Development of Antibodies against Hydroxyatrazine and Hydroxysimazine: Application to Environmental Samples

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An enzyme-linked immunosorbent assay (ELISA) selective for hydroxyatrazine and hydroxysimazine was developed as a means of monitoring environmental and microbial degradation of atrazine to hydroxyatrazine. This ELISA was tolerant to solvents, salts, and pH changes and functioned well in a series of matrices (water, soil, horse manure, urine, and fungal extracts). In preliminary bioremediation studies conducted in a drum impervious to UV light, the microbes endogenous to horse manure converted approximately 1% of the total initial amount of atrazine to hydroxyatrazine. Also, a UV-resistant strain of white rot fungus (*Phanerochaete chrysosporium*) was tested for its ability to degrade atrazine. Using the ELISA described here, approximately half of the time zero amount of atrazine (2 μg/35-mm Petri dish) was gone in 1 day and essentially all of the atrazine was converted to hydroxyatrazine, primarily due to UV irradiation. This ELISA provided a valuable method to quantify hydroxyatrazine and hydroxysimazine in a series of traditionally difficult matrices for the investigation of the bioremediation of atrazine.

INTRODUCTION

Atrazine and simazine are *s*-triazine herbicides used extensively for controlling broad-leaf weeds in agriculture and in the maintenance of rights of way. Both atrazine and simazine are converted to their nonphytotoxic 2-hydroxy forms in the environment (Jordan et al., 1970; Paris et al., 1973; Khun and Marriage, 1979). However, analysis is usually performed only for the parent compound. A more complete monitoring strategy, which includes not only the parent compound but also the environmental degradation products, would yield a better profile of the herbicide burden.

The large volume of *s*-triazines released into the environment, coupled with the identification of atrazine as a major pesticide contaminant of ground water (Parsons and Witt, 1988; Eisler, 1989; Belluck et al., 1991), makes this compound ideal for the evaluation of strategies for monitoring the fate of both parent and key degradation products under a variety of conditions. By using this approach, information on the ability of certain ecosystems to tolerate or degrade xenobiotics can aid in effective management of agrochemicals.

In the development of analytical techniques to determine *s*-triazine contamination, studies of enzyme-linked immunosorbent assays (ELISAs) for both atrazine (Hüber, 1985; Bushway et al., 1988; Wittmann and Hock, 1989; Schlaeppi et al., 1989; Dunbar et al., 1990; Harrison et al., 1990) and its hydroxylated form (Schlaeppi et al., 1989) have indicated that antibody-based methods are useful for rapid quantitative analysis of environmental samples. Techniques for determination of hydroxyatrazine or hydroxysimazine in environmental samples require extensive cleanup for classical sample analysis (Khan and Marriage, 1979; Ramsteiner and Horman, 1979). Either methylation or silylation of the hydroxytriazine is necessary prior to GC analysis (Khan and Marriage, 1979; Drozd, 1981); sample extraction and cleanup are required for HPLC analysis (Schlaeppi et al., 1989; Ramsteiner and Horman, 1979).

Here we report an ELISA selective for the detection of hydroxyatrazine and hydroxysimazine in water, soil, and a series of other environmental and biological matrices. The assay is suitable for direct use in water samples and requires minimal sample preparation for soil. In addition to the routine characterization of this assay in a variety of matrices, this assay was used to follow the degradation of atrazine. Manure samples from a biological digestion (Bio-Gest) machine spiked with atrazine were analyzed by ELISA to investigate the bioremediation of this herbicide and to evaluate the Bio-Gest instrument as a means of disposal for agrochemical waste. Approximately 1% of the initial dose was converted to hydroxyatrazine. When the UV-resistant strain of white rot fungus (*Phanerochaete chrysosporium*) was used with and without UV light to degrade atrazine, essentially all of the total initial amount of atrazine was converted to hydroxyatrazine. The $t_{1/2}$ of atrazine in this system was roughly 1 day; however, the hydroxylation of atrazine in this study was completely due to the UV irradiation. The use of this ELISA to follow the appearance of hydroxyatrazine circumvented the need for more arduous and slower analytical methods.

MATERIALS AND METHODS

Chemicals. Alkaline phosphatase (AP), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), *p*-nitrophenyl phosphate enzyme substrate tablets, and *N*-acetyl-L-cysteine were obtained from the Sigma Chemical Co. (St. Louis, MO), and affinity-purified goat anti-rabbit antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). All solvents and salts were purchased from J. T. Baker (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), or Aldrich Chemical Co., Inc. (Milwaukee, WI). A Sybron/Barnstead Nanopure II water system set at 16.7 MΩ cm provided water for

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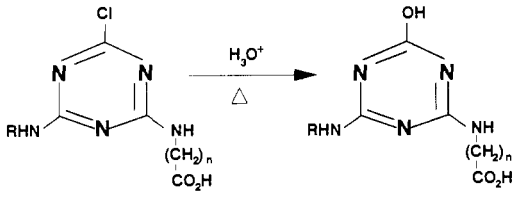

Figure 1. Schematic of the reaction used to make *N*-[4-(ethylamino)-6-hydroxy-1,3,5-triazin-2-yl)]-β-alanine (**III**), for use as the immunizing hapten, and 6-{[4-hydroxy-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}amino}hexanoic acid (**IV**), a hapten for use in the ELISA. Details of the reaction and hapten treatment can be found in the text.

all aqueous solutions. Microtiter plates were purchased from both Nunc (No. 4-42404, Roskilde, Denmark) and Dynatech Laboratories, Inc. (No. 001-012-9200, Chantilly, VA). Technical atrazine was donated by Shell Agriculture Chemical Co. (Modesto, CA), and the analytical *s*-triazine standards were a gift from the Ciba-Geigy Corp. (Greensboro, NC). The purities of the analytical standards used in this study were confirmed by observing single UV dense spots for each standard on a TLC plate (0.25-mm precoated silica gel 60 F254 plastic sheet from E. Merck, Darmstadt, Germany). Details of the synthesis of compounds **III** and **IV** are listed under Synthesis of Haptens **III** and **IV**. The syntheses of other haptens, **2a–c**, **4a**, and **2e**, are described in Goodrow et al. (1990) (see Figure 1 and Table I).

