technical atrazine is a white crystalline material with a melting
point of 173°C to 175°C. It is slightly soluble in water (70 ppm
at 27°C) and appreciably soluble in organic solvents such as chloro-
form (52,000 ppm at 27°C) and methanol (18,000 ppm at 27°C). Atra-
zine is stable in neutral, slightly acid, or slightly basic media.
When heated in acid or basic media, it hydrolyzes to 2-hydroxy-4-
ethylamino-6-isopropylamino-s-triazine which has no herbicidal
activity.

Gysin and Knusli reported the herbicidal properties and syn-
thesis of the s-triazines in 1956 (16, p. 615-623) and 1960 (17,
p. 289-358) and in 1963 by Gysin (18, p. 17-28). The synthesis be-
gins with cyanogen chloride to yield cyanuric chloride. Promising
herbicidal compounds were produced when two of the chlorine atoms
were replaced by aliphatic amine groups. Amine side chains con-
taining two or three carbon atoms yielded the most phytotoxic com-
pounds. Atrazine is an example of an asymmetrical mono-chloro-bis-
alkylamino-s-triazine. Its synthesis may be summarized as follows:

Cl-C≡N --→   cyanogen chloride → cyanuric chloride

CH₃CH₂NH₂ --→   ethylamine

2,6-dichloro-4-ethylamino-s-triazine

(CH₃)₂CHNH₂ ---→   isopropylamine

2-chloro-4-ethylamino-6-isopropylamino-s-triazine
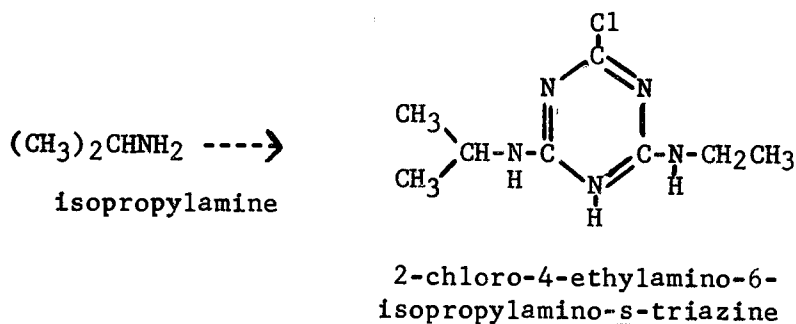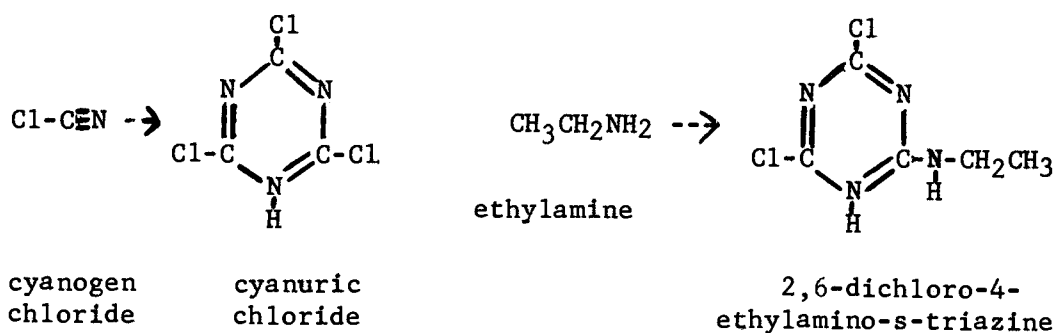
Atrazine can penetrate the leaves of grasses and small broad-leaf weeds and has shown considerable promise as a pre- and post-emergence herbicidal compound (18, p. 20; 23, p. 170). It is very effective for the control of perennial weeds such as quackgrass (Agropyron repens) and yellow nutsedge (Cyperus esculentus) at approximately four pounds per acre during the fall or early spring. Atrazine is a very popular herbicide for weed control in corn and rates of two to four pounds per acre are recommended as a pre- or early post-emergence application. At rates of one to two pounds per acre, it effectively controls many annual grasses and broadleaf weeds in perennial grasses grown for seed or forage. Chemical control of weeds during fallow, known as chemical fallow, is generally accom-plished with rates of one to two pounds per acre. Several weeds controlled at rates of one to four pounds per acre are barnyardgrass (Echinochloa crusgalli), mayweed (Anthemis cotula), downy brome

(Bromus tectorum), foxtails (Setaria spp.), common lambsquarters

(Chenopodium album), ragweeds (Ambrosia spp.) and redroot pigweed

(Amaranthus retroflexus). The many research reports giving the re-

sults of the use of atrazine are well summarized in a technical

bulletin by Geigy Chemical Corporation (15).

Effects of Herbicides on Soil Microorganisms

The physical and chemical properties of soils, the activity of

soil microorganisms, and the presence of herbicides are conducive

to many complex interactions. Two important interactions are the

effects of herbicides on the soil microflora and the effects of the

soil microflora on herbicides.

Evolution of CO_2, consumption of O_2, plate counts, and response

of pure cultures are methods that have been used by investigators

to study the effects of herbicides on soil microorganisms. The

numerous early works are reported in excellent reviews by Bollen

(3) and Fletcher (13).

The rhizosphere is a restricted area within the immediate area

of the root and contains a higher microbial population than the

surrounding soil. This concentration of microorganisms in the

rhizosphere indicates a zone of intense microbial activity, with

its possible effect(s) on the metabolism of the surrounding soil.

Rovira (29) reported a stimulation of short, gram negative rods; an

increase in the nitrification rate; and an increase in oxygen uptake

by the additions of root exudates to a soil system. These results

were thought to be indicative of the true rhizosphere effect, or

changes in the microbial population affected by root exudates.

Smirnova (34) found no significant effects from 2,4-D, simazine, or

atrazine on the total corn rhizosphere bacterial population or

specific physiological groups in the rhizosphere.

In general, there are no significant or lasting effects on the

soil population from recommended agronomic rates of herbicides.

Persistence of Atrazine in
Relation to Soil Microbial Activity

The disappearance of atrazine is generally correlated with

factors favorable to microbial activity. Talbert and Fletchall

(36) observed the greatest inactivation of atrazine and simazine

from May until September or when the soil environment was most suit-

able for microbial activity. They stated that increased temperature

and moisture could significantly increase chemical as well as bio-

logical reactions. Sheets and Shaw (33) studied the persistence of

14 s-triazines in four different soil types and indicated that vari-

ations in microbial populations or activity may account for some of

the differences in persistence. They found it tempting but perhaps

precarious to suggest that differences in hydrogen-ion concentra-

tions may affect the persistence.

Buchanan and Rodgers (6) found the inactivation of atrazine to

be correlated with increased soil temperatures. They suggested that

increased microbial activity at the higher soil temperatures may

account for the reduction in detectable atrazine concentrations.

Burnside (7) found the breakdown of atrazine to be extremely tem-

perature-dependent. He incubated 2 ppm at three different

temperatures in Wymore silty clay loam soil at field capacity.
Using soybeans as a bioassay, the time required to reduce the atra-
zine concentration below the detectable range was one month at 35°C,
two months at 25°C, and nine months at 15°C. Thus, the microbial
and/or chemical breakdown of atrazine was greatly influenced by
soil temperatures. Harris (19) reported hydroxysimazine after 32
weeks incubation in four soils. The mechanism of conversion is
being studied. Agundis and Behrens (1) suggested that atrazine
deactivation proceeded at a more rapid rate as the soil environment
became more anaerobic.

Degradation of s-triazines
by Specific Microorganisms

An important aspect of microbial degradation of chemicals is
the classical enrichment phenomenon. By repeated applications or
continuous exposure to a chemical, there is a build-up of micro-
organisms via mutations or adaptive enzymes that attack the chemi-
cal. Lees and Quastel (24) designed a soil perfusion column in
which continuous perfusion by an aerated solution of ammonia allowed
determination of nitrification rates by an "enriched" population.

Audus (2, p. 168) demonstrated the enrichment phenomenon in
the degradation of 2,4-D by the soil population with a soil perfu-
sion apparatus; however, according to Sheets and Danielson (31,
p. 171), there is no enrichment from repeated applications of
s-triazines. They indicated that the soil retains the same detoxi-
fication intensity over a long period of time and called it passive,
not selective or preferential detoxification. Sheets (32) later

suggested that the added herbicide as a substrate was not enough to cause an increase in the general soil bacterial population, but certain microorganisms might utilize these herbicides passively. Recently there have been reports of enrichment with repeated applications of s-triazines[1] (11, p. 59).

Several fungi and bacteria capable of metabolizing various s-triazines have been isolated in pure cultures. Burnside et al. (8) isolated five microorganisms that survived five serial transfers on agar containing mineral salts and simazine as the carbon source. However, degradation of simazine was not observed after 30 days incubation in a liquid medium. Couch et al. (10) reported the formation of hydroxyatrazine from the parent compound in the presence of Fusarium roseum. Degradation was not affected by sucrose concentrations from 0 to 10,000 ppm in liquid media. They did not find ring cleavage by F. roseum, Penicillium sp., Trichoderma sp., or Geotrichum sp. using ring-labeled atrazine; whereas, they observed evolution of $^{14}CO_2$ with all species using chain-labeled simazine.

Duke (11, p. 44) isolated a Penicillium sp. and a Bacillus sp. on inorganic agar medium containing atrazine as the sole source of carbon. Carbon dioxide evolution from soil inoculated with Penicillium sp. and treated with atrazine was significantly higher than endogenous respiration. Bryant (5, p. 38) observed a correlation between the alkylamino substituent groups on the number four

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[1]Personal communication with Dr. H. M. LeBaron, Geigy Chemical Corporation, Ardsley, New York.

carbon and suitability as a substrate source for various bacteria in studies of 2-R-4-R-6-isopropylamino-s-triazines. Compounds containing the diethylamino group were preferred by the Pseudomonas sp., whereas the Arthrobacter sp. preferred the ethylamino substituted compounds. The isopropylamino group was utilized equally well by both genera.

Ragab and McCollum (28) reported the evolution of $^{14}CO_2$ from ring-labeled simazine in a non-sterile soil system. MacRae and Alexander (25) found only a small amount of evolved $^{14}CO_2$ from ring-labeled atrazine after 16 weeks in a soil system. McCormick and Hiltbold (26) observed a direct correlation between atrazine inactivation and metabolism of soil organic carbon. Atrazine decomposition was closely associated with repeated additions of glucose as an energy source, indicating an incidental or nonpreferential degradation by microorganisms. They reported a greater loss of phytotoxicity using a bioassay than could be accounted for by evolution of $^{14}CO_2$ from ring-labeled atrazine. This suggested that inactivation was principally via alteration of the substituent groups and a low level of metabolic availability of the triazine ring carbon.

