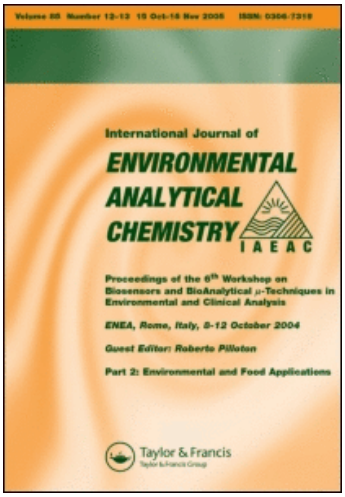

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A Laboratory and Field Evaluation of a Portable Immunoassay Test for Triazine Herbicides in Environmental Water Samples

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A LABORATORY AND FIELD EVALUATION OF A PORTABLE IMMUNOASSAY TEST FOR TRIAZINE HERBICIDES IN ENVIRONMENTAL WATER SAMPLES†

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The usefulness and sensitivity, of a portable immunoassay test for the semiquantitative field screening of water samples was evaluated by means of laboratory and field studies. Laboratory results indicated that the tests were useful for the determination of atrazine concentrations of 0.1 to 1.5 µg/L. At a concentration of 1 µg/L, the relative standard deviation in the difference between the regression line and the actual result was about 40 percent. The immunoassay was less sensitive and produced similar errors for other triazine herbicides. After standardization, the test results were relatively insensitive to ionic content and variations in pH (range, 4 to 10), mildly sensitive to temperature changes, and quite sensitive to the timing of the final incubation step, variances in timing can be a significant source of error. Almost all of the immunoassays predicted a higher atrazine concentration in water samples when compared to results of gas chromatography. If these tests are used as a semiquantitative screening tool, this tendency for overprediction does not diminish the tests' usefulness. Generally, the tests seem to be a valuable method for screening water samples for triazine herbicides.

KEY WORDS: Immunoassay, triazine herbicides, atrazine.

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†Use of brand, firm, or trade names in this paper is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey.

INTRODUCTION

Triazine herbicides, particularly atrazine (2-chloro, 4-ethylamino, 6-isopropylamino-s-triazine), are among the most extensively used herbicides worldwide. These herbicides have been highly useful in the control of weeds in corn and sorghum crops. Unfortunately, these compounds have been quantified in most forms of water in the environment (drinking water[1–2], agricultural field runoff water[5–6], ground water[7], rain[8], and fog[9]), and their prevalence is causing concerns of widespread chemical contamination. A wide variety of analytical methods have been developed to identify and quantify the triazine herbicides in water samples, including gas[10–11], liquid[12–13], and thin layer[14–15] chromatography, bioassays[16–17], and immunoassays[18–22]. Gas chromatography and high-performance liquid chromatography can produce quantitative results, whereas thin-layer chromatography and bioassays generally produce qualitative results. Immunoassay detection offers excellent possibilities as either a field-screening tool (semiquantitative) or as a laboratory method (quantitative) for the presence and quantification of triazines. Advantages of analysis by immunoassay include the relatively low cost (as compared to chromatographic analysis), the short time required (approximately 15 minutes), and the small sample volume required (less than 200 μL).

The first and most important step in the development of an immunoassay is the production of an antibody specific to the analytes of interest[23]. At present, most of the commercial pesticide-screening tests are based on polyclonal antibodies that are specific only for families of compounds rather than for an individual chemical. Because of this chemical nonspecificity, a particular compound cannot be identified (e.g., a positive result for a triazine test could indicate the presence of atrazine, simazine, propazine, other triazine herbicides, or a sum of any combination of triazines). Thorough reviews of the application of immunoassay technology to environmental chemicals can be found in Vanderlaan *et al.*[23], Monroe[24], and Hammock and Mumma[25].

This paper describes results of a study to evaluate the usefulness, accuracy, sensitivity, and robustness of a commercially available immunoassay test for the triazine herbicides. This particular test involves single test tubes coated with polyclonal rabbit antibodies. The test was originally designed as a portable, semiquantitative screening tool for field use. Laboratory and field studies were conducted using these test kits. The same immunoassay in a 96-well plate format for testing with laboratory instrumentation also is available for quantitative analysis but was not examined in this study.

