



Enhanced herbicide degradation and dissipation of clomazone in two Montana soils
by Eric Robert Gallandt

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Agronomy
Montana State University
© Copyright by Eric Robert Gallandt (1988)

Abstract:

Clomazone is a preplant herbicide that provides excellent control of many weeds which are common in fallow land however little is known about the residual period or potential for injury to subsequent wheat crops. Clomazone dissipation was examined at two locations in Montana. Clomazone at 2.2 kg/ha applied to a loam soil dissipated to below 0.1 ppmw in 3 months and applied to a silty clay loam soil dissipated to 0.2 ppmw 6 months after application. Half-lives, determined from first order rate plots, were 33 and 37 days in the Willow Creek loam and Bozeman silty clay loam, respectively. Thus, clomazone residue from labeled-use rates should not inhibit wheat in a wheat-fallow-wheat cropping system in Montana.

Enhanced herbicide degradation is a phenomenon of accelerated herbicide decomposition in soil which is induced by prior treatment of that soil with the herbicide.

Enhanced degradation of EPTC was first reported in 1979 and is caused by an adaptation of the constitutive microbial population due to prior EPTC exposure. A soil inoculation technique was used to examine the geographic extent and degree of EPTC enhancement in 166 United States soils. Enhanced degradation of EPTC is a wide-spread phenomenon and EPTC half-lives ranged from less than 1 day to greater than 20 days in the 166 soils examined.

A soil inoculation technique was also used to determine if soils exist which contain microorganisms with the ability to enhance the rate of degradation of persistent soil herbicides such as chlorsulfuron, picloram, atrazine, or clomazone. None of the 166 soils tested contained microorganisms able to enhance the degradation of atrazine or clomazone however, several soils significantly altered the rate of chlorsulfuron or picloram degradation.

A minimal medium plating screen was conducted to isolate microorganisms capable of metabolizing persistent herbicides. Soil extracts from 166 soils were plated on minimal medium which contained chlorsulfuron, picloram, atrazine, clomazone, or EPTC as a sole carbon source.

Twenty-five isolates rapidly grew in liquid medium containing chlorsulfuron, picloram, or atrazine as a sole carbon source. HPLC was used to measure herbicide concentration in liquid cultures. Although attempts to show disappearance of the parent herbicide molecule failed repeatedly, several isolates appeared to produce unidentified metabolite peaks.

ENHANCED HERBICIDE DEGRADATION AND
DISSIPATION OF CLOMAZONE IN TWO
MONTANA SOILS

by

Eric Robert Gallandt

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Agronomy

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 1988

N 378
G 1354

Cap 2

ii

APPROVAL

of a thesis submitted by

Eric Robert Gallandt

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

11-21-88
Date

Pete K Fay
Chairperson, Graduate Committee

Approved for the Major Department

11/21/88
Date

Charles F. McChure
Head, Major Department

Approved for the College of Graduate Studies

12/12/88
Date

Henry L. Parsons
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of source is made.

Permission for extensive quotation from or reproduction of this thesis may be granted by my major professor, or in his absence, by the Director of Libraries when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature Eric R. Gallant

Date 11/22/88

ACKNOWLEDGEMENTS

Foremost, I would like to thank Dr. Peter K. Fay for his patience, guidance, and advice during the difficult role of advisor and friend.

I also thank the other members of my committee, Dr. Gary Strobel, Dr. James Nelson, and Dr. William Dyer for their valuable insight into this work.

I also owe many thanks to fellow students, Greg Bunkers, Leslie Harrison, Tim Braun, Sue Schulze, Pam Borden, Martina Willens, and Kevin Murray for their ideas and, in many cases, long hours of help with this project. I also thank Drs. David Sands and Doug Kenfield for their invaluable technical assistance.

I wish to thank the other members of the weed group, Mark Stannard, Gary Fellows, and especially Edward Davis for their help, friendship, and inspiration. And, Jennifer Atchley for her help in many areas, her ideas, and above all, her friendship.

TABLE OF CONTENTS

	Page
APPROVAL.....	ii
STATEMENT OF PERMISSION TO USE.....	iii
VITA.....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	viii
LIST OF FIGURES.....	x
ABSTRACT.....	xii
Chapter	
1. LITERATURE REVIEW.....	1
General Herbicide Degradation in Soil.....	1
Clomazone.....	6
Enhanced Thiocarbamate Degradation.....	10
Enhanced Degradation of Other Herbicides..	15
Enhanced Degradation of Insecticides and Fungicides.....	17
2. DEGRADATION OF CLOMAZONE IN TWO MONTANA SOILS.....	20
Introduction.....	20
Materials and Methods.....	20
Results and Discussion.....	25
3. THE INCIDENCE AND MAGNITUDE OF ENHANCED EPTC DEGRADATION IN 166 SOILS.....	35
Introduction.....	35
Materials and Methods.....	36
Results and Discussion.....	41

Chapter	Page
4. THE INCIDENCE OF ENHANCED DEGRADATION OF FIVE PERSISTENT HERBICIDES IN 166 SOILS.....	48
Introduction.....	48
Materials and Methods.....	49
Results and Discussion.....	54
5. ISOLATION AND TESTING OF HERBICIDE DEGRADING MICROORGANISMS.....	62
Introduction.....	62
Materials and Methods.....	63
Results and Discussion.....	70
LITERATURE CITED.....	77

LIST OF TABLES

Table		Page
1	Weed control results obtained with clomazone in chemical fallow field trials conducted at 3 locations in Montana from 1984 through 1986...	9
2	Physical and chemical characteristics of soils used in clomazone dissipation experiments.....	21
3	Physical and chemical characteristics of soil used in the clomazone leaching experiment.....	26
4	Dry weight of winter wheat plants in the two leaf stage 205 days after application of clomazone at Bozeman and Willow Creek.....	26
5	Plant dry weight as percent of control for 5 species compared as bioassay indicators for clomazone residues in soil.....	27
6	Lentil biomass produced per plant when grown in incubated soil originally treated with 40 ppbw chlorsulfuron and inoculated with 10 g of a test soil.....	57
7	Pinto bean biomass produced per plant when grown in incubated soil originally treated with 40 ppbw picloram and inoculated with 10 g of a test soil.....	58
8	Oat biomass produced per plant when grown in incubated soil originally treated with 2 ppmw atrazine and inoculated with 10 g of a test soil.....	59
9	Oat biomass produced per plant when grown in incubated soil originally treated with 1.5 ppmw clomazone inoculated with 10 g of a test soil...	60
10	Composition of basal salts medium (BSM).....	64

Table		Page
11	Technical grade herbicide, % purity, and manufacturer of the products used in the isolation and testing of herbicide degrading microorganisms.....	65
12	Thin-layer chromatography solvent systems and corresponding Rf values used to confirm herbicide identity.....	68
13	Bacterial and fungal isolates obtained from extracts of 166 soil samples that were plated on agar plates containing one of five herbicides at a concentration of 500 ppm as a carbon source.....	71
14	Herbicide remaining in media originally containing 20 ppm herbicide which was inoculated with HBSM isolates and grown to a maximum cell density.....	73
15	Bioassay indicator species injury in herbicide treated soil inoculated with 6 HBSM isolates and incubated in the greenhouse for 60 days.....	75

LIST OF FIGURES

Figure		Page
1	Structure of clomazone.....	7
2	Structural comparison of EPTC and cycloate...	12
3	Standard curves for oat and wheat biomass production for plants grown 14 days in soil containing 10 rates of clomazone.....	28
4	Standard curves for oat percent chlorosis by height using soil from Bozeman and Willow Creek with 10 rates of clomazone.....	29
5	Clomazone detected in soil sampled monthly for 6 months after application of 0.6, 1.1, and 2.2 kg/ha clomazone at Bozeman.....	30
6	Clomazone detected in soil sampled monthly for 5 months after application of 0.6, 1.1, and 2.2 kg/ha clomazone at Willow Creek.....	31
7	First-order rate plots for clomazone applied at 0.6, 1.1, and 2.2 kg/ha at Bozeman and Willow Creek.....	32
8	Test soil locations for soils used in enhanced EPTC degradation studies.....	37
9	EPTC concentration detected in base soils initially treated with 10 ppmw EPTC and inoculated with 10 g of soil from 166 test soils. EPTC concentration was estimated using an oat bioassay seeded 30 days after herbicide application and test soil inoculation.....	42
10	EPTC detected in a base soil using a delayed planting oat bioassay. Oats were planted 0, 3, 6, and 9 days after herbicide application and inoculation with a small amount of autoclaved or nonautoclaved soil from enhanced soil 235 (A) and non-enhanced soil 71 (B)....	43

Figure		Page
11	First-order rate plots for EPTC degradation test soil number 140 with a half-life of 1.7 days and for test soil number 108 with a half-life of 23 days.....	45
12	EPTC half-life ($t_{1/2}$) in a base soil inoculated with 166 test soils and in a uninoculated soil (*).....	46
13.	Molecular structures of the herbicides used in enhanced degradation studies.....	50
14.	Herbicide detected in inoculated base soils 60 days after test soil inoculation and application of 40 ppbw of chlorsulfuron or picloram.....	55
15.	Herbicide detected in inoculated base soils 60 days after test soil inoculation and application of 2 and 1.5 ppmw of atrazine and clomazone respectively.....	56
16.	Growth curves for atrazine 313, chlorsulfuron 168, and picloram 78 isolates grown in liquid culture.....	72
17.	HPLC chromatograms of a HBSM standard which contains 20 ppm chlorsulfuron (above) and, 20 ppm HBSM after 5 days of growth with chlorsulfuron isolate G168. Arrows indicate elution of the parent chlorsulfuron molecule.....	74

ABSTRACT

Clomazone is a preplant herbicide that provides excellent control of many weeds which are common in fallow land however little is known about the residual period or potential for injury to subsequent wheat crops. Clomazone dissipation was examined at two locations in Montana. Clomazone at 2.2 kg/ha applied to a loam soil dissipated to below 0.1 ppmw in 3 months and applied to a silty clay loam soil dissipated to 0.2 ppmw 6 months after application. Half-lives, determined from first order rate plots, were 33 and 37 days in the Willow Creek loam and Bozeman silty clay loam, respectively. Thus, clomazone residue from labeled-use rates should not inhibit wheat in a wheat-fallow-wheat cropping system in Montana.

Enhanced herbicide degradation is a phenomenon of accelerated herbicide decomposition in soil which is induced by prior treatment of that soil with the herbicide. Enhanced degradation of EPTC was first reported in 1979 and is caused by an adaptation of the constitutive microbial population due to prior EPTC exposure. A soil inoculation technique was used to examine the geographic extent and degree of EPTC enhancement in 166 United States soils. Enhanced degradation of EPTC is a wide-spread phenomenon and EPTC half-lives ranged from less than 1 day to greater than 20 days in the 166 soils examined.

A soil inoculation technique was also used to determine if soils exist which contain microorganisms with the ability to enhance the rate of degradation of persistent soil herbicides such as chlorsulfuron, picloram, atrazine, or clomazone. None of the 166 soils tested contained microorganisms able to enhance the degradation of atrazine or clomazone however, several soils significantly altered the rate of chlorsulfuron or picloram degradation.

A minimal medium plating screen was conducted to isolate microorganisms capable of metabolizing persistent herbicides. Soil extracts from 166 soils were plated on minimal medium which contained chlorsulfuron, picloram, atrazine, clomazone, or EPTC as a sole carbon source. Twenty-five isolates rapidly grew in liquid medium containing chlorsulfuron, picloram, or atrazine as a sole carbon source. HPLC was used to measure herbicide concentration in liquid cultures. Although attempts to show disappearance of the parent herbicide molecule failed repeatedly, several isolates appeared to produce unidentified metabolite peaks.

CHAPTER 1

LITERATURE REVIEW

General Herbicide Degradation

The ultimate fate of a herbicide molecule in soil is regulated by properties of the herbicide molecule, and environmental and edaphic conditions. Some herbicide molecules are volatile and are lost to the atmosphere. Others bind tightly to clay surfaces or soil organic matter and become unavailable for plant uptake. Molecules which do not interact strongly with soil constituents may leach. While volatilization, binding, and leaching reduce herbicide activity, the herbicide molecule remains intact.

Degradation of the herbicide molecule in soil can be caused by both biological and nonbiological mechanisms. Some herbicide molecules absorb ultraviolet light and undergo photochemical degradation. Absorbed ultraviolet energy is released by spontaneous bond cleavage with the formation of free radicals (45). Free radicals are often unstable and react immediately with the solvent system or other reactants. The amount of light energy which penetrates soil is very low; therefore, photodegradation of most soil-active herbicides is insignificant. Alternatively,

photodegradation is very significant for picloram [4-amino-3,5,6-trichloro-2-pyridine carboxylic acid], and napropamide [N,N-diethyl-2-(1-naphthalenyloxy)propanamide] if they are not leached into soil by precipitation (26). Pesticide photodegradation has been reviewed by Plimmer (45).

Chemical hydrolysis represents a major abiotic mechanism of degradation. Hydrolytic reactions involving pesticides are often facilitated by binding to soil reactive sites which favor catalysis (5). Armstrong and Chesters (4) examined the effect of model sorbents and soil on atrazine [6-chloro-N-ethyl-N'-(1-methylethyl)1,3,5-triazine-2,4-diamine] hydrolysis. Cellulose acetate, montmorillonite clay, carboxylic resin, and a phenolic resin had high adsorption capacities for atrazine however only the carboxylic resin was more effective in catalyzing atrazine hydrolysis than soil. The authors proposed that the triazine molecule is weakly bound to carboxylic acid functional groups of soil organic matter. The adsorbed chloro-s-triazine is then susceptible to hydrolysis by nucleophilic substitution of -Cl by -OH forming hydroxy-s-triazine (4).

Studies designed to elucidate the degradative pathway of a herbicide in soil often fail to adequately separate biotic (microbial) and abiotic (photolysis/hydrolysis) factors. Separation of these factors is difficult because of the interaction of microbial populations and soil

chemistry. Microbial activity in soil is dependent upon soil pH, organic matter content, temperature, moisture content, and texture.

Soil microorganisms have the ability to partially degrade and/or mineralize an impressive array of natural and man-made organic compounds including herbicides. While microorganisms occupy approximately 0.1% of the soil volume, bacterial cell density can reach levels as high as 10^9 cells per g of soil (55).

Soil microorganisms may directly metabolize a herbicide, or indirectly affect degradation by inducing changes in the chemical environment (55). Direct catabolism occurs when a molecule is utilized as both a carbon and an energy source. This is typically a growth-linked process and often results in complete reduction of the molecule to carbon dioxide and water (1).

Early research on microbial degradation of synthetic organic molecules focused on catabolic metabolism. Considerable research was conducted with microorganisms growing on minimal media containing the chemical of interest as the sole source of energy for the organism. Lanzelotta and Pramer (36) used an enriched soil to select a strain of Fusarium solani capable of growth in a minimal salts medium containing the herbicide propanil [3',4'-dichloro-propionanilide] as the sole carbon source. The selected

fungus degraded propanil more rapidly when additional nutrients were added to the culture. The major metabolite, 3,4-dichloroaniline, eventually accumulated to levels which inhibited additional catabolism.

Hartman et al. (25) used a chemostat and stepwise selection to isolate a Pseudomonas spp. capable of utilizing 3,5-dichlorobenzoate as a sole carbon source. A chemostat system permits a culture to remain in the logarithmic phase of growth indefinitely. Fresh media is metered into the culture vessel as used media containing dead cells is removed. The growth rate of the bacterial culture increased significantly during the stepwise selection process as the sole carbon source was changed from 3-chloro- to 4-chloro-, and finally to 3,5-dichlorobenzoate. Unfortunately, the selected bacteria was not tested for its ability to degrade the compound in soil or water.

Daughton and Hsieh (13) used a chemostat to select microorganisms capable of using the insecticide parathion [O,O-diethyl O-P-nitrophenyl phosphorothionate] as a sole growth substrate. They selected two bacterial species which symbiotically metabolized parathion. P. stutzeri cometabolically hydrolyzed parathion to diethyl-thiophosphate and p-nitrophenol. P. aeruginosa utilized p-nitrophenol as a sole energy source. This culture was tested for its ability to degrade parathion in soil. Barles et al. (8) found that the culture obtained by Daughton and

Hsieh was capable of complete degradation of parathion with initial concentrations as high as 5000 ppmw in soil. The degradation rate of the commercial emulsifiable concentrate formulation was much slower than for technical grade parathion, or for rates of technical parathion above 5000 ppmw.

