

AN ABSTRACT OF THE THESIS OF

Lesley A. Smith for the degree of Master of Science in Soil Science presented on November 28, 1995. Title: Detection of the Herbicide Alachlor and its Sulfonic Acid Metabolite in Soil.

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Abstract approved: _____

Peter J. Bottomley

The chloroacetanilide herbicide alachlor is the second most widely used herbicide in the US, where it is used on major crops such as corn and soybean. Although alachlor is mineralized slowly in soil, metabolites of alachlor have been found in soil indicating that alachlor is subject to chemical or biological transformation. A water-soluble, non-carcinogenic metabolite of alachlor, 2-[(2,6-diethylphenyl) (methoxymethyl) amino]-2-oxoethane-sulfonic acid (ESA), has been detected in groundwater. Because ESA contains a sulfur atom it is likely that it was formed as a result of a glutathione-based transformation mechanism found in many organisms. The objectives of this study were to evaluate the use of an Enzyme-Linked ImmunoSorbent Assay (ELISA) to detect the herbicide alachlor and its ESA metabolite in soil, and to determine if alachlor and its ESA metabolite could be measured in a Willamette silt loam soil both before the planting, during the growth and after harvest of a sweet corn crop. Alachlor was analyzed by gas chromatography and ESA was analyzed by a commercial ELISA kit after separation of the two compounds by solid phase extraction (SPE). The SPE and ELISA systems were evaluated for efficiency of recovery and day-to-day variability. Although satisfactory

recoveries were achieved with the SPE system, there was some amount of variability in quantifying ESA with the ELISA system. Measures taken to minimize variability included frequent calibration of the system and the inclusion of appropriate controls in each analysis. Alachlor levels were below the detection limit ($<100 \mu\text{g}/\text{kg}$ soil) two months after application, whereas ESA was detected throughout the soil profile (0-100 cm) at 13.7-61.2 $\mu\text{g}/\text{kg}$ between July and September. In December the level of ESA dropped substantially, and was below the detection limit in soil collected in April and June of the following year. A laboratory study provided evidence that the transformation of alachlor to ESA in Willamette soil was microbiologically mediated, but the question as to what extent plants, bacteria, or fungi carry out the transformation under field conditions remains unresolved.

Detection of the Herbicide Alachlor and its Sulfonic Acid Metabolite in Soil

by

Lesley A. Smith

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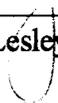
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Dean of Graduate School

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TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION.....	1
Problem Statement.....	1
Objectives.....	5
MATERIALS AND METHODS.....	6
Reagents.....	6
Methodology Overview.....	6
Soil Collection, Preparation and Extraction.....	8
Solid-Phase Extraction (SPE).....	9
Enzyme-Linked ImmunoSorbent Assay (ELISA).....	10
Gas Chromatographic Analysis of Alachlor.....	14
Laboratory Incubations.....	16
Confirmation of ESA in Soil by HPLC.....	17
RESULTS.....	18
Method Development: Calibration Curves.....	18
Recovery of Alachlor and ESA from Soil and Water.....	18
Analyses Conducted on Field Samples.....	20
Soil Incubations.....	27

TABLE OF CONTENTS (Continued)

	<u>Page</u>
DISCUSSION.....	29
Presence of ESA in Oregon Soil.....	29
Mass Balance.....	30
Strengths and Limitations of ELISA Kits.....	31
Microbiology of the Transformation of Alachlor to ESA.....	32
CONCLUSION.....	34
Results of the Study.....	34
Recommendations for Further Study.....	34
BIBLIOGRAPHY.....	35
APPENDICES.....	38
Appendix A. Using Softmax® Software.....	39
Appendix B. Quattro Pro Worksheet for an ELISA Calibration Curve.....	41

LIST OF FIGURES

	<u>Page</u>
Figure 1. Structures of Alachlor and ESA.....	2
Figure 2. Method Overview.....	7
Figure 3. Calibration Curves for Alachlor and ESA.....	12
Figure 4. Overview of ELISA Procedure.....	13
Figure 5. Distribution of ESA in Surface (0-20 cm) Soil During 1994-95.....	22
Figure 6. Distribution of ESA with Depth in 1994.....	23

LIST OF TABLES

	<u>Page</u>
Table 1. Distribution of Alachlor and ESA with Soil Depth in 1995 ($\mu\text{g}/\text{kg}$).....	24
Table 2. A Comparison of ESA Determinations by ELISA and HPLC.....	25
Table 3. Alachlor and ESA ($\mu\text{g}/\text{kg}$) in Soil Incubations.....	28

LIST OF APPENDICES

	<u>Page</u>
Appendix A. Using Softmax [®] Software.....	39
Appendix B. Quattro Pro Worksheet for an ELISA Calibration Curve.....	41

Detection of the Herbicide Alachlor and its Sulfonic Acid Metabolite in Soil

INTRODUCTION

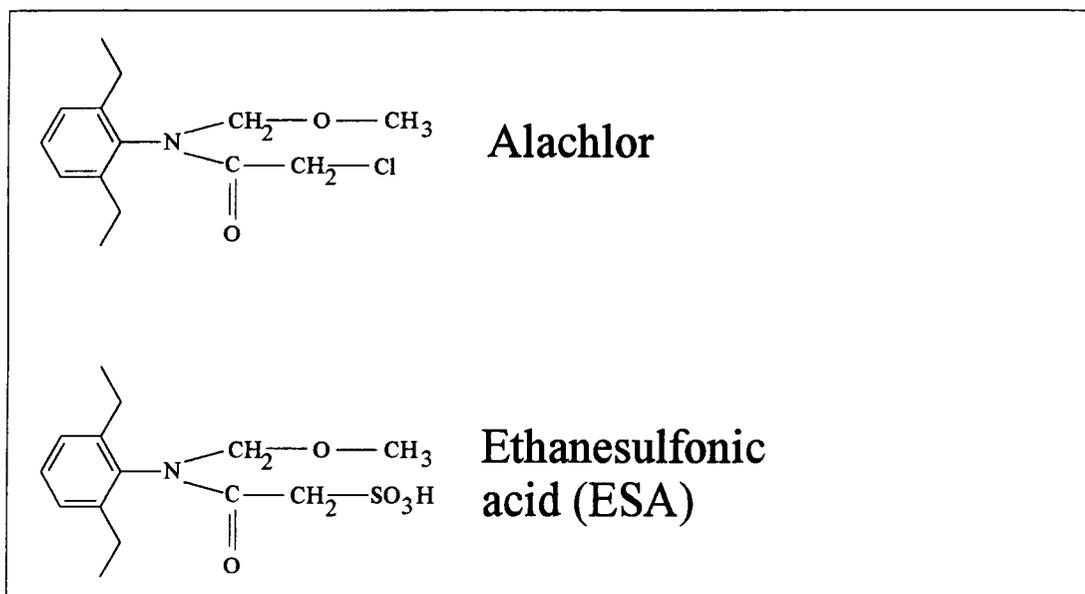
Problem Statement

The chloroacetanilide herbicides alachlor, metolachlor, and propachlor are widely used as broad-spectrum, pre-emergent herbicides to control weedy grass and broadleaf species (Sharp, 1988). Chloroacetanilides are used extensively on major crops such as corn and soybean. Alachlor [2-chloro-2,6-diethyl-N-(methoxymethyl)-acetamide] is manufactured by Monsanto Co. and is the active ingredient in the commercial products Lasso and Bronco. Alachlor is the second most widely used herbicide in the United States (~55000 lb. AI/YR, Herbicide Use in the United States, National Summary Report, 1991).