METHODS

Reagents and standards

Herbicide standards were obtained from the U.S. Environmental Protection Agency (Research Triangle Park, North Carolina, USA) and Ciba-Geigy Corporation* Agricultural Division (Greensboro, North Carolina, USA). All standards were greater than 98 percent pure. Standard solutions were prepared by dissolving a known weight (20–30 mg) of a given

triazine in methanol (Burdick & Jackson, Muskegon, Michigan, USA) followed by repeated dilutions in water (Milli-Q, Millipore Water Purification Systems, Boston, Massachusetts, USA). Standard solutions containing hydroxy-atrazine were first dissolved in water at pH less than 2 (HCl added). All final solutions contained less than 0.01 percent methanol except the alachlor and metribuzin solutions, which contained up to 5 percent methanol.

The immunoassay triazine test kits were manufactured by ImmunoSystems (Scarborough, Maine, USA). The specifics of the development of this test are described in Bushway *et al.*[18] and Thurman *et al.*[19]. Each test kit came with seven dropper vials containing enzyme conjugate, substrate, chromogen, negative control, 1.0 µg/L atrazine solution, 10.0 µg/L atrazine solution, stop solution (2.5 N sulfuric acid), and 20 antibody-coated tubes.

Immunoassay procedure

Test kits were stored at 4° C but were allowed to equilibrate to room temperature before use. Up to four water samples could be tested at any one time. A negative control had to be tested concurrently with each set of water samples. The negative control was used to standardize the water sample's absorbance measurements. Stated as a percentage of the negative control, the standardized absorbance measurements were used for intertest comparison. The manufacturer's recommended procedure for this analysis was followed.

Initially, 160 µL of the negative control and 160 µL of the water sample to be tested were added to different enzyme-coated reaction tubes. Next, the enzyme conjugate solution was added to each of the reaction tubes, and the tubes were shaken. The enzyme conjugate was added one drop at a time in each tube in turn until each received four drops (160 µL). The tubes were allowed to incubate for 5 minutes. After the 5-minute incubation period, all of the test tubes were emptied simultaneously and rinsed three times with triazine-free water (Milli-Q). After the rinsing, four drops of the substrate solution was added to each tube and was immediately followed by four drops of the chromogen solution. The test tubes were again shaken immediately and were incubated for 2 minutes. After 2 minutes, one drop of the stop solution was added, and the test tubes were shaken to completely "stop" the reaction. Following the addition of the stop solution, the solution in the tube turned from blue to yellow. In order to have enough volume for measurement on the spectrophotometer used in the laboratory portion of this study (Hitachi 100-20), 1.4 mL of Milli-Q water was added to each test tube before transfer to the cuvette. The absorbance was measured at 450 nm.

Absorbance and triazine concentration are inversely related; thus, the negative control and samples that were free of triazine herbicides yielded the highest absorbance readings. The difference in absorbance units (optical density, OD) between a sample or standard (OD_{sample} and the negative control (OD_{nc}) was defined as ΔOD ($\Delta OD = OD_{nc} - OD_{sample}$). Ideally, the relation between ΔOD and the log of the concentration of the triazine will be linear within a specified range. The differences in optical density between various sample runs caused by the external variables of time and temperature were standardized by dividing the change in optical density of the samples by the change in optical density of the negative control ($OD_{standardized} = \Delta OD/OD_{nc}$)[19]. The linear regression line between $OD_{standardized}$ and log concentration for a series of standard solutions was then used to predict triazine concentrations in water samples on the basis of absorbance measurements.

Laboratory experiments

A number of solutions with triazine compounds and other herbicides (Table 1) were prepared by serial dilution in Milli-Q water at known concentrations to test for the detection limit and the range of the immunoassay. Accuracy and precision also were tested with these solutions.

Aqueous solutions of atrazine, deethyl-atrazine (DEA), deisopropyl-atrazine (DIA), and hydroxy-atrazine (HA) were prepared at six pHs (1,3,4,5,7,9) by the addition of either HCl or NaOH to test the response of the immunoassay.

Most tests were done at room temperature (22° C), but several tests were done at 4° C to examine the effect of temperature on the immunoassay results.

Several steps in the test procedure involve specific incubation periods. Because the testing procedure depends on human judgment and the results of kinetically dependent reactions, variations on the recommended incubation periods also were examined.

Aqueous solutions of atrazine along with triazine-free solutions were examined under a variety of ionic conditions. Solutions of NaCl, $NaNO_3$, or $CaCl_2$ at concentrations ranging from 0.0063 g/L to 30 g/L were tested for interferences in test results.

The reagents supplied with the test kits came in plastic "eye-dropper" vials. The standard procedure for dispensing reagents is by the drop. By use of water at a known temperature and an analytical balance, the dropper vials were tested for variance in drop size from a single plastic vial and among several different plastic vials.