Instruments. Infrared (IR) spectra were recorded on an IBM IR/32 FTIR spectrometer (Danbury, CT). The ^1H and ^{13}C NMR spectra were obtained using a GE-300 spectrometer (General Electric NMR Instrument, Fremont, CA) operating at 300.1 and 75.5 MHz, respectively; chemical shifts are expressed in parts per million downfield from tetramethylsilane. Fast atom bombardment low- and high-resolution mass spectra (FAB-MS, FAB-HRMS) were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.), using xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix. Polyethylene glycol 300 was added to the matrix as a mass calibrant for FAB-HRMS. The low-resolution electron impact mass spectrum (EI-MS) was obtained on a Trio 2 (VG Masslab, Altrincham, U.K.) instrument at 70 eV.

Enzyme-linked immunosorbent assays (ELISAs) were carried out in 96-well plates (Nunc) and read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). Inhibition curves used for data analysis were composed of a 12-point standard curve in quadruplicate, and the software package Softmax (Molecular Devices) was used for fitting a sigmoidal curve based on the four-parameter logistic method of Rodbard (1981).

Synthesis of Haptens III and IV. *N-[4-(Ethylamino)-6-hydroxy-1,3,5-triazin-2-yl]-β-alanine* (**III**). A solution of 0.491 g (2.00 mmol) of the 2-chloro *s*-triazine (**I**) in 4.0 mL of concentrated hydrochloric acid was heated under reflux for 4 h (Figure 1). Removal of the acid under reduced pressure provided 0.458 g (100%) of **III** as a white powder: mp 174 °C (dec); TLC R_f = 0.76 ($CHCl_3$–methanol–acetic acid 20:10:1, v/v/v); IR (KBr) 3270 (m, NH), 3127 (m, NH), 1757, 1717 (s, s, C=O), 1609 (vs, C=N), 1201 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.72 (t, J = 5.4 Hz, 1 H, NH), 8.47 (t, J = 5.6 Hz, 1 H, NH), 8.1 (br, 1 H, ArOH), 3.58 (dt, J = 6.0, 6.4 Hz, 2 H, CH_2-3), 3.40 (dt, J = 1.0, 6.5 Hz, 2 H, CH_2N), 2.56 (t, J = 6.5 Hz, 2 H, CH_2-2), 1.15 (t, J = 7.2 Hz, 3 H, CH_3) (with the addition of D_2O the 12.0, 8.72, 8.47, and 8.1 ppm peaks disappeared, the 3.58 ppm dt peaks resolved into a triplet centered at 3.59 ppm, J = 6.5 Hz, and the 3.40 ppm dt peaks became a quartet centered at 3.41 ppm, J = 7.2 Hz); ^{13}C NMR (DMSO-d_6) δ 172.5 (C=O, C_1), 156.5 (Ar-$C_{4\text{ or }6}$), 156.3 (Ar-$C_{4\text{ or }6}$), 146.8 (Ar-C_2), 37.1 (J_{CH} = 144 Hz, C_3), 36.1 (J_{CH} = 141 Hz, C_2), 33.3 (J_{CH} = 126 Hz, CH_2N), 14.2 (J_{CH} = 126 Hz, CH_3); EI-MS *m/z* (relative intensity) 227 (9, M^+), 209 (44, M-H_2O), 69 (62, $C_3H_5N_2$), 55 (77, $C_2H_3N_2$), 44 (100, C_2H_6N); FAB-HRMS *m/z* calcd for $C_8H_{14}N_5O_3$ 228.1097, obsd 228.1078.

6-{[4-Hydroxy-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl}amino}hexanoic Acid (IV). A heterogeneous mixture of 1.21 g (4.00 mmol) of the 2-chloro compound (**II**) and 8.0 mL of concentrated hydrochloric acid was heated slowly to reflux, whereupon dissolution occurred (Figure 1). After 1 h under reflux, the solution, on slow cooling, deposited fine white crystals, mp 109.5–114.5 °C. Recrystallization from acetonitrile provided 0.91 g (80%) of glistening white crystals, mp 105.0–113.0 °C. Sharp and consistent melting point ranges were unattainable: IR (KBr) 3260 (m, NH), 3121 (m, NH), 1740 (s, C=O), 1614 (vs, C=N), 1170 (m, CO) cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.9 (br, 1 H, OH), 8.72 (d, J = 7.5 Hz, 1 H, NH) 8.60 (t, J = 5.6 Hz, 1 H, NH), 4.08 (heptet, J = 6.6 Hz, 1 H, CH), 3.36 (m, 2 H, CH_2N, CH_2-6), 2.21 (t, J = 7.3 Hz, 2 H, CH_2-2), 1.52 (quin, J = 7.1 Hz, 4 H, CH_2-3 and CH_2-5), 1.3 (m, 2 H, CH_2-4), 1.20 (d, J = 6.3 Hz, 6 H, 2 CH_3) (the 11.9, 8.72, and 8.60 ppm peaks disappeared and the 3.36 ppm multiplet became a triplet, J = 6.6 Hz, with added D_2O); ^{13}C NMR (DMSO-d_6) δ 174.2 (C=O), 156.3 (Ar-$C_{4\text{ or }6}$), 155.5 (Ar-$C_{4\text{ or }6}$), 146.7 (Ar-C_2), 43.7 (CHN) 40.6 (C_6), 33.6 (C_2), 28.1 (C_5), 25.6 (C_4), 24.1 (C_3), 21.8 (2 CH_3); FAB-MS *m/z* 284 (M + H^+); FAB-HRMS *m/z* calcd for $C_{12}H_{22}N_5O_3$ 284.1723, obsd 284.1741.

