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River Analyzer for Chlorotriazines with a Direct Optical Immunosensor

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An optical immunosensor coupled to a FIA system was evaluated to monitor chlorotriazine pesticides in river water samples. The assay is based on a solid-phase fluorescence immunoassay with immobilized analyte derivative and free, fluorescence-labeled anti-atrazine or anti-simazine antibodies. The response of the immunoprobe in the presence of cross-reactants structurally similar to atrazine (simazine, deisopropylatrazine, and deethylatrazine) was studied. Limits of detection for atrazine and simazine varied between 0.06 and 0.2 μg L^{-1}, depending if Milli-Q water or river water samples were used. Monitoring of river water samples in the Ebre area (Tarragona, Spain) during 3 months was carried out. Eighteen measurements were carried out, and only eight samples could be detected with simazine and atrazine levels ranging from 0.18 to 1 μg L^{-1}. The results obtained with the immunosensor were validated by chromatographic techniques. The 50-mL water samples were preconcentrated by on-line solid-phase extraction (SPE) followed by liquid chromatography−atmospheric pressure chemical ionization−mass spectrometry (LC−APCI−MS). In this way, unequivocal confirmation of the environmental levels of the Ebre samples was accomplished. This RIver ANAlyzer (RIANA) with an optical immunosensor can be applied for the monitoring of chlorotriazines in river water samples with a total analysis time of 15 min. No false positive was detected.

Introduction

Environmental legislation has increased the interest in developing analytical devices that can afford the regular monitoring of organic pollutants in water. As a consequence, biosensors seem to be a promising tool to obtain fast, sensitive, specific, and reproducible measurements. Immunosensors are solid-phase immunoassay-based biosensors that combine the power of antibodies as recognizing agents and an appropriate physicochemical transducer to convert the recognition success to a readable signal. Immunosensors are generally used to perform indirect measurements by means of competitive immunoassay configurations or labels such as enzymes, fluorescent chemicals, or electrochemically active substances (*1, 2*).

Chlorotriazine herbicides are among the top 10 herbicides used in the United States and Europe. They are detected in estuarine waters as a result of the transport from agricultural fields to surface waters. Chlorotriazine herbicides have been detected in estuaries and coastal seawaters from the Mediterranean such as the Ebre (Spain), Po (Italy), Rhone (France), and Axios (Greece) Rivers (*4*). Environmental monitoring requires the use of fast, cheap, easy-to-use analytical techniques for trace level monitoring of pesticides and other pollutants in natural water. In this respect, the Environment and Climate Program of the European Union has promoted research in the area of biosensors for environmental monitoring from the 1990s till now. Results on that area dealing with the use of a flow immunosensor consisting of a fiber optic onto which an atrazine derivative is immobilized (*5*), or of a planar waveguide onto which the hapten is immobilized (*6*), or the use of an evanescent wave fiber optic sensor that operates according to a competitive heterogeneous format with fluorescent detection (*3*) were reported. Recently, one of our groups was involved in the development of a flow-through immunosensor based on the direct immobilization of antibodies on a support, and it was applied to the monitoring of irgarol in estuarine waters (*7*−*9*).

Due to the lack of application of immunosensors to monitor triazine levels in natural water samples, we have decided to determine such compounds by a recently developed immunosensor. A solid-phase fluoroimmunoassay combined with an optical transducer to achieve excitation and collection of fluorescence from fluorescently labeled antibodies locally bound at the planar interface (*10*) was developed. The excitation of the fluorophores by an evanescent field can be adaptable to an optical transducer configuration such as attenuated total internal reflection element (ATR), which allows the selective detection of surface-bound fluorophores. As a consequence, on-line monitoring of binding events is feasible. This work is part of the European Union Project RIANA (RIver ANAlyzer) that includes the development of a prototype with an optical immunosensor for the determination of chlorotriazines in river water samples

The main objectives proposed for the present work were (i) the application of the RIANA prototype based on an optical immunosensor to monitor chlorotriazines in river samples during a monitoring study of 3 months; (ii) to study the effect of cross-reactants on the response of the evanescent wave immunosensor by validating the immunosensor using on-line solid-phase extraction (SPE) followed by liquid chromatography−mass spectrometry (LC−MS); and (iii) to carry out the unequivocal confirmation of the results obtained by the RIANA prototype by using on-line SPE−LC−MS. To our knowledge, no previous immunosensor applied to the monitoring of chlorotriazines in natural water samples has been reported in the literature.

Experimental Section

Chemicals. The pesticide standards atrazine, simazine, deisopropylatrazine, and deethylatrazine were obtained from Promochem (Wesel, Germany). The LC-grade water and acetonitrile were purchased from Merck (Darmstadt, Germany) and were passed through a 0.45-μm filter before use. Potassium dihydrogen phosphate, potassium hydroxide, and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Cy5.5-dye bisfunctional reactive *N*-hydroxysuccinimide (NHS) ester was purchased from Amersham Life Science (Braunschweig, FRG). Pepsine was purchased from Sigma Aldrich (Madrid, Spain). Deuterated atrazine was purchased from Cambridge Isotopes (Cambridge, U.K.).