In general, the chloroacetanilides, alachlor in particular, are known to be rather persistent and slowly mineralized in soil (Novick and Alexander 1985). Alachlor is sparingly soluble in water and is considered a potential human carcinogen (National Library of Medicine, 1992). Alachlor is under increasing scrutiny because of its widespread use, low rate of mineralization and potential human carcinogenicity. However, the economic consequences of replacing the chloroacetanilide compounds with other herbicides makes it important that alachlor's fate and transport in soil and water be accurately described.

In recent tests of ground water in Indiana, Kentucky and Ohio, alachlor was detected by immunoassay at levels exceeding the drinking water standard of 2.0 µg/L

Figure 1. Structures of Alachlor and ESA.



(Baker et al., 1993). However, on further analysis, it was found that these samples did not contain alachlor, but rather a compound that cross-reacted with the antibodies that had been raised against alachlor. The compound was a metabolite of alachlor, (2-[(2,6-diethylphenyl) (methoxymethyl) amino]-2-oxoethane-sulfonic acid) or ESA (Figure 1). This metabolite is water soluble, non-mutagenic, does not bioconcentrate, yet is persistent and mobile in groundwater (Baker et al., 1993). Unlike alachlor, ESA is non-chlorinated and contains a sulfonic acid group in place of alachlor's chlorine group (Figure 1). Because routine screenings for alachlor in ground water are often carried out by immunoassay and because ESA cross-reacts in these assays, false detections of alachlor are possible (Feng et al., 1990).

The detection of alachlor metabolites such as ESA in environmental samples is important for several reasons. It indicates that alachlor can be transformed in the environment into other compounds. In particular, ESA is interesting because it contains a sulfonate moiety. Sulfonates result from conjugation reactions between electrophilic xenobiotic chemicals such as herbicides and naturally-occurring nucleophilic thiols. An example of such a nucleophilic thiol is the compound glutathione. Glutathione is found in plants, mammals, fish, insects, bacteria and fungi (Caldwell, 1986; James, 1986; Lamoreaux and Russness, 1981; Wilkinson, 1986) and plays a major role in plant resistance to herbicides (Sharp, 1988). Glutathione reacts with a variety of xenobiotics such as alkylhalides, triazine herbicides and organophosphorus insecticides through nucleophilic substitution reactions (Seutter-Berlage et al., 1979; Lamoueux and Bakke,

1984; Motoyama and Dauterman, 1978). These reactions give rise to an enormous variety of metabolites which are either excreted by the organism or further metabolized.

Evidence exists for the involvement of glutathione conjugation in the transformation of chloroacetanilides by soil microorganisms (Feng, 1991). Glutathione conjugation reactions with propachlor gave rise to as many as fourteen metabolites (Lamoureux and Rusness, 1989). The major soil metabolites of metolachlor include oxanilic, sulfinylacetic, and sulfonic acids (Feng, 1991). At the end of a nine-day study conducted by Feng, 27% of the acetochlor remained, 57% was converted into the oxanilic metabolite, and 14% was converted to a mixture of the sulfinylacetic and ethane sulfonic acid metabolites (Feng, 1991). In the case of alachlor, the potential to cause false positives for alachlor in immunoassays was displayed mainly by its ethane sulfonic acid metabolite (Baker et al., 1993; Aga et al., 1994). Although the oxanilic metabolite of alachlor and the chloroacetanilide herbicide metolachlor also are known to cross-react in immunoassays, cross-reactivity with the alachlor-produced antiserum occurs to a lesser extent than with alachlor (Feng et al., 1990; Aga et al., 1994).

The presence of ESA in soil indicates that either plants or microbes, or a combination of the two are involved in the degradation of alachlor. An investigation of the distribution of ESA in soil with depth and over the growing season should provide clues as to whether alachlor is being transformed and whether the degradation products remain associated with surface soil or are transported downward into the soil profile.

Alternatively, detection of ESA in the subsurface soil may indicate that alachlor can be transformed if it was to penetrate into the subsurface environments.

An enzyme linked immunosorbent assay (ELISA) can be used to detect ESA. This technique is used for screening pesticides in various media including water, soils and foods (Baker et al., 1993). Commercially produced ELISA kits are now available for a variety of pesticides including 2,4,-D, aldicarb, isproturon, paraquat and chlorpyrifos. ELISA kits have certain advantages over traditional analytical techniques in that they offer simplicity, high sensitivity, and low cost. Although the alachlor ELISA kit can be used to detect and quantify both alachlor and ESA, a method is required to separate alachlor and ESA (Aga et al., 1994).

Objectives

1. The first objective of this study was to evaluate the use of an ELISA for detecting the herbicide alachlor and its ESA metabolite in soil.
2. The second objective was to determine the distribution of alachlor and its ESA metabolite in a soil which had been planted to sweet corn at the N. Willamette Experiment Station and which had received an application of alachlor.

