COMPARISON OF ENZYME-LINKED IMMUNOASSAY WITH GAS CHROMATOGRAPHY/MASS SPECTROMETRY FOR ANALYSIS OF THE COTTON HERBICIDE FLUOMETURON

***K. Chad Bastian, *E.M. Thurman, and **Richard A. Rebich**
***U.S. Geological Survey, WRD, Lawrence, Kansas**
****U.S. Geological Survey, WRD, Pearl, Mississippi**

INTRODUCTION

Fluometuron is a phenylurea herbicide used both as a pre- and post-emergence herbicide for broadleaf and grass control in cotton. Application of fluometuron in the United States totaled 1.5 X 10^6 kg (kilograms) (3.3 million pounds) in 1996 at an average rate of approximately 0.81 kg of active ingredient (ai) per hectare (kg per ha) (0.72 pounds ai per acre) (National Agricultural Statistics Service 1997). Although the average field half-life of fluometuron is relatively long (90 days), substantial photodegradation can occur, depending upon application rate and weather conditions. Fluometuron's solubility in water is approximately 100 mg/L (milligrams per liter); its octanol-water partition coefficient (K_{ow}) is approximately 240 (Ahrens 1994). Surface-water fluometuron concentrations in cotton-growing areas have been reported in the sub-part-per-billion to part-per-billion range (Pereira and Hostettler 1993; Coupe et al. 1998).

Cotton is a crop of major economic importance in the Mississippi Alluvial Plain, commonly referred to as the Delta. The Delta includes parts of Arkansas, Louisiana, Mississippi, and Missouri. Cotton matures slowly and is very susceptible to yield loss from competing weeds. Therefore, through a combination of mechanical and chemical control, cotton is kept as weed-free as possible throughout its growing period. Indeed, unlike corn and soybeans, which receive only one or two applications of herbicides at the beginning of the growing season, cotton may need herbicide applications for up to 12 weeks after planting. Widespread use of cotton herbicides in the Delta has raised concerns regarding offsite movement of these compounds, especially for those such as fluometuron that are relatively long lived and are fairly water soluble.

The U.S. Geological Survey (USGS), in cooperation with the Mississippi Department of Environmental Quality, began operating a streamflow and water-quality sampling network in the fall of 1995 as part of the Mississippi Delta Management Systems Evaluation Areas (MDMSEA) project (Rebich 1997). The determination of concentrations and loads of herbicides in runoff are a major component of the work of the USGS as part of the MDMSEA project. Fluometuron is a primary herbicide of interest in the MDMSEA project because of its use by the participating farmers. Not only do all of the participating farmers use this particular compound, but it is also likely that they will continue using the compound throughout the duration of the MDMSEA project, which should allow for adequate trend analyses at the conclusion of the project.

Traditional methods of herbicide analyses include high-pressure liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). Both of these methods require sophisticated equipment and are fairly labor intensive, thus raising the cost of analyses. In addition, both methods generally require a fair amount of time and a large volume of sample to physically conduct the analyses. Because about 200 to 300 samples are collected each year for herbicide analyses as part of the MDMSEA project, a method was needed that would provide results that were cost effective, timely, and required a smaller sample volume.

One method that has the potential to meet the requirements of the MDMSEA project for herbicide analyses is enzyme-linked immunosorbent assay (ELISA). This procedure, which is based on immunological responses to target analytes, was chosen because of its low cost, quick turnaround times, and small sample-volume requirements. ELISA has been used in similar ways to examine triazine herbicides and metabolites in surface water (Thurman et al. 1990) and rainfall (Pomes et al. 1998), acetanilide herbicides and metabolites in surface water (Aga et al., 1994), and other herbicides in soil and water (Aga and Thurman 1996). In typical environmental ELISA protocols, the target analyte (in this case, fluometuron) and an enzyme conjugate, which mimics the action of the target analyte, compete for binding sites on polyclonal antibodies bound to a well in a microtitre plate or to a magnetic particle (Aga and Thurman 1996). After the analyte(s) and enzyme conjugate have been given time to bind to the antibodies, the remaining solution is washed away and the conjugate is allowed to catalyze a colorimetric reaction, allowing for spectrophotometric quantitation of analyte concentration. As these are competitive assays with color production caused by the enzyme conjugate rather than the presence of the analyte, color intensity varies inversely with analyte concentration.

A commercially available ELISA kit was not available for fluometuron, however, when the MDMSEA project began. Therefore, the USGS initiated a cooperative agreement with private industry to manufacture a fluometuron ELISA kit. As part of the agreement, the manufacturer would provide the antibody for the ELISA, and the USGS would provide the confirmatory GC/MS analyses. Additional funding for the agreement was provided by the Department of Plant and Soil Sciences at Mississippi State University in Starkville, Mississippi.