Kaufman et al. (20) used a soil solution enrichment technique to obtain microorganisms capable of degrading simazine. They isolated Aspergillus flavipes, A. fumigatus, A. ustus, Fusarium moniliforme, F. oxysporum, Penicillium purpurogenum, P. sp., Rhizopus stolonifer, Stachybotrys sp., Trichoderma viride, three species of Streptomyces, and four bacterial isolates believed to belong in the genus Arthrobacter. These microorganisms could utilize simazine as

a sole or supplementary source of carbon. $\underline{A}$. $\underline{fumigatus}$ was the most

effective in the degradation of simazine and loss of radio-activity

was greater from culture solutions containing sucrose as a supple-

mental carbon source. Chromatographic evidence indicated that $\underline{A}$.

$\underline{fumigatus}$ may possess a degradation scheme unlike that which occurs

in corn plants where simazine is hydrolysized to hydroxysimazine.

In later studies, Kaufman $\underline{et}$ $\underline{al}$. (21) reported no evolution of

$^{14}CO_2$ from ring-labeled simazine, whereas, approximately 40 percent

of the chain-labeled simazine was evolved as $^{14}CO_2$ after 12 days of

incubation by $\underline{A}$. $\underline{fumigatus}$. A metabolite was identified as 2-chloro-

4-amino-6-ethylamino-$\underline{s}$-triazine. A second ^{36}Cl-labeled metabolite

possessed an intact ring but no alkyl substituents (22). Their

data indicated that the degradation of simazine occurred by dealky-

lation, deamination, or both, of the side chains, and that hydroxy-

simazine was not an intermediate. Fungi were listed as the dominant

group in the degradation of triazines.

The microorganisms reported as capable of degrading triazine

herbicides by various investigators are summarized by Ercegovich

(12).

Inoculation with Specific Micro-
organisms for Protection Against Chemicals

Freeman $\underline{et}$ $\underline{al}$. (14) studied the absorption and distribution of

labeled-simazine in pine seedlings grown in mycorrhizae-free Prairie

soil and inoculated Prairie soil. Autoradiograms and count data

showed a much higher concentration of ^{14}C in the non-inoculated

white pines ($\underline{Pinus}$ $\underline{strobus}$). They suggested that the presence of

mycorrhizae may enhance the resistance of white pine to simazine. There were no apparent differences in simazine absorption between inoculated and non-inoculated red pine (Pinus resinosa).

Paul and Polle (27) injected a Nitrobacter sp. suspension beneath the soil surface with a hypodermic syringe to prevent accumulation of nitrites. Rovira (30) suggested the possibility of biological control of root diseases by inoculation of seed with microorganisms antagonistic to the pathogens. MacRae and Alexander (25) inoculated alfalfa seed with a 4-(2,4-DB)-utilizing Flavobacterium. In sterile Honeoye silt loam soil, the alfalfa grew normally in the presence of 10 ppm 4-(2,4-DB), whereas in non-sterile soil there was little protection. The protection in Williamson silt loam soil was marginal in either sterile or non-sterile soil. If the Flavobacterium sp. was allowed to develop in the sterile Williamson soil for three weeks prior to planting, the resulting population detoxified the herbicide to permit normal plant growth. The success of using seed inoculation to establish susceptible plants in herbicide-treated soil must await better means of introducing and establishing a specific beneficial microorganism in the natural ecosystem.

EXPERIMENTAL METHODS AND MATERIALS

Isolation of Soil Microorganisms

Three "enrichment" methods were used for the isolation of soil microorganisms. Major emphasis was placed on bacteria because of lack of previous work and their high titer in soils. The objective was to obtain microorganisms that were at least tolerant of atrazine and possibly capable of degrading atrazine. These "enriched" cultures would be used to inoculate seeds in atrazine-treated soil for greenhouse studies and for degradation of labeled-atrazine in laboratory experiments.

The basic medium used for these studies was a glucose nutrient broth (GNB) consisting of 5.0 g glucose, 5.0 g peptone, and 3.0 g beef extract per liter of distilled water. Glucose nutrient agar (GNA) had the addition of 15.0 g agar (1.5%) per liter of broth. Sterilization was achieved by autoclaving for 20 minutes at 121°C. Various atrazine concentrations were made by dilutions from stock solutions at room temperature for the broth and approximately 50°C for the agar media. The media were allowed to set for several days to check for any contamination from the atrazine. Subsequent use of media and culture transfers were made aseptically.

Soil samples were taken from the top two inches of Woodburn silty clay loam soil in February, 1965. The treated sample had received 2.4 pounds atrazine per acre in October, 1964, and the non-treated sample was taken from the check plots. The samples were air-dried at room temperature and stored until used.

The exposure of an aliquot of soil to a liquid medium + atrazine would favor microorganisms that could utilize atrazine for growth or were at least tolerant of it. Such a soil solution enrichment contained 1.0 g of treated or non-treated soil in the initial flasks of 100 ml GNB + 100 ppm atrazine. After vigorous shaking, one ml aliquots were transferred to additional flasks. This scheme was repeated twice to obtain a series of dilutions from the initial flasks. The flasks were shaken at 30°C in a rotary shaker. Upon visible growth, a loop of inoculum was then streaked on GNA + 100 ppm atrazine plates. Subsequently, colonies were transferred to GNA slants.

A second method consisted of selecting colonies from GNA + 100 or 1000 ppm atrazine pour plates used for counting the microbial population of the soil samples. Pour plates were made by adding an aliquot of the sample to be counted to a sterile petri dish. Approximately 15 ml GNA at 45°C was added to each plate. The plates were swirled to insure uniform dispersion of the sample and allowed to cool at room temperature. Colonies were selected at random and transferred to GNB + 100 or 1000 ppm atrazine and shaken at 30°C in a rotary shaker. Upon subsequent growth, a loop of inoculum was streaked on GNA slants. Transfers were later made to GNA + 1000 ppm atrazine slants.

The third series of isolations came from the rhizosphere of corn, oats, tomatoes, or soybeans grown in Newberg sandy loam soil containing 0.00 or 0.25 ppm atrazine for eight weeks. Plants from both concentrations were treated in the same manner. The roots from

one plant of each species were shaken almost free of soil and placed
in 99 ml sterile water and shaken. A one ml aliquot was added to
99 ml sterile water and again shaken. A loop of rhizosphere inoculum was then transferred to GNB and GNB + 100 ppm atrazine and
shaken at room temperature on a rotary shaker for six days. Loop
inoculums were streaked on GNA + 1000 ppm atrazine and after seven
days colonies were transferred to GNA + 1000 ppm atrazine slants.

Cultural Maintenance

In the initial stages, cultures were maintained on GNA slants.
However, subsequent maintenance was on GNA + 1000 ppm atrazine
slants to insure continuous exposure of the cultures to atrazine
and favor any adaptive enzymes. The cultures were kept at room temperature and fresh transfers made at approximately six-week intervals.

Microbial Growth Studies with Atrazine

To test the ability of isolated soil microorganisms to utilize
atrazine as a source of carbon, an experiment was designed to compare growth of microorganisms in a medium containing no carbon versus growth in a medium containing atrazine as the sole source of
carbon. The index of comparison was viable cell counts.

The medium without a carbon source (NCB) contained 1.5 g
NH_4NO_3, 0.25 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g yeast
extract and 1.0 g K_2HPO_4 per liter distilled water. The medium was
sterilized by autoclaving at $121^{\circ}C$ for 20 minutes. Technical

atrazine (98.2%) as the carbon source was added to the NCB medium

at a concentration of 2500 ppm or approximately 110 mg of carbon per

100 ml of broth.

Selected cultures were transferred to GNB + 1000 ppm atrazine

and shaken at 30°C for 48 hours in a rotary shaker to provide fresh

inoculum for the growth study. One ml aliquots were then trans-

ferred to 100 ml of NCB or NCB + 2500 ppm atrazine broth in 250 ml

Erlenmeyer flasks. The flasks were shaken by hand to insure uniform

dispersion of the inoculum. One ml aliquots were taken and diluted

to obtain the initial viable cell counts per flask.

All cultures were shaken at 30°C in a rotary shaker during the

growth study. One ml aliquots were taken at 3 and 5 days and di-

luted for the viable cell counts. Adequate dilutions were made to

obtain approximately 30 to 300 colonies per plate. Triplicate plates

were counted after 36 hours growth in GNA.

In a qualitative experiment, visual growth of microorganisms on

GNA + 1000 ppm atrazine or agar + 2500 ppm atrazine (80% WP) was com-

pared. At four to six week intervals, loop inoculums from the

latter plates were transferred to fresh GNA + 1000 ppm atrazine or

agar + 2500 ppm atrazine. Subsequent growth was compared. The se-

quence was repeated twice to give a total of three serial transfers

on agar + 2500 ppm atrazine.

Selection of a Bioassay for Greenhouse Studies

The soil used in greenhouse studies was Newberg sandy loam soil.

The analyses are given in Table 1. Soil was taken from the top six

inches, screened through a 7 mm screen, air-dried at room tempera-
ture and stored until used.

Table 1. Chemical and mechanical analyses of
Newberg soil

Chemical Analysis

% organic matter	% total N	CEC me/100 g	Soil pH
2.66	0.093	18.7	6.1

Mechanical Analysis

% sand	% silt	% clay
67.42	20.66	11.92

Atrazine was incorporated into the soil by means of a cement
mixer. As the soil tumbled, the desired concentration of atrazine
on a dry weight basis was applied with a hand sprayer. The atrazine
concentrations were applied in the amount of water required to bring
the soil to field capacity which was approximately 20 percent mois-
ture.

A preliminary tolerance trial was established to select a sen-
sitive and simple bioassay. Two replications of atrazine at 0.00,
0.25, 0.50, 1.00, 1.50, 2.00, and 4.00 ppm were planted to corn,
oats, wheat, soybeans, cucumbers and tomatoes. The plants were
grown in quart cans containing 900 g of treated soil and sub-irri-
gated every two days in watering trays. A growth reduction of 50
to 70 percent within a two-week growing period was desired. Growth
reduction was assessed by visible observation. Any benefits from
seed inoculation in later studies should be detectable within the

above selected range of growth reduction.

In a certain soil and a given concentration of atrazine, the number of plants per can could affect the subsequent reduction in growth. Assuming a limited amount of atrazine is available for plant uptake, a large number of plants would reduce the amount of atrazine available per plant. Thus, there would be less growth reduction per plant. Three studies were conducted with 5, 10, 20, and 50 oat seeds grown in 0.00 or 0.25 ppm atrazine-treated soil. Experimental design was a randomized block with three replications. Plants were grown under greenhouse conditions and sub-irrigated every three days. After two weeks, the plants were harvested at the soil surface and dry weights obtained for comparisons.

Seed Inoculation with Isolated Bacteria

If isolated cultures had the ability to degrade atrazine, then one possible method of protecting susceptible species in atrazine-treated soils would be to inoculate the susceptible seeds with a suspension of atrazine-degrading microorganisms. Degradation by the inoculum would provide an atrazine-free zone for the germinating seed. Upon emergence of the root system, the microorganisms could establish themselves on the surface of the roots, rhizoplane, or within the immediate root zone, rhizosphere, and provide continuous protection for the susceptible species.

The concept of inoculation was explored in two experiments with atrazine-resistant bacteria. Three bacterial isolates were selected on the basis of their apparent resistance and rapid proliferation in

the presence of atrazine.