Rache and Lichtenstein (46) found that soil microorganisms could degrade unextractable, bound residues of ^{14}C parathion in soil. The addition of glucose to soil reduced the amount of nonextractable ^{14}C residues and increased $^{14}\text{CO}_2$ evolution. While the concept of cometabolism and pesticide degradation is receiving increased attention, systems utilizing cometabolism are exceptionally difficult to study (34).

The occurrence of microorganisms capable of normal growth on a single substrate such as a pesticide molecule is rare. Cometabolic degradation, in which an organism derives its energy from one substrate and incidentally metabolizes a pesticide molecule, is far more common both in pure cultures and in naturally occurring microbial populations (34). Wang et al. (59) examined the microbial degradation of monuron [$\text{N}'-(4\text{-chlorophenyl})\text{-N,N-dimethylurea}$], linuron [$\text{N}'-(3,4\text{-dichlorophenyl})\text{-N-methoxy-N-methylurea}$], and diuron [$\text{N}'-(3,4\text{-dichlorophenyl})\text{-N,N-dimethylurea}$] at several concentrations in several environments. All three compounds

were mineralized (converted to CO₂ and H₂O) when added to sewage at 10 ug/L. However, when the concentration of monuron was increased to 10 mg/L the compound was cometabolically altered, not mineralized.

Lappin et al. (37) washed soil adhering to wheat (Triticum aestivum L.) roots into a minimal salts media containing the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid] as a sole carbon source. They isolated a microbial community containing five species capable of growth on mecoprop. While none of the organisms could grow alone, combinations of two or more species were able to degrade the compound. Maximum culture growth and mecoprop degradation occurred when all five species were present.

Clomazone

Clomazone [2-(2-chlorophenyl)methyl-4,4-dimethyl-3-isoxazolidnone] (Figure 1) is a broad spectrum herbicide discovered in 1979. It is being developed by the FMC Corporation under the trade name Command^R. Clomazone is currently labeled for use as a preemergence or preplant incorporated herbicide in soybeans (Glycine max L. Merr.) and as a fallow herbicide. The herbicide controls many problem weeds of soybeans including barnyardgrass (Echinochloa crus-galli L.), fall panicum (Panicum dichotomiflorum Michx.), foxtails (Setaria spp.), velvetleaf

(Abutilon theophrasti Medico.), common lambsquarters (Chenopodium album L.), and black nightshade (Solanum nigrum L.).

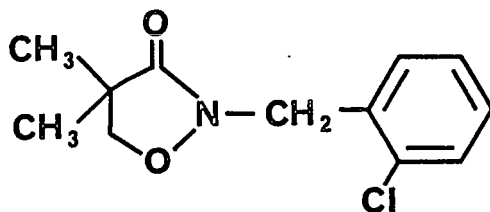


Figure 1. Structure of clomazone.

Bellman et al. (10) found that clomazone provided better residual control of giant foxtail (Setaria fabre Herrm.) than several other grass herbicides commonly used in soybean production including trifluralin [2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine], metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide], and alachlor [2-chloro-N-(2,6-diethylphenyl)N(methoxymethyl) acetimide]. Velvetleaf was most susceptible to clomazone followed in order by giant foxtail, common lambsquarters, and redroot pigweed (Amaranthus retroflexus L.) (10). Clomazone alone or in combination with metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one] controlled

velvetleaf and common lambsquarters for a longer period of time than metribuzin alone or metribuzin in combination with the grass herbicides mentioned above.

Clomazone has been tested for use in chemical fallow in Montana for several years. Results from field trials established in 1984 through 1986 indicated good to excellent control of several common weed species associated with fallow (18) (Table 1).

Soil factors influence the activity of clomazone. In leaching studies, the herbicide demonstrated low mobility in sandy loam, silt loam, and clay loam soils, and intermediate mobility in fine sand (2). In field studies, the half-life was 15 to 45 days depending upon soil type (2).

Tymonko and Guscar (39) applied clomazone to 21 soil types and examined its effect on the growth of ten plant species in the greenhouse. High levels of soil organic matter reduced activity. Activity was also reduced in soils with high clay content or high cation exchange capacity. Activity was highest in sandy soils. There was no correlation between soil pH and herbicide activity.

Loux and Slife (41) compared the activity of clomazone in sandy clay loam (2.7% organic matter) and sandy loam (1.3% organic matter) soils using sorghum (Sorghum bicolor L.) as a bioassay indicator species. Initial sorghum injury was greater in low organic matter soil. Herbicide incorporation did not reduce activity.

Table 1. Weed control results obtained with clomazone in chemical fallow field trials conducted at 3 locations in Montana from 1984 through 1986 (18)..

Clomazone Rate	No. of Trials	Bare Ground	Vol. Wheat	Downy- Brome ²	Russian thistle ³
(kg ai/ha)		-%-		Percent Control ¹	
0.6	4	85	70	63	73
0.8	3	89	98	65	88
1.1	2	94	100	100	88
1.4	1	90	90	100	85

¹Control was rated visually with 0 = no control and 100 = complete control.

²Bromus tectorum L.

³Salsola iberica L.

They found that mobility in soil columns varied with soil texture and organic matter content. Significantly greater movement occurred in a soil with 1.3% organic matter compared to a soil with 5.7% organic matter. These findings are supported by the results of other studies which used soil thin-layer chromatography and soil leaching columns to examine clomazone mobility (20).

Keifer and ElNagger (33) also examined the role of soil organic matter on the activity of clomazone. The herbicide was applied to seven field soils and three clays and allowed to dry overnight. The herbicide was extracted from soil using 5mM CaSO₄ to determine the partition coefficient between soil and water. Soil/water partition coefficients ranged from 8 for a kaolin clay to 60 for a muck soil with 76% organic matter indicating that the herbicide is

partitioned into organic matter. The average of soil organic matter/water partition coefficients was 960 over a range of field soils which indicates that on a weight basis clomazone has more affinity for organic matter than clay.

Aerobic degradation of ^{14}C -methyl-labeled clomazone led to the evolution of $^{14}\text{CO}_2$ and unidentified soil-bound residues (20). Under anaerobic soil conditions the herbicide was rapidly degraded to an experimental compound, FMC 65317 (N-[(2-chlorophenyl)methyl]-3-hydroxy-2,2-dimethylpropanamide) (20).

Enhanced Thiocarbamate Degradation

Enhanced herbicide degradation is the phenomenon where accelerated herbicide decomposition in soil is induced by prior treatment of that soil with the herbicide (51). Enhanced degradation was first reported by Audus (6) in 1949. Soil pretreated with 2,4-D [2,4-dichloro-phenoxy acetic acid] showed an enhanced degradation rate of subsequent 2,4-D applications. Enhanced degradation in soil has also been shown for several other herbicides that have foliar but not soil activity (35).

Enhanced herbicide degradation was first reported for the soil applied herbicide EPTC [s-ethyl dipropyl-carbamothioate], in 1979 (49). EPTC is a preemergence herbicide introduced in 1959 to control broadleaf and grass weeds in corn (Zea mays L.), potatoes (Solanum tuberosum

L.), alfalfa (Medicago sativa L.), and many vegetable crops. Rhaman et al. (49) found that EPTC applied to plots which had previously received three annual EPTC applications failed to control bristly foxtail (Setaria verticillata L. Beauv.). The herbicide provided excellent control in adjacent plots which had no prior EPTC history. The authors proposed that the herbicide failure was caused by rapid degradation from enhanced microbial activity, a theory which has been supported by many studies in the United States (23,38,41,43,54,60).

Obrigawitch et al. (43) measured accelerated $^{14}\text{CO}_2$ evolution when ^{14}C -carbonyl-labeled EPTC was applied to a soil with a history of EPTC use. In other studies a single EPTC application was sufficient to enhance the degradation of subsequent applications in some soils (42,43). The half-life of EPTC was reduced from 13 to 3 days in a soil in which EPTC had been applied for eight consecutive years (42).

Applications of EPTC may induce enhanced degradation of other thiocarbamate herbicides, a phenomenon termed cross-enhancement (54). While EPTC degradation was greatly accelerated in a soil which received 9 annual EPTC applications, the rate of degradation of butylate [s-ethyl bis(2-methylpropyl)carbamothioate], a thiocarbamate analog of EPTC, was accelerated far less than EPTC (42).

Obrigawitch et al. (42) examined butylate degradation in a soil with zero to six prior applications of EPTC. The rate of butylate degradation increased as the number of EPTC applications increased however, the the degree of enhancement was less than for EPTC.

Cross-enhancement has been reported for EPTC, butylate, and vernolate [s-propyl dipropylcarbamothioate]. In all cases the compounds show greater enhancement when self-enhanced, and the degree of cross-enhancement appears to vary with herbicide structure (52,53,57,60). One thiocarbamate herbicide, cycloate [s-ethyl cyclohexyl-ethylcarbamothioate], is not susceptible to enhanced degradation (24). Enhanced degradation may be prevented by steric enzyme interference caused by the cyclohexane group of cycloate (60) (Figure 2).

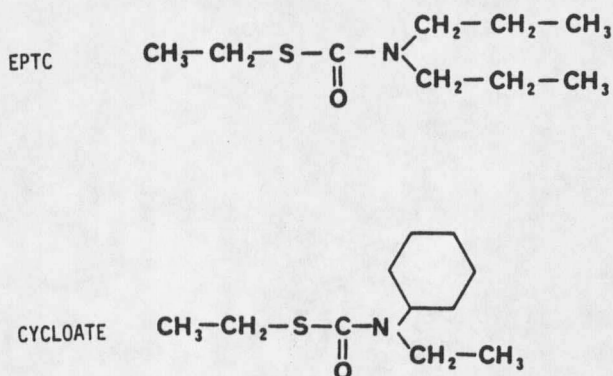


Figure 2. Structural comparison of EPTC and cycloate.

Microorganisms cause enhanced EPTC degradation and resultant weed control failures. Microbial activity is affected by soil moisture, temperature, and carbon source availability. Obrigawitch (43) found that EPTC degradation in an enhanced soil was no longer enhanced if soil moisture content was maintained below 3%. The rate of EPTC degradation in an enhanced soil was reduced when incubated at 5 C however, the degradation rate increased when the soil was incubated at 15 or 25 C due to temperature effects on soil microorganism growth. Autoclaving, propylene oxide, sodium azide, and sodium cyanide have been used as soil sterilents to substantiate the fact that microorganisms cause enhanced degradation (23,54).

The sterilization techniques mentioned above are not practical options to overcome enhanced degradation in field situations. There are, however, compounds termed extenders which specifically inhibit the enzymes responsible for thiocarbamate degradation when applied to soil. Kaufman et al. (32) found that several soil-applied methyl carbamate insecticides would increase the half-life of a phenyl carbamate herbicide in soil. The carbamate insecticides have been shown to competitively inhibit the phenylcarbamate-hydrolyzing enzyme of several soil microorganisms (32).

Additional compounds have been tested for use as extenders with considerable success. Dietholate [O,O-diethyl O-phenylphosphorothioate] extended the half-life of EPTC in an enhanced soil from 9 to 18 days (44). The extender was only effective in soils which were considered to be enhanced (44,48). Dietholate also extended the persistence of butylate and vernolate whose degradation had been enhanced by a prior application of EPTC (42).

Many species of microorganisms are responsible for EPTC degradation. Lee (38) sampled soil from plots which had a 4 year history of EPTC use. He used an enrichment technique where 10 g of soil was added to a glucose-salt medium containing 200 ppm EPTC. After several transfers to fresh medium the solution was plated and the microbial isolates were tested for their ability to degrade the herbicide. Twenty-nine fungal and nine bacterial isolates degraded EPTC however most isolates lost the ability after extensive subculturing on nutrient agar. Lee proposed that the loss of degradative ability may have been due to the loss of plasmid-encoded degradative genes. Other attempts to isolate organisms capable of utilizing EPTC as a sole carbon-source have failed (41).

A thorough knowledge of the enzyme(s) responsible for EPTC metabolism is important in order to understand the phenomenon of enhanced EPTC degradation. Partially purified preparations of EPTC-degrading enzymes have been reported

however, the specific enzyme(s) responsible for degradation has not been identified (54). Moorman (41) examined a population of EPTC-degrading microorganisms in an enhanced field before and after an application of EPTC. The number of EPTC-degrading microorganisms did not increase after herbicide application suggesting that enhanced degradation is caused by a physiological adaptation process rather than an increase in the number of degrading microorganisms.

Enhanced Degradation of Other Herbicides

In 1949 Audus (6) found that subsequent applications of 2,4-D to soil were degraded more rapidly than the first application. Since then, many herbicides have been tested for enhanced degradation however the phenomenon appears to be restricted to the phenoxy and thiocarbamate herbicides (51).

Torstensson et al. (56) examined MCPA [(4-chloro-2-methylphenoxy)acetic acid] and 2,4-D degradation in soils which had received MCPA or 2,4-D annually for 1 or 19 years. Two,4-D and MCPA applied to soil with 1 year of pretreatment were degraded in 10 and 20 weeks respectively. The same compounds were degraded in 4 and 7 weeks, respectively, in soil which received 19 annual applications. These results are consistent with others who found that 2,4-D and MCPA degradation in soil could be enhanced by pretreatment (6,21). Torstensson et al. (38) isolated microorganisms which could utilize 2,4-D or MCPA as a sole carbon source.

It appears that cross-enhancement also occurs with MCPA and 2,4-D. MCPA and 2,4-D degradation was increased regardless of which herbicide was used as the pretreatment (55,60). Kirkland et al. (35) examined the degradation of MCPB [4-(4-chloro-2-methylphenoxy)butanoic acid], dichloroprop [(±)-2-(-,4-dichlorophenoxy)propanoic acid], mecoprop, fenoxaprop [(±)-2-[4-[(6-chloro-2-benzoxazolyl)-oxy]phenoxy]propanoic acid], and dicamba [3,6-dichloro-2-methoxybenzoic acid] in soil which had 9 annual MCPA applications. MCPA and MCPB degradation was enhanced in this soil, however, the degradation rates of the other herbicides were unchanged. Fryer et al. (21) examined the degradation rate of MCPA, triallate [s-(2,3,3-trichloro-2-propenyl)bis(1-methylethyl)carbamothioate], simazine [6-chloro-N-N'-diethyl-1,3,5-triazine-2,4-diamine], and linuron after 5 annual applications of each herbicide. Only MCPA showed enhanced degradation. MCPA residues were below the limit of detection by 3 weeks in a soil with 3 previous MCPA applications. In a soil with 10 prior MCPA applications it took only 4 days to reach the same level. Accelerated MCPA degradation was shown to be stable for 5 years after the final application (22).

The effect of annual herbicide applications on the degradation of other soil applied herbicides has been examined. Horowitz et al. (29) examined the efficacy and persistence of bromacil [5-bromo-6-methyl-3-(1-

methylpropyl)-2,4(1H,3H)-pyrimidinedione], chlorthal-dimethyl, diphenamid [N,N-dimethyl-a-phenylbenzene acetamide], diuron, fluometuron [N,N-dimethyl-N'-[3(trifluoromethyl)phenyl]urea], neburon, prometryn [N,N'-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine], pyranazon [5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone], simazine, and trifluralin. Each herbicide was applied at a labeled application rate in spring and in autumn for 4 consecutive years. The 10 herbicides either accumulated in soil or remained at a similar level throughout the experiment. None of the herbicides exhibited enhanced degradation.

Enhanced Degradation of Insecticides and Fungicides

Enhanced degradation of several insecticides and fungicides has been reported. Carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate] is a soil applied carbamate insecticide used to control corn rootworm larvae (19). Early failures of this insecticide were discounted as insect resistance, a common problem with many insecticides. Although low level carbofuran resistance had been reported, Felsot et al. (19) felt that it did not adequately explain the reduced corn rootworm control. They showed that carbofuran degradation was enhanced in soils with a history of carbofuran use and they isolated a bacteria (Pseudomonas sp.) which could rapidly degrade carbofuran in nutrient broth. Read (50) reported that carbofuran failed to control

root maggots on rutabagas (Brassica napus L.) in 2 fields which had received carbofuran 1 or 2 years earlier. Read found that 60 ppmw carbofuran was degraded to 5 ppmw in 120 days after the first application, while in the second year, the same rate was degraded to less than 0.2 ppmw in 60 days.