As part of the confirmatory analyses, the USGS would identify potential concerns in using the ELISA kit as a screening method. One potential interference with accurate measurement of fluometuron is cross reactivity of similar compounds with the antibody. Whereas the antibodies depend on specific binding mechanisms to recognize the target analyte, a particular sample may contain additional compounds which are chemically similar to the target compound. These compounds can compete for the antibody binding site, causing the ELISA to indicate higher concentrations of the target analyte than are present. When a concentration measured with ELISA is significantly higher than the concentration obtained with a confirmatory method such as GC/MS, cross reactivity is often the cause.

The purpose of this paper is to describe the measurement of fluometuron concentrations in edge-of-field MDMSEA surface-runoff samples using a magnetic-particle fluometuron ELISA kit. ELISA accuracy was examined using a comparison of fluometuron ELISA concentrations to fluometuron concentrations measured with GC/MS. Cross reactivity of ELISA antibodies with similar compounds and fluometuron metabolites was also determined.

MATERIALS AND METHODS

Sample-Collection

Data were collected from runoff sites in three MDMSEA watersheds. The first site is in the Thighman Lake watershed (Site A, Figure 1). This edge-of-field site is on the east side of Thighman Lake and drains approximately 5.7 ha (hectares) of conventionally tilled cotton. The second site is in the Beasley Lake watershed (Site B, Figure 1) on the north side of Beasley Lake. This site drains approximately 8.1 ha of conventionally tilled cotton. On the downstream side of the drainage basin, a berm and slotted-board riser have been installed for erosion and sedimentation control. The third site is in the Deep Hollow watershed (Site C, Figure 1) on the south side of Deep Hollow Lake. This site drains approximately 6.9 ha of cotton and 3.2 ha of soybeans. Runoff drains from the cotton field through the soybean field to the sampling site. Conservation tillage, winter cover crops, and precision farming are used on both crops in this watershed. Fluometuron is applied primarily as a pre-emergent in all three watersheds at a typical application rate of 0.75 kg/ha.

Both streamflow data (or total runoff volume) and water-quality samples were collected at each site. Streamflow was measured using flow-control structures such as flumes and weirs. Discrete and flow-weighted composite samples were collected at each site by automatic samplers for nearly every runoff event in 1997; however, only the discrete samples were used for this study. The discrete samples were collected in 1-L (liter) polypropylene bottles that were fluorinated to render the porous container as inert as possible so that compounds were not absorbed by the container. Laboratory-assured pesticide-free water was pumped through the entire system and collected in sample bottles prior to use and shortly after a sampling event to check cleaning procedures (blank samples) as a means of quality assurance and quality control. No detections of fluometuron were reported in the equipment blank samples. All samples and blanks were filtered using a 0.45-μm (micrometer) cartridge filter and then shipped to the USGS laboratory in Lawrence, Kansas, for analysis.

ELISA

The fluometuron RaPID Assay kit (Strategic Diagnostics, Inc., Newtown, PA) was used for all ELISA analyses. A 200-μL (microliter) aliquot of standard or sample and 250 μL of a solution containing a horseradish peroxidase-labeled fluometuron analog (the fluometuron enzyme conjugate) were combined in a polystyrene test tube. A 500-μL volume of rabbit anti-fluometuron antibody-coupled paramagnetic particles was added to the mixture, which then was incubated at room temperature for 30 minutes. The test tubes, held in place by a plastic rack, were placed in a magnetic separation apparatus, which immobilized the paramagnetic particles and any compounds bound to them; the solution was decanted, and the tubes were rinsed twice with a deionized water/detergent mixture. The test-tube rack was removed from the magnetic separator, and 500 μL of color solution (a mixture of hydrogen peroxide and the chromogen (3,3'-5,5'-tetramethylbenzidine) were added to the test tubes and allowed to incubate for 20 minutes at room temperature. Finally, 500 μL of an 0.5% sulfuric acid solution was added, stopping the catalytic reaction and changing the characteristic color of the chromogen from blue to yellow.

Analysis of absorbance of 450 nm (nanometer) light was performed using a RPA-I RaPID Analyzer (Strategic Diagnostics, Inc., Newtown, PA). A standard curve was developed by performing a linear regression with standard concentrations of 0, 0.25, 2.0, and 10 μg/L using a natural log/logit transformation. Absorbance of samples was

compared with this standard curve for calculation of ELISA concentrations, which were reported in micrograms per liter as fluometuron. Due to the potential for large relative error at the low end of the concentration curve, the lowest positive standard (i.e., 0.25 μg/L) was used as the limit of quantitation, giving a quantification range of 0.25 to 10.0 μg/L before dilution. Samples causing absorbance representing concentrations exceeding the highest standard (10.0 μg/L) were diluted with distilled water and reanalyzed.

For comparison with cross reactivity data provided by the manufacturer and found in the scientific literature, the lowest detectable dose (LDD) was defined as the concentration that produced an absorbance of 90% of the absorbance of the blank sample. The LDD for fluometuron using this definition was 0.08 μg/L. An additional parameter, the IC50, was defined as the concentration which caused an absorbance of 50% of the absorbance of the blank. LDD and IC50 values for linuron and diuron, two other phenylurea herbicides, were obtained from commercial literature (Strategic Diagnostics, Inc. 1997). Analysis of LDD and IC50 for demethylfluometuron, 3-trifluoromethylphenylurea, and 3-trifluoromethylaniline (USDA Agricultural Research Service, Stoneville, MS), three products of fluometuron degradation, was performed as part of this research.