Triplicate bottle plates of the selected cultures were grown for 48 hours at room temperature on GNA + 1000 ppm atrazine bottle plates, which were 200 ml bottles containing 50 ml of the agar and lying on one side. Ten ml of sterile water were added to the first bottle and five ml were added to the remaining two bottles of each culture. The bottles were shaken gently and the resulting bacterial suspension from all three cultures were poured together. The latter suspension served as the inoculum for this experiment.

Ten oat seeds were soaked briefly in the bacterial inoculum and then planted in Newberg soil containing 0.00, 0.25, 0.50, or 1.00 ppm atrazine. Another set of seeds was placed on the soil surface and sprayed with five ml of the inoculum using a hand atomizer. A third set did not receive any inoculum. Ten seeds of each inoculation treatment were counted to obtain the viable cell counts per seed.

Experimental design was a randomized block with two replications. The cans were sub-irrigated every three days. Greenhouse temperatures were approximately $16^\circ C$ ($60^\circ F$) at night and $24^\circ C$ ($75^\circ F$) during a 14-hour day by supplemental light. The plants were harvested at the soil surface after two weeks and dried in glass-stoppered weighing bottles at $100^\circ C$ for 24 hours to obtain dry weights.

In a second inoculation experiment, the same three bacteria were grown on triplicate plates of GNA or GNA + 1000 ppm atrazine bottle plates for 96 hours at room temperature. Ten ml of sterile

water were added to each bottle, gently shaken, and poured together to give 90 ml of the respective bacterial inoculum. Forty ml of each inoculum was centrifuged at approximately 5000 rpm for ten minutes and resuspended in 40 ml sterile water. The washing procedure was repeated to remove any GNA or atrazine that had dissolved when removing the cells.

Ten oat seeds were soaked briefly in the washed GNA, washed GNA + 1000 ppm atrazine, or nonwashed GNA + 1000 ppm atrazine inoculum. Experimental design, greenhouse conditions and harvest were as previously described, with the exceptions of three replications and a modified irrigation system.

Two large (No. 2) soda straws were inserted to different soil depths in the cans.[2] The soil was brought to field capacity every three days by adding water through the straws (Plate 1). Such a scheme would maintain the soil moisture at a more optimum level for microbial activity and eliminate saturation that resulted from sub-irrigation.

Soil Incubation Studies

Often the persistence of herbicides is related to environmental conditions unfavorable for microbial and/or chemical degradation. Therefore, soils were brought into the greenhouse or laboratory to follow degradation under a more defined environment.

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[2]Personal communication with Drs. L. Boersma and M. E. Harward, Department of Soils.

Atrazine at 0.00 and 0.75 ppm was added to Newberg soil in sufficient water to bring the soil to 18 percent moisture. On a dry weight basis, 600 g were dispensed in quart cans. The cans were covered with a plastic (Parafilm) that had five holes punched with a toothpick. All treatments were incubated in a water tank held at 30 $\pm$ 3°C (Plate 2). Photoperiod was a 14-hour day with the aid of fluorescent light.

At intervals of 0, 1, 2, 3, and 4 weeks, twelve oat seeds were planted in each treatment and later thinned to ten plants per can. Straws were inserted for irrigation and approximately 30 g of vermiculite were dispersed on the soil surface to reduce evaporation. The cans were brought to the initial weight every three days by adding water through the straws and also systematically rotated in the tanks. After 16 days growth, the plants were harvested and dried as previously.

To separate microbial from chemical degradation, an identical sterile set was established. The soil and cans were autoclaved at 121°C for 24 hours. Cement mixer and hand sprayer were thoroughly washed with 70 percent alcohol just prior to use.

A set of standards were grown under the same conditions for both sterile and non-sterile soil treatments. Rates were 0.00, 0.05, 0.25, 0.50, and 0.75 ppm atrazine. Experimental design for the experiments was a randomized block with four replications.
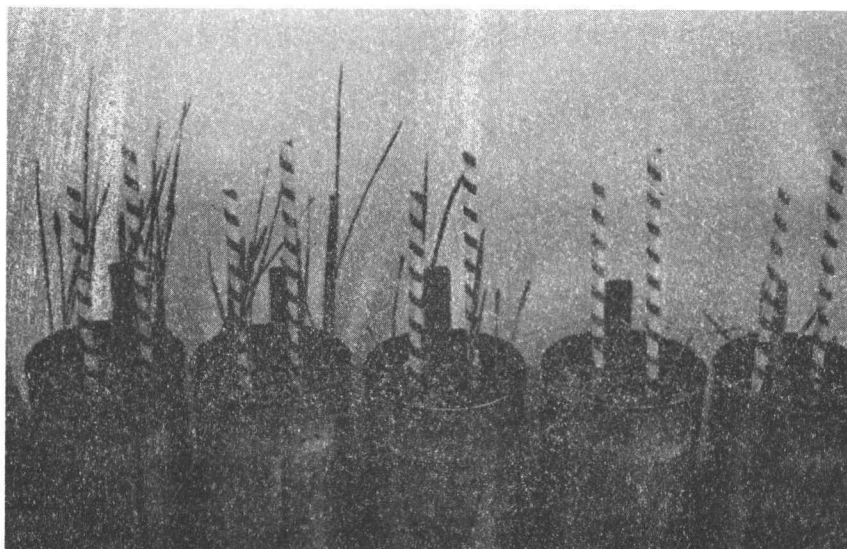
Plate 1. Growth of oats at 0.00, 0.05, 0.25, 0.50 and 0.75 ppm
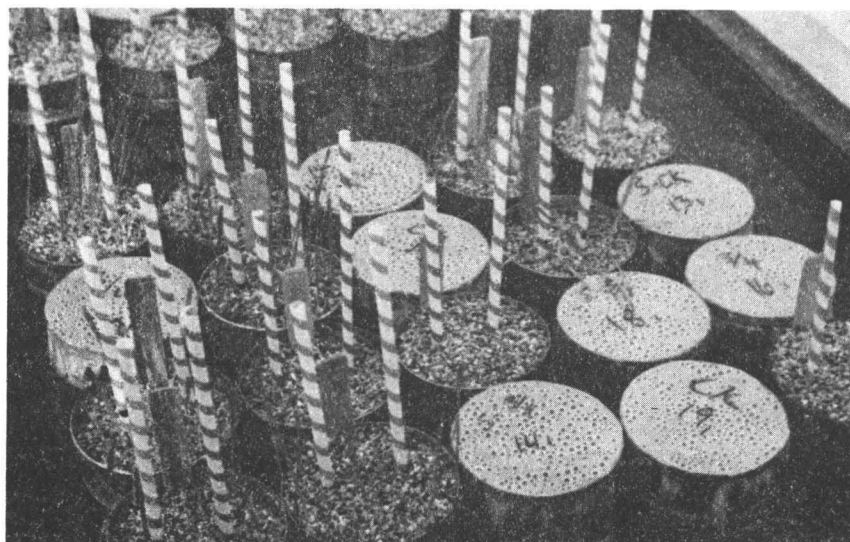atrazine from left to right using straws for
irrigation.

Plate 2. The atrazine treatments incubated for 0, 1 and 2 weeks
have been planted with treatments under Parafilm to be
planted after 3 and 4 weeks incubation.

Bacterial Degradation of
^{14}C-atrazine in Synthetic Media

The degree of atrazine utilization by microorganisms may be assessed by evolution of $^{14}CO_2$ from ^{14}C-labeled atrazine. Moreover, degradation of ring- versus chain-labeled atrazine would indicate the point of initial attack and the possible breakdown pathways.

The specific activity of ring-labeled atrazine for these studies was 7.8 µc/mg and for chain-labeled atrazine was 6.6 µc/ milligram. The chain-^{14}C was located adjacent to the amino group in the ethylamino side chain ($-NH-^{14}CH_2-CH_3$) and the ring was uniformly labeled.

A modified Warburg apparatus described by Wang et al. (37) was used to follow the evolution of respiratory $^{14}CO_2$ by selected bacterial cultures, known as radiorespirometry. The previous NCB medium was modified slightly to contain 1.0 g NH_4NO_3, 0.25 g $MgSO_4$, 0.25 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g yeast extract, and 0.5 g K_2HPO_4 per liter of distilled water. The medium was autoclaved at 121°C for 20 minutes.

Bacterial cells for degradation studies were grown in GNB + 50 ppm atrazine in a rotary shaker at 30°C for 24-30 hours just prior to use. The cells were centrifuged at approximately 5000 rpm and resuspended in 10 ml of NCB medium. The washing sequence was repeated a second time to remove the glucose nutrient broth + atrazine and to obtain the desired amount of cells in a small volume.

The reaction mixture contained 20 mg of cells, on a dry weight basis, in 10 ml NCB; 4 ml of cold atrazine in NCB; 16 ml of NCB;

and 10 ml of ring- or chain-labeled atrazine in sterile water for a total volume of 40 milliliters. Ring- and chain-labeled solutions had a total activity of 1.43 μc and 1.19 μc, or 4.58 and 4.50 ppm, respectively. The 25 ppm cold atrazine concentration + 4.5 ppm of labeled gave approximately a 30 ppm concentration of atrazine.

Reaction vessels were 125 ml Erlenmeyer flasks with ground-glass tops to insure a closed system. The flasks were connected to the modified Warburg apparatus and shaken at $30^{o}C$. Sterilized compressed air was passed through the flasks and the CO_2 absorbed in 15 ml of a 2:1 ethanol-ethanolamine solution.

The trapping solution was changed at 12-hour intervals during a 72-hour period. Five ml aliquots of the trapping solutions were added to 10 ml of a scintillation solution, which contained 6.0 g PPO (2,5-diphenyloxazole) and 0.1 g dimethyl-POPOP [1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene] per liter of toluene. Samples were counted on Packard's Tri-Carb Liquid Scintillation Spectrometer Model 3000 series for a preset time of 20 minutes or 10,000 counts. Counting efficiency was checked by counting a sample of ^{14}C-naphthalene with known activity.

After 72 hours the cells were centrifuged and the media decanted and saved. Cells were washed twice with water and resuspended in five ml of water. Media and cells were counted by adding 0.5 ml aliquots to a scintillation gel, which contained 40 g of thixotropic gel (Thixcin) per liter of scintillation solution. Samples were mixed on a Vortex mixer and counted as described.

Slight modifications were made in additional flasks by adding

1000 ppm atrazine, 0.1 percent glucose, or 1.0 percent glucose to
see if an additional carbon source would influence the evolution of
$^{14}CO_2$.

Degradation of ^{14}C-atrazine in Soils

The determination of microbial degradation of atrazine by pure
cultures in synthetic media is relatively simple and provides evi-
dence of the possible breakdown pathways. Nevertheless, the essen-
tial experiment is to study degradation within the more complex eco-
system of the soil, where the chemical and microbial aspects of deg-
radation are at work.