Field study

A total of 141 samples were analyzed: 107 from surface water sites, 15 from field-drainage tiles, and 19 from observation wells. All samples were collected within the Cedar River watershed, Iowa and Minnesota, USA, from April through July 1988. Samples were collected in glass bottles that had been baked at 350° C and stored at 4° C until the immunoassay analysis was done (normally within 3 or 4 days after sample collection). The surface-water samples were depth integrated from a single vertical section of the river at the centroid of water flow[26]. Collection of samples from field drainage-tile was accomplished by filling the sample bottles from the discharge pipe. Samples from the aquifer were collected after three volumes of water in the well casing were removed with a stainless-steel bailer.

The immunoassay sample analysis normally was performed by two people on four samples at a time. A stopwatch was used to ensure accurate timing of incubation periods, but temperature was not carefully regulated. Standards were selected to bracket the concentrations detected in the samples. Either a Beckmann Model B or a Milton Roy Spectronic 40 spectrophotometer was used to determine the absorbance of the sample at a wavelength of 450 nm. Triazine concentrations were determined as described previously under "Immunoassay-Procedure". Gas-chromatography techniques were used to compare and evaluate the immunoassay results.

Gas-chromatography analysis was done by standard U.S. Geological Survey technique (National Water Quality Laboratory, Denver, Colorado, USA)[27]. Three extractions with dichloromethane were performed on an unfiltered, 1-L water sample. The dichloromethane

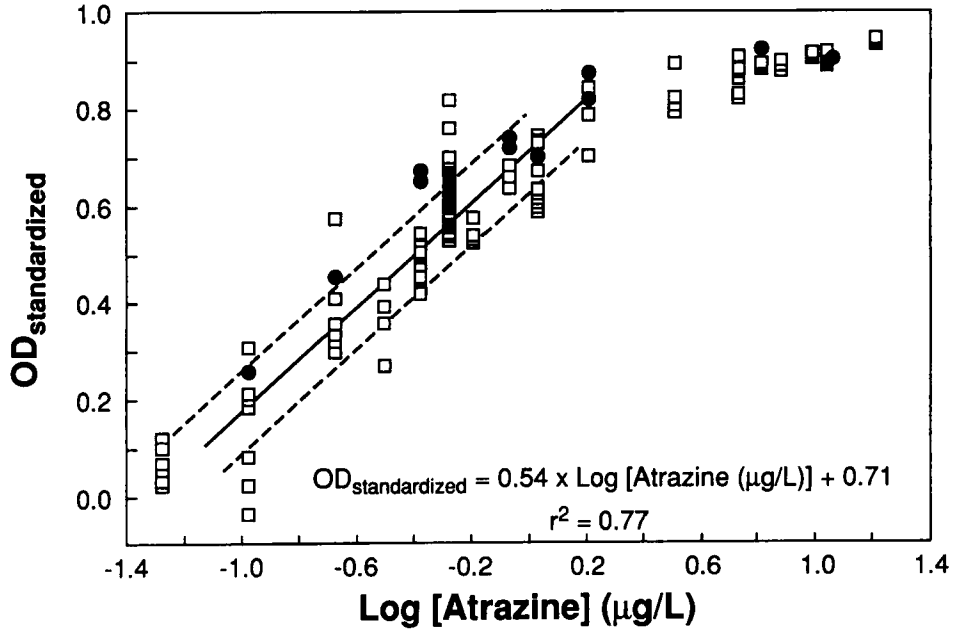

Figure 1 The standardized absorbance ratio ($OD_{standardized}$) as a function of the log of atrazine concentration for 110 tests (□, at 22° C; ●, at 4° C). The solid line delineates the concentration range within which a semiquantitative estimate can be made. The dashed lines represent ±1 standard deviation from the regression line.

extractions were combined and reduced in volume in a Kudurna-Danish apparatus. Final volume reduction to 1 mL with a concurrent solvent exchange to hexane was done under a gentle stream of dry nitrogen. Analysis was done with a Hewlett-Packard 5880 gas chromatograph equipped with dual 25-m columns (methyl-silicon and 5 percent phenyl-methyl-silicon) and nitrogen/phosphorus detector. The gas-chromatography reporting limit was 0.1 µg/L for each of the following compounds: ametryne, atrazine, cyanazine, metribuzin, prometon, prometryn, propazine, simazine, and simetryn. Terbutylazine was used as a surrogate. Recovery was 80 to 90 percent.