Field treatments of carbofuran after fensulfothion [O,O-diethyl O-[4-(methylsulfonyl)phenyl]-phosphorothioate] or fensulfothion after carbofuran indicate that cross-enhancement occurs between these two insecticides. However, as shown with the thiocarbamate herbicides, the degree of enhancement is less than that observed for self enhancement.

Enhanced degradation has also been reported for isophenphos [1-methylethyl 2-[[ethoxy[(1-methylethyl)-amino]phosphinothioyl]oxy]benzoate], an organophosphorus insecticide used in corn to control soil-dwelling insects. Racke and Coats (47) used uniformly ring labeled ^{14}C -isophenphos to measure degradation in soils with and without a prior history of isophenphos use. In 4 weeks an application of 5 ppmw isophenphos had dissipated to 0.7 to 2 ppmw in an isophenphos history soil while in a comparable soil without prior isophenphos use, 3.2 to 3.8 ppmw were recovered.

Metalaxyl [N-[2,6-dimethylphenyl]-N-[methoxyacetyl]-alanine methylester], a systemic fungicide, in subject to

enhanced degradation due to prior metalaxyl treatments. Bailey and Coffey (7) examined metalaxyl degradation in soils with and without prior metalaxyl use. A bioassay performed 70 days after treatment indicated nearly 100% of the metalaxyl originally applied remained in soils without prior metalaxyl exposure. In three of five soils which had received prior metalaxyl applications, no fungicide was detected. The metalaxyl applications apparently did not predispose the soil microorganisms to enhance the degradation of other related members of the acylanilide family, including two fungicides and the herbicide metolachlor.

CHAPTER 2

DEGRADATION OF CLOMAZONE IN TWO MONTANA SOILS

Introduction

Approximately 2 million ha of cropland in Montana is under a wheat-fallow-wheat rotation in Montana (40). Chemical fallow is an attractive alternative to tillage because it promotes soil and moisture conservation, and reduces production costs associated with dryland wheat production.

Ideally, a herbicide to be used in fallow land would provide long term control of a broad spectrum of weeds with no carryover into subsequent crops. Results from field tests with clomazone in Montana indicate good to excellent residual control of downy brome and volunteer wheat which are common weed problems on fallow land. The residual period and half-life of clomazone under field conditions in the Northern Great Plains is presently unknown.

Materials and Methods

A study was initiated in the spring of 1986 to examine clomazone dissipation in 2 Montana soils (Table 2). Clomazone was applied to fallowed ground at 0, 0.6, 1.1, and

2.2 kg ai/ha. Application was made using a CO₂-pressurized backpack sprayer at 276 kPa pressure, with 289 and 125 L of water/ha, on April 4, and March 26, 1986 at Willow Creek and Bozeman, respectively. Each treatment was replicated 3 times and arranged in a randomized complete block design. Soil samples were taken to a depth of 8 cm with a 6 cm diam by 10 cm deep bulb planter from 5 random locations per plot. Soil samples were taken immediately after application and at monthly intervals for 6 months. Samples were bulked and placed in a freezer at -5 C within 8 h.

Table 2. Physical and chemical characteristics of soils used in clomazone dissipation experiments.

Soil series and texture	Soil pH	Organic matter	Sand	Silt	Clay	CEC
			percent			meq/100g
Bozeman silty clay loam	8.1	2.3	14	57	29	18.1
Willow Creek loam	8.0	1.8	43	34	23	20.1

A Field Bioassay for Clomazone

A field bioassay was conducted in the above plots to determine if spring applications of clomazone would injure winter wheat planted the following fall. 'Winridge' and 'Redwin' winter wheat was planted on September 12 and 13, 1986 in rows spaced at 15 and 30 cm apart, at seeding rates of 87 and 60 kg/ha in Bozeman and Willow Creek respectively.

Wheat in the 2 leaf stage was harvested 1 month after planting at each location to measure crop injury. Plants in 30 cm of a row were clipped at the soil surface, oven dried at 60 C for 4 days and weighed. Samples were harvested from 5 random locations in each plot.

Evaluation of a Bioassay Indicator Species

Corn, oat (Avena sativa L. 'Otana'), radish (Raphanus sativus L.), sunflower (Helianthus annuus L.), and wheat were tested to find a suitable indicator species for a clomazone bioassay.

Standards were prepared using untreated soil from the Bozeman site. Soil was removed to a depth of 8 cm, air dried, and passed through a 10-mm sieve. A stock herbicide solution was prepared by mixing 2.3 ml of 4 EC commercial clomazone with water to a final volume of 1 L which produced a final concentration of 1.1 g clomazone/L. Thirteen kg of Bozeman soil was spread on a clean plastic sheet and stock herbicide solution was applied at 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1.2, 1.6, and 2.0 ppmw clomazone. Each treated soil was placed in a 5 L plastic container, sealed, and placed on a rolling mixer for 1.5 h. Two hundred g of treated soil was placed in 6.5 cm diam by 9 cm deep waxed paper cups and planted. Four oat, 4 wheat, or 2 sunflower seeds were planted in individual cups by placing the embryo end of the seed into the soil and pushing the seed flush with the soil surface. Two corn seeds were planted 1.5 cm

deep in each cup, embryo end down. Four radish seeds were planted 0.5 cm deep in each cup.

Cups received 15 ml water twice daily with an automatic pipet. The greenhouse temperature was maintained at 21 ± 4 C. Plants were grown under natural light supplemented with metalarc halide lamps which supplied $140 \text{ uEm.}^{-2}\text{s.}^{-1}$ artificial lighting for a 12 h photoperiod. The experiment was arranged in a completely randomized design with 6 replications per treatment. Each species was harvested 14, 21, 28, and 36 days after seeding to determine the optimum growth response to clomazone. At harvest, plants were cut at the soil surface and oven dried at 60 C for 4 days prior to weighing. A standard curve was constructed by regression analysis of the herbicide concentration against plant dry weight.

In addition to dry weight, the chlorotic symptoms observed in oat plants were quantified and evaluated as an indicator of herbicide rate. Oat symptoms included chlorotic tissue which started at the base of the plant and progressed acropetally in response to rate. The distinct interface between chlorotic and green tissue, termed the chlorotic margin, was easily measured. The height from the soil surface to the chlorotic margin divided by the total plant height was used to measure herbicide activity and is referred to as the percent chlorosis by height.

Bioassay of soil samples

Standard curves using untreated soil from the Willow Creek and Bozeman sites were prepared as described above. Standard herbicide solutions were applied to soil in 20 ml of water at 10 rates from 0 to 2.0 ppmw clomazone, prepared as described above. Treated soil was placed in a 2 L plastic container, sealed, and placed on a rolling mixer for 1.5 h. Following mixing, 100 g treated soil was placed into 6.5 cm diam by 4 cm deep styrofoam cups.

Field samples taken at monthly intervals were thawed overnight, thoroughly mixed, and 100 g of soil was placed into styrofoam cups. Field samples were weighed at ambient field moisture levels to avoid potential volatility losses of clomazone during air drying.

Cups containing field soil to be analyzed for clomazone and clomazone treated standards were placed in the greenhouse, planted with 4 oat seeds, and grown under the greenhouse conditions and watering regime described above. Each treatment was replicated 6 times and arranged in a completely randomized design. The experiment was repeated once. To overcome variability in greenhouse conditions, cups were randomized at 5 and 3 day intervals in the first and second experiments, respectively.

Sixteen days after planting, the percent chlorosis by height was measured for 2 randomly selected plants per cup. Eighteen days after planting, oat plants were clipped at the

soil surface and oven dried as described above. Standard curves were constructed by regression analysis of herbicide rate against the percent chlorosis by height, and against plant dry weight.

Leaching of Clomazone

An experiment was conducted to determine if clomazone leached in soil. Seven months after application, soil samples were removed from the field plots described above treated with 0 or 2.2 kg/ha clomazone at Willow Creek. Soil cores 6.5 cm diam by 24 cm deep were removed with a Giddons soil probe from 20 random locations per plot. Each core was divided into 0-8, 8-16, and 16-24 cm soil depths which were bulked and stored at -5 C within 8 h of sampling. Samples were bioassayed using the percent chlorosis by height method described above. Appropriate standards were constructed as before using untreated soil collected from each of the 3 soil depths. The soil characteristics at each depth are shown in Table 3.

Results and Discussion

Field Bioassay

The dry weight of wheat planted in plots that were previously treated with 1.1 and 2.2 kg/ha of clomazone was significantly reduced at Bozeman (Table 4). At Willow Creek wheat dry weight was reduced only at the highest rate tested, 2.2 kg/ha which is 2 to 4 times the proposed use rate for chemical fallow (3).

Table 3. Physical and chemical characteristics of soil used in clomazone leaching experiment.

Soil series and texture	Depth	Soil pH	Organic matter	Sand	Silt	Clay	CEC
	(cm)			percent			meq/100g
Willow Creek loam	0-3	8.0	1.8	43	34	23	20.1
	3-6	8.0	1.5	35	36	29	20.5
	6-9	8.3	1.3	35	30	34	19.8

Table 4. Dry weight of winter wheat plants in the two leaf stage 205 days after application of clomazone at Bozeman and Willow Creek.

Clomazone	Wheat Dry Weight ¹	
Rate	Bozeman	Willow Creek
(kg/ha)		mg/plant
0.0	44a	49a
0.6	37ab	47a
1.1	28bc	45a
2.2	22c	41b

¹Numbers in columns followed by the same letter are not significantly different at the 5% level using the LSD test.

Evaluation of Plant Species for the Bioassay

A suitable indicator species for a bioassay should respond to reasonable amounts of a herbicide with clear, easily measured symptoms (28). The growth of sunflower,

radish, and corn responded erratically to the rates of herbicide applied (Table 5).

Table 5. Plant dry weight as percent of control for 5 species compared as bioassay indicators for clomazone residues in soil¹.

Clomazone Rate	Oat	Wheat	Percent of Control ²		Sunflower
			Corn	Radish	
(ppmw)					
0.1	86a	70a	112a	74bcd	92a
0.2	75b	52bc	103a	86abc	79ab
0.3	66c	55b	69cd	90ab	80ab
0.4	63c	44cd	75c	63cd	77ab
0.5	55d	44cd	83bc	73bcd	80ab
0.8	48e	33e	45e	56d	61bc
1.2	39f	37de	53de	29e	57bc
1.6	34f	34e	37e	32e	41c
2.0	35f	31e	38e	22e	54bc

¹Oat and wheat were grown for 14 days, corn and radish for 28 days, and sunflower for 21 days.

²Numbers in columns followed by the same letter are not significantly different at the 5% level using the LSD test.

Oat dry weight was curvilinear in response to herbicide rate with a steep slope from 0.1 to 0.6 ppmw which provides accurate detection within this range (Figure 3).

Oat percent chlorosis by height provided the most acceptable standard curve with a curvilinear response and a steep slope in the range of 0.1 to 1.2 ppmw which provided accurate detection of clomazone over a range twice that provided by a dry weight standard curve (Figure 4).

Dry Weight (percent of control)

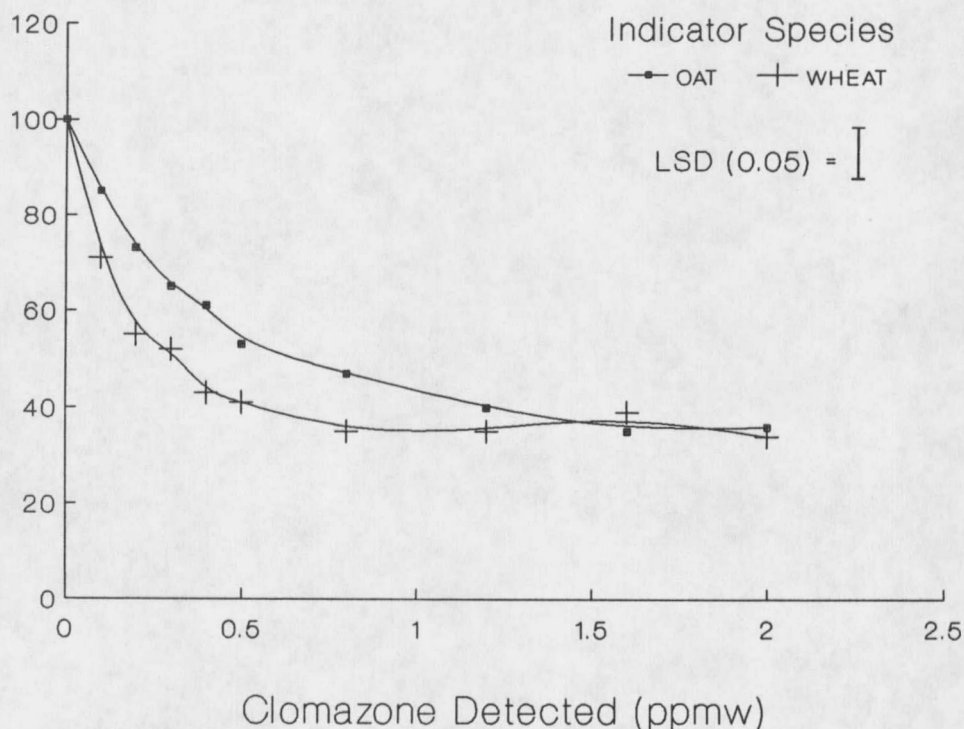


Figure 3. Standard curves for oat and wheat biomass production for plants grown 14 days in soil containing 10 rates of clomazone.

The fit of the regression line as determined by the F test was significant at the 5% level with an r^2 of 0.99. The percent chlorosis method takes only 16 days, and does not require harvesting, drying, and weighing plant material.

Greenhouse Bioassay Results

There were no injury symptoms on oat plants grown in soil 3.5 and 6 months after application of 0.6 and 1.1 kg/ha clomazone at Bozeman.

Percent Chlorosis by Height

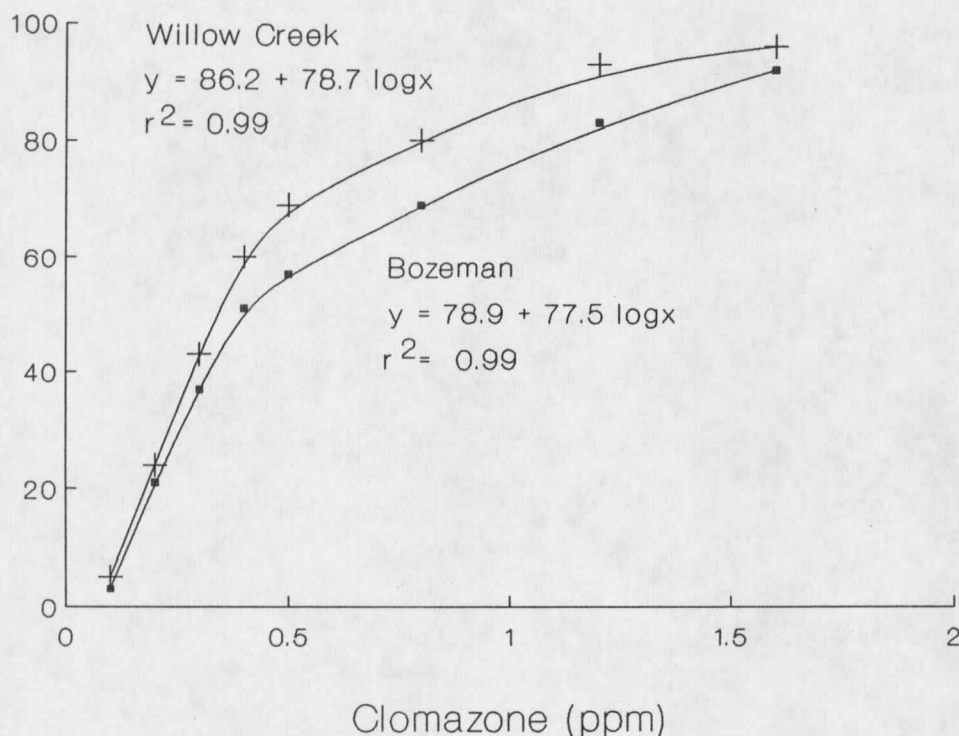


Figure 4. Standard curves for oat percent chlorosis by height using soil from Bozeman and Willow Creek with 10 rates of clomazone.

By comparison to the standard curve (Figure 4), approximately 0.2 ppmw clomazone was detected 6 months after application of 2.2 kg/ha at Bozeman (Figure 5). Lower levels of clomazone were recovered at Willow Creek (Figure 6). No herbicide was detected 1 month after application in soil from plots treated with 0.6 kg/ha at Willow Creek.