To facilitate radiorespirometric studies in soils, a new system
was developed (Plates 3 and 4). Reaction vessels were 125 ml Erlen-
meyer flasks with ground-glass tops. Seventy-five grams of soil,
on a dry weight basis, and labeled atrazine were added to each flask.
The flasks were placed in a temperature bath at 30°C. Compressed
air was passed over the soil at a slow flow rate and the CO_2 ab-
sorbed in 15 ml of the trapping solution. Moisture in the air was
removed by Drierite columns inserted between the flasks and trapping
solutions. Five ml aliquots were counted at a preset time of 50
minutes or 100,000 counts for a standard deviation of less than 1
percent.

Experiments were designed to follow evolution of $^{14}CO_2$ from
labeled-atrazine by pure cultures as compared with the native soil
population in non-sterile soils. Sterilized soil, rather than syn-
thetic media, was used for pure culture studies. Sterilized soils
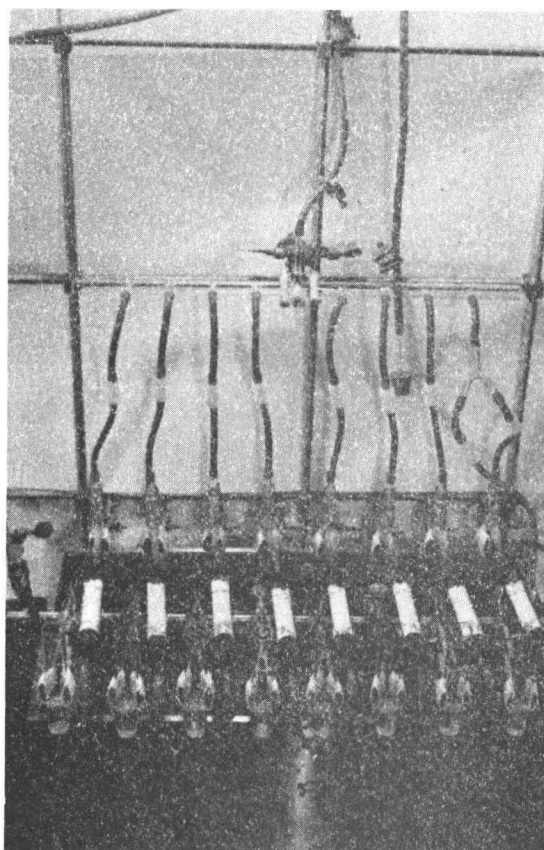
Plate 3. Radio-
respirometric
apparatus for
determining
labeled-atrazine
degradation in
soils.
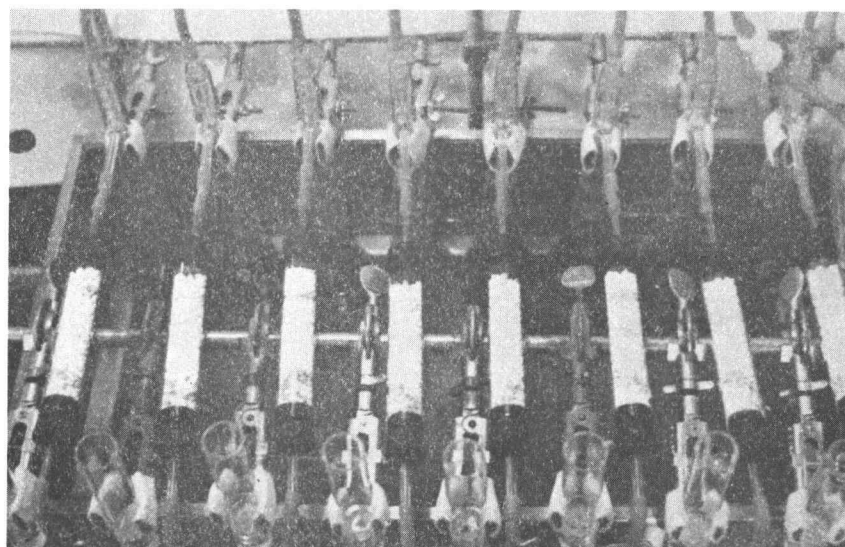
Plate 4. Close-up view of apparatus showing from top to
bottom: flask + soil in water bath, Drierite
column to remove moisture and traps to remove
carbon dioxide.

would be more favorable for microbial activity. The soils were
autoclaved at 121°C for 12 hours. Forty mg of cells, prepared as
previously described, were added to the flasks in sterile water.
Six bacterial isolates and one mold, Aspergillus fumigatus,[3] were
used in these studies.

In addition to the Newberg soil, Chehalis silty loam soil was
used in these studies. The analyses are given in Table 2.

Table 2. Chemical and Mechanical Analyses
of Chehalis silty loam soil

Chemical Analysis

% organic matter	% total N	CEC me/100 g	Soil pH
8.02	0.28	35.83	6.3

Mechanical Analysis

% sand	% silt	% clay
10.78	46.99	42.23

For these experiments, ring-labeled atrazine had a specific
activity of 17.3 μc/mg and the chain-labeled was 6.6 μc/milligram.

Approximately 2 μc of labeled-atrazine from stock ethanol solu-
tions, or 1.6 ppm ring and 3.5 ppm chain, were added to the soils
in a solution designed to "spark" microbial activity. The solution
gave a final concentration of 0.1 percent glucose and 0.0005 percent
nitrogen as NH_4Cl. Moisture level was adjusted to 20 percent and 28

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[3]Courtesy of Dr. D. D. Kaufman, U.S.D.A., Beltsville, Maryland

percent for the Newberg and Chehalis soil, respectively. Sterilization of the compressed air was achieved by passage through a millipore filter. The trapping solution was changed after the first 24 hours and every three days thereafter for a period of 12 days.

Assessment of degradation by the native population was made in similar experiments with non-sterile soils. After two to four weeks the soils were extracted with 150 ml of methanol at $60^{\circ}C$ for two hours on a rotary shaker. The solution was decanted through filter paper and evaporated at $65^{\circ}C$ to a volume of ten milliliters. A 0.1 ml aliquot was chromatographed with a solvent system of isoamyl alcohol saturated with 3N HCl.[4] The radiochromatograms were scanned with a Vangard Autoscanner Model 880. Primary concern was the presence of hydroxyatrazine. The evolution of $^{14}CO_2$ and possible formation of hydroxyatrazine would present a more complete picture of the total degradation of atrazine in soils. Sterile samples were run concurrently to detect for $^{14}CO_2$ evolution and also possibly hydroxyatrazine formation via chemical hydrolysis.

Assuming there would be hydroxyatrazine formed, then its rate of degradation would have important implications. A fast rate of hydroxyatrazine degradation would indicate the hydrolysis of Cl-atrazine to OH-atrazine as the rate limiting step in the dissipation of atrazine in soils. The slow hydrolysis of Cl-atrazine would account for its residual life.

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[4]Personal communication with M. L. Montgomery, Department of Agricultural Chemistry.

To investigate the degradation of hydroxyatrazine in soils, ring-labeled hydroxyatrazine with a specific activity of 10.4 μc/mg was used in these studies. Approximately 1 μc or 1 ppm was applied to the above soils. Evolution of $^{14}CO_2$ was followed.

Under field conditions, the soil environment may become saturated with moisture and thus suitable only for anaerobic microorganisms. Therefore, a small scale experiment was designed to explore the role of anaerobic microorganisms in the degradation of atrazine and hydroxyatrazine. Helium was passed through the radio-respirometric system rather than compressed air and $^{14}CO_2$ was trapped as previously described.

EXPERIMENTAL RESULTS

The results of the various experiments will be presented under the following headings: Isolation and growth of microorganisms, seed inoculation and incubation studies, and degradation of ^{14}C-atrazine.

Isolation and Growth of Microorganisms

Three methods were used to isolate microorganisms: (1) a broth containing an aliquot of soil, or soil solution enrichment, (2) random selection of isolates from pour plates of atrazine-treated and non-treated soil, and (3) isolation of microorganisms from the rhizosphere of various plant species.

The soil solution technique was used only to a limited degree. However, after 48 hours growth, there was a marked increase in visible turbidity of the solution containing the atrazine-treated aliquot of soil over the non-treated soil. A loop of inoculum was taken only from the atrazine-soil solution and streaked on GNA + 100 ppm atrazine. Subsequent growth yielded only one bacterium. Thus, the enriched atrazine solution had produced a pure culture.

Eleven bacteria and one mold were selected at random from pour plates of the atrazine-treated and non-treated soil. Cultures from both soil samples appeared to grow equally well in GNB + 100 or 1000 ppm atrazine. All cultures were resistant to atrazine under these conditions.

A variety of microorganisms were isolated from the rhizosphere of corn, oats, tomatoes, and soybeans. Each plant species yielded

different microorganisms, i.e., different bacteria or different molds. Both rates of atrazine-treated soil (0.00 or 0.25 ppm) also produced differences. Moreover, growth in GNB or GNB + 100 ppm atrazine yielded a different microflora. In all cases, the subsequent growth on GNA + 1000 ppm atrazine gave one culture, or two at the most. Twelve bacteria and two molds were eventually isolated from the rhizosphere.

Eight bacterial isolates were grown in NCB + 2500 ppm atrazine or NCB only to compare viable cell counts at 0, 3, and 5 days. The data are reported in Table 3. There were no marked increases in viable cell counts with atrazine as the sole source of carbon. A consistent trend was not apparent and occasionally the count from NCB was slightly higher than with atrazine as a carbon source.

Visible growth of all isolated cultures was compared on GNA + 1000 ppm atrazine or agar + 2500 ppm atrazine. Growth on the former medium was abundant; however, growth on the latter medium was very slow and sparse. Bacterial colonies were observed only after 3-4 weeks. Molds developed a limited mycelial mass. Nevertheless, when inoculum from the agar + 2500 ppm atrazine plates was transferred to fresh GNA + 1000 ppm atrazine, the resulting growth was again abundant. After three serial transfers on agar + 2500 ppm atrazine, the cultures grew prolifically when transferred to fresh GNA + 1000 ppm atrazine plates.

Table 3. Viable cell counts of bacteria (ten millions)*
grown in NCB + 2500 ppm atrazine or
NCB only at 0, 3, and 5 days

Isolate no.	0	3	5
1	9.3	37.0	27.0
1-ck	12.5	19.8	6.0
6	11.1	47.0	34.0
6-ck	10.5	54.0	22.2
9	11.8	25.9	11.6
9-ck	9.3	94.0	19.2
20	8.2	70.0	50.0
20-ck	10.8	60.0	38.0
22	7.9	79.0	72.0
22-ck	8.4	87.0	73.0
23	11.4	49.0	45.0
23-ck	11.4	32.0	35.0
25	9.6	75.0	69.0
25-ck	11.5	38.6	105.0
26	11.1	94.0	74.0
26-ck	10.6	81.0	85.0

*Average of triplicate plates

Two of the mold isolates were tentatively identified as a

Pencillium species and a Rhizopus species.[5]

Seed Inoculation and Incubation Studies

From the preliminary screening trial to select a plant species

for bioassay studies, oats were selected because of their sensiti-

vity, ease of handling and a high percentage of seed germination.

Soybeans, cucumbers and tomatoes appeared to be equally sensitive,

but lacked the handling or germination qualities.