Synthesis of Hapten–Protein and Hapten–Enzyme Conjugates. Hapten **III** was conjugated to BSA or KLH in 0.05 M borate buffer, pH 8, using the active ester method of Bauminger and Wilchek (1980), and each was used as an immunogen. Following dialysis of the conjugate against PBS (0.1 M phosphate-buffered saline), the solution was scanned spectroscopically from 220 to 600 nm. The hapten–conjugate spectra obtained were compared to the spectra of the hapten and of the protein alone to qualitatively confirm that conjugation had occurred. A slight shift in the λ_{max} from 366 nm for the hapten alone to 276 nm for the hapten–BSA conjugate and to 352 nm for the hapten–KLH conjugate was seen. An analysis using an ELISA (Harrison et al., 1990; the assay using antibody 354) previously shown to have reasonable affinity for hydroxyatrazine and hydroxysimazine was performed to estimate the amount of hapten per milligram of protein. The hapten loading was estimated, using hydroxysimazine as the standard, at 0.11 ng/mg of protein for the KLH conjugate and 0.225 ng/mg for the BSA conjugate. Using the active ester method cited above, **IV** (Figure 1) and **2b** (Table I) (Goodrow et al., 1990) were conjugated to alkaline phosphatase (AP) and BSA for use in a competitive ELISA.

Immunization and Serum Collection. Six female New Zealand white rabbits (2.2 kg) were immunized intradermally, three with the **III**–BSA conjugate and three with the **III**–KLH conjugate. The hapten–protein conjugates were prepared by dissolving separately 100 μg of each immunizing hapten–protein conjugate in 0.25 mL of PBS and mixing 1:1 with Freund's complete adjuvant for each rabbit. Subsequent boosts were given to the animals every 4 weeks using the same immunizing preparation with the exception that Freund's incomplete adjuvant was used. Rabbits were bled 7–10 days after each boost. The blood was allowed to clot overnight. The following day, the antisera were removed, separated from any remaining red blood cells by centrifugation, and stored at −20 °C. The sera were tested for titer using the method outlined by Gee et al. (1988) and used without further purification.

ELISA. Following the principles of solid-phase immunoassay (Voller et al., 1976), the ELISA was conducted using a competitive format. Three different formats were used in screening for the best serum and in optimizing assay conditions. Prior to using any format for inhibition studies, antibody, enzyme tracer, and coating antigen dilutions were optimized by using the two-dimensional titration method as outlined by Gee et al. (1988). The general assay conditions are given below.

Coated Antigen Format. Plates were coated with 100 μL/well of a hapten–protein conjugate in a carbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, 0.2 g/L NaN_3) and allowed to stand overnight at 4 °C. The following day, the plates were washed with PBSTA [phosphate-buffered saline with Tween 20 and sodium azide: 8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) NaN_3]. Then 50 μL/well of the anti-triazine antibody and 50 μL/well of the analyte were placed on the plate and incubated for 2 h. The dilution for each hapten–protein conjugate and antibody combination was chosen after a two-dimensional titration was run to optimize concentrations. After the plates were washed again

Table I. Cross-Reactivity of Antibody 2266 for a Series of s-Triazines[a]

inhibitor	structure R_1	R_2	R_3	cross-reactivity
atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	2
hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	38.4
simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	2.4
hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$	100
atrazine mercapturate	*N*-acetyl-L-cysteine	$NHCH_2CH_3$	$NHCH(CH_3)_2$	1.6
ametryne	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	<0.1
simetryne	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$	1
propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	<0.1
prometryne	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	<0.1
prometon	OCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	3.4
terbutryne	SCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$	<0.1
cyanazine	Cl	$NHCH_2CH_3$	$NHCCN(CH_3)_2$	<0.1
cyanazine acid	Cl	$NHCH_2CH_3$	$NHCCOOH(CH_3)_2$	<0.1
deethylatrazine	Cl	NH_2	$NHCH(CH_3)_2$	<0.1
deisopropylatrazine	Cl	NH_2	$NHCH_2CH_3$	<0.1
didealkylated atrazine	Cl	NH_2	NH_2	<0.1
cyanuric acid	OH	OH	OH	<0.1
ammeline	NH_2	OH	OH	<0.1
ammelide	NH_2	NH_2	OH	3
melamine	NH_2	NH_2	NH_2	<0.1
2a	Cl	$NHCH_2CH_3$	$NHCH_2COOH$	7
2b	Cl	$NHCH_2CH_3$	$NH(CH_2)_2COOH$	2
2c	Cl	$NHCH_2CH_3$	$NH(CH_2)_3COOH$	1
2d	Cl	$NHCH_2CH_3$	$NH(CH_2)_4COOH$	10
4a	SCH_2CH_2COOH	$NHCH_2CH_3$	$NHCH_2CH_3$	<0.1
2e	Cl	$NHCH_2CH_3$	$NH(CH_2)_5COOH$	96