GC/MS

GC/MS analysis generally followed the procedure described by Thurman et al. (1992). Herbicides were extracted from water samples using an automated workstation by filtering the samples through C-18 cartridges (Sep-Pak Millipore, Cambridge, MA) and eluting with ethyl acetate. An internal standard (deuterated phenanthrene) was added to the extracts, which then were evaporated to approximately 100 μL. These concentrated extracts were analyzed for herbicides using a Hewlett Packard (HP) gas chromatograph (5890 Series II) with an HP Ultra 2 capillary column and an HP mass selective detector (Model 5970 or 5972) in selected-ion monitoring mode. Quantitation was performed by comparison of response to fluometuron with that of the internal standard; confirmation of peak identity was obtained using retention-time comparisons and ion-fragment ratios for at least one fragment ion in addition to that which was used for quantitation. Fluometuron was quantified with the response at 72 amu and confirmed with the 232-amu response; demethylfluometuron was quantified with the 161-amu response and confirmed with responses at 58 and 142 amu. The quantification range for GC/MS analysis prior to dilution was 0.05 to 5.0 μg/L; comparison with an external curve showed that fluometuron recovery was approximately 90%.

Statistical Methods

Bayes's rule provides a method for comparing results from a screening test such as an ELISA with those of a confirmatory method (Remington and Schork 1985). Primary parameters used in this analysis include the following: (a) confirmed positives, or samples in which fluometuron was detected by ELISA and GC/MS; (b) confirmed negatives, or samples in which fluometuron not detected by either method; (c) false positives, or samples in which fluometuron was detected by ELISA but not GC/MS; and (d) false negatives, or samples in which fluometuron was not detected with GC/MS but was detected by ELISA. From these primary parameters, generalized indicators of test validity were defined. These indicators consisted of prevalence (total number of detections by GC/MS divided by the total number of samples), sensitivity (number of detections by both methods divided by total number of detections), specificity (number of samples in which neither method detected fluometuron divided by number of samples in which fluometuron was not detected by GC/MS), and yield (confirmed positives divided by total number of detections by ELISA) (Pomes et al. 1996). An ideal screening test (e.g., all positives and negatives confirmed) would have specificity, sensitivity, and yield approaching values of 100%.

RESULTS

Data-set overview

Approximately 200 samples were analyzed with the fluometuron ELISA. Fluometuron concentrations ranged from undetected to 193 μg/L. Comparative analyses by GC/MS were performed on 36 of the 200 samples that were analyzed using the ELISA. The distribution of concentrations determined by ELISA are compared in figure 2 to the distribution of concentrations determined by both ELISA and GC/MS on a site-by-site basis. None of the data sets or subsets had a normal distribution, and there were some differences between the distributions of the overall data sets and the subsets chosen for GC/MS analysis.

To determine ELISA repeatability, replicate ELISA analyses were performed for 32 samples, and percent differences were computed for all of the replicate analyses. A difference of 20 percent or less is typically considered acceptable for ELISA data. Of the 32 replicate analyses, the average percent difference was 5.73%, the median percent difference was 4.76%, and the standard deviation of the percent difference was 4.77%. In only one case was the difference greater than 20%.

Cross reactivity

The chemical structures of fluometuron and several other molecules that could potentially cross react with the fluometuron antibodies are shown in Figure 3. Diuron and linuron, two phenylurea herbicides, are the closest parent compound analogs to fluometuron; the primary difference between these compounds and fluometuron is the presence of chlorine atoms rather than the trifluromethyl group. Demethylfluometuron (DMFM), 3- trifluoromethyl-phenylurea (TFMPU), and 3-trifluoromethylaniline (TFMA) are degradation products of fluometuron and are distinguished from the parent compound by the absence of one or two methyl groups or the entire side chain containing the methylurea group. As noted in Figure 3, the LDD value for fluometuron is 0.08 μg/L, and the IC50 is 2.0 μg/L. Although diuron, linuron, and demethylfluometuron all have LDDs below 1.0 μg/L, the response of the antibodies to these compounds at higher concentrations, as represented by the IC50 values, was much smaller than the response due to fluometuron. It was not expected, therefore, that these compounds would have a significant effect on ELISA results.

Comparison of ELISA and GC/MS Concentrations for Edge-Of-Field Samples

Application of Bayes's Rule to the ELISA and GC/MS results for the MDMSEA samples is detailed in Table 1. Calculations for prevalence, sensitivity, and yield produced values of 100%, and the false negative rate was 0%. These 'ideal' results are, however, misleading, as the nature of the sampling protocol, calling for edge-of-field samples during runoff periods, almost assured detectable fluometuron in each sample. This was indeed observed, leading to a specificity and a false positive rate which were indeterminate. Furthermore, no sample had a fluometuron concentration less than 0.44 μg/L by either method, suggesting that this study did not address the effectiveness of the ELISA as a screening method at fluometuron levels less that approximately 0.5 μg/L.