RESULTS AND DISCUSSION

Precision and accuracy: Laboratory study

Although a definite relation was observed between the known solution concentration and the standardized immunoassay test results, the results were variable. Within a single test run of up to four replicate samples and a negative control, the relative standard deviation was approximately ±20 percent (atrazine at 1 µg/L). The $OD_{standardized}$ ($OD_{standardized} = \Delta OD/OD_{nc}$) of a large population (n = 110) of immunoassays as a function of the known atrazine concentration ranged from 0.05 to 16 µg/L (Figure 1).

Table 1 Chemical structure and immunoassay test response of test compounds.

Compound	*Substituents* *R1*	*R2*	*R3*	*Manufacturer's reported detection limit (μg/L)*	*Concentration range of linear relation (μg/L)* $0.1 < OD_{standardized} < 0.8$	*Range of concentration at 1 μg/L ±1 SD*	*Number of samples tested*
Atrazine	Cl	C_3H_7	C_2H_5	0.1	0.074 – 1.4	0.70 – 1.4	110
Desethyl-atrazine (DEA)	Cl	C_3H_7	H	.4	.82 – 210	.47 – 2.1	12
Desisopropyl-atrazine (DIA) (at 10 μg/L)	Cl	C_2H_5	H	—	7.8 – 750	7.8 – 13	12
Hydroxy-atrazine (HA)	OH	C_3H_7	C_2H_5	1.0	.61 – 540	.65 – 1.5	7
Simazine	Cl	C_2H_5	C_2H_5	1.0	.12 – 12	.55 – 1.8	12
Simetryn	S-CH_3	C_2H_5	C_2H_5	.3	.20 – 2.7	.66 – 1.5	11
Propazine	Cl	C_3H_7	C_3H_7	.1	.0063 – 1.2	.40 – 2.5	50
Prometryn	S-CH_3	C_3H_7	C_3H_7	.1	.060 – 1.9	.73 – 1.4	12
Metribuzin	NA	NA	NA	>1,000	>1,000	NA	6
Alachlor	NA	NA	NA	>1,000	>1,000	NA	6

SD: standard deviation; OD: optical density; NA: not applicable.

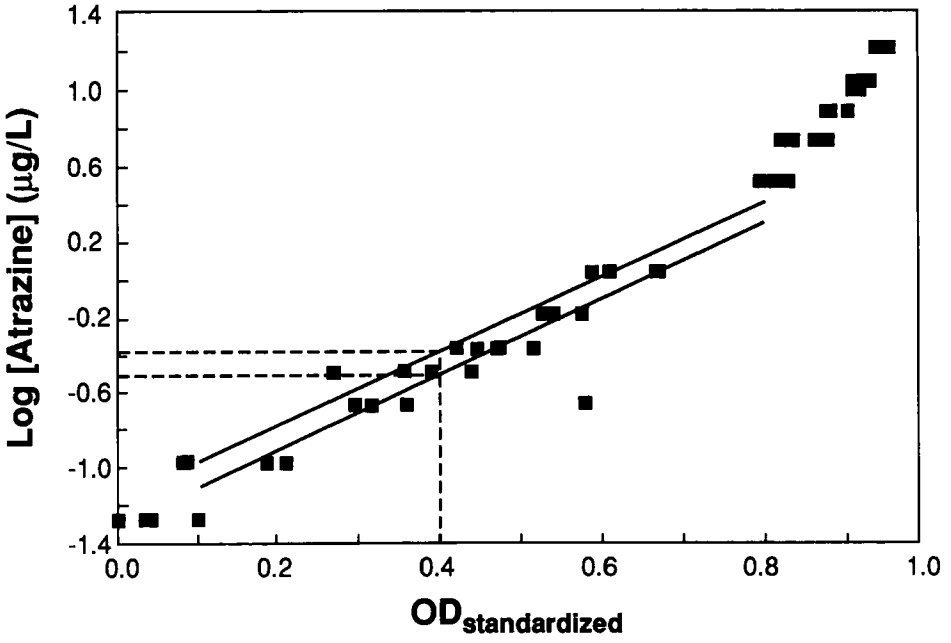

Figure 2 Log atrazine concentration (μg/L) as a function of $OD_{standardized}$ for twelve sets of four tests that could have been potential "standard concentration curves." The solid lines denote the highest and lowest results. The dashed lines demonstrate how differences between the two sets translate into differences in the predicted atrazine concentration.