MATERIALS AND METHODS

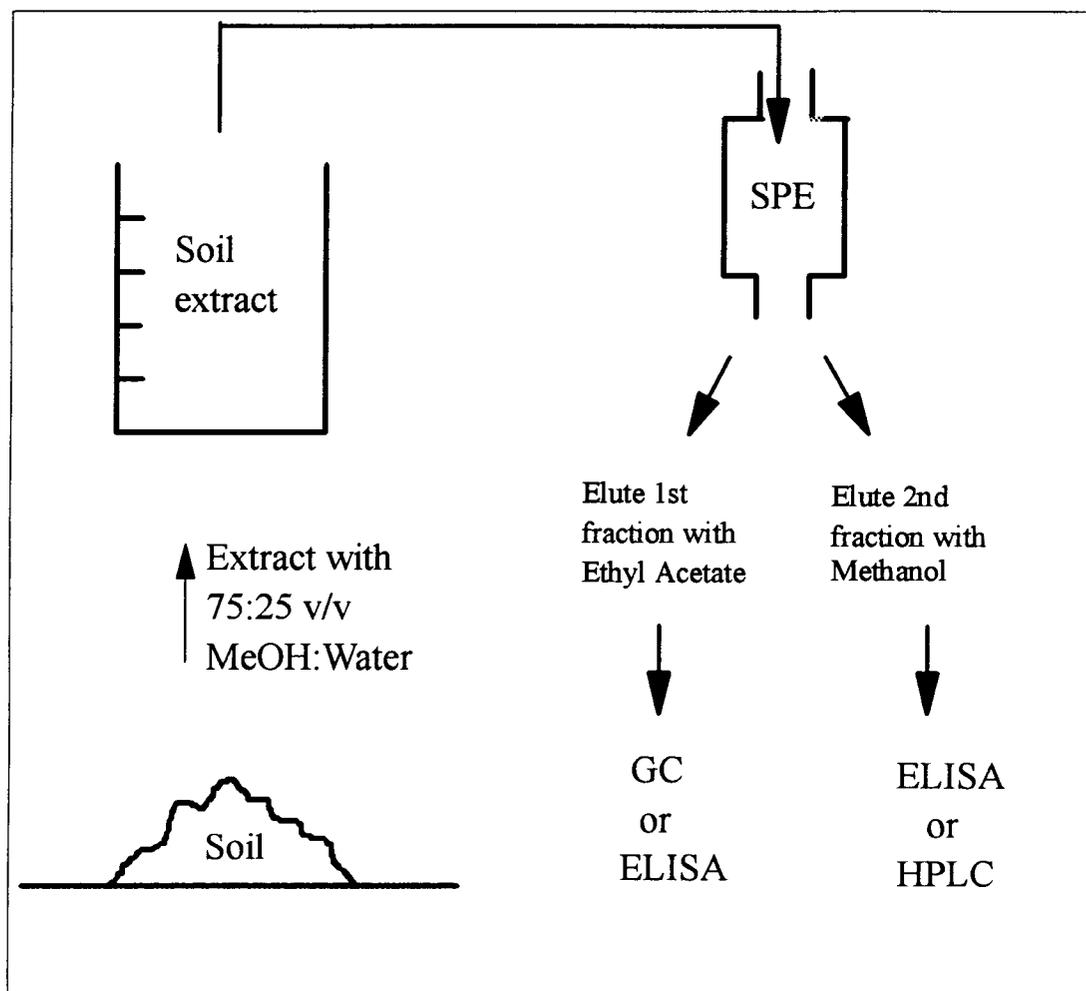
Reagents

Alachlor (99.0 % purity) was obtained from Chem Service Co. (West Chester, PA). An ESA standard was obtained from Dr. E. M. Thurman at the US Geological Survey (Lawrence, KS). An internal standard of 2-chlorolepidine was obtained from Aldrich Chem Co. (Milwaukee, WI). High Performance Liquid Chromatography (HPLC)-grade methanol and ethyl acetate were obtained from Oregon State University, Chemistry Stores (Supplier: EM Industries, Gibbstown, NJ). Sources of other materials are listed directly in the text.

Methodology Overview

A schematic representation of the analytical method used in this study is presented in Figure 2. Soil containing alachlor and ESA is extracted with a mixture of 75:25 vol/vol methanol and water (Mills and Thurman, 1992). The compounds are separated from the methanol/water solution using a solid phase extraction (SPE) cartridge containing 360 mg of C-18 bonded-phase silica (Aga et al., 1994). While both of the compounds bind to the SPE cartridge, alachlor and ESA are sequentially eluted with ethyl acetate and methanol, respectively. The ethyl acetate fraction is analyzed either by ELISA or gas chromatography (GC). The methanol fraction containing the ESA is analyzed either by HPLC or ELISA.

Figure 2. Method Overview.



Soil Collection, Preparation and Extraction.

Samples of a Willamette silt loam soil were collected from the North Willamette Experiment Station, Aurora, OR during the 1994 and 1995 growing seasons. Soil samples were collected in 1994 from plots containing either a sweet corn-winter fallow rotation or a sweet corn-winter rye cover crop rotation. Samples were collected from these plots in April, July, September, and December 1994 and in April and June 1995. Alachlor was applied to the plots at a rate of 1.5 kg/ha in May 1994, immediately before the corn was planted. The plots were irrigated by drip irrigation and received a total of 38 cm of water. In the summer of 1995, the above mentioned plots were not planted to corn and no alachlor was applied. In order to follow the fate and distribution of alachlor and ESA in the soil during a second year, soil was collected from a nearby field that had been planted to corn in May 1995 and to which alachlor was applied at a rate of 3.0 kg/ha, in May 1995 before the corn was planted.. Soil samples were collected from this field in June, July, and August 1995. The field was irrigated by an overhead sprinkler system and received a total of 28 cm of water.

Soil was sampled using a soil tube auger (2.5 cm in diameter) and cores were taken in 20 cm increments to a depth of 100 cm. Ten cores were taken per plot. All core sections from a given depth increment were combined, homogenized, placed in freezer bag, and stored at 4 °C. Before analysis, 500 gm sub-samples of soil were air-dried overnight, homogenized with a pestle on heavy-duty brown paper, and passed through a No. 25 size sieve (0.7 mm). Samples of the soil (2 gm) were placed in 50 mL polypropylene centrifuge tubes and brought up to field wetness (27% of soil dry weight)

with the addition of 0.54 mL of water. The samples were allowed to sit for 10 min to become uniformly wet before extraction. Extraction commenced with the addition of a mixture of 0.5 mL of water and 1.5 mL of methanol to each tube. The tubes were capped, vortexed and placed in a 70 °C water bath for 30 min. During this period, the tubes were monitored to prevent overheating and periodically vortexed to maintain sample homogeneity. The tubes then were placed on a mechanical shaker to cool for 5-10 min and centrifuged at 6,000 rpm for 10 min. Centrifugation was repeated if the supernatant appeared cloudy. The supernatant was decanted into a vial and reserved. Another 0.5 mL of water and 1.5 mL of methanol were added to the soil pellet and the procedure was repeated. The supernatant from the second centrifugation was combined with the first and the soil pellet was discarded. Because the supernatant contained a significant amount of soil solids, the particulates were allowed to settle for ~2 hrs until the supernatant was reasonably clear. The supernatant was pipetted into a second vial and more water added to the first vial, which was shaken and allowed to settle. This step was performed a total of three times. The supernatant and the additional water rinses were reduced under a stream of nitrogen at 45 °C to remove the methanol, and produce an aqueous extract suitable for separation by SPE.

Solid-Phase Extraction (SPE).

Thealachlor and ESA in soil extracts were removed from the soil extracts using Sep-pak[®] Plus cartridges purchased from Waters/Millipore[®]. The cartridges were filled with 360 mg of 40- μ m C-18 bonded-phase silica. Polypropylene reservoirs (75 mL) were

attached to the top of the cartridges and the entire assembly was placed on a vacuum manifold (Supelco, Bellefonte, PA). Cartridges were sequentially preconditioned by rinsing with 2 mL of methanol, 6 mL of ethyl acetate, 2 mL of methanol and 2 mL of water. *Note:* At no point in the preconditioning process was the cartridge allowed to go dry. The soil extract was added to the cartridge and the sample vial rinsed with 10 mL of water that also was added to the cartridge. The sample was drawn through the cartridge under full vacuum (25 mm Hg). Once the sample had passed through the cartridge, the vacuum was turned off and a collection vial was placed under the cartridge. Alachlor was collected by adding 3 mL of ethyl acetate to the reservoir, drawing a small amount into the cartridge, allowing it to soak for about 1 min., before drawing the rest of the 3 mL through. The eluate was collected in a vial placed below the cartridge. The vacuum was turned off and the collection vial removed and replaced with a fresh one. The ESA was collected in a manner similar to that used for alachlor, except that methanol was used as the solvent instead of ethyl acetate.

Enzyme-Linked ImmunoSorbent Assay (ELISA).

The methanol fraction containing the ESA is blown down to dryness at 70 °C, reconstituted by the addition of 1 mL of water, and analyzed by ELISA. The assay is conducted using a commercial kit from Waters/Millipore, the Envirogard alachlor Plate Kit®. Although the kit is designed specifically for alachlor, ESA also binds to antibodies raised against alachlor. However, the alachlor kit can be used to detect ESA provided that alachlor and ESA are separated prior to analysis. The ELISA system consisted of a

96-well microtiter plate to which antibodies raised against alachlor have been bound. The microplate is divided into eight removable strips of 12 wells each. The reagents included in the kit are the alachlor-enzyme conjugate, the enzyme horseradish peroxidase, the substrate for horseradish peroxidase, and a dye that turns the product of the enzyme and substrate blue. Also included is a solution of 2M sulfuric acid, which is used to stop the reaction between the dye and the enzyme-substrate product.