Clomazone Detected (ppmw)

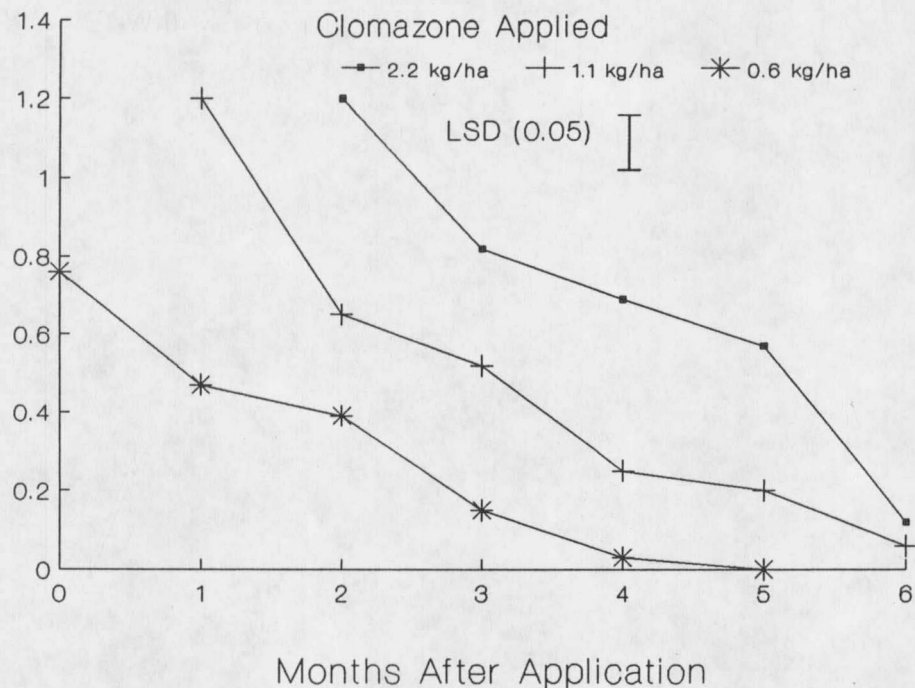


Figure 5. Clomazone detected in soil sampled monthly for 6 months after application of 0.6, 1.1, and 2.2 kg/ha clomazone at Bozeman.

The two highest rates tested, 1.1 and 2.2 kg/ha, dissipated to levels below the detection limit of the bioassay 3 months after application, a level similar to that detected 6 months after application at Bozeman.

The levels of clomazone detected over time were used to examine the kinetics of clomazone degradation.

First order reaction plots were prepared for each herbicide rate at each location according to the equation:

$$\ln \frac{[A]_x}{[A]_0} = -kt$$

where $[A]_0$ and $[A]_x$ were the concentrations of herbicide determined from the bioassay at initial time = 0 and sampling time = x , k = first order rate constant and t = time. A plot of $-\ln[A]_x/[A]_0$ vs time has a slope = k .

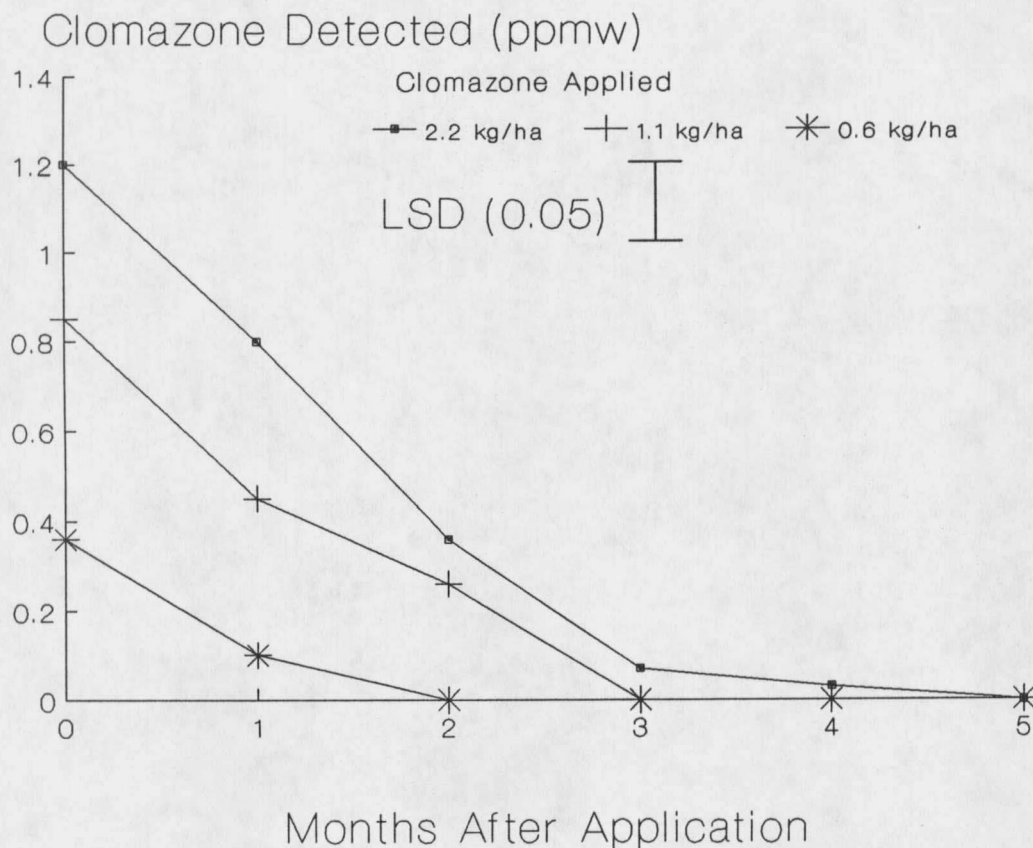


Figure 6. Clomazone detected in soil sampled monthly for 5 months after application of 0.6, 1.1, and 2.2 kg/ha clomazone at Willow Creek.

The slope is used to calculate the half-life based on the equation $t_{1/2} = 0.693/k$ (31). Regression analysis indicates a significant fit of the values for herbicide dissipation to first order kinetics where the r^2 values are ≥ 0.89 for all treatments at Bozeman and Willow Creek (Figure 7).

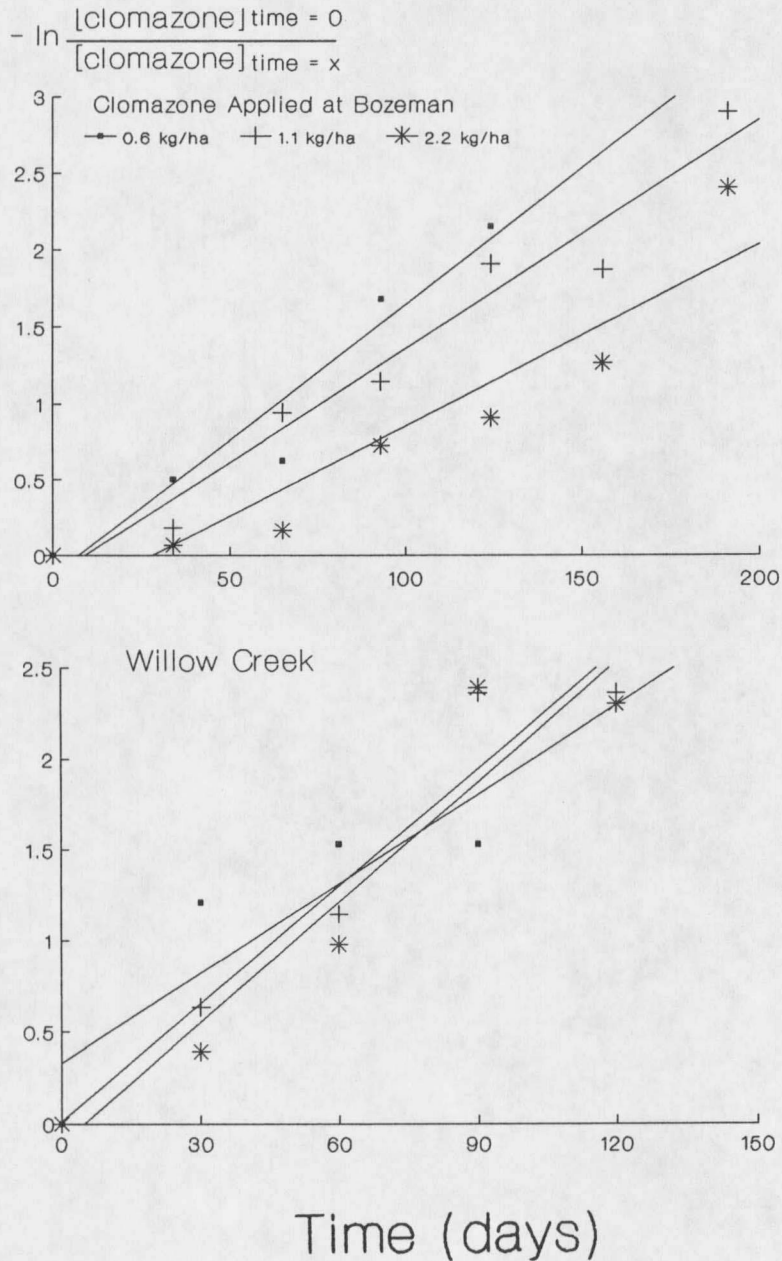


Figure 7. First-order rate plots for clomazone applied at 0.6, 1.1, and 2.2 kg/ha at Bozeman and Willow Creek.

The half-lives calculated from the rate constants averaged 32.9 days at Willow Creek and 37.4 days at Bozeman, values consistent with the 15 to 45 day range previously

reported for this herbicide (2). The rapid rate of dissipation at Willow Creek may be a result of lower organic matter and higher sand content than the soil at Bozeman since binding of clomazone to soil is positively correlated to organic matter content (33,39,58). Reduced binding would permit greater volatility, and increased availability for degradation.

Leaching of Clomazone

There were no differences in dry weight production or chlorotic symptoms produced on oats planted in soil from any depth indicating little or no leaching occurred (data not shown). These results are consistent with the work of others who found that the mobility of clomazone in soil is low (20).

The chlorotic tissue symptoms observed in oat from clomazone permitted development of an accurate and facile bioassay technique using a measurement of percent chlorosis by height. The nondestructive percent chlorosis by height method accurately measured levels of clomazone from 0.1 to 1.2 ppmw and took only 16 days.

Clomazone dissipated more rapidly in a sandy, low organic matter content soil. Clomazone applied at 2.2 kg/ha dissipated to levels below 0.1 ppmw in 3 months in the Willow Creek loam soil. Clomazone at 2.2 kg/ha, applied to the finer textured Bozeman silty clay loam dissipated to a level of 0.2 ppmw 6 months after application. It appears

that the residual period of active clomazone residues in 2 Montana soils will not interfere with the planting of wheat in a wheat-fallow-wheat cropping system.

CHAPTER 3

THE INCIDENCE AND MAGNITUDE OF ENHANCED EPTC DEGRADATION IN 166 SOILS

Introduction

EPTC degradation has been enhanced in many soils since the first commercial utilization of the compound in the 1950^s however the phenomenon was not reported until 1979 (49). Since the initial report many authors have reported enhanced EPTC degradation in some soils with a history of thiocarbamate use (42,43,48,49,51,54). The published research has been conducted at relatively few locations, so the geographic extent and rate of occurrence of the phenomenon is unknown.

Enhancement of EPTC degradation appears to be the result of adaptation of the constitutive microbial population due to prior EPTC exposure. The adapted microbes are able to degrade the compound much more rapidly than microbes that have not been exposed to the herbicide (41). Adaptation is not limited to EPTC since cross-enhancement, in which one compound induces enhanced degradation of another compound, has also been reported for several

thiocarbamate analogs (52,53,57,60).

This research was designed 1) to determine the rate of occurrence and geographic extent of enhanced EPTC degradation and 2) to examine the degree of enhancement in different soils.

Materials and Methods

Soil Sampling

Three hundred twenty weed scientists with an interest in herbicides and soil, or herbicide degradation, were selected from the 1985 membership directory of the Weed Science Society of America. Letters were sent to the selected weed scientists in July, 1987, requesting a soil sample from a location where pesticides had been used. The recipients were asked to fill an enclosed 14 by 47 cm tyvek sample bag with soil and return the sample by mail. Each sample included an information card requesting site history and exact location information which was filled out by the sample collector. One hundred sixty six samples weighing an average of 1.5 kg each were received by October 1, 1987 (Figure 8). Each soil sample was passed through a 10-mm sieve sterilized with 70% ethanol to avoid sample cross-contamination. Sieved soil was placed into a polyethylene bag and stored at 15 C.



Figure 8. Test soil locations for soils used in enhanced EPTC degradation studies.

Soil Inoculation Screen

A soil inoculation technique was developed to identify soils with the ability to degrade EPTC rapidly.

A Bozeman silty-clay-loam, (fine-silty, mixed, frigid, agric Pachic cryoboroll), (pH 8.1, 2.3% organic matter, 14% sand, 57% silt, and 29% clay) was chosen to serve as a "base soil". The base soil was inoculated with a small amount of each test soil after being treated uniformly with 10 ppmw EPTC.

Following inoculation the sample was mixed, incubated in the greenhouse, and bioassayed to measure EPTC residues remaining in the soil. The EPTC degradation rate in the base soil was considered to be a standard or normal rate. Inoculation with a test soil served to introduce microorganisms to the base soil which may have the ability to enhance EPTC degradation and should not have significantly altered soil properties.

A 1 L EPTC stock solution was prepared by adding 9.5 ml of commercially formulated herbicide (7EC, Stauffer Chemical Co., Westport, CT) to 990.5 ml water. An aerosol TLC atomizer was used to apply 12.5 ml of EPTC stock solution to 10 kg of base soil to give a final application rate of 10 ppmw, the upper limit of EPTC detection using the oat bioassay described below. The treated soil was placed into a 5 L plastic container and mixed in a tumbling soil mixer

for 1 h. After mixing, 380 g of herbicide treated "base soil" was placed in a 12 cm diam by 12 cm deep pot and inoculated with 20 g of a "test" soil. Tight fitting lids were placed momentarily on the pots which were tumbled thoroughly by hand to thoroughly mix the base soil and inoculum. Twenty ml of water was added and the pots were placed in the greenhouse. The greenhouse was maintained at 21 ± 4 C with natural light supplemented with metalarc halide lamps ($140 \text{ uE.m}^{-2}.\text{s}^{-1}$). Pots were watered daily and thoroughly mixed by hand as described above once a week. After 30 days, five Otana oat seeds were planted in each pot by placing the embryo end of the seed into the soil and pushing the seed flush with the soil surface. Oats were grown under the greenhouse conditions described above for 28 days after which the shoot biomass was clipped at the soil surface, oven dried for 4 days at 60 C and weighed.

The EPTC remaining in each pot was estimated using a standard curve. The standard curve was constructed by diluting base soil containing 10 ppmw EPTC with an appropriate amount of untreated soil to give final EPTC rates of 0, 2, 4, 6, 8, and 10 ppmw. The soils used for determination of the standard curve were prepared, incubated and planted under the same conditions as described above for the test soils. Linear regression analysis of biomass produced per plant versus EPTC concentration in soil

resulted in a significant fit with an r^2 of 0.97.

Confirmation of Microbial Activity

The ability of microorganisms in enhanced soil sample number 235, and a non-enhanced soil, number 71, to alter EPTC degradation was examined using the delayed planting bioassay described above. Base soil was prepared and treated with EPTC as described above to give a final EPTC concentration of 10 ppmw. One g of each "test" soil was autoclaved for 20 min at 121 C prior to inoculation. Herbicide treated base soil (99 g) was placed in 6.5 cm diameter by 4 cm deep styrofoam pots with one g of autoclaved or non-autoclaved inoculum from soils 71 and 235. The soil mixture was thoroughly incorporated, and pots were placed in a growth chamber operating at 25 C day (14 h), and 20 C night (10 h) temperatures. Incandescent and fluorescent light intensity was $165 \text{ uE.m}^{-2}.\text{s}^{-1}$. Four Otana oat seeds were planted as described above at 0, 3, 6, and 9 days after herbicide application and inoculation. Oats were grown for 28 days and harvested as described above. Each treatment was replicated 4 times and the experiment was conducted twice. Results are presented as the mean of two experiments.