A linear relation was observed for atrazine concentrations of 0.074 μg/L to 1.4 μg/L: however, for concentrations greater than 1.4 μg/L where $OD_{standardized}$ was greater than 80 percent of OD_{nc}, the relation ceased to be linear and the semiquantitative usefulness of the test was limited. The range of linear relation varied with each analyte (Table 1).

For atrazine, the relative standard deviation in the difference between the regression line and the actual result over its linear range was about 40 percent at 1 μg/L (Table 1). Generally, the relative deviation increased as the concentration approached the detection limit. Relative standard deviations for other triazine herbicides were generally greater.

To test the reproducibility of standard concentration curves obtained from the immunoassay, 12 sets of tests were performed with known atrazine concentrations ranging from 0.05 to 16 μg/L. A regression of the log of atrazine concentration as a function of $OD_{standardized}$ was calculated for each of five sets that had all of their concentrations within the previously described linear range of the immunoassay. The highest and lowest regression lines are shown in Figure 2.

The slopes of the two regression lines were nearly identical, but the intercepts were different by about 0.08 units on the $OD_{standardized}$ scale. For an $OD_{standardized}$ of 0.4, the concentrations determined from the highest and lowest regressions differed by about 0.13 log concentration units (concentration in μg/L), which represents an approximate error of 30 percent at a concentration of 0.35 μg/L. The variations in results from these tests confirmed the semiquantitative nature of the immunoassay.

The immunoassay test responses were established for atrazine, DEA, DIA, HA, simazine, simetryn, propazine, and prometryn (Table 1). The responses of metribuzin and alachlor also were tested (Table 1) and shown that they will not influence the results of the triazine immunoassay under typical environmental conditions. Similar results for this same test have been presented by Thurman *et al.*[19] for these and other herbicides. These two compounds can be assumed to be representative of other non s-triazine herbicides in their interference with the immunoassay test. The standard deviations for standardized negative controls were calculated, and a difference of three standard deviations above the zero (5.2 percent OD_{nc}) was established as the detection limit of this test; but, for semiquantitative results, $OD_{standardized}$ must be greater than 10 percent OD_{nc} and less than 80 percent OD_{nc}. Linearity was questionable outside this range.

Accuracy: Field study

The gas-chromatography analysis of 141 water samples indicated that triazine herbicides were present in concentrations greater than the 0.1-μg/L gas-chromatography reporting limit in all but 15 samples. Atrazine was present in all but one of the samples that gave positive chromatographic results. Cyanazine and simazine were present in only a few samples. None of the other triazine herbicides examined by gas chromatography were detected. The degradation products of the herbicides were not included in the gas-chromatography analysis, but it is known that the concentrations of degradation products are lower than the concentrations of parent compounds in the surface water and ground water in the area.

The concentrations of the triazine herbicides determined by immunoassay were compared to those determined by gas chromatography (Figures 3A and 3B).