[a] Numbers are expressed as a percent relative to the IC_{50} for hydroxysimazine. The assay parameters are detailed in the text. Cross-reactivity is determined by expressing the ratio of the IC_{50} of the chemical assigned to be 100% (hydroxysimazine) to the IC_{50} of the other compounds and expressed as a percent.

with PBSTA to remove any unbound material, 100 μL/well of a commercially available goat anti-rabbit alkaline phosphatase (AP) conjugate was added. Two hours later, the plate was washed and a substrate solution of 1 mg/mL *p*-nitrophenyl phosphate in 10% diethanolamine was added to each well. Approximately 1 h thereafter, the plates were read spectroscopically in a dual-wavelength mode at 405 nm minus 650 nm. The development of a yellow color was inversely proportional to the amount of triazine present.

Double-Antibody Format. This format involved coating 100 μL/well of a 1:2000 dilution of a goat anti-rabbit antibody in carbonate buffer into plate wells and incubating overnight at 4 °C. The following day, in a separate uncoated plate, anti-triazine antibody, analyte, and the enzyme tracer (**IV** or **2b** conjugated to AP) were allowed to equilibrate for 1 h. Individual antibody and enzyme tracer concentrations were determined by a two-dimensional titration. An aliquot of this mixture was then added to the coated antibody plate wells. After any unbound material was washed away, *p*-nitrophenyl phosphate substrate solution was added. After sufficient color development (OD of 0.5, approximately 30 min) the plates were read at 405 minus 650 nm.

Double-Coated Antibody Format. For this format, a 1:2000 dilution of a goat anti-rabbit trapping antibody in carbonate buffer was coated into plate wells and incubated overnight at 4 °C. After any unbound material was washed away, the plates were recoated with an anti-triazine antibody (1:5000) in carbonate buffer overnight at 4 °C. The following day, 50 μL/well of analyte and 50 μL/well of enzyme tracer (**VI**–AP or **2b**–AP) were added. Antibody and enzyme tracer dilutions were chosen after a two-dimensional titration was performed to estimate optimal concentrations. Thirty minutes later the wells were washed to remove any unbound components. Thereafter, addition of the substrate, 1 mg/mL of *p*-nitrophenyl phosphate, yielded color development inversely proportional to the amount of analyte present.

Sample Extraction and Preparation Methods. *Soil.* On the basis of the work done by Stearman and Adams (1992), who compared extraction solvents and methods for soil samples to be analyzed by an ELISA method, soil samples were extracted by shaking 25 g of soil with 50 mL of acetonitrile. Four different soil types were examined (Auburn, a sandy loam with 3.4% organic matter; McCarthy, a sandy loam with 18.8% organic matter; Yolo 1, a silt loam with 1.7% organic matter; and Yolo 2, a sandy loam with 1.4% organic matter). The following day, extracts were filtered through Whatman No. 1 filter paper, evaporated under a gentle stream of nitrogen, and reconstituted with PBSTA buffer before assaying. To minimize solvent effects on the assay, the evaporated soil extracts were also reconstituted in PBSTA and then partitioned with $CHCl_3$ before analysis.

Water. Waters from three different sources were used to evaluate directly any potential matrix effects. Tap water from Davis, CA, distilled water, and water from Putah Creek (a small oxbow lake with minimal flow and turbid with algae) were compared to the standard assay buffer of 0.1 M, pH 7.4, PBSTA. Water was added directly to the assay with no prior cleanup or preparation.

Bio-Gest Manure Samples. In a study to investigate the bioremediation of pesticides using microorganisms which thrive in horse manure, 1000 ppm of atrazine was placed into the sump of a Bio-Gest machine (Advance Manufacturing and Development, Willits, CA). This instrument contained a sump where water can be added and where water from the entire system is collected for recycling. The material to be degraded was introduced into the sump. The aqueous slurry of this material was then pumped to a large drum containing manure. As water percolated through the drum, returning to the sump for recycling through the manure again, metabolites and parent compound in the sump were monitored. Side sampling ports in the manure drum were used to evaluate the microbial population and compound degradation. Water that evaporated from the manure and sump area was collected using a refrigerated condenser and was also analyzed to monitor for any volatilized metabolites of microbial degradation. Complete details of this study utilizing the Bio-Gest machine to degrade atrazine, as well as a series of other pesticides, are described in a final report to the California Environmental Protection Agency (California EPA, 1992).