Effect of Inoculum Source on EPTC Degradation

The rate of EPTC degradation in base soil individually inoculated with 166 test soils was determined using the

delayed planting bioassay technique described above. One kg of herbicide-treated base soil containing 10 ppmw EPTC was inoculated with 10 g of test soil as described above.

Following inoculation the sample was placed in a 5 L plastic container which was placed on a rolling mixer for 30 min.

Inoculated soil (100g) was placed into 6.5 cm diameter by 4 cm deep pots which were watered with 20 ml water, and placed in the greenhouse. Four Otana oat seeds were planted as described above 2, 4, 8, and 16 days after herbicide application and test soil inoculation. Oat biomass was harvested as described above 28 days after planting, dried, and weighed as before. A standard curve was prepared as described above. There were 6 replications and the experiment was conducted twice. Linear regression analysis of biomass produced per plant versus EPTC concentration resulted in a significant fit with r^2 values of 0.92 to 0.98 for the 4 planting dates. The equations of these lines were used to estimate the EPTC concentration remaining in each soil.

Results and Discussion

Soil Inoculation Screen

The amount of EPTC remaining in inoculated base soils varied significantly after a 30 day incubation period (Figure 9). Forty-seven of 166 soils contained less than 1 ppmw EPTC after 30 days. More than two-thirds of the soils

contained less than 3 ppmw EPTC. The results indicated that the rate of degradation varies significantly in 166 soils from across the United States.

Number of Test Soils

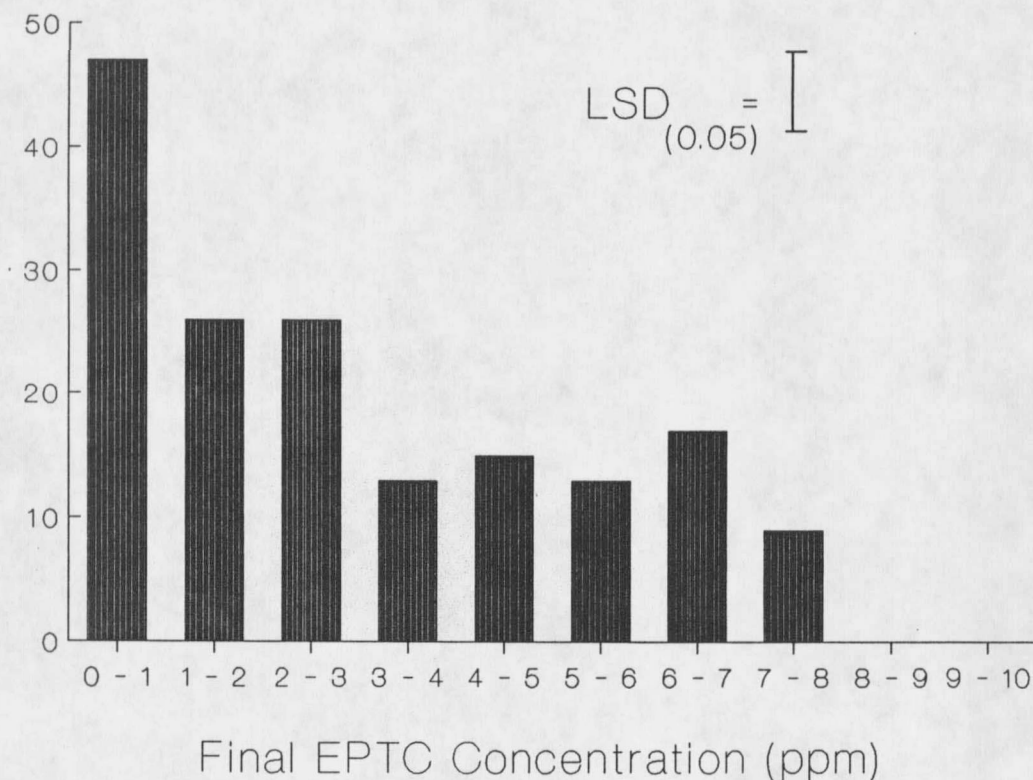
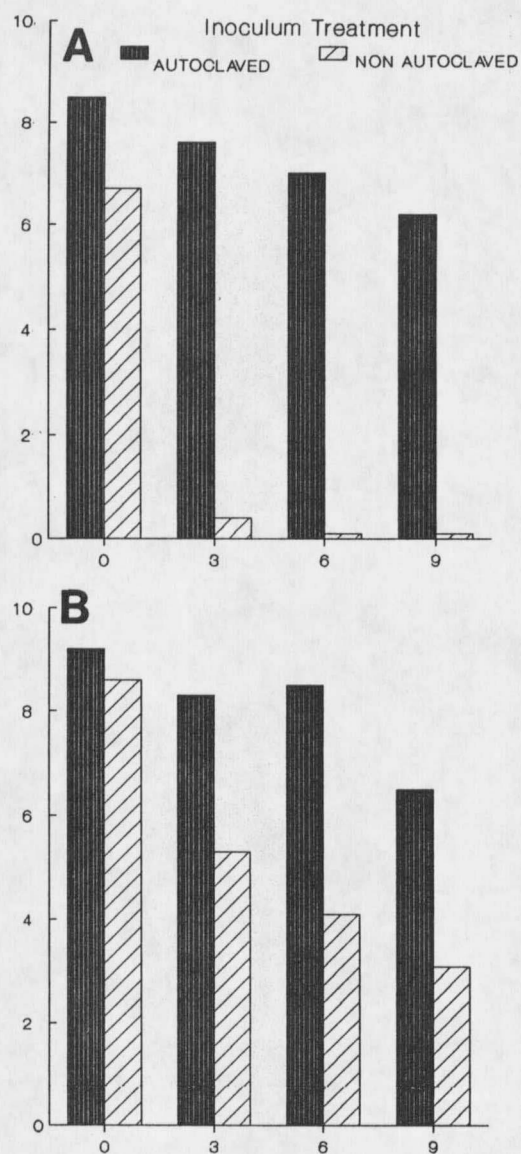


Figure 9. EPTC concentration remaining in base soils initially treated with 10 ppmw EPTC and inoculated with 10 g of soil from 166 individual test soils. EPTC concentration was estimated using a delayed planting oat bioassay seeded 30 days after herbicide application and test soil inoculation.

Confirmation of Microbial Activity

Autoclaving inoculum significantly reduced the rate of EPTC degradation (Figure 10).

Final EPTC Concentration (ppmw)



Incubation Period (days)

Figure 10. EPTC detected in a base soil using a delayed planting oat bioassay. Oats were planted 0, 3, 6, and 9 days after application of 10 ppmw EPTC and inoculation with a small amount of autoclaved or non-autoclaved soil from enhanced soil 235 (A) and non-enhanced soil 71 (B).

No EPTC was detected 6 days after application of 10 ppmw EPTC and inoculation with soil number 235. Approximately 4 ppmw EPTC was detected in soil number 71 when oats were planted 9 days after herbicide application.

There was no difference in the rate of EPTC degradation between the two test soils when soil inoculum was autoclaved prior to incorporation into herbicide treated soil. These results support the results of Moorman (41) who found that microbial adaptation was the cause of enhanced EPTC degradation.

Effect of Inoculum Source on EPTC Degradation

The kinetics of EPTC degradation in 166 test soils were examined individually. First order reaction plots were constructed by plotting estimated EPTC concentration versus time according to the equation:

$$\ln \frac{[A]_x}{[A]_0} = -kt$$

where $[A]_0$ and $[A]_x$ are the estimated concentration of EPTC determined by oat bioassay at initial time = 0 and sampling time = x, k = the first order rate constant, and t = time. A linear plot of $-\ln [A]_x/[A]_0$ vs time has a slope = k which is used to calculate EPTC half-life ($t_{1/2}$) based on the equation $t_{1/2} = 0.693/k$ (44).

The results suggest that EPTC degradation is accurately

described by first order kinetics in 152 of 166 test soils. Soils in which first-order kinetics described EPTC degradation had an r^2 value of 0.80 or greater, 72% of these soils had an r^2 greater than 0.90. First order plots for test soils with half-lives of 1.7 days and 23 days are shown (Figure 11).

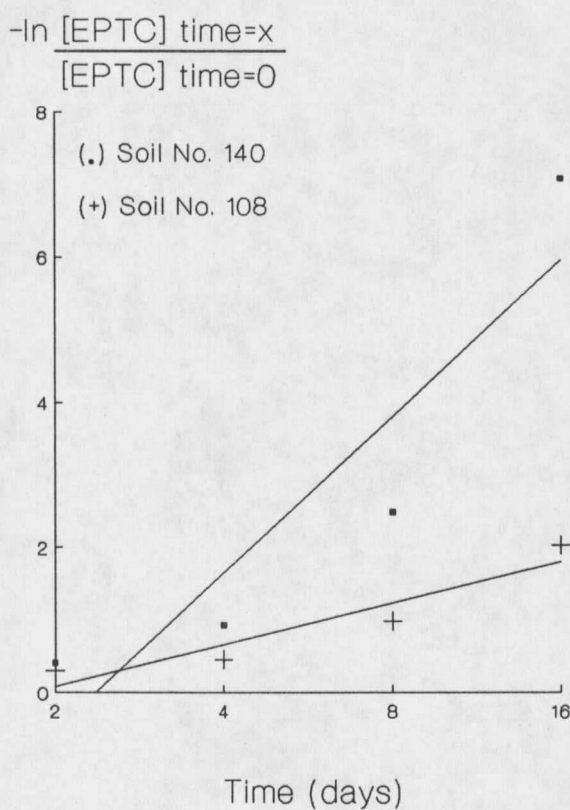


Figure 11. First order rate plots for EPTC degradation in test soil number 140 with a half-life of 1.7 days and for soil number 108 with a half-life of 23 days.

The half-life for EPTC ranged from less than 1 day to greater than 20 days (Figure 12). EPTC half-life in the pasteurized base soil without inoculation was 11.4 days. Six test soils were able to rapidly degrade EPTC with a $t_{1/2}$ of 0.5 to 2.0 days. Approximately 50% of the test soils had a half-life less than 7 days (Figure 12).

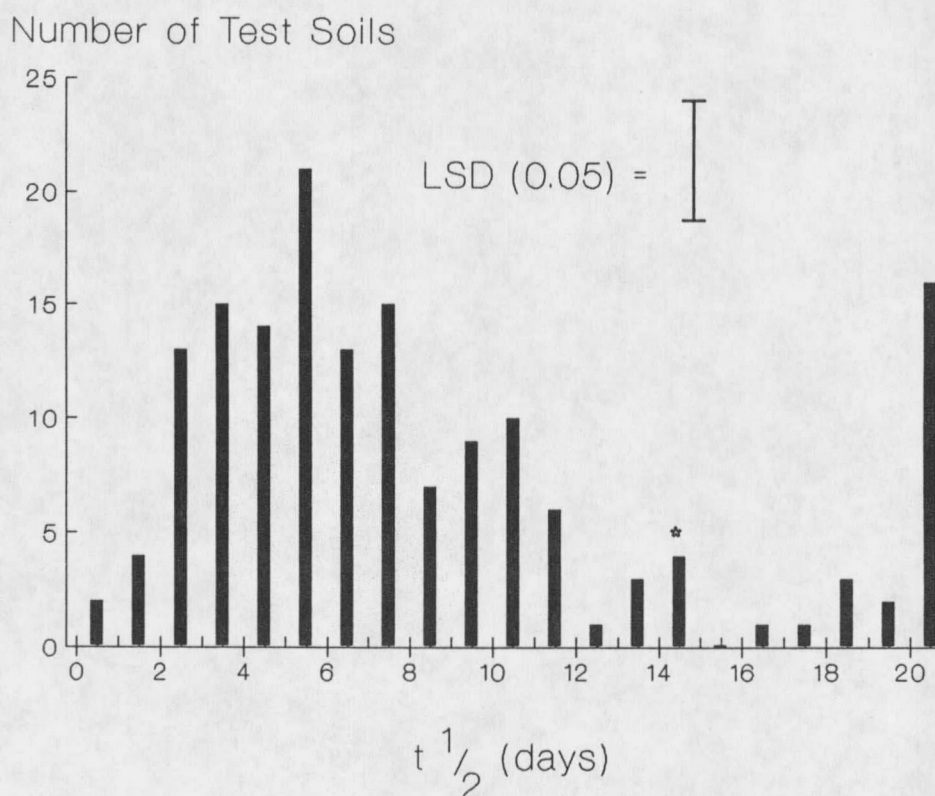


Figure 12. EPTC half-life ($t_{1/2}$) in a base soil inoculated with 166 test soils and in an uninoculated soil (*).

Sixteen test soils contained microorganisms which were able to increase the half-life of EPTC in the base soil to greater than 20 days. Soil microorganisms found in certain

test soils may be able to out-compete or consume constituent EPTC-degrading microorganisms thus increasing persistence.

Discovery that enhanced degradation was occurring in the field took many years despite the fact that the results presented here clearly show it to be a frequent phenomenon. The range of EPTC degradation rates shown here indicate that EPTC residues required for weed control will not be satisfactory if weeds germinate over a long time period. Microbial adaptation and environmental conditions determine whether or not an enhanced soil is identified in the field.

CHAPTER 4

THE INCIDENCE OF ENHANCED DEGRADATION OF FIVE PERSISTENT HERBICIDES IN 166 SOILS

Introduction

The usefulness of persistent herbicides would increase if the rate of degradation could be selectively enhanced. Currently, the concentration of many soil-applied herbicides required for acceptable weed control may persist too long and injure subsequent rotational crops.

The only herbicides that have been shown to be susceptible to enhanced degradation are certain thiocarbamate and phenoxy herbicides (1). There are no published reports of enhanced degradation of persistent herbicides. Persistent herbicides such as chlorsulfuron, clomazone, atrazine, or picloram may be susceptible to enhanced degradation, however, if the degree of enhancement is small the phenomenon would remain undetected in the field.

Microorganisms that cause enhanced degradation may be used as soil inoculum to enhance the degradation of residual herbicides in the field. Edgehill and Finn (17) showed that the half-life of pentachlorophenol (PCP) was reduced from 2

weeks to less than 1 day by inoculating PCP-containing soil with a culture of PCP-degrading bacteria. Daughton et al. (13) reported a significant reduction in technical grade parathion residue levels in soil after inoculation with parathion-degrading microorganisms. While persistent herbicides are degraded by microorganisms relatively little has been done to exploit this ability.

The objective of this research was to discover soil microorganisms which could enhance the rate of degradation of chlorsulfuron, atrazine, clomazone, or picloram (Figure 13).

Materials and Methods

Soil Sampling

Soil samples were obtained from weed scientists in the United States as described in Chapter 3.

Green Island Screen

The soil inoculation technique described in Chapter 3 was used to identify soils which contained microorganisms capable of enhanced degradation of chlorsulfuron, atrazine, clomazone, or picloram in soil. The base soil was the same as in Chapter 3 and was steam pasteurized and passed through a 10-mm sieve. The base soil was then uniformly treated with atrazine, clomazone, chlorsulfuron or picloram at 2 ppmw, 1.5 ppmw, 40 ppbw, or 40 ppbw respectively. The rate of herbicide degradation in the uninoculated base soil was considered "normal". Our assumption was that any alteration

of the rate of degradation would be due to the introduction of microorganisms from the test soil and would result in healthy bioassay indicator plants or "green islands".