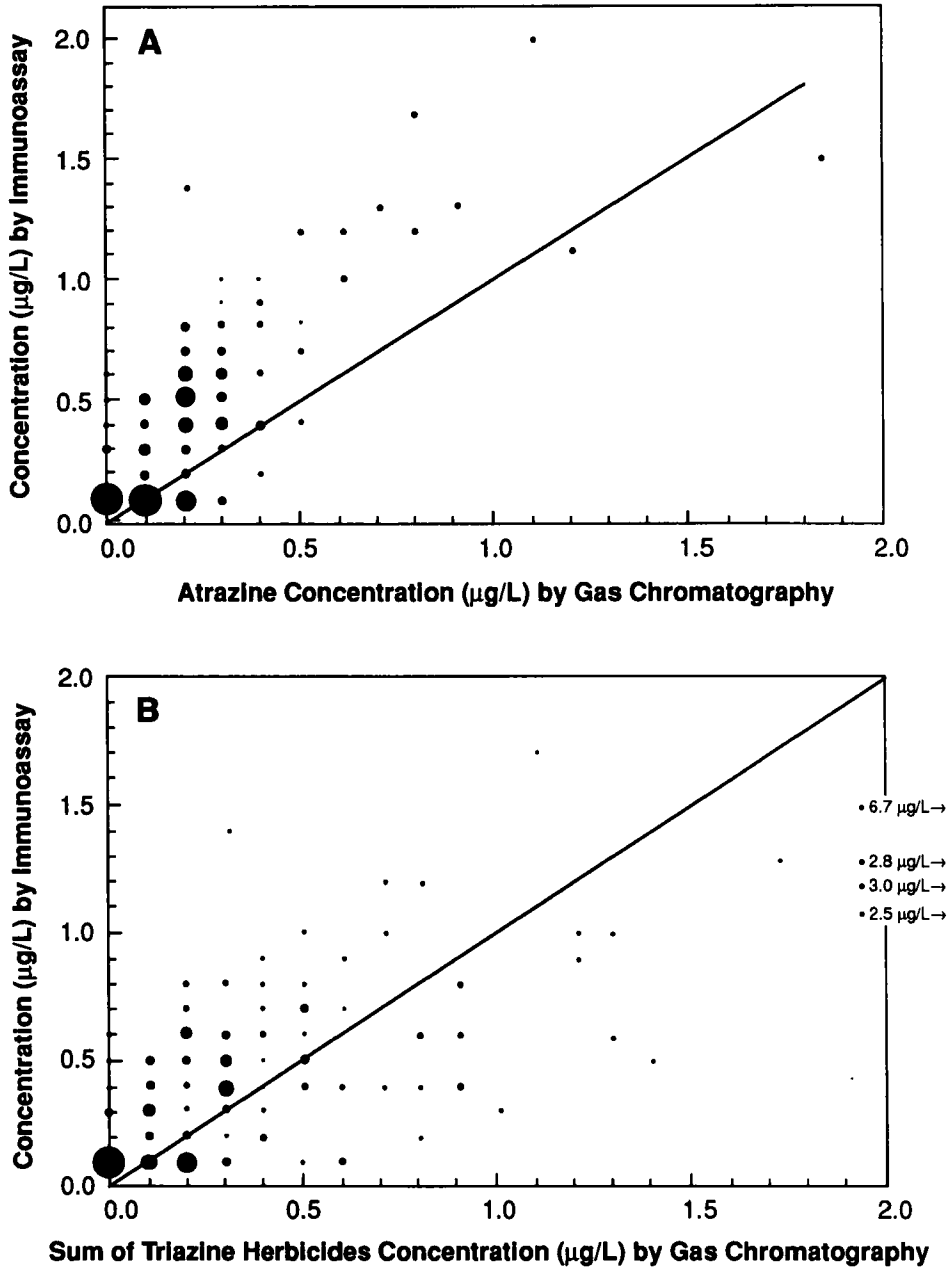

Figure 3 Triazine herbicide concentration measured by the immunoassay (based on atrazine sensitivity) as a function of (A) atrazine concentration and (B) sum of the triazine herbicide concentrations determined by gas chromatography. The line represents 1:1 agreement in concentration. The size of the symbol is proportional to the number of data (from 1 to 9).

Table 2 Comparison of results of immunoassay and gas chromatography of surface- and ground-water samples.

Concentration determined by gas chromatography (μg/L)	*Concentration determined by immunoassay (μg/L)*		
	<0.2	*0.2–0.5*	*>0.5*
ATRAZINE ONLY			
<0.2	13	16	1
0.2–0.5	9	29	26
>0.5	0	0	7
SUM OF THE TRIAZINE HERBICIDES			
<0.2	12	14	1
0.2–0.5	7	26	17
>0.5	1	7	16

Numbers presented are in terms of percentage of total data points. The sum exceeds 100 percent because of rounding.

In all samples, the immunoassay test predicted the presence of triazines with no false negatives in agreement with the findings of Thurman *et al.*[19]. For atrazine alone (Figure 3A), almost all (124 out of 141) of the concentrations of the triazine herbicides determined by immunoassay analysis (on the basis of atrazine's sensitivity) were larger than concentrations determined by gas chromatography. The concentrations of the sum of the triazine herbicides (atrazine, cyanazine, simazine) determined by immunoassay analysis (Figure 3B), although closer to 1:1 agreement, also were generally greater than the concentrations determined by gas chromatography. This differs from the findings of Thurman *et al.*[19] which reported almost a 1:1 agreement between the concentrations from the immunoassay test and the sum of the concentrations by gas chromatography. For atrazine alone, the median difference between the concentrations determined by immunoassay and gas chromatography was 0.2 μg/L, with a standard deviation of 0.3 μg/L. For the sum of the triazine herbicides, the median difference between concentrations determined by the two methods also was 0.2 μg/L. The concentrations of the triazine herbicides determined by immunoassay and gas-chromatographic analysis were grouped into three ranges to compare the two analytical methods (Table 2).