Manure (10 g) was extracted with 50 mL of a 1:1 (v/v) methanol–ethyl acetate solution and filtered. Sump (10 mL) and condensed water (1 L) samples were extracted by vacuum filtration through washed C_{18} solid-phase extraction (SPE) cartridges, followed by elution with 20 mL of ethyl acetate to recover the analyte. One milliliter of these organic extracts was brought to dryness under a gentle stream of nitrogen, reconstituted with 1 mL of PBSTA, and partitioned with 1 mL of $CHCl_3$. Analysis was performed on the remaining aqueous layer. For confirmation, selected manure extracts were also brought to dryness and then derivatized with BSTFA. Analysis of these derivatized samples was per-

Table II. Results of Two-Dimensional Titrations of the Antibodies Raised against III When Run against III and IV as Coating Antigens[a]

antibody	immunizing conjugate	1/Ab for either III-KLH or III-BSA	1/Ab for IV-BSA
2266	III-KLH	64 000	16 000
2264	III-KLH	32 000	8 000
2263	III-KLH	16 000	16 000
2257	III-BSA	8 000	2 000
2256	III-BSA	16 000	8 000
2242	III-BSA	16 000	2 000

[a] The values are expressed as the reciprocal antibody dilution necessary to obtain a good color development (optical density of approximately 0.5 at 405–650 nm at 15 min).

Table III. IC_{50} Value for the Antiserum When Used in a Double-Antibody Format with IV-AP as the Enzyme Tracer and Hydroxysimazine as the Analyte

antibody	IC_{50}, μM	slope	curve fit
2266	1.03	0.774	0.985
2264	46.5	0.281	0.960
2263	1.26	1.26	0.989
2257	132	0.137	0.993
2256	no recognition		
2242	1.06	0.563	0.997

formed by GC/MS in selective ion monitoring mode for m/z 2852, 905, 882, and 327.2.

White Rot Fungus. The development of the UV-resistant strain of this fungi and the procedure used to degrade pollutants are detailed in Katayama and Matsamura (1991). Briefly, cultures of *P. chrysosporium* were grown in nitrogen-deficient media and incubated in 35-mm-diameter polystyrene Petri dishes. Two micrograms of atrazine was introduced to 3 mL of culture media in these plates in 20 μL of acetone after they had been preincubated at 27 °C for 3 days. Selected cultures were irradiated for 2 h/day with 254-nm light, while control cultures were not exposed to UV light. The effect of the UV light alone on atrazine was evaluated by placing 2 μg of atrazine in a Petri dish without any microorganism and treating it concurrently with the UV-irradiated fungal cultures. Additionally, dead micellae plus atrazine was used as a control for any effects which may arise in this particular system. After incubation, the culture was extracted using a hand homogenizer with an equal volume of 1:10 methanol to water. The final 6 mL of extract was measured directly using the ELISA described here for hydroxyatrazine or using an assay previously detailed (Lucas et al., 1991) for atrazine.

RESULTS AND DISCUSSION

Screening and Selection of Antisera. Antisera were screened using the two-dimensional titration method with a coated antigen format. Those rabbits immunized with **III**–KLH (2263, 2264, 2266) showed titers consistently equal to, or higher than, those immunized against **III**–BSA (2257, 2256, 2242) when examined against coating antigen **III** or **IV** (Table II). Attempts to design a homologous system (using the same ELISA hapten as immunizing hapten) with **III**–BSA as the coating antigen for antiserum 2266, 2264, or 2263 yielded no inhibition curves when hydroxyatrazine or hydroxysimazine were used as the competitor.

In constructing a heterologous system (utilizing an ELISA hapten other than that used for immunizing) using **IV**–BSA in a coated antigen format, rabbits 2257, 2256, and 2242 generated some inhibition at the low ppm level (above 5 μM). **IV**–AP, in a double-antibody format, had IC_{50} levels in the micromolar range (high ppb) (Table III). Preincubating the analyte (hydroxyatrazine or hydroxysimazine) with the antibody in a double-coated antibody format yielded a slightly more sensitive assay but one still not acceptable for residue analysis (TableIV). Although the difference between **III** and **IV** consisted of changing one of the alkyl groups from ethyl to isopropyl and lengthening the side chain conjugated to the protein, the difference in affinity for the ELISA hapten was not significantly dissimilar to the immunizing hapten to generate a sensitive assay.

Table IV. IC_{50} Value (Micromolar) for the Antisera 2263 and 2266 When Preincubated with either Hydroxyatrazine or Hydroxysimazine from 30 to 120 min prior to Addition of the Enzyme Tracer IV–AP

		preincubation time of analyte, min			
antibody	analyte	30	60	90	120
2266	hydroxyatrazine	0.267	0.236	0.328	0.279
	hydroxysimazine	0.305	0.313	0.338	0.250
2263	hydroxyatrazine	0.509	0.423	0.489	0.455
	hydroxysimazine	0.477	0.388	0.417	0.417

Table V. IC_{50} Value, Slope, and Fit of the Curve Generated When the Antiserum Was Assayed in a Double-Coated Antibody Format Using 2a–AP as the Enzyme Tracer with either Hydroxyatrazine or Hydroxysimazine as the Analyte[a]

antibody	analyte	IC_{50}, μM	slope	curve fit
2266	hydroxyatrazine	0.0636	0.470	0.979
	hydroxysimazine	0.0421	0.678	0.987
2264	hydroxyatrazine	0.312	1.16	0.836
	hydroxysimazine	0.010	1.36	0.646
2263	hydroxyatrazine	0.112	0.517	0.966
	hydroxysimazine	0.0548	0.948	0.829
2242	hydroxyatrazine	0.114	0.798	0.997
	hydroxysimazine	0.087	0.910	0.993

[a] Although 2264 appears to be quite sensitive for hydroxysimazine, the curve generated had a very poor fit and high background absorbance. Antibodies 2257 and 2256 showed no comparable inhibition under these conditions.