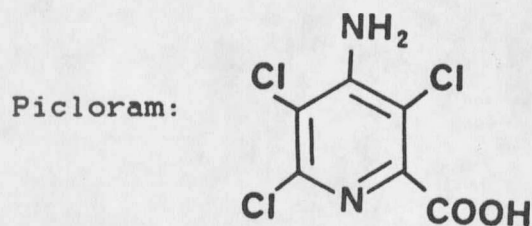
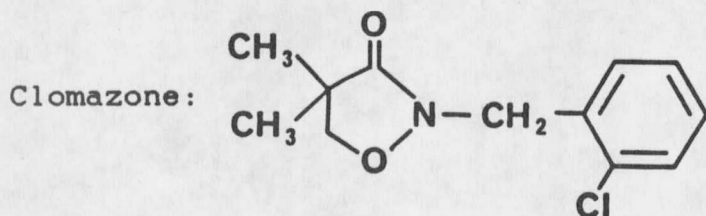
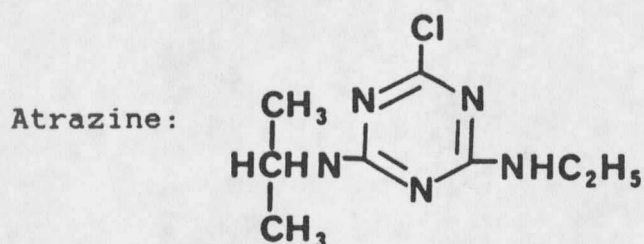
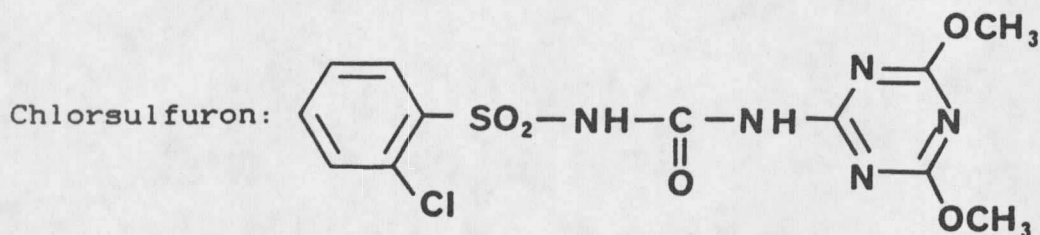


Figure 13. Molecular structures of the herbicides used in enhanced degradation studies.

An atrazine stock solution was prepared by placing 0.2 g of an 80 WP (wetable powder) commercial formulation of the herbicide in 1 L water. Four hundred twenty seven ml of stock solution was sprayed onto 32.5 kg base soil which corresponded to a soil treatment of 2.0 ppmw. Herbicide-treated soil was placed in a tumbling soil mixer for 1 h. A standard curve was prepared by placing 0, 4.2, 8.4, 12.6, 16.8, 21, or 25 ml of stock solution, brought to a total volume of 25 ml with water, on 1.9 kg soil. Each treatment was placed in a 5 L plastic container and mixed on a rolling mixer for 1 h. Standard herbicide treatments corresponded to atrazine soil rates of 0, 0.3, 0.7, 1.0, 1.3, 1.7, and 2.0 ppmw respectively.

A clomazone stock solution was prepared by placing 250 ul of 4 EC (emulsifiable concentrate) commercial clomazone in 1 L water. The base soil was uniformly treated with 1.5 ppmw clomazone by spraying 32.5 kg soil with 427 ml of clomazone stock solution as described above. Standard curve soils were prepared by spraying 1.9 kg of base soil with 0, 4.2, 8.3, 12.5, 16.7, 20.8, or 25 ml of clomazone stock solution each brought to a total volume of 25 ml. Standard soil treatments were mixed as described above and corresponded to a soil application of 0, 0.3, 0.5, 0.8, 1.3, and 1.5 ppmw clomazone.

Stock solutions for chlorsulfuron and picloram were prepared by placing 0.42 g chlorsulfuron 75 DF (dry

flowable) or 13.4 ml of picloram 22 K (liquid), both commercial formulations, in 1 L of water. Base soil treatments for both herbicides corresponding to 40 ppbw were made by applying 427 ml of stock solution to 32.5 kg of soil as above. Standard soil treatments for chlorsulfuron and picloram were prepared by applying 0, 6.3, 12.5, 15.6, 18.8, 21.9, or 25 ml of the appropriate stock solution, brought to a total volume of 25 ml with water, to 1.9 kg of base soil. These applications corresponded to 0, 10, 20, 25, 30, 35, and 40 ppbw for each herbicide.

Following herbicide application the soils were placed separately into a tumbling soil mixer for 1 h. Following mixing, 380 g of treated soil was placed into 166 11.5 cm diam. by 11.5 cm deep pots for each of the 4 herbicides. Each pot was then inoculated with 20 g of an individual test soil, the pot was capped, thoroughly mixed by hand tumbling, and placed in the greenhouse for incubation. The greenhouse temperature was maintained at 21 ± 4 C with natural lighting only. The pots were watered every 4 days and the soil was mixed by hand tumbling every 14 days to redistribute soil microorganisms throughout the pot.

Sixty days after test soil inoculation and herbicide application a bioassay was performed to determine the amount of herbicide remaining in each soil. Five Otana oat seeds were planted in atrazine and clomazone-treated soil by placing the embryo end of the seed into the soil and pushing

the seed flush with the soil surface. Oats were grown for 28 days, clipped at the soil surface, oven dried at 60 C for 5 days, and weighed. Four pinto bean seeds were planted 3 cm deep into picloram-treated soil 60 days after inoculation. Chlorsulfuron-treated soil was planted with 4 lentil seeds 2 cm deep. Pinto beans and lentils were grown for 30 days at which time the first true leaves of the beans, and the above ground portion of the lentils were harvested. They were dried and weighed as described above. The herbicide concentration remaining in the soil was determined by comparison to the standard treatments. The green island screen had one replication and was conducted only once.

Confirmation of Green Islands

To confirm the green islands found in the above screen, the 10 soils with the greatest least amount of herbicide degradation respectively, were bioassayed again. One hundred twenty days after the original test soil inoculation and herbicide application, 20 soils were selected for confirmation for each herbicide. The soil in each 400 g pot was thoroughly mixed and divided into five 75 g subsamples each placed in a 6.5 cm diam by 4.5 cm deep styrofoam pot. The 20 soils for each herbicide and the standard soils for atrazine, clomazone, chlorsulfuron, and picloram were bioassayed as described above. Herbicide degradation was measured by comparing biomass production of indicator

species grown in inoculated base soil to the biomass of the same species grown in a non-inoculated soil originally treated with the same amount of herbicide.

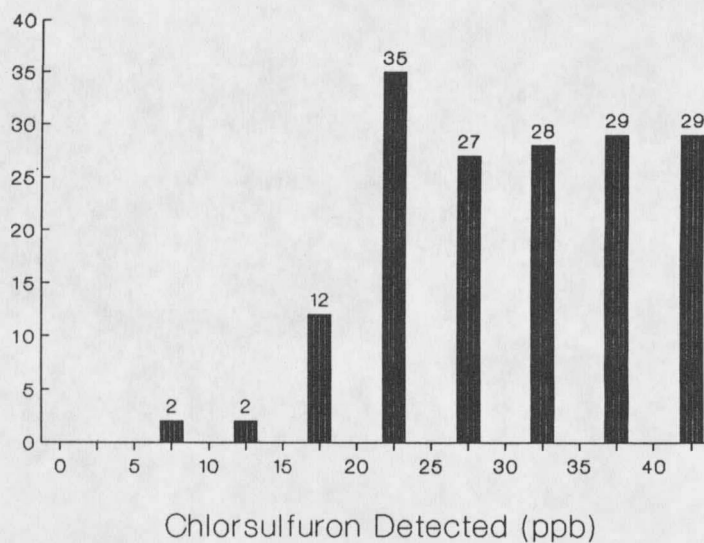
Results and Discussion

The rate of herbicide degradation varied considerably in the 166 test soils examined (Figures 14 and 15). Sixteen test soils inoculated into chlorsulfuron-treated base soil and 3 inoculated into picloram-treated soil contained microorganisms that reduced herbicide levels by 50% or more (Figure 14). The range of degradation of atrazine and clomazone detected 60 days after inoculation and herbicide application varied much less than for chlorsulfuron and picloram (Figure 15).

Confirmation of the Green Island Screen

Soils 4 and 9, treated with chlorsulfuron, exhibited enhanced degradation in the green island screen since lentil biomass production was significantly greater than in uninoculated soil treated with 40 ppbw chlorsulfuron (Table 6). While several of the soils which tested positive for enhanced degradation in the green island screen produced more biomass than the control in the confirmation experiment, only soils 4 and 9 were significantly different according to the LSD test. Results of the confirmation experiment indicated that much of the variation in herbicide levels detected 60 days after inoculation (Figures 14 and 15) was due to inherent bioassay error.

Number of Soils in Screen



Number of Soils in Screen

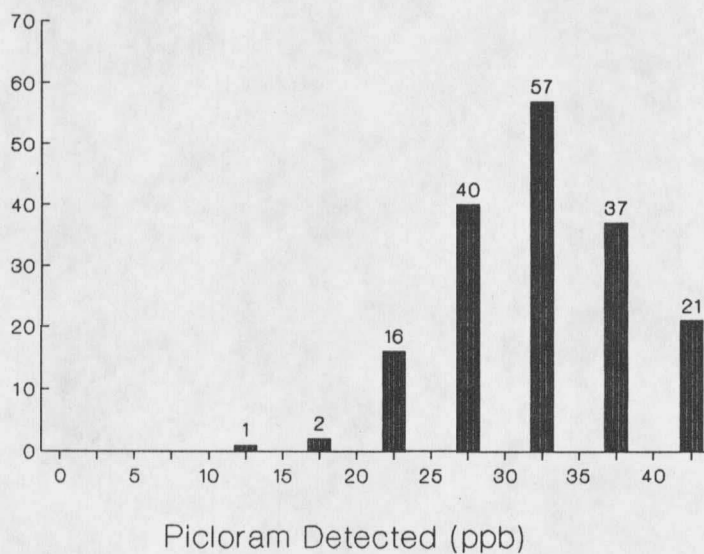
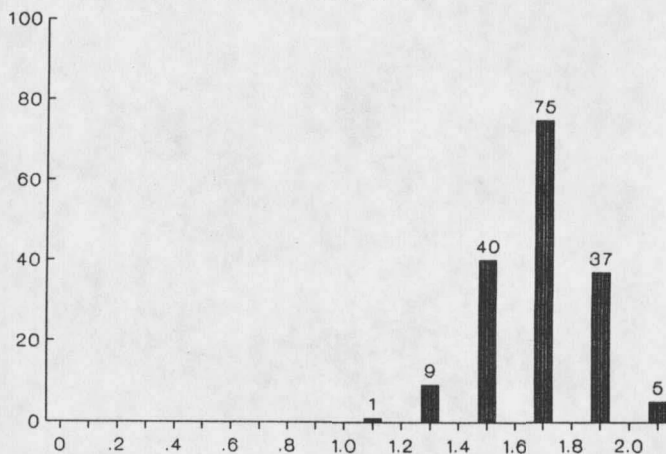


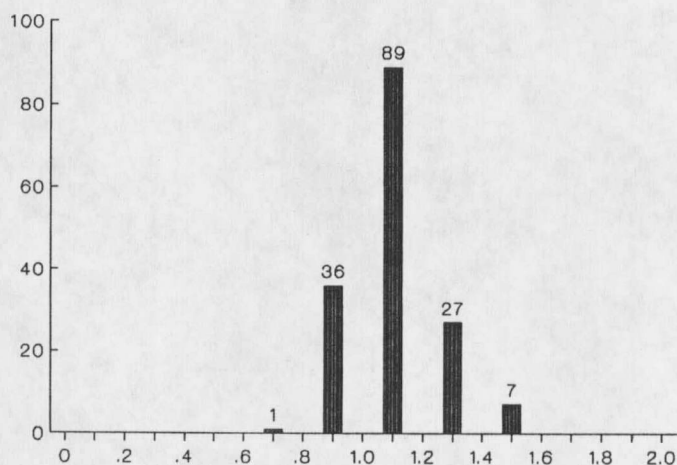
Figure 14. Herbicide detected in inoculated base soils 60 days after test soil inoculation and application of 40 ppb of chlorsulfuron or picloram.

Number of Soils in Screen



Atrazine Detected (ppm)

Number of Soils in Screen



Clomazone Detected (ppm)

Figure 15. Herbicide detected in inoculated base soils 60 days after test soil inoculation and application of 2 and 1.5 ppm of atrazine and clomazone respectively.

Microorganisms in test soil number 7 significantly reduced the level of picloram in soil (Table 7). Two additional

soils, numbered 14 and 18, also contained microorganisms which reduced the rate of picloram degradation. While most of the soils which tested positive for enhanced degradation in the green island screen produced greater plant biomass than the uninoculated control, degradation was enhanced significantly only in soils 7, 14, and 18.

Table 6. Lentil biomass production per plant when grown in incubated soil originally treated with 40 ppbw chlorsulfuron and inoculated with 10 g of a test soil.

Test Soil No.	Green Island Result ¹	Biomass Production
		mg/plant
7	+	9.2
11	-	10.7
6	+	11.1
15	-	11.1
18	-	11.5
16	-	11.9
13	-	12.4
17	-	12.9
20	-	13.1
12	-	13.5
10	+	14.3
14	-	14.8
uninoculated control	-	15.1
5	+	15.6
2	+	16.3
8	+	16.8
1	+	17.2
3	+	18.2
19	-	21.2
4	+	33.8 ^{*2}
9	+	44.9 [*]

¹Inoculum sources were determined to enhance (+) or to not enhance (-) chlorsulfuron degradation using the green island screen.

²Values denoted with (*) were significantly different from the uninoculated control.

Table 7. Pinto bean biomass production per plant when grown in incubated soil originally treated with 40 ppbw picloram and inoculated with 10 g of a test soil.

Test Soil No.	Green Island Result ¹	Biomass Production
		mg/plant
14	-	11.3 ^{*2}
18	-	15.5 [*]
6	+	19.3
3	+	19.9
12	-	20.3
16	-	20.5
15	-	20.9
19	-	21.2
1	+	21.3
17	-	22.2
uninoculated control	-	22.5
11	-	22.8
2	+	23.0
4	+	23.6
13	-	23.6
8	+	25.0
5	+	26.2
9	+	27.4
10	+	29.6
7	+	31.2 [*]

¹Inoculum sources were determined to enhance (+) or to not enhance (-) picloram degradation using the green island screen.

²Values denoted with (*) were significantly different from the uninoculated control.

Results of the confirmation experiment in which test soils were added to atrazine- and clomazone-treated base soils indicated that inoculation with the test soils did not

significantly reduce herbicide residues (Tables 8 and 9).

These results support the green island screen results

(Figure 15) which showed a narrow range of degradation rates for the 166 test soil inoculum sources.

Table 8. Oat biomass production per plant when grown in incubated soil originally treated with 2.0 ppmw atrazine and inoculated with 10 g of a test soil.

Test Soil No.	Green Island Result ¹	Biomass Production
		mg/plant
13	-	8.2 ²
20	-	9.1
17	-	9.2
18	-	9.3
11	-	9.6
10	+	9.6
16	-	10.0
uninoculated control	-	10.1
15	-	10.3
9	+	10.4
2	+	10.6
3	+	10.8
19	-	11.1
12	-	11.2
8	+	11.4
4	+	11.6
1	+	11.6
14	-	11.7
5	+	12.5
7	+	12.5
6	+	12.8

¹Inoculum sources were determined to enhance (+) or to not enhance (-) atrazine degradation using the green island screen.

²None of the values listed were significantly different from the uninoculated control.

Table 9.. Oat biomass production per plant when grown in incubated soil originally treated with 1.5 ppmw clomazone and inoculated with 10 g of a test soil.

Test Soil No.	Green Island Result ¹	Biomass Production
		mg/plant
14	-	11.3 ²
18	-	11.5
6	+	11.3
3	-	11.9
12	+	12.0
16	-	12.0
15	-	12.1
19	-	12.2
1	+	12.4
17	-	12.5
uninoculated control	-	12.5
11	-	12.5
2	+	12.6
4	+	12.6
13	-	12.6
8	+	12.7
5	+	12.7
9	+	12.7
10	+	12.9
7	+	13.1

¹Inoculum sources were determined to enhance (+) or to not enhance (-) clomazone degradation using the green island screen.

²None of the values listed were significantly different from the uninoculated control.

Our results suggest that the green island screen is an effective technique of identifying soils which contain microorganisms capable of enhanced degradation. This technique was proven to be valuable because enhanced

degradation was identified in soil. Microorganisms in soil which have the ability to rapidly degrade herbicides are environmentally fit which enables them to compete in soil. Environmental fitness is an important consideration as many microorganisms lose this desirable quality during growth on artificial media.

Two test soils increased the rate of chlorsulfuron degradation, and one test soil accelerated picloram degradation. These three soils, and two soils which apparently reduce the rate of picloram degradation need to be studied further to determine if these attributes have commercial potential that can be exploited.

CHAPTER 5

ISOLATION AND TESTING OF
HERBICIDE DEGRADING MICROORGANISMSIntroduction

Persistent herbicides could be used more widely if the residual period could be reduced. Soil microorganisms exist which enhance the rate of herbicide degradation however little work has been done regarding the intentional use of microorganisms to degrade herbicides in soil. Microorganisms be found which could significantly shorten the residual period of persistent herbicides when added to soil.