The concentrations determined by the two methods were in the same range for about half of the observations. The concentrations determined by immunoassay tended to be higher than the concentrations determined by gas chromatography for most of the remaining observations. The presence of target-compound degradation products or non-target triazine herbicides at concentrations about one order of magnitude higher than that of the target compounds could create a situation in which the sum of triazine herbicides actually present in the water sample, as determined by immunoassay analysis, could exceed that measured by gas chromatography. This situation could explain the field observations, but the observed differences in test results could also be caused by errors inherent in the immunoassay. The tendency of the immunoassay to predict concentrations slightly higher than those determined by gas chromatography makes the immunoassay a conservative screening test for the presence of total triazines but does not diminish its usefulness for this purpose.

Effects of environmental variables

The effects of environmental variables such as pH, ionic content and species, temperature, and duration of chromophore response were examined. The response of the immunoassay to these variables was evaluated at conditions within and outside the ranges for these variables normally found in natural waters. One environmental variable, the concentration of dissolved organic carbon (DOC), was not examined in this study. Thurman *et al.*[19] have reported that DOC does not affect results of this immunoassay.

The effects of pH on the response of the immunoassay were examined for atrazine along with its degradation products, DEA, DIA, and HA. At pH less than 1, the test was chemically destroyed and the immunoassays indicated a very high concentration ($OD_{standardized} = OD_{nc}$). The predicted atrazine concentration decreased slightly as pH increased. The predicted concentration of a 0.53-μg/L atrazine solution decreased linearly by one standard deviation as pH was increased from 4 to 8. This error is within the overall variability of the immunoassay. For pHs of 4 to 10, pH had no statistically significant effect on the result of the immunoassay ($OD_{standardized}$) for the tested degradation products of atrazine.

The effects of ionic strength and species on the response of the immunoassay also were tested. The salts that were chosen included the common monovalent and divalent cations and nitrate, a common ground-water contaminant in agricultural areas. Solutions containing NaCl, $CaCl_2$, or $NaNO_3$ at concentrations of 0.0063 g/L to 30 g/L, with and without atrazine, were tested. The range of concentrations of these ions exceed the concentrations normally found in natural waters. None of the species at any of the concentrations tested affected the results.

The results of the immunoassays are based on the kinetics of a series of chemical reactions. Thus, temperature plays a significant role in test results. Although standardization of the optical density partly accounted for differences in temperature among test runs, measurements of solutions of a known atrazine concentration at 4° C yielded some $OD_{standardized}$ values that were outside the one-standard-deviation range measured at 22° C (Figure 1). Generally, tests conducted at 4° C show a tendency, even after standardization, to predict higher concentrations than predicated at 22° C. Errors in predicting the triazine herbicide concentration in a water sample can occur if the water sample is tested at a different temperature than that of the standards. The manufacturer recommends that the immunoassays and reagents be stored at 4° C. If they are not allowed to equilibrate with the ambient temperature before their use, the first tests will tend to yield different results than later tests. Handling of the reagent bottles could also heat the contents and change conditions as testing continues. Variances in temperatures are one of the reasons that a negative control must be included with each set of tests. All samples, standards, and negative controls need to be tested under identical conditions for optimum accuracy and precision.

The final product of the test is a colored solution whose absorbance varies inversely with the triazine concentration. The final absorbance response was tested to see if it would change with time when stored at room temperature in a sealed container (to prevent evaporation). No substantial change was noted after 6 days. Thus, tests can be performed in the field, stored in sealed containers, and measured for absorbance at a later date in the laboratory.