A representation of ELISA concentrations at levels above 0.5 μg/L is presented in Figure 4, which displays fluometuron ELISA concentrations versus GC/MS concentrations for all three sites included in the study (N=36). Fluometuron concentrations by ELISA ranged from 0.67 to 165 μg/L, with a median value of 9.0 μg/L; concentrations by GC/MS ranged from 0.44 to 127 μg/L, with a median concentration of 5.1 μg/L. The slope of the regression line (m) obtained by a least-squares fit with the regression line passing through the origin was 1.37, and the value of the coefficient of determination (R^2) was 0.79. The value of the slope, which is greater than 1.0, suggests that the fluometuron antibodies were reacting to something in addition to fluometuron, potential candidates being DMFM and diuron. DMFM concentrations were measured in all samples, but the median DMFM concentration for all samples was 1.12 μg/L, and only two samples had DMFM concentrations that were higher than 5 μg/L, indicating that DMFM was probably not cross reacting significantly with the fluometuron antibodies. A subset of samples was also analyzed for diuron, but no detections were found. Although diuron cannot be ruled out completely, it is also unlikely to have had significant effect on ELISA responses, and the cause of the apparent cross reactivity remains unknown.

The large site-to-site variations in field conditions and fluometuron concentrations, likely due to management practice differences, raised the possibility of site-to-site differences in ELISA performance. Individual least-squares regressions with regression curves passing through the origin were, therefore, calculated for each site, as shown in Figure 5. The highest fluometuron concentrations were observed in samples from site A in the Thighman Lake watershed. The slope (1.36) and coefficient of determination (0.65) mimicked the values of the overall regression (Figure 4), partly due to the effects of the samples with very high concentrations. The samples with the lowest fluometuron concentrations during this sampling period were from site B in the Beasley Lake watershed. The slope (1.93) was significantly higher than the slope for the overall regression, due in part to two samples having ELISA concentrations much higher than concentrations measured with GC/MS. These two samples also affected the coefficient of determination, which was only 0.015. Fluometuron concentrations in samples from site C in the Deep Hollow Lake watershed ranged from approximately 1.5 to 40 μg/L; although the slope (1.70) was higher than the slope of the overall regression, the coefficient of determination (R^2=0.89) was better.

Further examination of the data suggests that these differences in slope and goodness of fit from site to site are due to random analytical performance error or variation in ELISA performance rather than site-specific biases. Specifically, at each site, one or two data points lie outside the 95% confidence level limits and have marked effects on the slopes and coefficients of determination. When these points are left out of the regressions, the slopes all fall within approximately 15% of each other (1.47 +/- 0.22) highlighting the importance of confirmatory analysis and of careful examination of the data. Overall, however, the ELISA analysis was an effective screening test for fluometuron in these samples.

CONCLUSIONS

A magnetic particle-based ELISA procedure was used to examine fluometuron concentrations in edge-of-field

samples from the Mississippi Delta area, and confirmatory GC/MS analysis showed a reasonable fit between concentrations measured with ELISA and GC/MS (R^2=0.79). Statistical analysis according to Bayes's Rule demonstrated the effectiveness of the ELISA as a screen for fluometuron in these samples, due in part to the relatively high fluometuron concentrations that are characteristic of the edge-of-field, storm-runoff sampling scheme. Compounds other than fluometuron appeared to affect ELISA concentrations to a small extent, but cross reactivity due to the phenylurea herbicides linuron and diuron or the fluometuron degradation products demethylfluometuron, 3-trifluoromethylphenylurea, and 3-trifluoromethylaniline is unlikely. Site-to-site variations in the relation between fluometuron concentrations measured by ELISA and fluometuron concentrations measured by GC/MS likely were caused by random error rather than actual site-specific differences in ELISA response. When acceptable confirmatory analyses are performed, the fluometuron ELISA is an effective and specific screen for moderate to high concentrations of fluometuron in water samples.

DISCLAIMER

The use of brand names in this paper is for identification purposes only and does not constitute endorsement by the U.S. Government.