Behki and Khan (9) isolated three Pseudomonas species which were able to use atrazine as a sole carbon source from a soil with a long history of atrazine use. Cook and Hutter (12) isolated microorganisms from soil and sewage samples which were able to use s-triazine molecules as a sole source of nitrogen. Three strains of Pseudomonas and two of Klebsiella pneumoniae were able to degrade several triazine molecules to ammonium ion and carbon dioxide. Hogrefe et al. (27) immobilized a culture of s-triazine-degrading microorganisms on sand and placed the mixture in a column forming a fluidized bed reactor. When s-triazine-containing

industrial wastes were passed through the fluidized bed reactor the s-triazines were rapidly degraded.

While there are numerous reports of isolation of microorganisms which can degrade persistent compounds in pure or mixed culture, there are few reports of successful use of such organisms in natural environments. Since pesticides are usually found in low concentrations in the environment their biodegradation may be difficult. Boethling and Alexander (11) found that laboratory biodegradation studies which used pesticide concentrations higher than those found in nature did not correlate well with degradation rates in natural systems.

The objective of this research was to isolate pure cultures of microorganisms capable of metabolizing chlorsulfuon, atrazine, picloram, clomazone, or EPTC with the intent of reducing herbicide residues in field soils by direct inoculation.

Materials and Methods

Soil Sampling

Soil samples were obtained from weed scientists in the United States as described in Chapter 3.

Microorganism Isolation

A plating technique using selective medium was used in an attempt to isolate herbicide degrading microorganisms from 166 soils. Five ml of basal salts medium (BSM), pH 7.0 (Table 10) was added to a 5 by 20 mm tube containing 1 g of

an individual test soil. The tube was capped and placed on a horizontal shaker operating at 50 strokes/min for 8 h to place the microorganisms in solution. The tubes were allowed to settle for 15 min to reduce the amount of silt and clay in the supernatant. One hundred μ l of supernatant was spread evenly over the surface of each of six 9-cm diam petri plates containing BSM and 15 g/L Noble agar (Difco Corp., Detroit, MI). plus one of 5 herbicides as a carbon source.

Table 10. Composition of basal salts medium (BSM).

Salt	g/L	Salt	g/L
K_2HPO_4	1.6	$CaCl_2$	0.025
KH_2PO_4	0.4	NH_4NO_3	0.5
$MgSO_4 \cdot 7H_2O$	0.2	NH_4SO_4	0.1
NaCl	0.1	$FeCl_3 \cdot 6H_2O$	0.007

The BSM was autoclaved at 121 C for 20 min, allowed to cool to 60 C and filter-sterilized herbicide was added. The media was designated HBSM (herbicide containing basal salts media). Fresh herbicide stock solutions contained 0.5 g herbicide per ml of HPLC grade methanol. One ml of stock solution was added to 1 L of BSM to provide 500 ppm HBSM plates. Technical grade herbicides were obtained from their respective manufacturers (Table 11). A 100 μ l aliquot of each soil extract was placed on a BSM plate as a control. One ml of HPLC grade methanol was added to the BSM control

medium. Plates were incubated at 28 C for 20 days. Colony growth on the 5 HBSM plates was compared to the BSM control at 5 day intervals. Bacterial or fungal types present on an HBSM plate but not on the corresponding BSM control indicated possible use of the herbicide for growth. Single colony isolates from HBSM plates were purified by streaking onto fresh HBSM plates.

Table 11. Technical grade herbicide, % purity, and manufacturer of the products used in the isolation and testing of herbicide degrading microorganisms.

Herbicide	% Purity	Source
chlorsulfuron	98.9	E.I. DuPont DeNemours & Co.
EPTC	98.0	Stauffer Chemical Co.
atrazine	90.0	Ciba-Geigy Corp.
clomazone	98.7	FMC Corp.
picloram	90.0	The Dow Chemical Co.

Measuring Isolate Growth Rate

The growth rate of each bacterial isolate was determined in liquid culture by recording the change in culture absorbance over time. Isolates with the most rapid growth in herbicide medium were selected for further testing. Use of liquid media also eliminated isolates that utilized agar as an energy source. Bacterial isolates growing on atrazine, picloram, chlorsulfuron, EPTC, or clomazone HBSM plates were inoculated into the corresponding

liquid HBSM. Technical grade herbicide was added to a final concentration of 20 ppm after autoclaving the medium as described above. Five ml of liquid HBSM was placed into 5 by 100 mm borosilicate culture tubes that were capped with lids which allowed air exchange. The culture tubes were inoculated with a single colony from an HBSM plate and placed on an orbital shaker at 500 rpm at 28 C. The culture absorbance was monitored at 12 h intervals for 10 days using a Klett-Summerson colorimeter. Growth curves were prepared twice for each isolate.

Monitoring Herbicide Degradation in Culture

Each isolate in Table 13 was tested for the ability to degrade herbicide in liquid medium. Liquid HBSM containing 20 ppm technical grade chlorsulfuron, atrazine, or picloram was prepared as described above. While several isolates grew in liquid culture containing EPTC or clomazone, these isolates were not characterized further.

A 1.5 ml aliquot was removed from each tube at 12 h intervals for 7 days, and stored at -4 C to await high performance liquid chromatography (HPLC) analysis. Once a culture reached a maximum cell density, indicated by a growth curve, the samples were analyzed for the herbicide using HPLC.

Samples were thawed and centrifuged at 10,000 x g for 10 min to pellet the cells. A 25 ul aliquot of the supernatant was injected with an ISCO ISIS Auto sampler and

Auto injector into the HPLC. The herbicides and metabolites were separated from other culture constituents with a 25 cm long by 4.6 mm diam Regis Hi-Chrom reversed phase C-18 column with a 5 μ m particle size. The mobile phase was delivered to the system with a Kratos Spectroflow 400 pump.

Picloram and chlorsulfuron samples were analyzed using methanol:water at 6:4 with 0.1% trifluoroacetic acid (TFA) at 0.5 and 1.0 ml/min flow rate and 65 and 95 bar pressure respectively. Atrazine samples were injected into a mobile phase of methanol:water at 8:2 with 0.1% TFA delivered at 0.5 ml/min with 70 bar pressure. The herbicides were detected using a Kratos Spectraflow 747 UV detector at 254 nm. The herbicides were identified by comparison of retention time with standards and were quantified by peak area integration using a Shimadzu C-R3A Chromatopac. Separated compounds were individually collected at the detector and identity confirmed by comparison to standards using thin layer chromatography with 2 solvent systems (Table 12).

HBSM standards were injected at the beginning and end of each sample run. Regression analysis of peak area against herbicide concentration of HBSM standards was linear with r^2 values consistantly greater than 0.97. Each isolate was tested twice with 3 replications.

Table 12. Thin-layer chromatography solvent systems and corresponding Rf values used to confirm herbicide identity.

Herbicide	Solvent System		Rf
atrazine	10:6:2:2	benzene:CHCl ₃ :DEA ¹ :MEOH ²	.50
		CHCl ₃ with 2% MEOH	.68
chlorsulfuron	2:1:1	acetone:toluene:MEOH	.70
	1:1	acetone:toluene	.42
picloram	2:1:1	CHCl ₃ :MEOH:DEA	.80
	1:1	CHCl ₃ :MEOH	.20

¹Diethanolamine.

²Methanol.

Greenhouse Testing of Select Isolates

Six rapidly growing isolates were tested for their ability to degrade herbicide in soil. Isolates were obtained on picloram, atrazine, or chlorsulfuron HBSM plates and inoculated into soil which contained each herbicide at a field rate (x) and twice field rate (2x).

Picloram isolates P79 and P272, atrazine isolates A54 and A313, and chlorsulfuron isolates G168 and G374 were first inoculated into 500 ml of liquid HBSM in a one L flask containing 20 ppm of the respective herbicide. Culture flasks were placed on an orbital shaker operating at 150 rpm at 28 C and culture absorbance was measured daily with a

Klett colorimeter. The cultures were grown for 5 to 7 days to a reading of 100 Klett units which corresponded to ca. 7×10^8 colony forming units per ml. A colony forming unit (CFU) represents viable cells per ml of culture while absorbance measurements reflect total living and dead cells per ml. Each culture was centrifuged at $8000 \times g$ for 10 min, the supernatant was discarded, and the cell pellets combined and resuspended in 500 ml BSM. Samples were again centrifuged to remove all traces of the herbicide. After the final wash the cells were resuspended in 100 ml BSM corresponding to ca. 7×10^9 cells per ml.

The greenhouse soil used in this experiment was a silty-clay-loam, pH 8.1, 2.3% organic matter, 14% sand, 57% silt, and 29% clay which was pasteurized and passed through a 10-mm sieve. Herbicide stock solutions were prepared using commercial formulations of atrazine (80WP), picloram (22K), and chlorsulfuron (75DF) by placing 31.3 g, 2.1 ml, or 0.67 g of each compound respectively in a total volume of 1 L water. One or 0.5 ml of the appropriate stock solution was brought to a total volume of 10 ml and sprayed on 6.3 kg of soil for the twice label rate and label rate of application for each herbicide, respectively. Atrazine was applied at 4 and 2 ppmw, while picloram and chlorsulfuron were applied at 80 and 40 ppbw. The soil was placed in a 15 L plastic container and placed on a rolling mixer for 1 h. Pots

containing 600 g herbicide-treated soil were inoculated with 10 ml of the appropriate bacterial cell suspension described above corresponding to ca. 1×10^5 cells per g of soil. The pots were capped and thoroughly mixed by hand shaking, watered to field capacity, and placed in the greenhouse at 21 ± 4 C under natural light supplemented with $140 \text{ uE} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ artificial lighting from metalarc halide lamps. Inoculated soils were mixed every 10 days and incubated for a total of 60 days. Following incubation, soil treated with atrazine, picloram, and chlorsulfuron were planted with 4 Otana oat seeds, 3 pinto bean seeds, or 4 lentil seeds, respectively. The bioassay species were grown for 30 days at which time the lentils and oats were clipped at the soil surface and the first true leaves of the pinto beans harvested. The plants were oven dried at 60 C for 4 days and weighed.

Results and Discussion

Microorganism Isolation

Numerous bacterial and fungal isolates were obtained on HBSM plates for each herbicide tested (Table 13). There were 53 bacterial and 24 fungal isolates collected on HBSM plates containing chlorsulfuron that were not present on their respective BSM control plates. The lowest number of isolates, 28, was obtained on clomazone HBSM plates. All isolates visually detected on the HBSM but not on BSM plates were purified by streaking on fresh HBSM. The isolates were

assumed pure after three successive pure streak plates were prepared. Fungal isolates obtained in this study were not used in further tests.

Table 13. Bacterial and fungal isolates obtained from extracts of 166 soil samples that were plated on agar plates containing one of five herbicides at a concentration of 500 ppm as a carbon source.

Herbicide in Agar Plate	Bacterial Isolates		Fungal Isolates	
	Total	Types ¹	Total	Types
atrazine	41	26	14	6
EPTC	46	32	7	3
chlorsulfuron	53	31	24	5
clomazone	26	15	2	2
picloram	41	22	4	3

¹Types were distinguished by colony morphology.

Measuring Isolate Growth Rate

Each bacterial isolate was grown in liquid culture to select isolates with the most rapid growth rate in an attempt to reduce the number of isolates in further tests. An arbitrary value of 100 Klett units (7×10^8 CFU/ml) growth in 8 days was selected as acceptable growth. While most isolates reached 40 to 50 Klett units by 8 days after inoculation, only the 11 atrazine, 6 chlorsulfuron, and 8 picloram isolates shown in Table 14 were able to grow to an acceptable level in two separate growth curves. None of the EPTC or clomazone isolates examined met this criteria.

Representative growth curves for isolates grown in atrazine, chlorsulfuron, and picloram HBSM are shown in Figure 16.

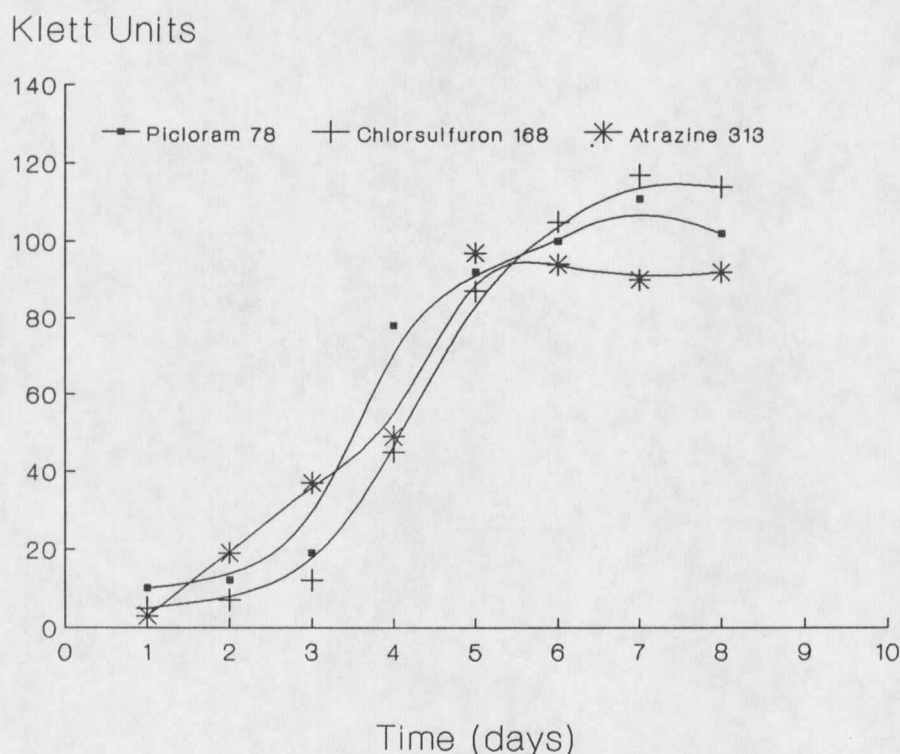


Figure 16. Growth curves for atrazine 313, chlorsulfuron 168, and picloram 78 isolates grown in liquid culture.

Monitoring Herbicide Degradation

Eleven isolates were examined for the ability to degrade atrazine, six for chlorsulfuron, and eight for picloram. None of the isolates examined were able to significantly reduce the amount of herbicide in the culture medium, at this level of detection, although the isolates grew rapidly in the herbicide containing minimal medium (Table 14). Although attempts to show disappearance of the

parent herbicide molecule failed repeatedly, several isolates appeared to produce metabolite peaks. Several chlorsulfuron isolates produced a uv-absorbing compound (Figure 17) which eluted 3 min prior to the parent herbicide molecule. Further testing must be done to determine if the compound(s) represented by these peaks are herbicide metabolites.

Table 14. Herbicide remaining in media originally containing 20 ppm herbicide which was inoculated with HBSM isolates and grown to a maximum cell density.

Herbicide in Liquid Medium (20 ppm)	Isolate (herbicide concentration) ¹		
Atrazine	A54 (20.1a)	A78 (21.2a)	A135 (20.0a)
	A163 (19.8a)	A168 (20.1a)	A226 (19.6a)
	A313 (18.9a)	A373 (19.8a)	A377 (18.5a)
	A162 (19.7a)	A272 (19.9a)	
Chlorsulfuron	G79 (21.1a)	G86 (21.2a)	G168 (19.7a)
	G272 (19.8a)	G374 (20.0a)	G202 (19.2a)
Picloram	P54 (20.1a)	P78 (19.4a)	P79 (20.2a)
	P168 (19.6a)	P202 (19.7a)	P272 (18.9a)
	P162 (19.9a)	P374 (21.0a)	

¹None of the isolates tested were able to reduce the initial herbicide concentration to a level significantly different from an uninoculated control.