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[5]Courtesy of R. C. Carlstrom, Department of Botany and Plant
Pathology.

When different rates of oats were planted in quart cans con-
taining soil treated with 0.25 ppm atrazine, there were differences
in the subsequent reduction in growth, as may be seen in Table 4.
The values for 5, 10, 20, or 50 plants per can, expressed as a per-
cent of the corresponding check, were 25.7, 27.8, 30.0, and 40.8
percent, respectively. Analysis of variance of the dry weight
yields revealed significant differences among all seeding rates.
Comparable results were found in two other identical experiments.

Data from the seed inoculation studies with a mixture of iso-
lates 1, 9, and 23 are presented in Tables 5 and 6. Seeds soaked
in the bacterial mixture had 15.0 X 10^7 bacteria per seed and the
sprayed seeds had 37.4 X 10^7. As shown in Table 5, the check weight
without the addition of bacteria is significantly higher than all
other treatments and is the only significant difference found.
Check weights with the addition of bacteria had been significantly
reduced. Since the bacteria were grown on GNA + 1000 ppm atrazine
and were not washed prior to inoculation, atrazine was probably
present in the bacterial inoculum.

The above isolates were used again in the second experiment.
Seeds soaked in glucose-washed cells had 6 X 10^8 bacteria per seed;
atrazine-washed had 4.8 X 10^8; and atrazine-non-washed had 32.8 X
10^8.

The data in Table 6 show no significant differences among
plants grown in atrazine-treated soil with or without the addition
of bacteria. However, the checks did show a response. The dry
weight of the check + glucose-washed cells was significantly higher

Table 4. Dry weight of oats treated with 0.25 ppm
atrazine at seeding rates of 5, 10, 20,
and 50 plants per can ($s_{\bar{x}}=8.6$)

Dry weight of oats in mg

Plants per can	Replications			Average	% of check*	
	1	2	3			
1. 5	26.6	32.1	32.4	30.4	25.7	a
2. 5-ck	120.6	118.8	115.1	118.2		
3. 10	61.6	65.2	49.7	58.8	27.8	b
4. 10-ck	216.7	207.6	210.4	211.6		
5. 20	122.0	94.0	122.0	112.7	30.0	c
6. 20-ck	388.0	352.0	388.0	376.0		
7. 50	245.0	300.0	275.0	273.3	40.8	d
8. 50-ck	670.0	670.0	670.0	670.0		

*Values followed by different letters are significantly different
at the 5% level using Duncan's multiple range test (35, p. 107)
(CV=6.4%)

Table 5. Dry weight of oats that had been soaked (so)
or sprayed (sp) with bacteria for protection
against atrazine residues and planted in
soil containing 0.00, 0.25, 0.50, or 1.00
ppm atrazine. ($s_{\bar{x}}=5.0$)

Dry weight of 10 oat plants in mg

Rate + bacteria	Replications		Average*	
	1	2		
1. 0.00	116.9	92.6	104.8	a
2. 0.00-so	52.9	54.6	53.8	b
3. 0.00-sp	68.2	60.4	64.3	b
4. 0.25	55.0	68.2	61.6	b
5. 0.25-so	48.5	52.9	50.7	b
6. 0.25-sp	52.9	50.6	51.8	b
7. 0.50	51.5	46.4	49.0	b
8. 0.50-so	50.1	51.2	50.6	b
9. 0.50-sp	52.7	51.0	51.8	b
10. 1.00	45.2	52.8	49.0	b
11. 1.00-so	47.9	59.8	53.8	b
12. 1.00-sp	55.4	54.2	54.8	b

*Values followed by different letters are significantly different at
the 5% level using Duncan's multiple range test. (CV=12.1%)

Table 6. Dry weight of oats inoculated with bacteria
and planted in soil containing 0.00, 0.25,
or 0.50 ppm atrazine ($s_{\bar{x}}$=8.4)

	Dry weight of 10 oat plants in mg			
	Replications			
Treatment[1]	1	2	3	Average*
1. 0.00	163.7	187.7	172.9	174.7 a
2. 0.00-g	214.4	265.2	200.2	226.6 b
3. 0.00-a	184.4	142.5	174.7	167.2 a
4. 0.00-w	220.0	201.5	182.3	301.3 c
5. 0.25	61.2	63.0	71.1	65.1 d
6. 0.25-g	74.3	77.2	47.0	66.2 d
7. 0.25-a	47.0	59.8	39.9	48.9 d
8. 0.25-w	64.0	59.3	44.4	55.9 d
9. 0.50	49.9	47.8	52.1	49.9 d
10. 0.50-g	54.9	48.7	30.2	44.6 d
11. 0.50-a	52.2	45.8	35.9	44.6 d
12. 0.50-w	53.1	46.3	39.0	46.1 d

*Values followed by different letters are significantly different
at the 5% level using Duncan's multiple range test. (CV=14.6%)

[1]Rates of 0.00, 0.25, and 0.50 ppm atrazine: g=glucose-washed cells;
a=atrazine-non-washed cells; w=atrazine-washed cells.

than the check + atrazine-washed cells which was higher than the
check or check + atrazine-non-washed cells. In general, the addi-
tion of bacteria did not significantly increase the growth of oats
grown in atrazine-treated soil.

Soil treated with 0.00 or 0.75 ppm atrazine and incubated at
30°C for 0, 1, 2, 3, or 4 weeks, was bioassayed to determine the
concentration of residual atrazine.

Results of the incubation study in non-sterile Newberg soil are
given in Table 7. The sterile set became contaminated with molds
early in the sequence and was discarded.

Table 7. Dry weight of oats to determine the residual
concentration of atrazine when grown in soil
containing 0.00 or 0.75 ppm atrazine initially
and incubated at 30°C for 0, 1, 2, 3, or 4
weeks. (sx̄=3.5)

		Dry weight of 10 oat plants in mg					
		Replications					
Time	Rate	1	2	3	4	Average	% of check*
1. 0	0.75	34.6	40.5	41.9	48.5	41.4	36.6 a
2. 0	ck	110.4	126.2	101.0	115.3	113.2	
3. 1	0.75	46.5	48.1	44.9	51.1	47.6	33.3 a
4. 1	ck	148.5	132.3	153.2	137.7	142.9	
5. 2	0.75	53.5	58.1	52.5	35.2	49.8	38.5 a
6. 2	ck	132.8	129.1	119.3	135.8	129.2	
7. 3	0.75	60.0	66.0	59.6	63.2	62.2	47.1 b
8. 3	ck	117.0	153.7	113.6	144.1	132.1	
9. 4	0.75	51.5	70.9	67.0	76.3	66.4	52.9 b
10. 4	ck	128.3	104.0	132.1	137.9	125.6	

*Values followed by different letters are significantly different at
the 5% level using Duncan's multiple range test. (CV=13.6%)

The disappearance rate of 0.75 ppm atrazine after incubation
was measured by the growth of oats. After 0, 1, 2, 3, or 4 weeks
of incubation, the growth, as a percent of the check, was 26.6,
33.3, 38.5, 47.1, and 52.9 percent, respectively. An analysis of
variance on the dry weight yields of the treated oats showed a sig-
nificant increase in yield after three weeks incubation. There was
no difference in dry weight between 3 or 4 weeks. From the standard
growth curve, based on dry weight matter at 0.00, 0.05, 0.25, 0.50,
and 0.75 ppm atrazine, illustrated in Figure 1, there was approxi-
mately 0.2 ppm of residual atrazine or 27 percent of the initial
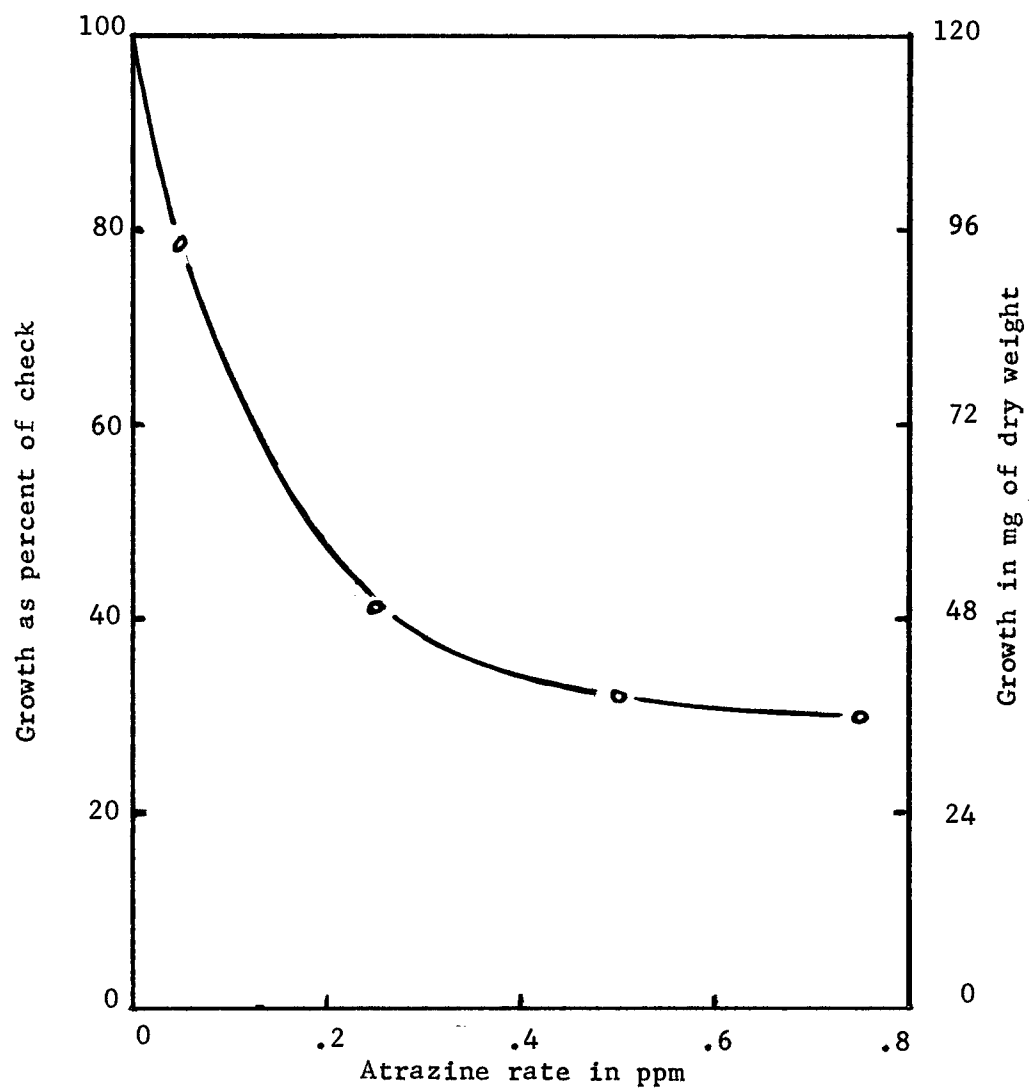0.75 ppm atrazine remaining after 3 or 4 weeks incubation.

Figure 1. Standard growth curve of oats grown
in atrazine-treated soil.