Samples containing different concentrations of alachlor are added to each of the wells. Immediately following, a fixed concentration of alachlor-enzyme conjugate (a molecule of alachlor to which an enzyme has been covalently bound), is added to the wells in the same order as that of the samples. Both alachlor and the alachlor-enzyme conjugate compete for sites on the antibody. The amount of enzyme-conjugate bound is inversely proportional to the amount of alachlor or ESA in the sample. After the plates are incubated for one hour, they are rinsed five times under gently running distilled water to remove unbound sample and enzyme conjugate. A substrate of the enzyme conjugate is added along with a dye that forms a blue product. The higher the concentration of alachlor in the original sample, the less enzyme conjugate binds to the antibody and the lower the intensity of color produced. Conversely, if the sample contains little alachlor, more enzyme conjugate is bound to the antibody, so that more substrate is attacked and hence more color is produced by reaction with the dye. The amount of color produced therefore is inversely proportional to the concentration of alachlor in the sample (See Figures 3 and 4).

Figure 3. Calibration Curves for Alachlor and ESA.

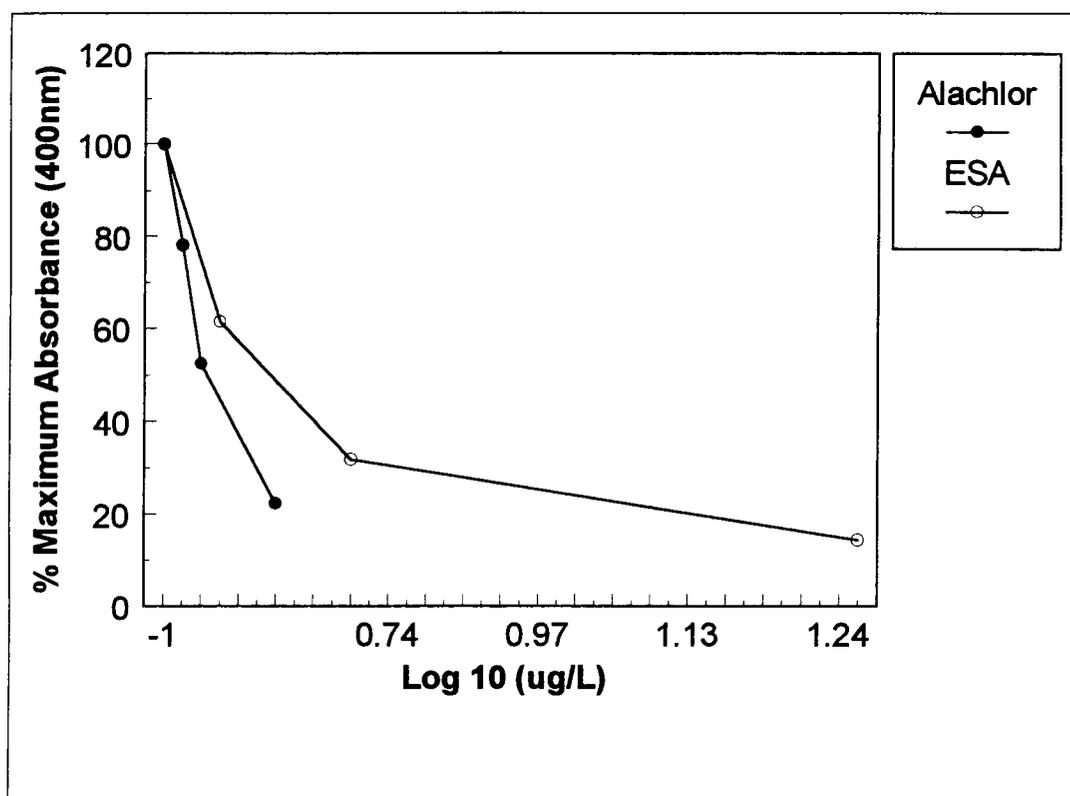
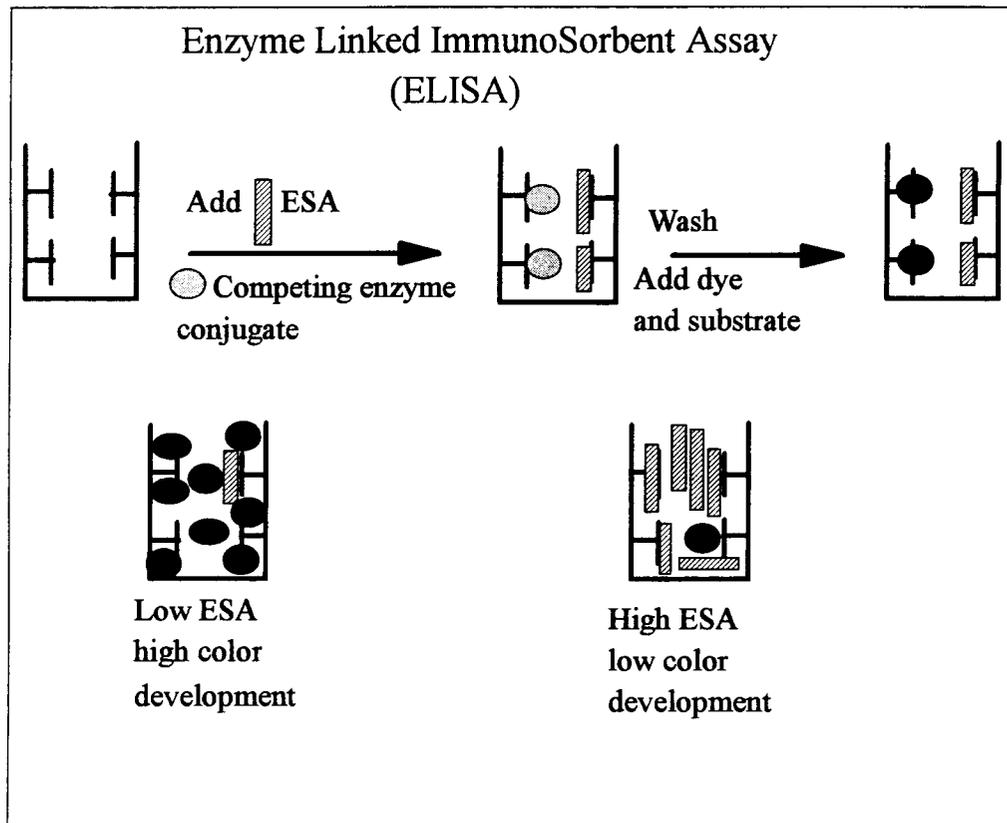


Figure 4. Overview of ELISA Procedure.



The ELISA kit contained pure water and three concentrations of alachlor, 0.1 µg/L, 0.5 µg/L and 2.5 µg/L. When the kit was used to analyze ESA, three ESA standards were prepared at concentrations of 0.9, 4.5, and 18.0 µg/L. Immunoassays were conducted following the directions provided in the kit: 80 µl aliquots of sample were placed in each well followed by 80 µl of alachlor-enzyme conjugate. The plate was shaken gently by hand, covered with parafilm[®], and allowed to incubate at room temperature for one hour with periodic shaking. After an hour, the wells were rinsed five times under gently flowing distilled water and all the water was shaken out after the final rinse. Substrate (80 µl) was added to each well followed by 40 µl of dye. The wells were shaken, covered with parafilm, and incubated for one-half hour. Stop solution (40 µl) was added to each of the wells which were shaken thoroughly. The plate was then read using a Titertek Multiscan Plus[®] plate reader and Softmax[®] software. (See Appendix A for details of software manipulation, and Appendix B for the spreadsheet used to convert absorbance readings to concentrations with the calibration curve.)

Gas Chromatographic Analysis of Alachlor.