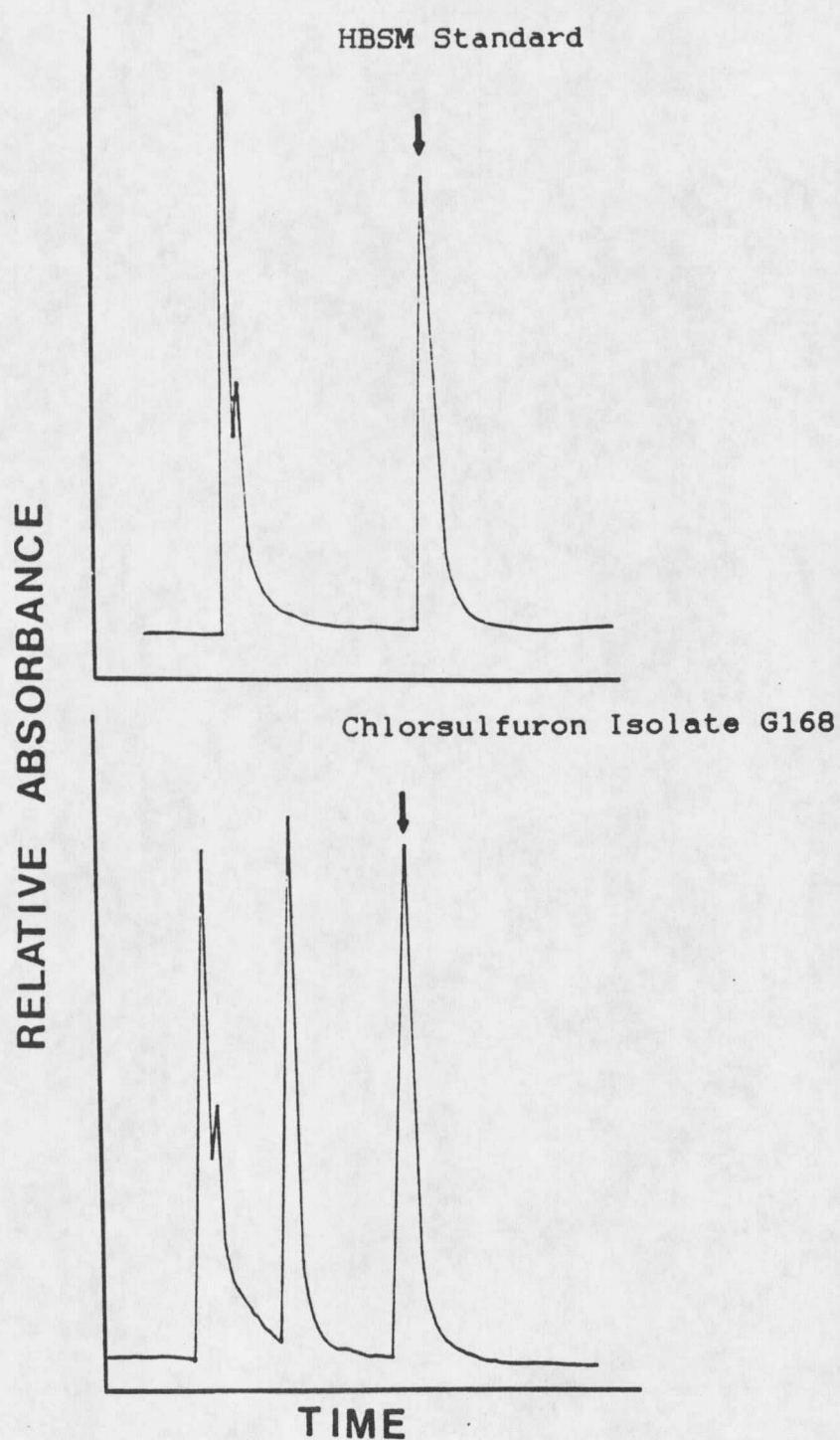


Figure 17. HPLC chromatograms of a HBSM standard which contains 20 ppm chlorsulfuron (above) and, 20 ppm HBSM after 5 days of growth with chlorsulfuron isolate G168. Arrows indicate elution of the parent chlorsulfuron molecule.

Greenhouse Testing of Select Isolates

The two most rapidly-growing isolates for picloram, atrazine, and chlorsulfuron were unable to significantly reduce the level of their respective herbicides in soil. Chlorsulfuron isolate G168 caused a slight increase in lentil dry weight at both herbicide rates tested however, the increase was not statistically significant (Table 15).

Table 15. Bioassay indicator species injury in herbicide treated soil inoculated with 6 HBSM isolates and incubated in the greenhouse for 60 days.

Bacterial Isolate	Herbicide in Soil	Plant Dry Weight (% of control) ¹	
		Herbicide Rate ²	
		Low	High
P79	picloram	13a	17a
P272	picloram	15a	7a
control	picloram	10a	8a
G168	chlorsulfuron	23a	18a
G374	chlorsulfuron	9a	11a
control	chlorsulfuron	13a	4a
A54	atrazine	15a	5a
A313	atrazine	7a	12a
control	atrazine	6a	4a

¹Numbers in columns followed by the same letter are not significantly different at the 5% level using the LSD test.

²Low and high herbicide rates were 40 and 80 ppbw for picloram and chlorsulfuron, and 2 and 4 ppmw for atrazine respectively.

The plating technique used in these studies effectively isolated microorganisms which appear to utilize herbicides as a sole carbon source. The herbicide-containing minimal medium screen identified 25 isolates which grew rapidly in liquid medium containing atrazine, chlorsulfuron, or picloram as a sole carbon source.

It is unclear why the isolates listed in Table 14 grew in HBSM and not in BSM yet were unable to reduce the herbicide concentration in the liquid media. The HPLC results are promising in that the isolates that grew rapidly in liquid culture also produced unidentified metabolites.

To date I have generated data which provides circumstantial evidence of herbicide metabolism, however, definitive proof of metabolism was not obtained.

LITERATURE CITED

1. Alexander, M. 1981. Biodegradation of chemicals of environmental concern. *Science*. 211:132-138.
2. Anonymous. 1986. Command herbicide technical information. FMC Corporation. Philadelphia, PA. 9 pages.
3. Anonymous. 1986. Command^R 4EC Herbicide (Label) Philadelphia, PA. 1 page.
4. Armstrong, D.E., and G. Chesters. 1968. Adsorption catalyzed chemical hydrolysis of atrazine. *Environ. Sci. Technol.* 2:683-689.
5. Armstrong, D.E. and J.G. Konrad. 1974. Non-biological degradation of pesticides. in "Pesticides in Soil and Water". R.C. Dinahauer ed. Soil Sci. Soc. of America. pgs 123-129.
6. Audus, L.J. 1949. Biological detoxification of 2,4-D. *Plant Soil*. 2:31-36.
7. Baily, A.M. and M.D. Coffey. 1985. Biodegradation of metalaxyl in avocado soils. *Phytopathology*. 75:135-137.
8. Barles, R.W., C.G. Daughton, and D.P.H. Hsieh. 1979. Accelerated parathion degradation in soil inoculated with acclimated bacteria under field conditions. *Arch. Environm. Contam. Toxicol.* 8:647-660.
9. Behki, R.M. and S.U. Khan. 1986. Degradation of atrazine by Pseudomonas: N-dealkylation and dehalogenation of atrazine and its metabolites. *J. Agric. Food Chem.* 34:746-749.
10. Bellman, S.K., H. Guscar, and M. Hillson. 1984. Residual weed control with FMC 57020 herbicide. *Proc. North Cent. Weed Cont. Conf.* 39:19.
11. Boethling, H. and M. Alexander. 1979. Effect of concentration of organic chemicals on their biodegradation by natural communities. *Appl. and Environ. Microbiol.* 37:1211-1216.

12. Cook, A.M. and R. Hutter. 1981. s-Triazines as nitrogen sources for bacteria. J. Agric. Food Chem. 29:1135-1143.
13. Daughton, C.G., and D.P.H. Hesieh. 1977. Parathion utilization by bacterial symbionts in a chemostat. Appl. Environ. Microbiol. 34:175-184.
14. Duke, S.O. and R.N. Paul. 1986. Effects of dimethazone (FMC 57020) on chloroplast development. I. Ultrastructural effects in cowpea (Vigna unguiculata L.) primary leaves. Pestic. Biochem. Physiol. 25:1-10.
15. Duke, S.O. and W.H. Kenyon. 1986. Effects of dimethazone (FMC 57020) on chloroplast development II. Pigment synthesis and photosynthetic function in cowpea (Vigna unguiculata L.) primary leaves. Pestic. Biochem. Physiol. 25:11-18.
16. Duke, S.O., W.H. Kenyon, and R.N. Paul. 1985. FMC 57020 effects on chloroplast development in pitted morninglory (Ipomoea lacunosa L.) cotyledons. Weed Sci. 33:786-794.
17. Edgehill, R.V., and R.K. Finn. 1983. Microbial treatment of soil to remove pentachlorophenol. Appl. and Environ. Microbiol. 45:1122-1125.
18. Fay, P.K. and E.S. Davis. 1987. Personal communication.
19. Felsot, A., J.V. Maddox, and W.Bruce. 1981. Enhanced microbial degradation of carbofuran in soils with histories of furadan use. Bull. Environmen. Contam. Toxicol. 26:781-788.
20. Froelich, L.W., T.A. Bixler, and R.A. Robinson. 1984. Soil metabolism and mobility of FMC 57020, a new soybean herbicide. Proc. North Cent. Weed Cont. Conf. 39:79.
21. Fryer, J.D. and K. Kirkland. 1970. Field experiments to investigate long-term effects of repeated applications of MCPA, triallate, simazine, and linuron: Report after 6 years. Weed Res. 10:90-95.
22. Fryer, J.D., P.D. Smith, and R.J. Hance. 1980. Field experiments to investigate long-term effects of repeated applications of MCPA, triallate, simazine, and linuron: II. Crop performance and residues 1969-78. Weed Res. 20:103-110.

23. Gallandt 1988. unpublished results.
24. Gray, R.A., and G.K. Joo. 1985. Reduction in weed control after repeat application of thiocarbamate and other herbicides. *Weed Sci.* 33:698-702.
25. Hartmann, J., W. Reineke, and H.J. Knackmuss. 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a pseudomonad. *Appl. Environ. Microbiol.* 37:421-428.
26. *Herbicide Handbook of the Weed Science Society of America.* 1983. Fifth Edition. Weed Science Society of America. Champaign, IL. pp 339, 380.
27. Hogrefe, W., H. Grossenbacher, A.M. Cook, and R. Hutter. 1986. Biotreatment of $\bar{s}$ -triazine-containing wastewater in a fluidized bed reactor. *Biotechnol. Bioeng.* 28:1577-1581.
28. Horowitz, M. 1976. Application of bioassay techniques to herbicide investigations. *Weed Res.* 16:209-215.
29. Horowitz, M., T. Blumenfeld, G. Herzlinger, and N. Hulin. 1974. Effects of repeated applications of ten soil-active herbicides on weed population, residue accumulation and nitrification. *Weed Res.* 14:97-109.
30. Horvath, R.S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. *Bacteriol. Rev.* 36:146-155.
31. Hurle, K. and A. Walker. 1980. Persistence and its prediction. in "Interactions Between Herbicides and Soil". R.J. Hance, ed. pgs. 83-85.
32. Kaufman, D.D., P.C. Kearney, D.W. VonEndt, and D.E. Miller. 1970. Methylcarbamate inhibition of phenylcarbamate metabolism in soil. *J. Agr. Food Chem.* 18:513-519.
33. Keifer, D.W. and S.F. El-Naggar. 1984. Interaction of FMC 57020 with the soil. *Proc. North Cent. Weed Cont Conf.* 39:79.
34. Kilbane, J.J., D.K. Chatterjee, J.S. Karns, S.T. Kellogg, and A.M. Chakrabarty. 1982. Biodegradation of 2,4,5-trichloro-phenoxyacetic acid in soil by a pure culture of Pseudomonas cepacia. *Appl. and Environ. Microbiol.* 44:514-516.

35. Kirkland, K. and J.D. Fryer. 1972. Degradation of several herbicides in a soil previously treated with MCPA. *Weed Res.* 12:90-95.
36. Lanzelotta, R.P., and D. Pramer. 1970. Herbicide transformation. I. Studies with whole cells of Fusarium solani. *Appl. Microbiol.* 19:301-306.
37. Lappin, H.M., M.P. Greaves, and J.H. Slater. 1985. Degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy)propionic acid] by a synergistic microbial community. *Appl. Environ. Microbiol.* 49:429-433.
38. Lee, A. 1984. EPTC (S-ethyl N,N-dipropyl thiocarbamate)-degrading microorganisms isolated from a soil previously exposed to EPTC. *Soil Biol. Biochem.* 16:529-531.
39. Loux, M.M., and F.W. Slife. 1984. Persistence and leaching of dimethazone in Illinois soils. *Proc. North Cent. Weed Cont. Conf.* 39:95.
40. Montana Agricultural Statistics Service. 1986. Montana Agricultural Statistics, Box 4369, Helena, MT 59604. Oct. Vol. XXIII. pg. 28.
41. Moorman, T.B. 1988. Populations of EPTC-degrading microorganisms in soils with accelerated rates of EPTC degradation. *Weed Sci.* 36:96-101.
42. Obrigawitch, T., A.R. Martin, and F.W. Roeth. 1983. Degradation of thiocarbamate herbicides in soils exhibiting rapid EPTC breakdown. *Weed Sci.* 31:187-192.
43. Obrigawitch, T., R.G. Wilson, A.R. Marlin, and F.W. Roeth. 1982. The influence of temperature, moisture, and prior EPTC application on the degradation of EPTC in soils. *Weed Sci.* 30:175-181.
44. Obrigawitch, T., F.W. Roeth, A.R. Martin, and R.G. Wilson Jr. 1982. Addition of R-33865 to EPTC for extended herbicide activity. *Weed Science.* 30:417-422. *Weed Sci.* 30:417-422.
45. Plimmer, J.R. 1970. The photochemistry of halogenated herbicides. *Res Rev.* 33:47-74.
46. Racke, K.D. and E.P. Lichtenstein. 1985. Effects of soil microorganisms on the release of bound 14-C residues from soils previously treated with [14-C]-parathion. *J. Agric. Food Chem.* 33:938-943.

47. Racke, K.D. and J.R. Coats. 1987. Enhanced degradation of isophenphos by soil microorganisms. J. Agric. Food Chem. 35:94-99.
48. Rahman, A., and T.K. James. 1983. Decreased activity of EPTC & R-25788 following repeated use in some New Zealand soils. Weed Sci. 31:783-789.
49. Rahman, A., G.C. Atkinson, and J.A. Douglas. 1979. Eradicane causes problems. N.Z.J. Agric. 139:47-49.
50. Read, D.C. 1983. Enhanced microbial degradation of carbofuran and fensulfothion after repeated applications to acid mineral soil. Agriculture, Ecosys. and Environ. 10:37-46.
51. Roeth, F.W. 1986. Enhanced herbicide degradation in soil with repeat application. Rev. Weed Sci. 2:45-66.
52. Rudyanski, W.J., R.S. Fawcett, and R.S. McAllister. 1987. Effect of prior pesticide use on thiocarbamate herbicide persistence and giant foxtail (Setaria faberi) control. Weed Sci. 35:68-74.
53. Skipper, H.D., E.C. Murdock, D.T. Gooden, J.P. Zublena, and M.A. Amakiri. 1986. Enhanced herbicide biodegradation in South Carolina soils previously treated with butylate. Weed Sci. 34:558-563.
54. Subba-Rao, R.V., T.H. Chromartie, and R.A. Gray. 1987. Methodolgy in accelerated biodegradation of herbicides. Weed Tech. 1:333-340.
55. Torstensson, L. 1980. Role of microorganisms in decomposition. in "Interactions Between Herbicides and the Soil". R.J. Hance ed. pg.159-176.
56. Torstensson, J. Stark, and B. Goransson. 1975. The effect of repeated application of 2,4-D and MCPA on their breakdown in soil. Weed Res. 15:159-164.
57. Tuxhorn, G.L., F.W. Roeth, A.R. Martin, and R.G. Wilson. 1986. Butylate persistence and activity in soils previously treated with thiocarbamates. Weed Sci. 34:961-965.
58. Tymonko, J.M. and H.L. Guscar. 1984. Influence of soil properties on the activity of FMC 57020. Proc. North Cent. Weed Cont. Conf. 39:76.

59. Wang, Y.S., E.L. Madsen, and M. Alexander. 1985. Microbial degradation by mineralization or cometabolism determined by chemical concentration and environment. J. Agric. Food Chem. 33:495-499.

60. Wilson, R.G. 1984. Accelerated degradation of thiocarbamate herbicides in soil with prior thiocarbamate herbicide exposure. Weed Sci. 32:264-268.

MONTANA STATE UNIVERSITY LIBRARIES



3 1762 10131794 7