Degradation of ^{14}C-atrazine

Although 24 bacterial and 3 mold isolates were obtained, only 7 of the bacterial cultures and A. fumigatus were used in the ^{14}C-atrazine studies. These were designated as isolates 1, 9, 23, 43, 45, 48, and 53. The origin of 1 was the soil solution; 9 and 23 were isolated from the pour plates; and 43, 45, 48, and 53 were isolated from the rhizosphere of corn, soybeans, tomatoes, and oats, respectively. The latter were grown in atrazine-treated soil. A general description and tentative identification (4) of the cultures are presented in Table 8.

Table 8. General description and tentative identification of bacteria used in degradation of ^{14}C-atrazine.

Isolate	Gram stain	Morphology	Genus	Pigmentation
1	Negative	Short rod		White
9	"	"	Alcaligenes	White
23	"	"	Pseudomonas	White
43	"	"		Pale yellow
45	"	"	Alcaligenes	White
48	"	"		Yellow
53	"	Long rod		Pale yellow

Preliminary experiments on the degradation of ^{14}C-atrazine in synthetic media indicated no detectable evolution of $^{14}CO_2$ when samples were taken hourly. Therefore, the sample period was expanded to 12 hours.

Isolates 1, 9, 23, 43, 45, and 53 were used in the synthetic studies. The $^{14}CO_2$ evolution from the chain-labeled atrazine was appreciable during the first 12-hour period. However, the count rate decreased rapidly thereafter. After 72 hours, the count rate was almost zero. Data relating to isolate 9 are shown in Figure 2. These data are representative of all cultures tested and account for approximately 0.1 percent of the input ^{14}C-activity. Addition of 1000 ppm atrazine, 0.1 percent,or 1.0 percent glucose did not influence the evolution of $^{14}CO_2$. Viable cell counts remained essentially constant throughout the test period except with the addition of 1.0 percent glucose. The initial cell count was generally 1 X 10^9 cells per ml, whereas,the final cell count had fallen below 1 X 10^6 with the high level of glucose after 72 hours. The pH had also dropped from 7.5 to 3.5. There was no detectable $^{14}CO_2$ evolved from ring-labeled atrazine by any of the bacteria used.

The degradation of chain-labeled atrazine by pure cultures in sterile Newberg soil presented a different picture from that observed in synthetic media. Evolution of $^{14}CO_2$ did not decrease rapidly after the first day. As may be observed in Table 9, there was a peak of evolved $^{14}CO_2$ at four days, perhaps from the sparking solution, and a gradual decrease thereafter. The bacterial isolates evolved 0.4-0.7 percent of the input ^{14}C-activity over a two-week span, whereas,A. fumigatus evolved 4.0 percent. As in the synthetic media, there was no detectable $^{14}CO_2$ evolved from ring-labeled atrazine.

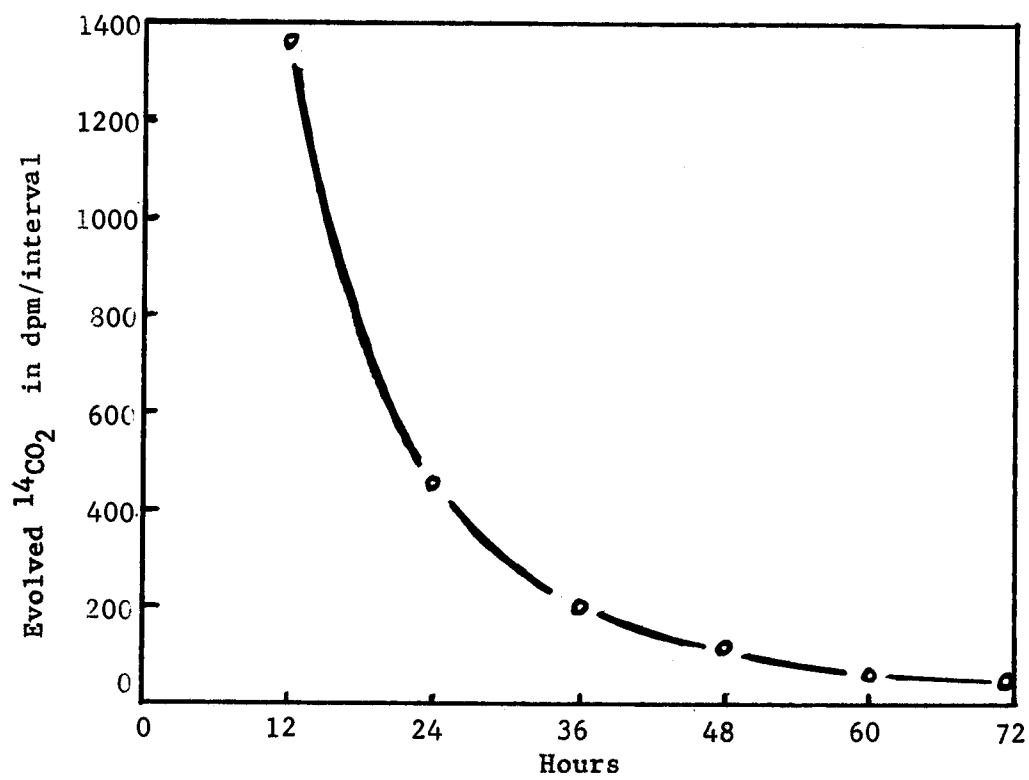Figure 2. Evolution of $^{14}CO_2$ from chain-labeled atrazine in synthetic media.

Table 9. Evolution of $^{14}CO_2$ from chain-labeled atrazine
in sterile Newberg soil inoculated with micro-
organisms.

	dpm per 3-day intervals					
Isolate	1	4	7	10	13	% of input
1	2,841	10,992	3,717	2,808	1,949	0.6
9	5,680	9,463	4,004	2,504	2,070	0.7
43	3,839	5,842	3,007	2,471	1,698	0.4
45	805	10,674	4,486	2,321	1,986	0.6
48	2,921	11,331	5.044	2,158	2,027	0.7
53	3,202	11,720	4,200	2,870	2,304	0.6
A. fum.*	4,029	80,103	30,152	16,744	13,643	4.0

*Sterile Chehalis soil

Table 10. Evolution of $^{14}CO_2$ from chain-labeled
atrazine in non-sterile soils

	dpm/3-day intervals for 2 soils			
	Newberg		Chehalis	dpm/sample hr.
Days	1	2	1	from Chehalis
1	3,514	3,571	5,451	227
4	20,774	19,183	15,629	216
7	11,331	10,287	13,842	193
10	12,755	8,518	10,891	151
13	11,763	7,829	9,859	137
*	1.6	1.4	1.6	
16	---	9,812	9,087	126
19	---	7,085	8,303	116
22	---	5,894	8,896	123
25	---	3,607	6,630	92
28	---	3,123	5,898	82
*		2.2	2.6	
ppm input	3.52	3.24	3.27	

*Percent of input ^{14}C-activity

The rate of $^{14}CO_2$ evolution from chain-labeled atrazine by the native soil population was higher than the test bacteria but lower than the mold species. Both soils respired approximately the same amount of $^{14}CO_2$ in two weeks. After four weeks the Chehalis soil had evolved a slightly higher amount of $^{14}CO_2$ than the Newberg soil, or 2.6 versus 2.2 percent of the input ^{14}C-activity, respectively (Table 10). The rate of gas evolution in both soils tapered off gradually. Figure 3 depicts the general scheme with dpm/sample hour plotted against time. The cumulative $^{14}CO_2$ evolved is shown in Figure 4.

A small amount of ring breakage occurred in non-sterile soils. Over a three-week period, approximately twice as much $^{14}CO_2$ was evolved from the ring-labeled atrazine in the Chehalis soil (1.0%) than from the Newberg soil (0.6%) (Table 11). The concentration of atrazine had no apparent effect on $^{14}CO_2$ evolution. Equal percentages of $^{14}CO_2$ (0.4-0.5%) were evolved from soils containing 1.64 or 3.08 ppm ring-labeled atrazine in two weeks (Table 12).

As shown in Table 13, the evolution of $^{14}CO_2$ from ring-labeled hydroxyatrazine was 1.1-1.6 percent of the input ^{14}C-activity. This rate was two to three times that from ring-labeled atrazine tested over a two-week period.