The ethyl acetate fraction containing alachlor contained some residual water that remained after cartridge pre-conditioning. The ethyl acetate (top) was separated from the water (bottom) by pipette. More ethyl acetate was added to the water and the vial was shaken and allowed to settle after which the ethyl acetate was removed. This step was conducted a total of three times. The ethyl acetate fractions were combined and reduced to about 0.5 mL at 45 °C under a stream of nitrogen. Samples were placed in 1-mL

autosampler vials and spiked with 1.17 μg of an internal standard, 2-chlorolepidine. The samples were analyzed using a Hewlett Packard Model 5890 Series II gas chromatograph equipped with a Alltech Econocap SE-54 column (30 m x 0.25 mm x 0.25 μm film thickness). The samples were run using a standard EPA-approved method for chlorinated pesticide separation by GC-ECD (EPA Method #8080), with some customization which depended on the instrument conditions available and purity of gases used. Details are listed herein. The initial oven temperature of 140 $^{\circ}\text{C}$ was held for 1 min and then increased at 25 $^{\circ}\text{C} / \text{min.}$ to 190 $^{\circ}\text{C}$, followed by a 10 $^{\circ}\text{C} / \text{min.}$ ramp to 205 $^{\circ}\text{C}$ where it was held for 3 min. This was followed by a final ramp of 20 $^{\circ}\text{C} / \text{min.}$ to a final temp of 300 $^{\circ}\text{C}$ where it was held for 1 min. An injection volume of 1 μl was used under splitless conditions with an injector temperature of 280 $^{\circ}\text{C}$ and helium was used as the carrier gas. The ^{63}Ni electron capture detector was operated at a temperature of 300 $^{\circ}\text{C}$. The retention time of the internal standard is 4.38 min and the retention time of alachlor is 7.0 min.

A calibration curve was constructed from 5 calibration standards of alachlor. Each calibration standard was spiked with 1.17 μg of internal standard. The calibration curve was created by comparing the ratio of the amounts of alachlor and the internal standard to the peak area ratio of alachlor and the internal standard. Because the amount of internal standard added to every sample is fixed, the amount of alachlor in any given sample can be determined from the peak area ratio of alachlor to the internal standard.

Laboratory Incubations.

To determine whether the transformation of alachlor to ESA was biologically mediated and to monitor the disappearance of alachlor and the appearance of ESA over time, a laboratory experiment was conducted. The experiment involved incubating soil with and without alachlor and also included a sterile soil control to which alachlor was added. The incubations were conducted over a period of four weeks with samples sacrificed at 7 day intervals. Soil was collected in July 1995 from plots at the North Willamette Experiment Station that had been treated with alachlor during the spring of the previous year. The soil contained no residual alachlor and a low level (12 $\mu\text{g}/\text{kg}$ soil) of ESA. Ten cores of soil were collected to a depth of 0-20 cm from each of four replicate plots of a rye cover crop experiment. The cores were homogenized and 500 gms were air-dried overnight and passed through a 0.7 mm sieve. Five tubes, each containing two grams of soil, were set up for each time point in the experiment. Of the five tubes, three contained non-sterile soil and 9.72 μg alachlor/gm of soil, one tube contained sterile soil and the same amount of alachlor, and one contained non-sterile soil without alachlor. Alachlor was applied to the soil by incorporation into the water that was used to bring the soil to 27% (wt/wt) water content. This aqueous solution of alachlor was placed in the bottom of the test tubes followed by the air dried soil. Sterile soil was prepared by autoclaving 150 gms of soil for one hour. Sub-samples (2gms) of soil were dispensed into the tubes using sterile technique. At weekly intervals, for four weeks, the five tubes were extracted and analyzed as described previously.

Confirmation of ESA in Soil by HPLC.

A select number of samples analyzed by ELISA were analyzed by HPLC by Dr. D. Aga at the USGS in Lawrence, KS. The method is described in Aga et al.(1994). In brief, the methanol fraction from SPE cartridges was reduced under a stream of nitrogen, and dissolved in 200 μ L of 20:80 MeOH:Sodium Phosphate buffer (pH 7.0). Aliquots (100 μ L) were placed in autosampler vials and analyzed with a HP Model 1090 series II liquid chromatograph with a photodiode-array detector (Hewlett Packard). The HPLC was equipped with a 2.1 mm X 100.0 mm narrow-bore, reverse-phase column, packed with 5- μ m Hypersil ODS (Hewlett Packard). The mobile phase consisted of 38.5% HPLC-grade methanol and 61.5% 10 mM Na_2HPO_4 (pH 7.0 buffer) that was prepared in Nanopure water. The flow rate of the mobile phase was 1.2 mL/min. The sample injection volume was 90 μ L. The elution of ESA was monitored at a wavelength of 200 nm with a 4-nm bandwidth. The reference wavelength was set at 450 nm with a 80-nm bandwidth. To confirm the identity of the ESA, the peaks were scanned from 190 to 400 nm and the spectra were matched with standard spectra in an automated library search.

RESULTS

Method Development: Calibration Curves.

A calibration curve was constructed in accordance with the manufacturer's specifications using the threealachlor standards provided (Figure 3). Day-to-day variability in the assay could be accounted for if optical density (OD) values were divided by the OD of a water blank run concurrently with the samples. The OD of the samples and the standards were thus expressed as % maximum absorbance. A new calibration curve was prepared whenever a new ELISA kit was opened. Additionally, one standard from the calibration curve was run during each analysis to ascertain that the sensitivity of the kit fell within acceptable limits. The relative standard deviation of replicate standard analyses was 8%. The %RSD was determined by taking the mean of the % maximum absorbance of all the standards and dividing by the standard deviation of the mean. The ELISA kits are more sensitive toalachlor than to ESA such that 3.5 times more ESA thanalachlor is required to generate the same ELISA response. A similar phenomenon was reported by Aga et al. (1994), where 0.06 $\mu\text{g/L}$ ofalachlor and 0.12 $\mu\text{g/L}$ of ESA gave the same response.

Recovery of Alachlor and ESA from Soil and Water.

The average recovery ofalachlor (0.9 $\mu\text{g/L}$) and ESA (9.0 $\mu\text{g/L}$) for three replicate samples was 87 +/- 20% and 100% +/- 15%, respectively. In addition, the efficiency of recovery of different mixtures ofalachlor and ESA was examined. A mixture containingalachlor (0.9 $\mu\text{g/L}$) and ESA (9.0 $\mu\text{g/L}$) representing the middle of the

respective calibration curves were effectively recovered (130% and 128%, respectively). The extraction efficiency of SPE depended upon the ratio of sample volume to the mass of sorbent in the SPE cartridge and elution efficiency depended on the volume of solvent applied to the cartridge (Jennifer Field, Personal Communication). A single cartridge rinse of ethyl acetate (3 mL) followed by methanol (3 mL) produced good separation of alachlor and ESA in most cases (Aga et al., 1994). In our study, situations arose where the separation of alachlor and ESA was not as good when for samples contained a large amount of alachlor relative to ESA. An experiment was conducted in this laboratory to determine exactly how much alachlor remained associated with the C-18 cartridge after it was rinsed with 3 mL of ethyl acetate. The amount of alachlor that remained associated with the cartridge depended on the sample matrix. When the experiment was conducted using water samples, between 0.06% and 1.0% of the alachlor added to the cartridge remained associated with the cartridge from samples containing between 100 and 300 µg/L of alachlor. The same experiment conducted on soil samples showed that between 2 and 3% was left behind on the cartridge from soil samples containing between 100 and 300 µg/kg of alachlor. Therefore, it is possible for this residual alachlor on the SPE to elute with methanol and result in an overestimation of ESA. This limitation of the method is considered when field levels of ESA are discussed in a later section.