ACKNOWLEDGMENTS

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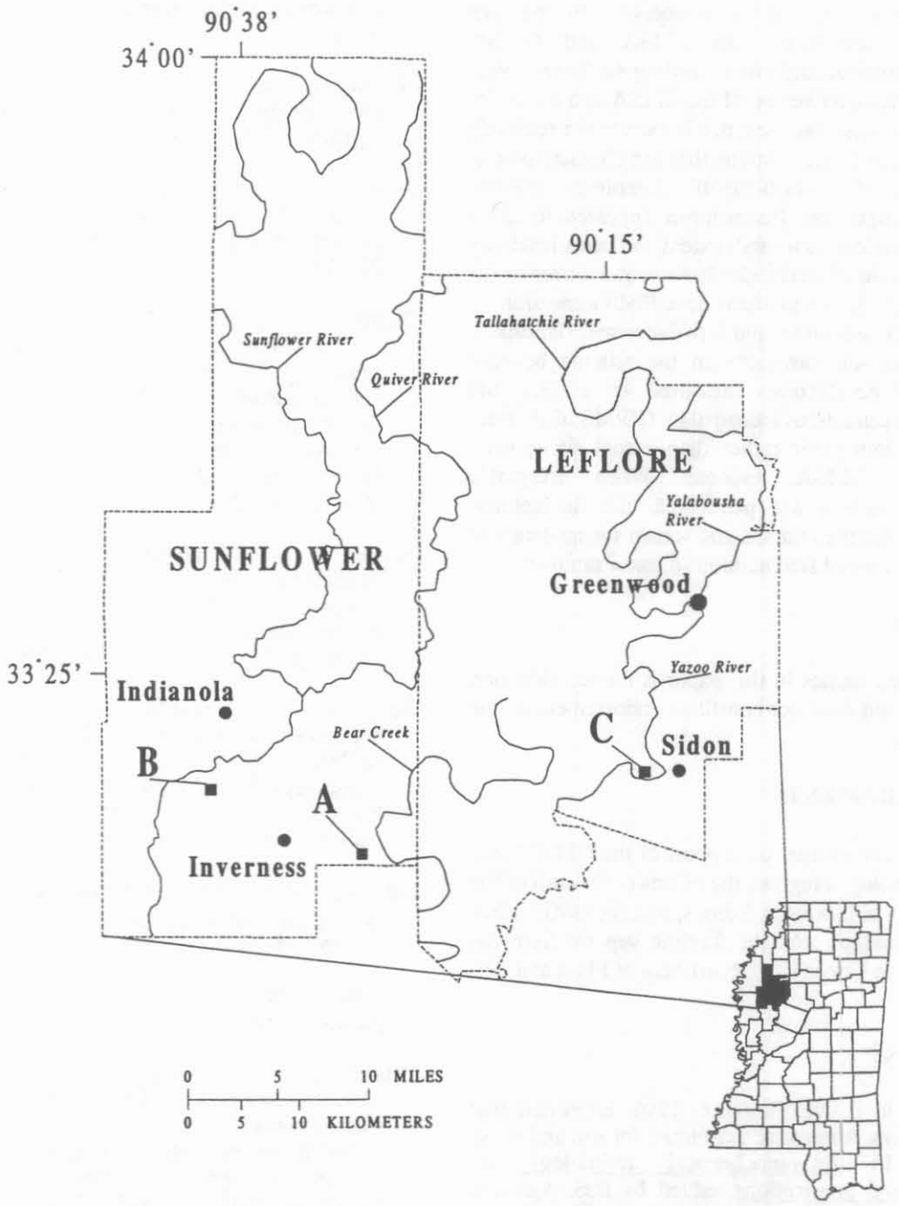


Figure 1. Mississippi Delta MSEA study watersheds and runoff monitoring site locations: A) Thighman Lake watershed; B) Beasley Lake watershed; C) Deep Hollow Lake watershed.

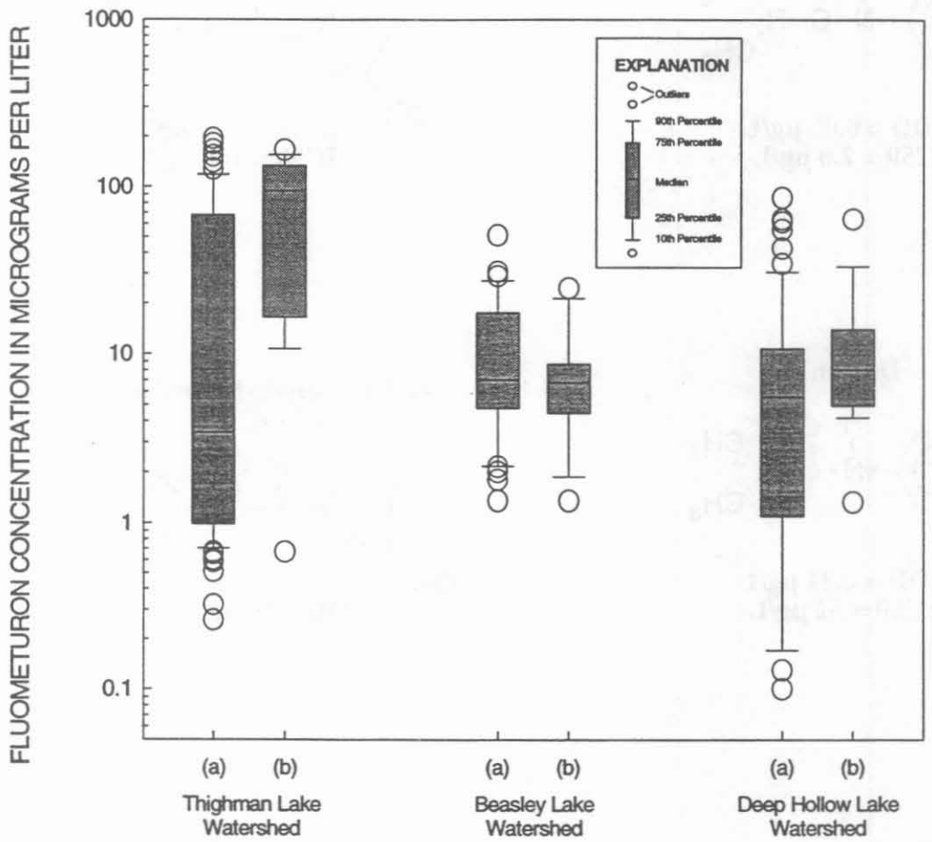
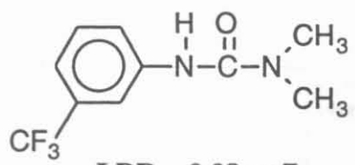