After three or four weeks, approximately 25 percent of the input ^{14}C-activity was extracted with methanol from non-sterils soils. Peaks were detected on the radiochromatograms at R_f values of 0.9 and 0.5, which corresponded to atrazine and hydroxyatrazine, respectively, in the isoamyl alcohol + 3N HCl solvent system. Based on the
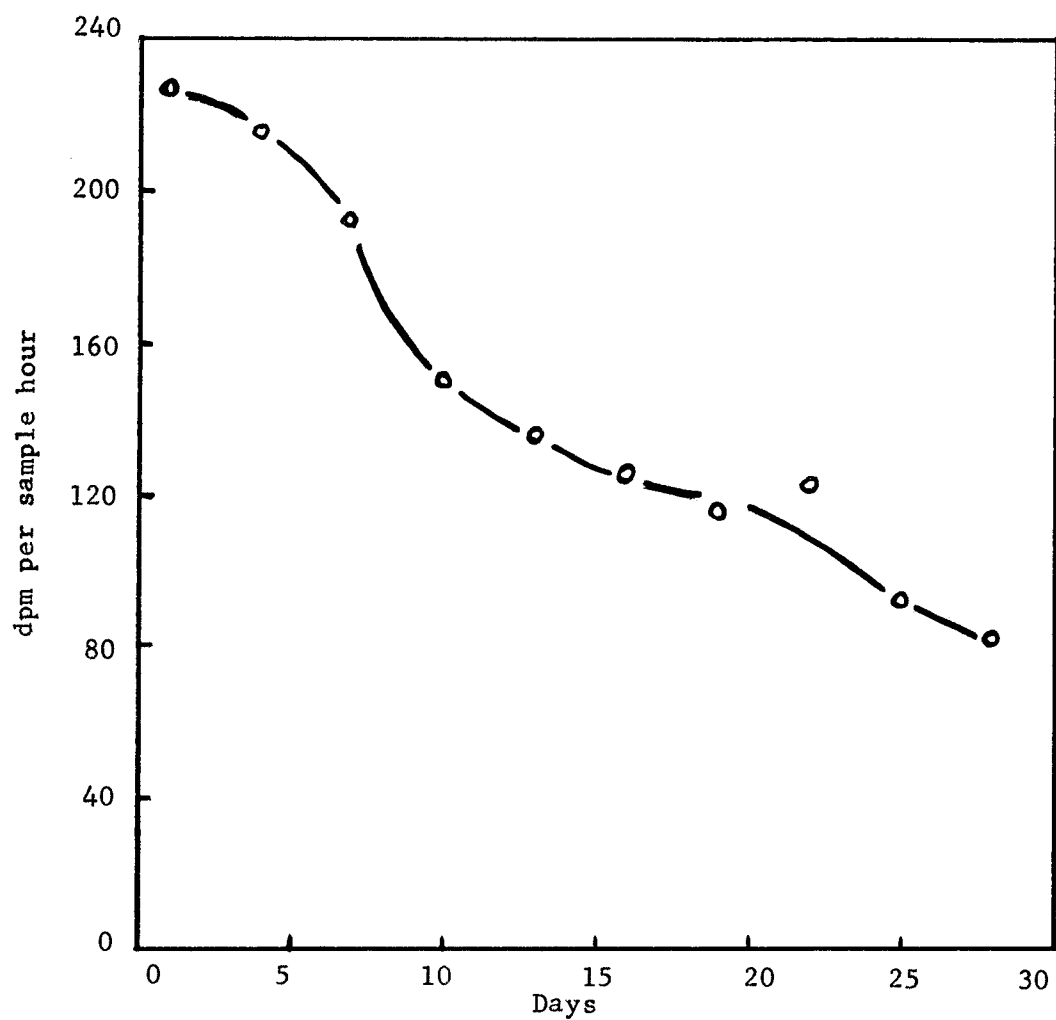
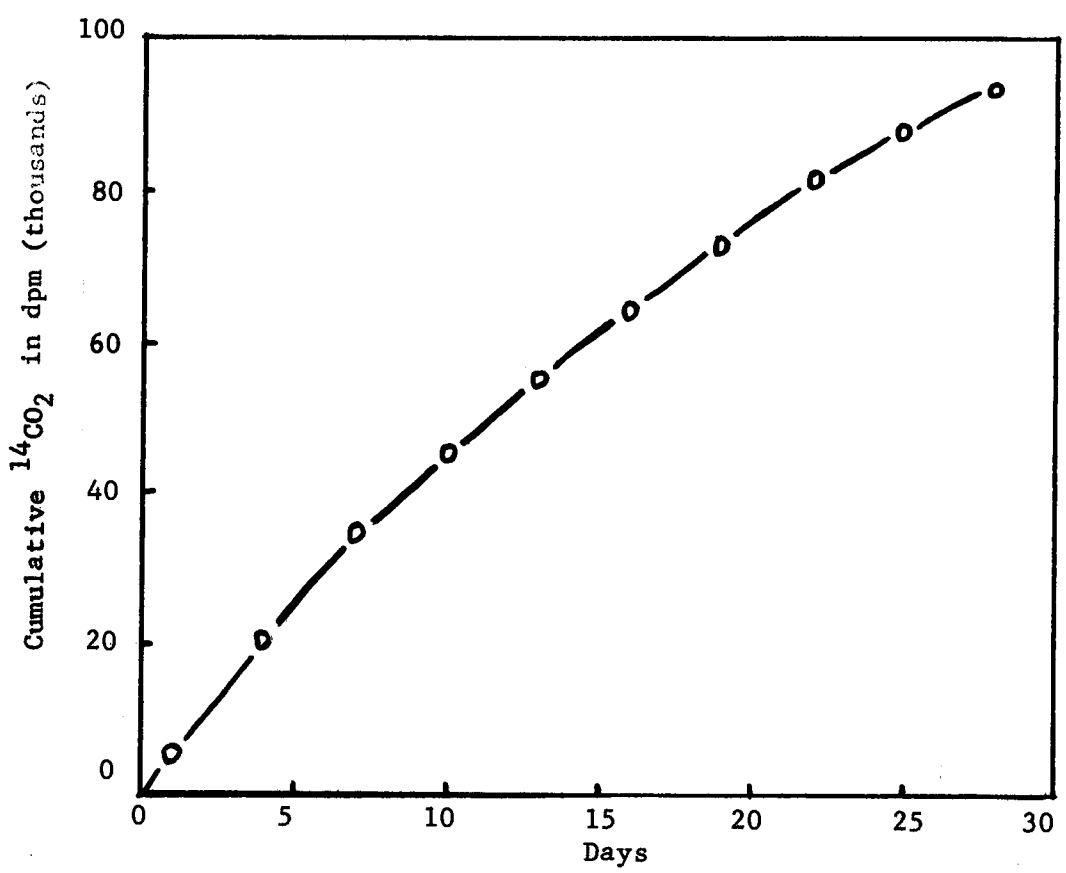Figure 3. Evolution of $^{14}CO_2$ from chain-labeled
atrazine in Chehalis soil.

Figure 4. Cumulative $^{14}CO_2$ evolved from chain-labeled atrazine in Chehalis soil.

Table 11. Evolution of $^{14}CO_2$ from ring-labeled atrazine
in non-sterile soils.

	dpm/3-day intervals for 2 soils				
	Newberg	Chehalis			
Days	1	1		2	
1	685	4,884		4,204	
4	4,535	9,890		9,607	
7	6,014	5,568		4,950	
10	3,572	4,291		4,197	
13	3,020 (0.4%)*	4,909	(0.7%)	4,912	(0.6%)
16	2,917	4,729		5,441	
19	2,608	4,593		4,613	
22	2,451 (0.6%)	5,228	(1.0%)	4,612	(1.0%)
25		4,726			
28		4,497	(1.2%)		
ppm input	1.61	1.51		1.61	

*Percent of input ^{14}C-activity

Table 12. Evolution of $^{14}CO_2$ from ring-labeled atrazine,
1.64 or 3.08 ppm (2.1 or 4.0 μc) in non-
sterile soils.

	dpm/3-day intervals for 2 soils			
	Newberg		Chehalis	
Days	1.64	3.08	1.64	3.08
1	625	820	3,169	5,271
4	4,394	6,824	9,172	16,915
7	5,914	10,672	4,168	8,761
10	4,117	8,316	3,941	5,926
13	3,177	6,216	3,550	5,980
% of input ^{14}C-activity	0.4	0.4	0.5	0.5

Table 13. Evolution of $^{14}CO_2$ from ring-labeled hydroxy-
atrazine, 1.12 ppm, in non-sterile soils.

	dpm/3-day intervals for 2 soils			
	Newberg		Chehalis	
Days	1	2	1	2
1	976	983	5,824	6,761
4	8,506	8,348	13,888	13,572
7	6,796	7,234	4,586	4,108
10	3,562	3,711	3,339	3,264
13	1,821	1,801	2,856	2,771
% of input ^{14}C-activity	1.1	1.1	1.6	1.6

area of the peak height, the hydroxyatrazine accounted for approxi-
mately 20 percent of the extracted activity in both soils. Similar
results were found in sterile soils. Thus, the conversion of
Cl-atrazine to OH-atrazine is a chemical phenomenon rather than de-
rived via microbial activity.

Under anaerobic conditions, there was essentially no $^{14}CO_2$
evolved from ring-labeled atrazine or hydroxyatrazine. A minute
amount was evolved from chain-labeled atrazine in both soils during
a seven-day period.

DISCUSSION AND CONCLUSIONS

Microbial Growth Studies

Viable cell count data from eight bacterial isolates indicated that there was no appreciable utilization of atrazine as the sole source of carbon. The endogenous respiration of the isolates supported approximately the same population numbers as the atrazine. Since these experiments were terminated after five days, the endogenous food supply may have been sufficient to maintain the isolates. Thus, a longer time lapse would be needed to deplete the endogenous materials and force the isolates to use atrazine as the carbon source. Other factors could be involved in these findings: such as, a concentration of 2500 ppm atrazine may have been inhibitory to the bacterial metabolism; the basal inorganic salt medium may have lacked an essential constituent(s) for bacterial growth; or a toxic metabolite was produced in the initial stages of bacterial growth.

Evolution of $^{14}CO_2$ occurred only from the labeled-ethylamino group since the isopropylamino group was not labeled. There was apparently none evolved from ring-labeled atrazine. Even so, there would have been approximately 30 mg of carbon from the chain constituent of atrazine, which would support appreciable microbial growth.

Growth or subsistence on agar + 2500 ppm atrazine was very limited. This indicated a low level of attack on the atrazine, the inert materials in 80 percent WP (atrazine), or the agar. A pink and yellow isolate were observed to grow on agar without an added carbon

source. Therefore, caution must be observed when stating that microorganisms can utilize atrazine as the sole source of carbon.

Seeding Rate

The data showed significant differences among 5, 10, 20, or 50 plants when grown in quart cans containing 900 g of soil treated with 0.25 ppm atrazine. Since bioassays are used quite often in herbicidal studies to report the percent of growth reduction by different rates of herbicides, the experimental procedures, i.e., the number of plants per unit of soil, should be similar in order to make adequate and fair comparisons of the reported data. Otherwise, bioassays with a high plant density would indicate less herbicidal residue than would a low plant density. This statement is made on the basis of a limited amount of herbicide available to the plant population at a given time. At the field level, Gysin and Knusli (17, p. 345) stated that in a weedy field there was less residue the following year. Moreover, perhaps a higher rate of herbicide would be needed to control weeds in a heavily infested field.

Seed Inoculation

No significant benefits were observed by inoculating seeds with bacteria resistant to atrazine. The rate of atrazine degradation was not sufficient to protect susceptible plants grown in atrazine-treated soil. These results agree with the low rate of $^{14}CO_2$ evolved from soils containing ^{14}C-atrazine. Consequently, seed inoculation must wait the isolation of a microorganism effective in the

degradation of atrazine.

Soil Incubation

After three or four weeks incubation at 30°C, the concentration of atrazine had been significantly reduced from 0.75 ppm to 0.20 ppm or a 73 percent reduction. Within a similar time period, only 2.2 percent of chain-labeled atrazine was evolved as $^{14}CO_2$. The inactivation of atrazine in the soil incubation study was 33-fold greater than could be accounted for by the gas evolution. Since 20 percent of the extracted ^{14}C-activity was hydroxyatrazine in non-sterile or sterile soils, the major pathway of atrazine degradation appears to be a chemical conversion to hydroxyatrazine rather than microbial attack.

^{14}C-atrazine Degradation

No detectable $^{14}CO_2$ was evolved from ring-labeled atrazine and only 0.1 percent from chain-labeled by the bacterial isolates used in synthetic media. The latter $^{14}CO_2$ evolution was essentially zero after 24 hours as shown in Figure 2. Perhaps the small amount of evolved ^{14}C-activity came from labeled impurities. On the other hand, the bacteria may have had the ability to attack atrazine in the initial stage, but lost the ability because of a deficiency in the basal medium. Moreover, a toxic metabolic may have inhibited further degradation.

In sterile soil, the bacterial isolates tested evolved 0.4-0.7 percent of the input chain-labeled atrazine over a two-week period.

The peak of gas evolution at four days (Table 9) is probably an increase in initial microbial activity from the sparking solution. The low rate of attack indicated a non-preferential utilization of the atrazine carbon. <u>A</u>. <u>fumigatus</u> with 4.0 percent gas evolution was the most effective microorganism tested. The latter isolate gave a 6-10 fold increase above the bacterial cultures. As in the synthetic media, there was no detectable evolution of $^{14}CO_2$ from ring-labeled atrazine by any of the isolates tested.

The native soil population produced an intermediate rate of $^{14}CO_2$ evolution. In four weeks non-sterile Newberg and Chehalis soils had respired 2.2 and 2.6 percent, respectively, of the input chain-labeled atrazine. These rates indicated that the significance of bacterial degradation may be greater than observed in pure cultures and mold degradation may be less. The significance of bacterial versus mold attack in the soil ecosystem would depend on the soil environment, i.e., pH, temperature, organic matter and moisture level.