To ensure that it was possible to recover ESA from soil, single samples of ESA was spiked into three soils containing different amounts of clay and organic matter. The soils included a Bashaw clay loam (54 % clay, pH 7.8), a Willamette silt loam (15% clay, pH 6.2) and a Quincy loamy sand (7 % clay, pH 6.8). The amounts of ESA recovered

were 35, 100, and 72% from the Bashaw, Willamette, and Quincy soils, respectively. Low ESA recovery from Bashaw could be due to the high organic matter and clay content of the Bashaw soil.

Alachlor was not effectively detected in the ethyl acetate fraction of these soil samples using an ELISA. It is possible that soil organic matter eluted in the ethyl acetate fraction along with alachlor and interfered with the binding of the antibody to alachlor. This phenomenon was observed in our laboratory and by Dr. Aga at the USGS in Lawrence, KS. On the basis of these findings, alachlor was measured in the ethyl acetate fraction by gas chromatography (GC). In contrast, organic matter did not seem to cause any interference in the ELISA of methanol fractions, presumably because the organic matter was removed primarily by the ethyl acetate rinse.

Analyses Conducted on Field Samples.

Although alachlor was applied to the plots at the field site on May 26, 1994 at a rate of 1.5 kg/ha, by July 12 it was below the limit of detection by GC (below 100 µg/kg) in soil samples from all depths (0-100 cm). This site was also sampled in September and December 1994 and in April and June 1995, and alachlor concentrations in the soil during these months remained below detection by GC at all depths. Alachlor was not applied to these plots in 1995. In contrast, ESA was detected in July 1994 in surface soil samples (0-20 cm) collected from four different replicate plot areas at the field site. In July and September 1994, the ESA concentration in surface soil (0-20 cm) was 61.4 and 80.4

$\mu\text{g}/\text{kg}$, respectively. By December 1994, ESA concentrations had dropped to $22.3 \mu\text{g}/\text{kg}$. In April and June 1995, they were 12.0 and $9.4 \mu\text{g}/\text{kg}$, respectively. (Figure 5).

When soil samples were examined to a depth of 100 cm, it was found that ESA distribution patterns in July and September 1994 were similar (Figure 6). The ESA concentration in these months at depths of 20-100 cm ranged from 19.0 to $85.0 \mu\text{g}/\text{kg}$ respectively. However, in December 1994, the amount of ESA in the soil profile (20-100 cm) dropped to between 2.8 and $8.2 \mu\text{g}/\text{kg}$, and remained between 0.54 and $8.9 \mu\text{g}/\text{kg}$ in April 1995.

To determine whether the distribution patterns seen in 1994 would be repeated in 1995, soil samples were collected from an area of the station which had been treated with alachlor on May 26, 1995 at a rate of $3.0 \text{ kg}/\text{ha}$ and had been planted to corn. Surface soil was collected on June 7 (0-1cm, 0-5 cm.) and July 19 (0-1cm, 0-5 cm, 0-20 cm). Although alachlor was detected in June samples, by July the levels had dropped (Table 1). In surface samples (0-1, 0-20 cm), ESA was detected in July 1995 by ELISA and was confirmed by HPLC. (Table 2). In general, the concentrations of ESA obtained by ELISA were less than those obtained by HPLC. However, the concentration of ESA obtained by HPLC in the 0-1cm soil ($125 \mu\text{g}/\text{kg}$) was lower than the concentration obtained by ELISA ($427 \mu\text{g}/\text{kg}$). Such a difference between ESA concentrations determined by HPLC and ELISA also was observed by Dr. Aga for surface soil samples (1-5 cm) (Dr. D. Aga, USGS, Lawrence, KS, Personal communication, July 1995). The overestimation of ESA concentrations by ELISA is not due to alachlor contamination of the methanol fraction

Figure 5. Distribution of ESA in surface (0-20 cm) soil during 1994-95.

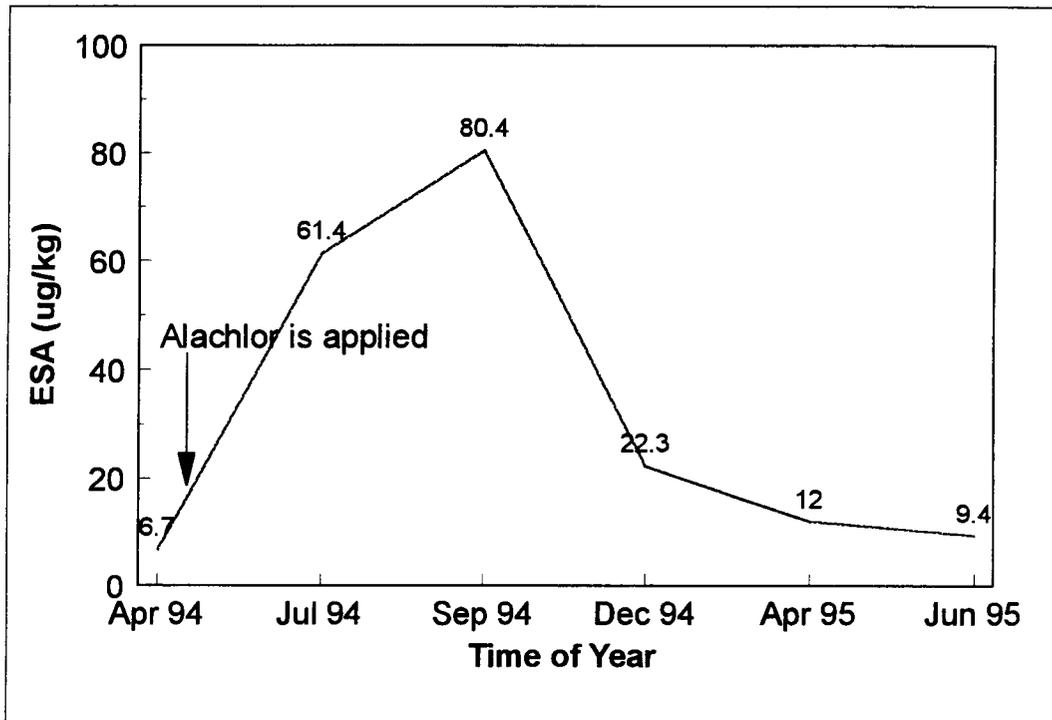


Figure 6. Distribution of ESA with Depth in 1994.