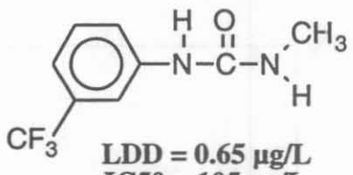
Figure 2. Distribution of: (a) all ELISA fluometuron concentrations per site and (b) ELISA fluometuron concentrations selected for GC/MS confirmation per site.

Fluometuron



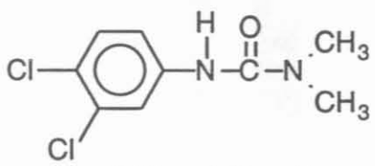
LDD = 0.08 µg/L
IC50 = 2.0 µg/L

Demethylfluometuron (DMFM)



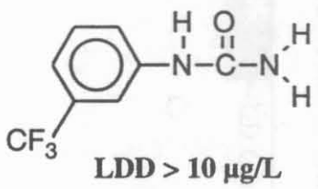
LDD = 0.65 µg/L
IC50 = 105 µg/L

Diuron



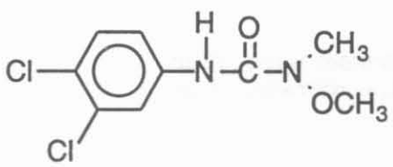
LDD = 0.31 µg/L
IC50 = 32 µg/L

3-Trifluromethylphenylurea (TFMPU)



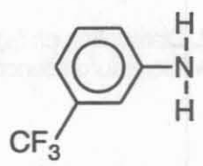
LDD > 10 µg/L

Linuron



LDD = 0.68 µg/L
IC50 = 79 µg/L

3-Trifluoromethylaniline (TMFA)



LDD > 10 µg/L

[LDD, lowest detectable doses; IC50, concentration of the compound that causes a 50% decrease in chromogen activity]

Figure 3. Structures of phenylurea herbicides and selected fluometuron degradation products.