Under anaerobic conditions, there was no detectable $^{14}CO_2$ evolved from ring-labeled atrazine or hydroxyatrazine. Only a minute amount was evolved from chain-labeled atrazine in seven days. Therefore, microbial degradation of atrazine by anaerobic microorganisms would be of minor importance. These results do not agree with those of Agundis and Behrens (1). Since they used a bioassay to test for atrazine in soils after being flooded and under nitrogen or helium atmospheres, the formation of hydroxyatrazine may have accounted for the degradation rather than microbial attack.

An important property of a herbicide is its residual life. A certain amount of residual activity is needed for season-long weed control; however, any remaining residual phytotoxicity in the next growing season(s) is generally undesirable. Therefore, a system to give a relative index of the residual life of a herbicide would be most beneficial in selecting and recommending new herbicides. The radiorespirometric system (Plates 3 and 4) developed in these studies, is proposed as a means to obtain data on the relative soil residual life of herbicides or pesticides in general.

In Table 14, three rates of $^{14}CO_2$ evolution from Figure 3 were selected for extrapolation. A high, medium and low rate were converted to percent $^{14}CO_2$ evolved per month or percent degradation of atrazine. The latter statement is made by assuming that dealkylation of the ethylamino group would render atrazine non-phytotoxic. The time required to reduce the initial 3.27 ppm to 0.25 ppm (92.3%) was then calculated. Assuming an equal attack on the isopropylamino group or a 2-fold increase in degradation, the time required is reduced by one-half. These data are shown in Figure 5.

The assumption of an equal attack on the isopropylamino group is made without direct evidence since atrazine labeled accordingly was not available for this study. However, Bryant (5, p. 38) reported that a _Pseudomonas_ sp. preferred a diethylamino group, an _Arthrobacter_ sp. preferred the ethylamino group, and both genera utilized the isopropylamino group equally well in studies of 2-R-4-R-6-isopropylamino-g-triazines.

The time required to dissipate 92.3 percent of the input

atrazine by microbial attack on the side chains would be 10, 15, and 29 months for a high, medium, and low rate of degradation, respectively. Under field conditions the normal time required for dissipation of atrazine is approximately 18-20 months. Thus, a medium rate of attack by microorganisms under optimum conditions gives a reasonable index of the residual life of atrazine under field conditions. However, there are other factors that should also be considered.

In the soil incubation study, 73 percent of the initial atrazine was detoxified and/or adsorbed so tightly as to be inactive in 3-4 weeks, whereas, only 4.4 percent (2 X 2.2%) of chain-labeled atrazine was evolved in the same period. Therefore, the degradation of atrazine must be largely dependent on chemical hydrolysis to hydroxyatrazine. This conclusion is supported by the appearance of hydroxyatrazine after 3-4 weeks incubation of labeled-atrazine in non-sterile and sterile soils. Moreover, the rate of hydroxyatrazine degradation was two to three times greater than atrazine. This indicated that microbial attack would be largely on the side chains with a low rate of degradation of the triazine ring upon hydrolysis to hydroxyatrazine.

Burschel and Freed (9) reported the decomposition of amitrol, IPC, and CIPC to follow a first order reaction, assuming adequate moisture for microorganisms. The results of these studies would support a first order reaction for the degradation of atrazine based on chemical and microbial participation.
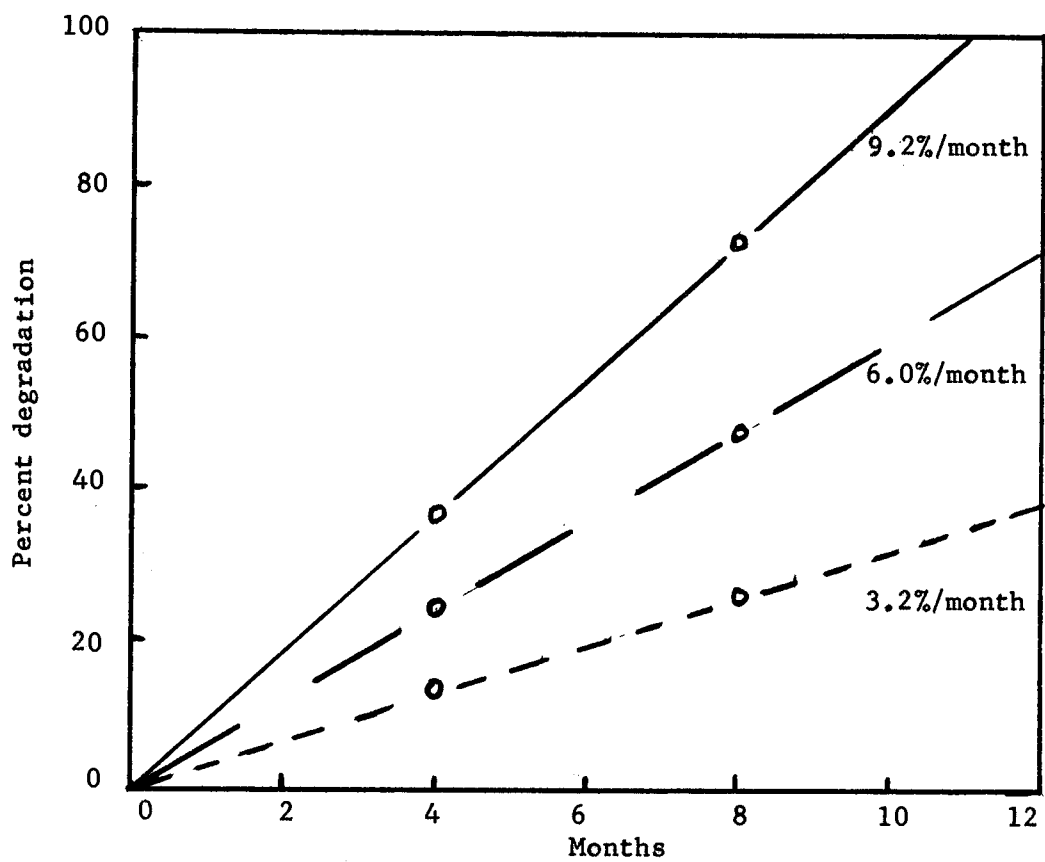
Figure 5. Extrapolation of $^{14}CO_2$ data to estimate
residual life of atrazine.

Table 14. Estimated time required to reduce atrazine
from 3.27 ppm to 0.25 ppm by extrapolation
of $^{14}CO_2$ data from Figure 3.

Rate, dpm/hr.	% per month	Time req. months	Plus equal % from isopropylamino	Time req. months
230	4.6	20	9.2	10
150	3.0	31	6.0	15
80	1.6	58	3.2	29

Decay constants were calculated using the first order equation:

$$k = \frac{2.3}{t} \log \frac{a}{a-x}$$

k = decay constant, ppm/month
t = time, month
a = initial concentration, ppm
a-x = concentration at time t

From the data in Table 10, with chain-labeled atrazine and assuming an equal degradation of the isopropylamino group, a decay constant of 0.05 ppm/month was calculated for both the Chehalis and Newberg soils or a half-life of 14 months. The ring data at two concentrations in both soils (Table 12) gave a decay constant of 0.02 ppm/month or a half-life of 34 months. By combining these two rates to obtain the total breakdown via ring and chain degradation, a decay constant of 0.07 ppm/month and a half-life of 10 months are obtained.

However, by using the greenhouse data in Table 7, a decay constant of 1.32 ppm/month and a half-life of 0.5 month are obtained. The greenhouse decay constant was 20-fold greater than that obtained

from the $^{14}CO_2$ data. Perhaps there was a greater microbial attack on the isopropylamino group than assumed in the latter calculations. Moreover, the bioassay would not be as sensitive as the radioassay and the presence of a plant system could enhance the microbial and/ or chemical degradation.

Under field conditions, the rate of chemical versus microbial degradation would depend on the soil environmental factors. With high temperatures ($30^{\circ}C$) and low pH values (5.5), the hydrolysis to hydroxyatrazine would probably be the dominant factor. Attack by molds would also be important under these conditions. Neutral pH values (7) would favor a limited attack by bacteria. At basic pH values (8.5) the hydrolysis would again be dominant. Low temperatures ($15^{\circ}C$) and low moisture levels would be unfavorable for both chemical and microbial degradation of atrazine in soils. Temperature and pH would only be two factors involved. Others, i.e., physical features, organic and inorganic nutrient supply, would also influence the degree of microbial and chemical participation.

SUMMARY

Experiments were conducted in the greenhouse and laboratory to study the degradation of atrazine by pure cultures and native soil microbial populations. Inoculation of seeds with bacteria for possible protection against atrazine was attempted. Evolution of $^{14}CO_2$ from ring- and chain-labeled atrazine and ring-labeled hydroxyatrazine under aerobic and anaerobic conditions in two soils was followed. A radiorespirometric system was designed for the latter studies. Soils were extracted with methanol and chromatographed to check for the formation of hydroxyatrazine. The following results were obtained:

1. Viable cell counts of bacterial isolates in an inorganic broth + atrazine as the sole source of carbon was not appreciably different from the controls without a carbon source. All isolates survived three serial transfers on agar + atrazine.

2. Seed inoculation did not increase the dry weight of oats grown in atrazine-treated soil as a bioassay.

3. The atrazine concentration was significantly reduced after three or four weeks incubation in Newberg soil at 30°C.

4. A small amount of $^{14}CO_2$ was evolved from chain-labeled atrazine by bacterial isolates in synthetic media during the first 24 hours and essentially none thereafter. In sterile soils, the rate of $^{14}CO_2$ evolution by the same isolates was appreciable and accounted for 0.4-0.7 percent of the input activity in a two-week period, whereas, A. fumigatus evolved 4.0 percent. There was no detectable ring breakage by any of the tested isolates.

5. In non-sterile Newberg and Chehalis soils, 2.2 and 2.6 percent, respectively, was evolved as $^{14}CO_2$ from chain-labeled atrazine in four weeks. From ring-labeled atrazine there was 0.4 and 0.6 percent, respectively, respired during two weeks. From ring-labeled hydroxyatrazine there was 1.1 and 1.6 percent, respectively, evolved in two weeks or a 2-3 fold increase above ring-labeled atrazine.

6. Hydroxyatrazine accounted for approximately 20 percent of the extracted ^{14}C-activity after 3-4 weeks in non-sterile and sterile soils.

7. Data of $^{14}CO_2$ evolution from chain-labeled atrazine was extrapolated to give an estimate of the residual life of atrazine based on microbial degradation.

8. Decay constants were calculated from the chain- and ring-labeled data as 0.05 and 0.02 ppm per month or half-lifes of 14 and 34 months, respectively. From greenhouse data, a decay constant of 1.32 ppm/month or half-life of 0.5 month were obtained, or a 20-fold increase.

9. Under anaerobic conditions there was only a minute amount of $^{14}CO_2$ evolved from chain-labeled atrazine.

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