When **2b**–AP was used in a double-coated antibody format, a substantial difference between ELISA and immunizing hapten recognition was present to generate an IC_{50} in the nanomolar range (low ppb) (Table V; see Table I for structure). Although 2242, 2263, 2264, and 2266 all recognized hydroxysimazine well, only 2266 showed reasonable sensitivity for hydroxyatrazine. Coating the antibody 2266 directly to the plate yielded IC_{50} values 2–5 times less sensitive than those when a trapping antibody was used. Since the primary objective of this project was to develop and apply an ELISA for hydroxyatrazine and hydroxysimazine, antibody 2266 with **2b**–AP as the enzyme tracer was chosen for further study. The final assay format used a 1:2000 dilution of goat anti-rabbit antibody to coat the plate, which was washed and recoated the following day with a 1:5000 dilution of antibody 2266. Addition of 50 μL of sample with 50 μL of a 1:3000 dilution of the enzyme tracer **2b**–AP to the coated plate for 30 min, followed by washing and then addition of substrate, provided adequate assay conditions for analysis of hydroxyatrazine and hydroxysimazine. The other antibodies could prove to be useful in future studies to distinguish hydroxyatrazine from hydroxysimazine.

Cross-Reactivity. Cross-reactivity values were determined as the ratio of the IC_{50} of hydroxysimazine to that of the test compound and expressed as a percentage. As presented in Table I, the assay showed remarkable specificity for hydroxysimazine and hydroxyatrazine. Replacement of the OH group (R_1) by other moieties or removal of one of the *N*-alkyl groups in the R_2 or R_3 position greatly reduced recognition of the analyte by the antibody. The only deviant result occurred when the R_3 side chain was lengthened to C_5 (compound **2e**).

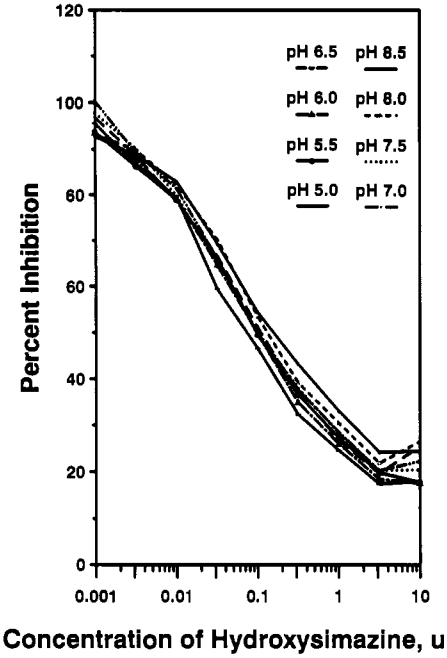

Figure 2. Effect of pH on assay performance. A series of standard curves using the average of quadruplicate well measurements, with an average CV of 10%, were constructed in PBSTA with the pH incrementally adjusted at 0.5 pH unit steps from 5.0 to 8.5 to assess assay operating parameters.

Time Effects. The effect of the order of addition in which the analyte and enzyme tracer were added and the length of time these components were allowed to incubate on the coated antibody plate was studied. Thirty minutes proved to be the optimal assay time for incubation on the coated plate. Longer incubation times showed no improvement in assay sensitivity, while shorter time periods yielded standard curves with large differences in IC_{50} and poor color development.

The order of addition and amount of time for preincubation of either the analyte or the enzyme tracer on the coated plate before the addition of the other assay constituent were examined. When the analyte, hydroxysimazine, was placed on a coated plate for 5, 15, or 30 min prior to the addition of the enzyme tracer (**2b**–AP), no significant improvement in the assay sensitivity was found. When the enzyme tracer was placed in the well first, and the hydroxysimazine added after 5, 15, or 30 min, the 15- and 30-min standard curves showed a decrease in IC_{50} and the curves had poorer fits to the four-parameter equation, as expected. Because preincubation of the individual components generated no significant improvement in assay performance, either premixing the analyte and the enzyme tracer prior to addition to the coated plate or addition of the components in rapid succession was adopted as standard operating procedure.

Salt, Solvent, and pH Effects. No significant difference was observed in standard curves generated in buffer and those in up to 12.5% acetone, acetonitrile, or 2-propanol of the sample volume or up to 10% methanol. Assay performance in various concentrations of salt was also evaluated. Standard curves prepared in 0.1–1.0 M PBS showed no significant deviation from the standard assay buffer of 0.1 M PBS. Additionally, this assay proved to be resistant to sample pH. As seen in Figure 2, there was no difference in assay performance from pH 5.0 to 8.5.

It was noted that subtle effects do occur when solvent or salt concentration was varied in this assay, such as poorer correlation coefficient, slower color development, and higher background noise. For this reason, a corresponding amount of modifier (salt, solvent, etc.) was added to the standard curve, as was done for the samples being analyzed unless a constant background level was established and could be subtracted.