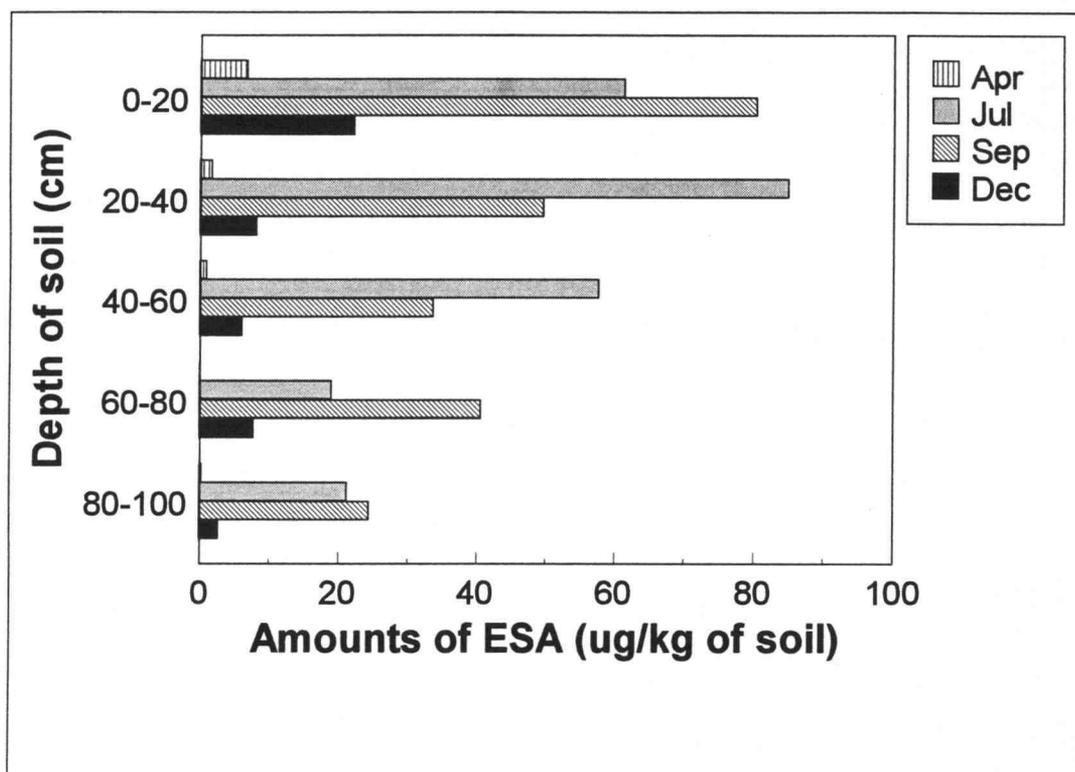


Table 1. Distribution of Alachlor and ESA with Soil Depth in 1995 (ug/kg).

Depth (cm)	June		July	
	Ala	ESA	Ala	ESA
0-1	3,800	438*	233	427
0-5	2,800	240*	114	229*
0-20			165	83

* ESA concentrations not confirmed by HPLC.

Table 2. A Comparison of ESA Determinations by ELISA and HPLC

Year and soil depth (cm)	ESA by HPLC ($\mu\text{g}/\text{kg}$)	ESA by ELISA ($\mu\text{g}/\text{kg}$)
July 95 (0-1)	125	427
July 95 (0-20)	82	83
July 94 (20-40)	110	85
July 94 (40-60)	92	57
July 94 (60-80)	73	19
July 94 (80-100)	71	21

because the amount of alachlor present in the July 1994 soil is too low. It is possible that other metabolites of alachlor are present in the soil that cross react with the ELISA.

In order to compare the distribution of alachlor and ESA with depth, a profile (0-100 cm) from the plot described above was taken in August 1995. While alachlor remained below detection at all depths, ESA levels in 0-20 cm were comparable to the previous year's concentrations (83 $\mu\text{g}/\text{kg}$). In contrast to 1994, ESA was not detected in 1995 below 20 cm in the soil profile.

The %RSD of alachlor and ESA recovery from field soil collected from the North Willamette Experiment Station was determined on four replicate samples of soil from the same plot that were analyzed on the same day. The %RSD for alachlor was 19.5% and for ESA, 4.7%. Based on my evidence that some alachlor might elute from the SPE in the methanol fraction, the possibility exists that some of the ESA detected by ELISA is due to the presence of alachlor in the methanol (ESA) fraction. However, the amount of alachlor in all 1994 soil samples was below 100 $\mu\text{g}/\text{kg}$, so approximately 3 $\mu\text{g}/\text{kg}$ of alachlor could be expected to be present in the methanol (ESA) fraction. Furthermore, because the ELISA is 3.5 times more sensitive to alachlor than to ESA, 3 $\mu\text{g}/\text{kg}$ of alachlor would evoke the same response as approximately 10 $\mu\text{g}/\text{kg}$ of ESA. Therefore, to conservatively estimate the amount of ESA in the soil samples, 10 $\mu\text{g}/\text{kg}$ should be subtracted from all ESA values determined by ELISA.

Soil Incubations.

Laboratory incubations confirmed the biological transformation of alachlor to ESA in soil. At the end of a four week experiment, 80 $\mu\text{g}/\text{kg}$ of ESA was detected by HPLC in non-sterile soil samples incubated with 9,720 $\mu\text{g}/\text{kg}$ of alachlor (Table 3). This amount of ESA (80 $\mu\text{g}/\text{kg}$) represents 0.8% of the total amount of alachlor added. No ESA was detected in sterile soil samples supplemented with alachlor, nor was any detected in non-sterile soil that was not supplemented with alachlor. The amount of alachlor in the non-sterile sample decreased by 3,889 $\mu\text{g}/\text{kg}$ during the course of the experiment, while the amount of alachlor in the sterile control decreased by 1,831 $\mu\text{g}/\text{kg}$. The loss in the sterile control could be due to irreversible sorption to soil, abiotic (physical/chemical) transformation, or to the possibility that the soil was no longer sterile and microbial transformation occurred.

Table 3. Alachlor and ESA ($\mu\text{g}/\text{kg}$) in Soil Incubations.

Analyte ($\mu\text{g}/\text{kg}$)	Incubation Time	Sample (+Ala)	Sterile (+Ala)	Control (-Ala)
Alachlor	0	5,360	5,170	<LLD
	1	3,712	4,649	<LLD
	4	1,471	3,339	<LLD
ESA	0	---		
	1	---		
	4	83	<LLD	<LLD

Note: LLD for Alachlor = 100 $\mu\text{g}/\text{kg}$, ESA = 0.1 $\mu\text{g}/\text{L}$.

DISCUSSION

Presence of ESA in Oregon Soil.

Because ESA is water soluble, its movement in soil should be influenced by water content and flow. The fact that ESA was distributed down to a depth of 100 cm in a field in the Willamette valley in the summer suggests that irrigation moved the metabolite into the profile. These plots were irrigated using drip irrigation and supposedly received a total of 38 cm of water. On the other hand, the concentration of ESA was substantially lower in the profile in December 1994, suggesting that ESA had been further leached during the winter rainy season. Alternatively, ESA could have been further metabolized to another compound either unreactive or less reactive in the ELISA. In the summer of 1995, ESA was found in 0-20 cm soil but was not distributed through the profile below 20cm as in summer 1994. In 1995, the plots were irrigated using overhead sprinklers, and received a total of 28 cm of water. The differences in ESA distribution between the two years could be due to spatial and temporal variation during sampling, the method in which the plots were irrigated (drip in 1994 vs. overhead in 1995) or to the total amount of water applied each year (38 cm in 1994 vs. 28 cm in 1995). Further studies are required to determine the effect that irrigation has upon the fate of alachlor in agricultural areas devoted to sweet corn production.

Mass Balance.

The total amount of any compound present in a system at any given time depends on a combination of the sources and sinks for the compound. Many natural systems including plants, animals, fungi and bacteria are capable of converting xenobiotic compounds to sulfonated metabolites (Lamoureux et al., 1993; Feng, 1991; Sharp, 1988). The sinks for ESA include plant uptake, further chemical or biological degradation and leaching to groundwater. Under the field conditions of this study, more ESA was found in 1994 than was found in 1995 (25% vs 2.8 % of the amount of alachlor applied to the field). In the summer of 1994, the amount of irrigation may have been sufficient to cause a downward movement of ESA through the soil profile, thereby escaping further transformation. In the summer of 1995, ESA remained in the surface layer of soil where it was susceptible to further biological attack by bacteria and fungi. Results from laboratory incubations using a closed system (no leaching) indicated that alachlor is biologically transformed in soil and that the amount of ESA formed is about 1% of the amount of alachlor added to the system. However, alachlor had disappeared from the soil in all field situations in this study. Both in 1994 and 1995, alachlor was below detection within two months of application in the field, and the levels of alachlor in laboratory incubations had dropped within four weeks to 25% of the original amount added. The fate of the remainder of the alachlor needs to be resolved. This point is worthy of note in the case of alachlor because it is a toxic herbicide used in major crops such as corn and soybean and the persistence of herbicides is particularly important in crops used for food production.