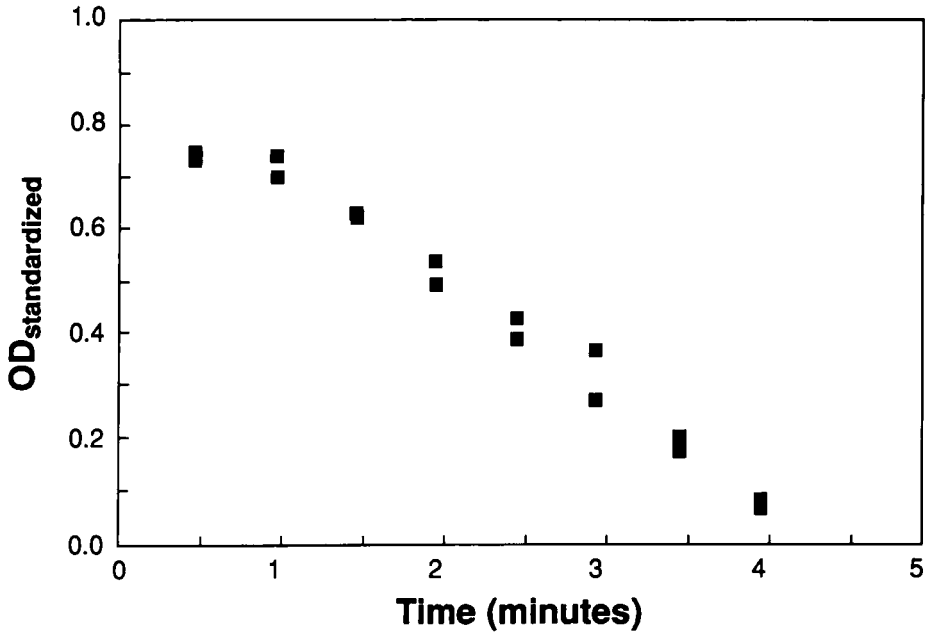

Figure 4 The variation of $OD_{standardized}$ as a function of time for the final incubation step in the immunoassay analysis. The manufacturer's specified period is 2 minutes.

Errors in the method

Tests were made of the consistency in the volume of the drops produced by the droppers supplied with the reagent vials. The average drop volume was 39.1 μL. Although this volume is slightly less than the 40 μL stated in the manufacturer's literature, the difference should not be a significant source of operator error. The average relative standard deviation in drop volume was 6.96 percent for any given bottle and 10.61 percent between different bottles.

Reagents are added to the sample tubes by putting a drop of a reagent into each tube, in turn, until each tube receives four drops. Because of this, it is easy to leave a drop stuck on the side of the tube or miscount the drops. Variances in the number of drops of enzyme conjugate, chromogen, and substrate added during a test were examined for their effects on test results. In general, as the number of drops added of either enzyme conjugate or chromogen decreased, the measured absorbance decreased. Failure to add two drops (50 percent) of enzyme conjugate raised the predicted concentration of an atrazine solution by approximately one standard deviation. The relative standard deviation was 40 percent at 1 μg/L atrazine. Failure to add one drop (25 percent) of chromogen increased the predicted concentration of the same solution by 75 percent of a standard deviation (40 percent at 1 μg/L). Failure to add up to three of the four drops of the substrate had no effect outside the usual variance.

Just as variations in the number of drops of the reagents could influence the results of the test, variances in the duration of the two incubation steps could yield erroneous predicted

concentrations. The first step is a 5-minute incubation period after the enzyme-conjugate is added to the water sample. This incubation period was altered from 2 to 8 minutes in a series of tests to observe the effect of the incubation period on the final results. For incubation times within ±1 minute of the correct 5-minute incubation period, the results were within one standard deviation of the correct results. An incubation period of 2 minutes increased absorbance. An incubation period of 8 minutes decreased absorbance. The envelope of at least ±1 minute allows a large margin for error in the tests. The second incubation period (2 minutes) follows the addition of chromogen and is concluded by the addition of the "stop" solution. This 2-minute incubation period was examined over the range of one-half minute to 4 minutes. The $OD_{standardized}$ values demonstrate a strong dependence on this incubation time (Figure 4). Errors in time keeping during this step will cause serious errors in results of the test; thus, time keeping must be critically controlled.

CONCLUSIONS

Field immunoassay methods have significant potential for the semiquantitative screening of triazine herbicides in water samples. The accuracy of the method was verified in laboratory and field studies. The precision was verified in the laboratory portion of the study. The results reported here support the usefulness of this method as a field-screening technique. The method has proved relatively insensitive to a wide range of environmental variables (pH, ionic strength and species, and concentration of DOC) and can, therefore, be used for a wide range of natural waters. Although changes in temperature had a minor effect on the test results, the utility of the immunoassay as a screening tool was not diminished. Variations in the length of the final incubation procedure could be a significant source of error in the final results.

These immunoassays can be useful semiquantitative additions to environmental field studies. They are useful primary as screening tools to detect the presence or absence of triazine herbicides in a given sample. When used in the field, these tests have the potential to guide sampling location and frequency. A sample could be screened in the field to decide whether or not the sample needs to be analyzed in the laboratory. In this way, a large percentage of the samples with concentrations below the analyte's detection limit could be eliminated. These tests can also provide real-time information about the presence or absence of the triazine herbicides in water samples. The immunoassay tests are not meant to be quantitative, and they do not replace laboratory analyses for water samples that test positive for these herbicides in the field.

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