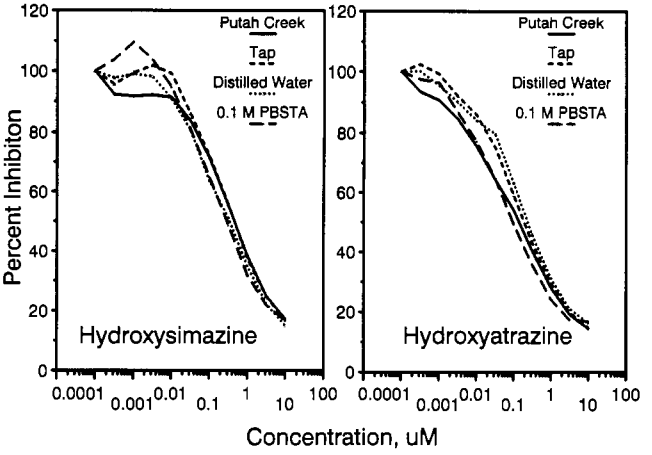

Figure 3. Performance of the ELISA in water from various sources for both hydroxyatrazine and hydroxysimazine. Waters from three different sources were used directly to evaluate any potential matrix effects. Tap water from Davis, CA, distilled water, and water from Putah Creek (a small oxbow lake with minimal flow and turbid with algae) were compared to the standard assay buffer of 0.1 M, pH 7.4, PBSTA. The curves represent the average of quadruplicate well measurements with an average CV of 6.3%.

Soil and Water Matrix Effects. Results from the four different sources of water used in constructing standard curves of hydroxyatrazine and hydroxysimazine indicated no significant matrix effect in assay performance (Figure 3). A series of 25 tap water samples fortified with hydroxyatrazine was analyzed in a blind fashion to validate the data generated using this assay. A linear regression comparing these data generated a correlation coefficient of 0.96, with a slope of 0.9 and an intercept at −0.108.

The soils were extracted in 100% organic solvent. To discriminate between an effect due to the solvent and an effect due to some component of the soil, a comparison was made between soil extracts diluted to 25% using PBS and solvent diluted to 25%. As seen in Figure 4, the matrix effects could be attributed almost completely to the solvent, except in the case of the second Yolo county soil.

Bioremediation Results. Direct analysis of the solvent extracts of manure in the ELISA assay required dilutions of 10-fold to obtain a usable ELISA curve (Figure 5). Evaporating the extract, reconstituting with PBSTA buffer, and then partitioning with $CHCl_3$ allowed the assay to function with greater sensitivity, because no dilution was then required. Previous studies of the partitioning behavior of atrazine and hydroxyatrazine demonstrate 90 ± 5% of the atrazine and 3 ± 1% of the hydroxyatrazine are found in the $CHCl_3$ layer when partitioned against an aqueous solution at pH 7 (Lucas et al., 1993). Although there is a matrix effect above the 6.25% dilution, the background noise is constant. On the basis of this consistent overestimation of 30%, recovery studies from 0.25 to 5 μM (50 ng/mL to 1 μg/mL) (triplicate fortification levels of a total of 12 non-zero samples) hydroxyatrazine in manure yielded an average recovery of 130% and an average CV of 10%.

Analysis of sump and manure samples from the Bio-Gest study revealed the appearance of hydroxyatrazine after 4 days, with an apparent plateau of concentration after 15 days (Figure 6). The sump sample consisted in

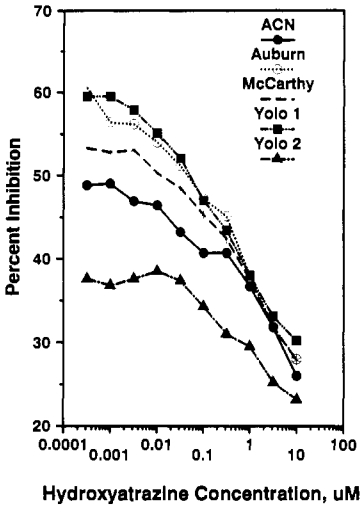

Figure 4. Effect of four different soil types (details in the text) on assay performance. A 25% dilution of the acetonitrile soil extracts in PBSTA was compared to a 25% dilution of acetonitrile alone to discriminate between an effect due to the solvent and an effect due to some component of the soil.

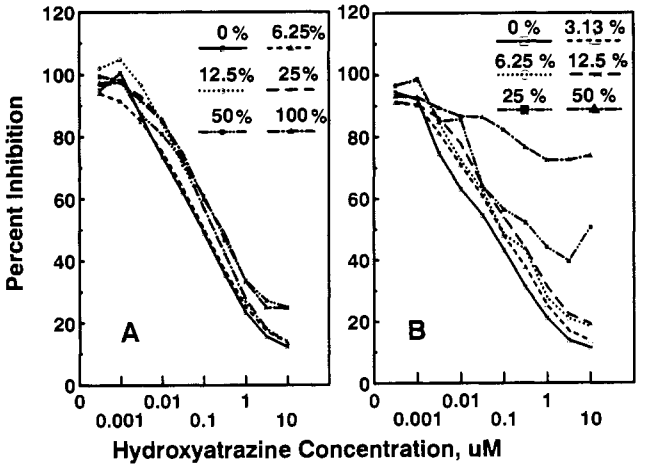

Figure 5. Assay performance in (A) blank horse manure extracts (1:1 methanol–ethyl acetate) after evaporation to dryness, reconstitution with buffer, and partitioning with $CHCl_3$ and (B) the same extracts without evaporation, reconstitution with buffer, and $CHCl_3$ partitioning. Direct analysis of the solvent extracts of manure in the ELISA assay (B) required dilutions of 10-fold to obtain a usable ELISA curve. The evaporation, reconstitution, and partitioning gave greater sensitivity to the assay because no dilution was then required (A). Although there is a matrix effect above the 6.25% dilution, the background noise is constant. The curves represent the average of quadruplicate well measurements with an average CV of 11.5%.

part of the water that had percolated through the manure, in addition to the remnants of the initial solution placed in the sump for bioremediation. The presence of some hydroxylated metabolite in this solution was expected. The concentration of hydroxyatrazine found in the sump was small compared to the concentration found in the manure. Hydroxyatrazine was a minor metabolite from the degradation of atrazine in horse manure, comprising approximately 1% of the initial amount of atrazine placed into the machine. Complete details of the metabolites detected and the rate of disappearance of atrazine can be found in California EPA (1992). Derivatization and GC/MS analysis of selected samples in selective ion monitoring mode for m/z 2852, 905, 882, and 327.2 were performed to confirm the presence of hydroxyatrazine.