Strengths and Limitations of ELISA Kits.

The use of commercially-produced ELISA kits is becoming increasingly popular not only in the disciplines of biology and chemistry but also in those of engineering and physics (Baker et al., 1993). Such pesticide screening kits are available from several manufacturers (e.g., Waters/Millipore, Ohmicron) for products such as isoproturon, atrazine and simazine. The kits are very sensitive and concentrations as low as 0.1 $\mu\text{g/L}$ can be detected. They are usually calibrated to detect the substance of interest at concentrations that meet USEPA levels for drinking water. For example, the range of the Waters/Millipore kit is 0.1 to 2.5 $\mu\text{g/L}$ for alachlor, and the USEPA drinking water standard for alachlor is 2.0 $\mu\text{g/L}$ (Aga et al, 1994). Although in most cases the kits are very specific, false positives are possible when the kit is used with a soil matrix or with water samples containing a large amount of particulate matter. False positives possibly are due to non-specific reactions with soil organic matter. Furthermore, cross reactions with metabolites of the compound in question and with other pesticides are possible. For instance, the oxanilic acid metabolite of alachlor and another chloroacetanilide herbicide, metolachlor, also produced a response in an alachlor ELISA, but to a lesser extent (Aga et al, 1994). An example of a cross reaction with other pesticides was found at the former site of a grain storage warehouse (Powell, 1995, Washington State University, Personal communication). The area was screened using an alachlor ELISA kit and positive responses for alachlor were found. Upon further analysis, it was found that the positive responses were not due to alachlor, but rather to the insecticides aldrin and dieldrin. Such cross reactivities can be used to one's advantage as in the case of the current study where

antibodies to alachlor cross react with ESA. When the kit is coupled with other separation techniques such as SPE the risk of cross reactions and false positives decreases. However, the use of the ELISA coupled with SPE as advocated by Aga et al., (1994) is not possible in all situations and should be treated with caution. Results from this study showed that at high alachlor concentrations, separation of alachlor and ESA by SPE is incomplete.

Because the ELISA cannot differentiate between the two compounds, incomplete separation can give rise to artificially elevated levels of ESA and artificially lower concentrations of alachlor. It is possible to use the ELISA coupled with SPE in the analysis of water samples because herbicide concentrations in water are potentially lower than those in soil. If ELISA is to be used for soil analyses, care should be taken to confirm ESA levels by other analytical techniques such as HPLC. Further, it is recommended that surface soils be analyzed for alachlor by GC rather than ELISA, because surface soils usually contain a significant amount of organic matter that can bind in a non-specific manner to the ELISA.

Microbiology of the Transformation of Alachlor to ESA.

The presence of a metabolite of alachlor indicates that although alachlor is poorly mineralized (converted to CO₂), it can be transformed to other less toxic by-products. This study has shown that under laboratory conditions, organisms in the Willamette soil have the potential to biologically transform alachlor to ESA. Plants (Lamoureux and Rusness, 1989), fungi (Levanon, 1993; Pothuluri et al., 1990) and soil bacteria (Zablotowicz et al., 1995) have been shown to transform alachlor into different products by different

mechanisms. For example, alachlor is transformed in fungi by the hydroxylation of the aryloethyl side chain, (Pothuluri et al., 1993), whereas in plants and bacteria, alachlor is transformed by the enzyme glutathione-S-transferase resulting in metabolites containing sulfur atoms (Lamoureux and Rusness, 1989, Zablotowicz et al., 1995). However, the relative contributions of plants, fungi and bacteria to the transformation of alachlor under field conditions has yet to be elucidated.

CONCLUSION

Results of the Study.

In conclusion, I found that commercial ELISA kits can be used to detect the herbicide alachlor and its sulfonic acid metabolite in soil. However, frequent calibration of the system and confirmation of ELISA results by HPLC is recommended.

Additionally, I found that the herbicide alachlor is not persistent in Willamette silt loam because alachlor levels dropped below the detection limit ($<100 \mu\text{g/kg}$ soil) two months after application in May whereas the sulfonic acid metabolite of alachlor, (ESA) was detected throughout the soil profile (0-100 cm) at 13.7-61.2 $\mu\text{g/kg}$ between July and September. In December the level of ESA dropped substantially and was below detection in soil recovered in April and June of the following year. A laboratory study provided evidence that the transformation of alachlor to ESA in this soil was biologically mediated, but the question as to what extent plants, bacteria or fungi carry out the transformation under field conditions remains unresolved.

Recommendations for Further Study.

1. A study of groundwater samples using lysimeters under the plots at the North Willamette Experiment Station is recommended.
2. A study of the relative contributions of plants, bacteria, and fungi on the transformation of alachlor is recommended.

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APPENDICES

APPENDIX A. Using Softmax[®] Software

1. Write up a template on a sheet of paper detailing which wells contain which samples, including the locations of the water and the standards. Randomize the placement of these every time you run a strip.
2. Turn on the computer and plate reader (switch on back of reader). Allow to warm up for at least 10 mins but do not leave on.
3. Insert 450 nm filter (green).
4. Open hard drive of computer (called "The World" or "Bad Duck")
5. Open the folder titled "ELISA (OLD)". (This has a frothing beer stein on the cover.)
6. The logo for Softmax[®] Software should appear. Go to *Template* and choose *Enter / Edit template*. Enter the contents of the wells and from the same menu, choose *Define segments*. Click and drag to encircle your strips. If you are reading an entire plate, you don't need to do this, but for a few strips, it keeps the machine from reading the entire strip and subtracting the OD of the empty wells from your wells. If it does this, you can sometimes have negative OD's. Save your template to a floppy disk choosing *Save Template and Desktop*.
7. When you are ready to read a plate, place plate on rack and push down firmly to make sure it is properly inserted. Then go to *Plate* and choose *Read plate*. On the first screen under this option you may enter pertinent plate information, but this is not mandatory. Choose *OK* when done. The next screen gives you a choice of types of runs, Kinetic or Endpoint, choose *Endpoint* with a 450 nm filter. Then choose *Read plate*.

8. Once the plate is read, the OD readings will automatically be displayed on your template on the screen. Record them in your lab book on your paper template. Save data on disk choosing *Save Plate and template* and *Desktop*.

9. The OD readings can now be entered into a Quattro Pro spreadsheet and the concentrations determined.

APPENDIX B. Quattro Pro Worksheet for an ESA Calibration Curve.

1. Enter OD's for samples, standards, and the water blank. OD for the water blank should be the maximum absorbance, i.e., the maximum OD.
2. Divide all OD's by the OD of the water blank and multiply by 100. This will convert them into a % of maximum absorbance.
3. Make a list of the concentrations of your standards and take their logarithms.
4. To create a calibration curve, choose *Tools / Numeric Tools / Regression*.
5. Independent variables are the % Max absorbance of your standards, dependent variables are the log concentrations of your standards. Specify where you want your output.
6. From the Output, *Constant* is "*b*" from the equation of a straight line: $y = mx + b$ and *X Coefficient* is "*m*". Log concentration or "*x*" = $(y-b)/m$, where "*y*" is % Max absorbance for each sample. Take the antilog of all "*x*'s" to get concentrations.