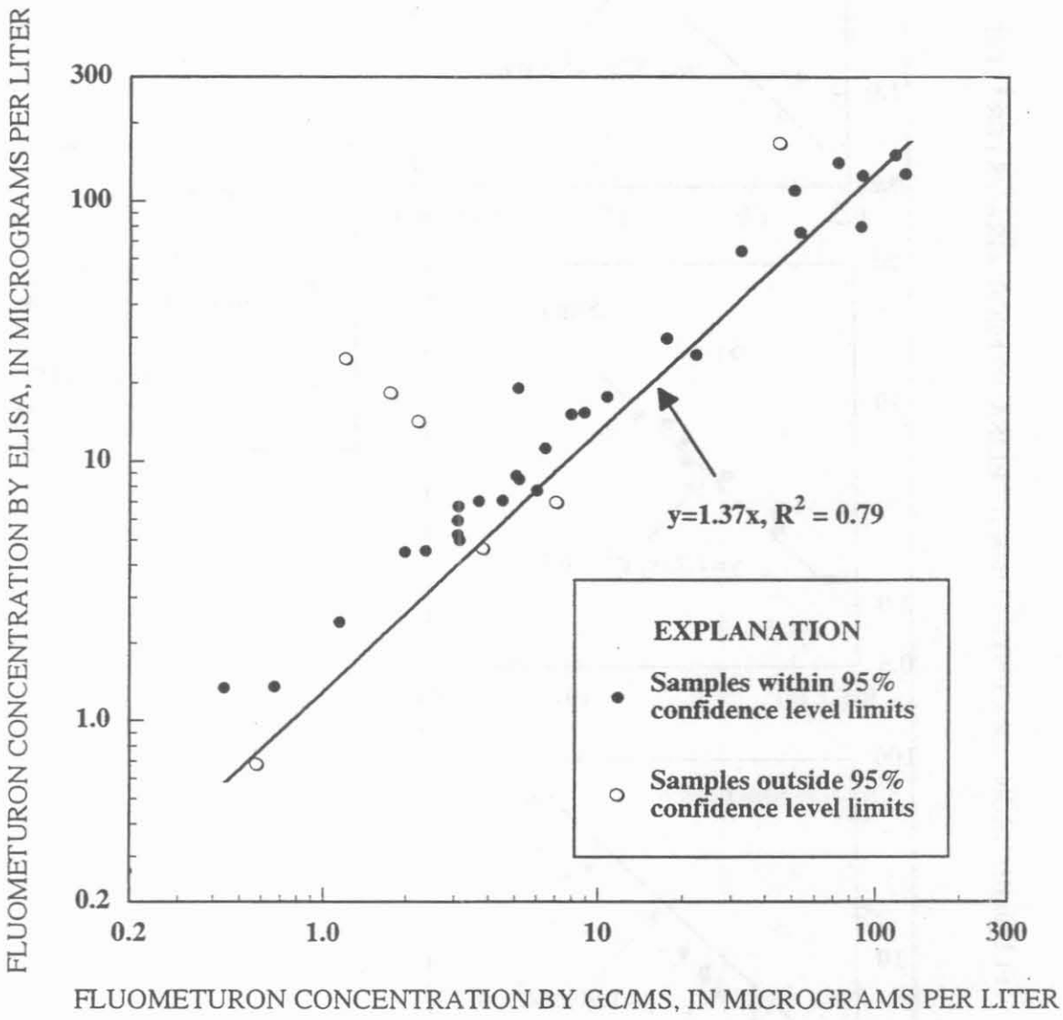


Figure 4. Comparison of fluometuron concentrations determined by ELISA and GC/MS.

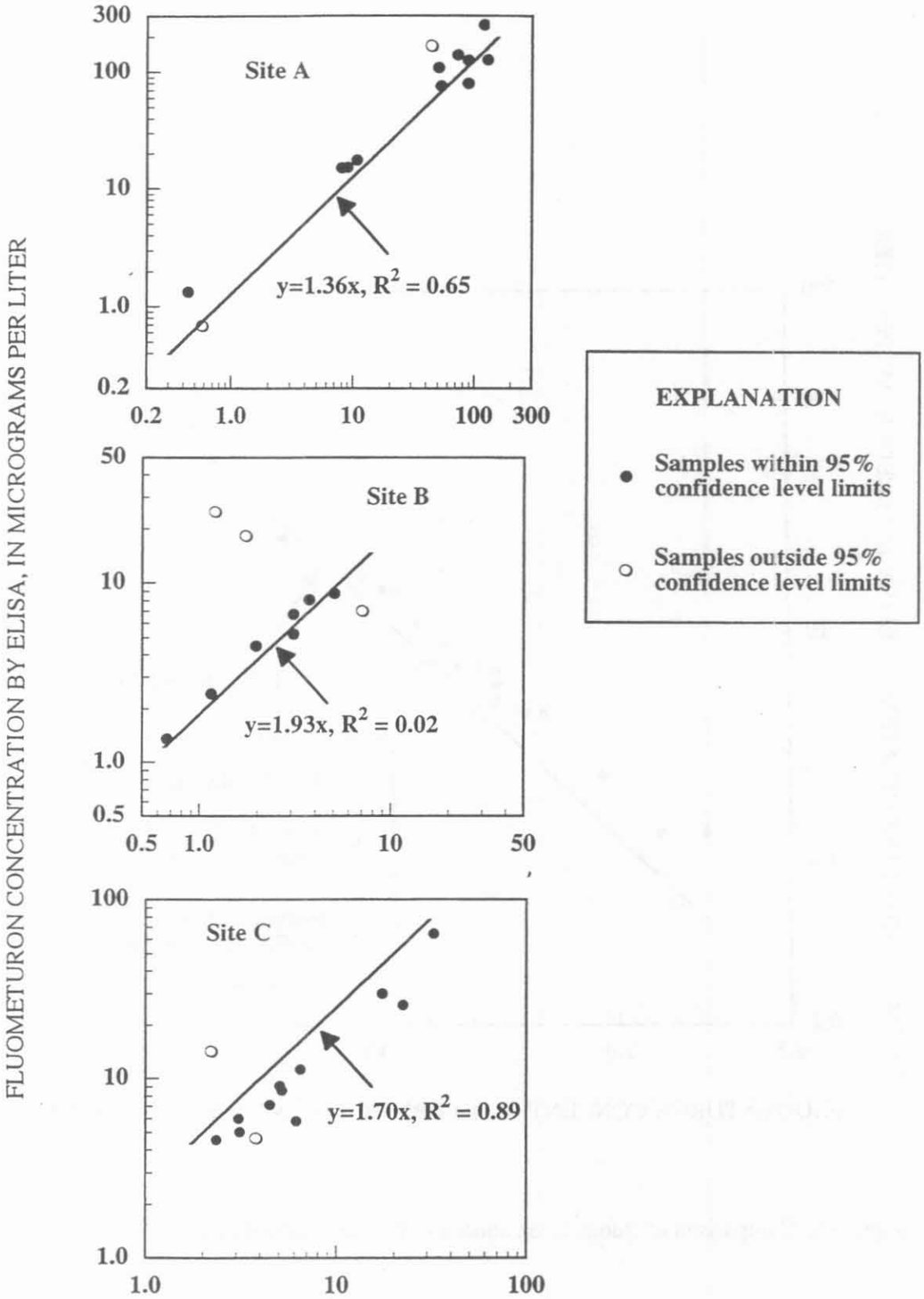


Figure 5. Relation of fluometuron concentrations determined by ELISA to concentrations determined by GC/MS for three MDMSEA runoff sites.