White Rot Fungus Results. Homogenized white rot fungus showed no adverse effect on assay performance

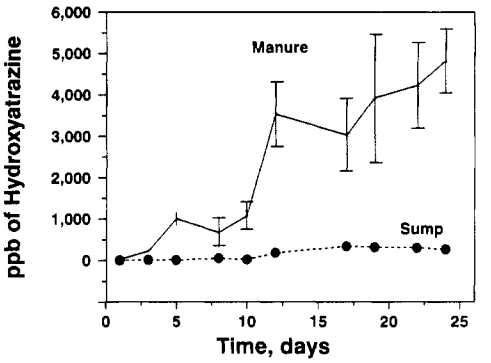

Figure 6. Amount of hydroxyatrazine, in nanograms per gram for the manure samples and in nanograms per milliliter for the sump samples, detected by ELISA over time from various samples generated by the Bio-Gest bioremediation study. The sump sample consisted in part of the water that had percolated through the manure, in addition to the remnants of the initial solution placed in the sump for bioremediation. The concentration of hydroxyatrazine found in the sump was small compared to those in the manure. Derivatization and GC/MS analysis of selected samples were performed to confirm the presence of hydroxyatrazine.

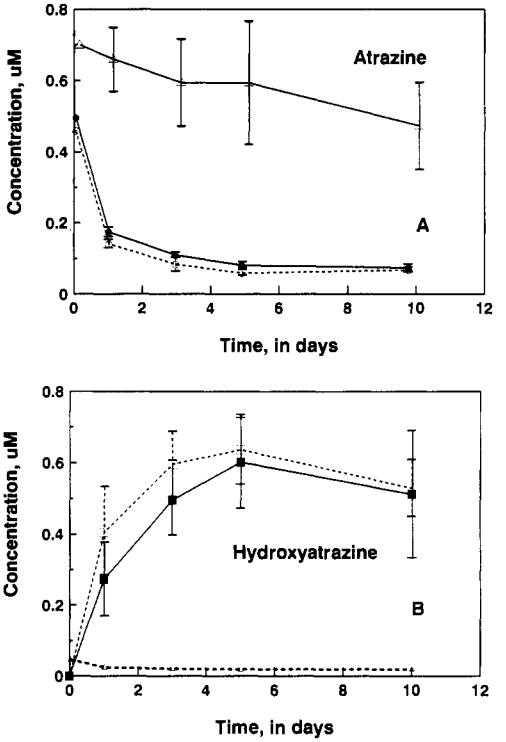

Figure 7. Conversion of atrazine (A) to hydroxyatrazine (B) when 2 μg of atrazine was irradiated with UV 254 nm for 2 h/day. Solid squares (hydroxyatrazine) and solid circles (atrazine) indicate the effect of UV exposure and incubation with *P. chrysosporium*. The open circle and squares represent the effect of UV light on atrazine alone. The error bars represent one standard deviation from the mean of quadruplicate well measurements of duplicate incubations. The triangles represent the controls using *P. chrysosporium* without UV irradiation. No detectable amount of atrazine was degraded.

when the organism in its medium was ground with a hand homogenizer and the aqueous suspension placed directly into the ELISA. No further preparation of samples was needed, except vigorous shaking and sonication just prior to analysis to prevent the fungal mat from re-forming. Atrazine was readily degraded to hydroxyatrazine under the conditions described here where UV light was used (Figure 7); the presence of the fungus did not affect atrazine hydroxylation. Dead micellae given an identical dose of

atrazine showed no significant loss of atrazine response throughout this experiment. When the fungus was incubated with atrazine, but no UV light was used, no breakdown of atrazine was observed. Further studies are being performed to see if after UV-catalyzed hydroxylation, this particular fungus can metabolize the resulting hydroxyatrazine.

Conclusions. The need to dispose of agrochemicals, and other waste matter, in an efficient, intelligent manner has been an area of growing concern over the past few decades. The two bioremediation systems presented here demonstrate application of microbes to degrade atrazine quickly and inexpensively. In general, using an ELISA to monitor the breakdown of parent and appearance of metabolites in these systems provides a great advantage in terms of minimal time and sample preparation needed for the rapid assessment of a large number of samples. Also, samples of biological origin often only need simple dilution prior to ELISA analysis.

The value of ELISA analysis for compounds that are not readily amenable to GC analysis or which require extensive sample preparation prior to HPLC determination, such as hydroxyatrazine and hydroxysimazine, has established the immunoassay as an essential tool in the repertoire of the analytical chemist. In addition, potential erroneous analytical results from long-term sample storage, if either the parent compound or the metabolites degrade, are minimized. In monitoring agrochemical levels in the environment, identification of not only the parent but also major metabolites can provide information on the persistence, efficacy, and potential toxicity of a given compound.

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