Table 1. Bayes's rule matrix for evaluation of fluometuron magnetic particle ELISA

	Fluometuron detected by GC/MS	Fluometuron not detected by GC/MS	Total
ELISA positive	36	0	36
ELISA negative	0	0	0
Total	36	0	36
False-positive rate		**0%**	
False-negative rate		**0%**	
Prevalence rate		**100%**	
Sensitivity		**100%**	
Specificity		**100%**	
Yield		**100%**	

ECOLOGICAL INTEGRITY OF WETLAND SOILS: TESTING OF SOIL ORGANIC MATTER AND TOTAL ORGANIC CARBON AS PARAMETERS FOR RESILIENCY OF WETLAND SOILS

J. A. Balducci, M. M. Holland, and R. S. Maul
Department of Biology
University of Mississippi

INTRODUCTION

Worldwide, natural resources are diminishing (Mitsch 1993). Humans are using more resources which results in stressed systems (Lubchenco et al. 1991). Information about resilience, the ability of a system to respond to stress, is needed. A resilient system is one that is able to return to its original characteristics within a reasonable time after a disturbance (Holland 1996). Developing indicators would aid in evaluating stressed areas and would allow them to be monitored for improvement or regeneration (Lubchenco et al. 1991).

According to Rapport et al. (1985), the health of an ecosystem is based on parameters that are significant to that ecosystem. Many factors are involved in determining the state of the ecosystem. Some changes in these factors are not recognized; therefore, characteristic values for a group of indicators are needed rather than relying on a single factor (Rapport et al. 1985). Monitoring indicators, such as biogeochemical indicators, can lead to early detection of stress, to protection of the integrity of the ecosystem, and to maintenance of the ecosystem. Using biogeochemical indicators can provide a systematic approach including both the organismal and environmental aspects of an ecosystem (Smith 1997). The need for knowledge about wetland resilience after disturbance is becoming more critical (Mitsch and Gosselink 1993).

Wetlands play an important role in landscape biogeochemical processes as they are linked to terrestrial and aquatic ecosystems (Walbridge and Lockaby 1994). Bottomland hardwood forests represent one of the most prevalent types of riparian ecosystems in the United States (Mitsch and Gosselink 1993). The majority of these wetlands have been highly subjected to timber harvesting and agricultural usage. Southeastern bottomland hardwood wetlands are characterized by increased organic matter accumulation due to higher clay content commonly found in these systems and highly variable decomposition rates (Patrick 1981; Griffin et al. 1992). These characteristics are tightly linked to primary productivity and the capacity of the forested wetlands to recover from disturbance (Griffin et al. 1992). The unique biogeochemical functions of bottomland hardwood wetlands are driven mostly by hydrology, biotic processes, and soil chemistry (Walbridge and Lockaby 1994). However, the driving factors regulating decomposition and nutrient processes in forested wetlands are poorly understood (Lockaby et al. 1996).

The need for additional baseline information about ecosystem resilience has also been documented by Brinson and Rheinhardt (1996). Reference wetlands are defined as sites within a specified geographic region that range in ecological integrity and successional stage (Brinson and Rheinhardt 1996). Reference sites should be representative of typical wetlands of the same class; they should not be unusual or unique (Brooks and Hughes 1988). According to Bailey (1980), Mississippi and Virginia are within the same ecoregion, Ecoregion 2 or the subtropical ecoregion (Figure 1). Therefore, we have made the assumption that all wetlands within this area may exhibit similar characteristics; another assumption that can be made is that reference wetlands can be used throughout an ecoregion for standards against which created or restored wetlands will be compared.

The purpose of this project is twofold. The first part focuses on determining whether mature forested wetlands within two separate watersheds located in the subtropical ecoregion are, in fact, similar in regard to soil nutrients. Mature wetlands refer to wetlands that have not been timber-harvested for an extended period of time. The mature wetlands in the Chowan River watershed have not been cut in over 80 years. The mature wetlands in the Yazoo-Tallahatchie River watershed have not been cut in over 60 years. The comparison of the mature wetlands will determine if, in fact, all mature wetlands within this study are similar. The second focus is to compare biogeochemical differences among wetlands of different successional stages within the Yazoo-Tallahatchie watershed, Mississippi. By determining if timber-harvested wetlands of different successional stage are different from mature wetlands, then some estimate of resiliency can be made. The objectives of this study were to: (1) determine if there are significant differences in soil organic matter (SOM) and total organic carbon (TOC) content between mature wetlands located in the Chowan River watershed Virginia, and mature wetlands of the Yazoo-Tallahatchie River watershed, Mississippi; and (2) determine if there are significant differences in SOM and TOC content among wetlands of different successional stages located in the Yazoo-Tallahatchie watershed.