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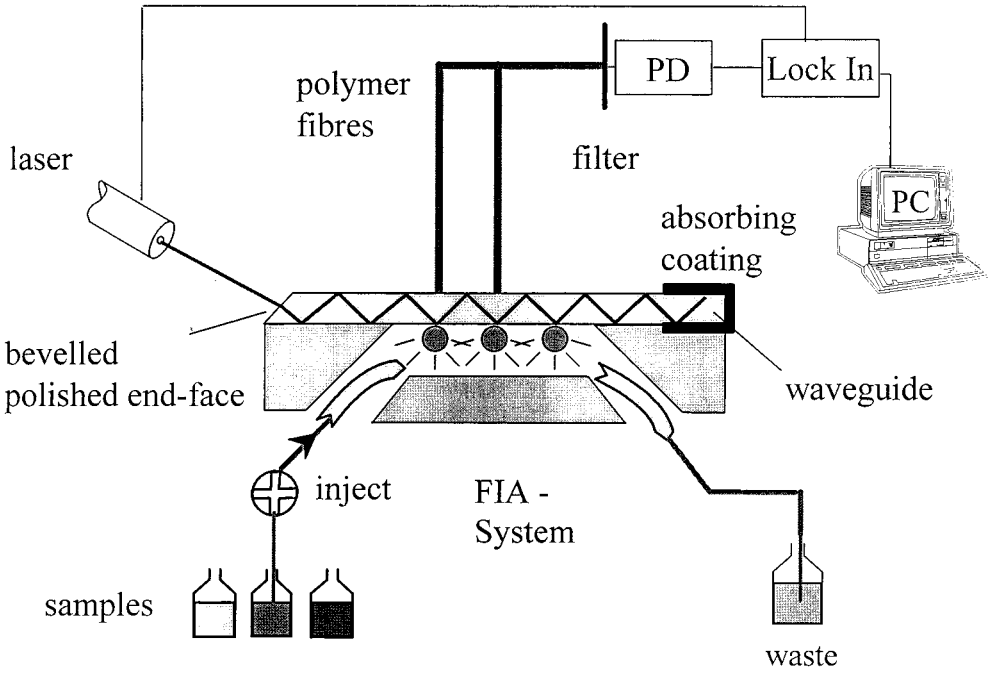

FIGURE 1. Scheme of the immunosensor. The FIA system equipped with a six-port valve and a 1-mL syringe pump delivers either the buffer solution, the sample, or the regeneration solutions to the flow cell. The transducer, a 1.5 mm thick surface polished sheet glass, is mounted on the flow cell and is sealed with an O-ring. The excitation light consists of a He−Ne laser. The collected light is filtered, detected with photodiodes, and acquired by lock-in detection.

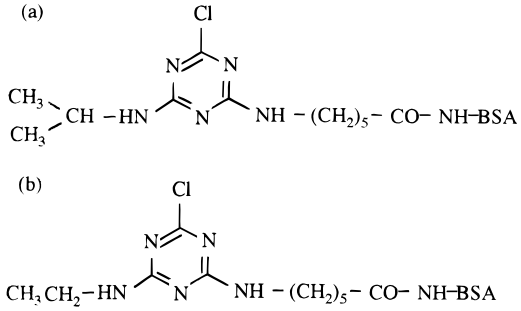

FIGURE 2. Chemical structures of the hapten derivatives used for the obtention of (a) anti-atrazine and (b) anti-simazine antibodies.

Biosensor. A detailed description of the immunosensor has been recently published by Brecht et al. (*10*). A scheme of the FIA system is shown in Figure 1. The device consists of a six-way distribution valve equipped with a 1-mL syringe pump and connected through a Teflon tubing to the flow cell. The transducers were mounted in this flow cell with a flow channel of 1.7 mm width and 0.1 mm depth and sealed by an O-ring. The FIA system corresponded to the FIAS 3000 device from Perkin-Elmer (Ueberlingen, Germany). The excitation of fluorescently labeled antibodies was accomplished using a collimated He−Ne laser that was directly launched into the transducer using a beveled edge. The collected light was subsequently filtered to avoid any collected pump radiation, detected with photodiodes, and acquired by lock-in detection. An autosampler AS90/91 from Perkin-Elmer was used to deliver the samples to the FIA system.

The hapten structures used to produce simazine and atrazine antibodies are shown in Figure 2. Simazine and atrazine caproic acid were coupled to a carrier protein (bovine serum albumin, BSA) via terminal carboxylic acid functions. The coupling step was carried out by first activating the carboxylic acid groups by conversion to NHS esters followed by mixing with an alkaline solution (pH 8.6) of the carrier protein. The immunogen was purified by dialysis. Sheep were immunized with 3.5 mg of immunogen at monthly intervals for 8 months.

Solid-phase immunoassay involves the immobilization of either the antibodies or the analyte derivative. In this approach, the immobilization of the analyte derivative was chosen in agreement to the highest stability of the immobilization structure. Immobilization was performed on clean glass slides treated with aminodextran (according to ref *11*) and addition of a DMF (dimethylformamide) solution of atrazine caproic acid, 1:2 (w/w), and 150% mol/mol of DIC (diisopropyl carbodiimide) (*10*).

To perform binding measurements, 1 μg/mL anti-atrazine or anti-simazine antibodies labeled with Cy5.5, 200 μg/mL of ovalbumin, and a water sample was placed in a 1-mL Eppendorf tube, which was placed in the autosampler. Prior to the measurement, the premixed samples were incubated for at least 15 min. After rinsing the flow cell with phosphate buffer solution, samples were injected into the flow cell for 270 s. During this time, the laser was switched off to avoid photobleaching of the dyes. Subsequently, and after a brief rinse with PBS (30 s), the fluorescence signal was recorded, and the signal difference before and after the binding was measured to obtain an estimation of the number of molecules binded to the transducer layer. Regeneration of the transducer is performed by rinsing with pepsin solution (2 mg/mL, pH 1.9) and acetonitrile:water (50:50). The total analysis time for a single measurement was 15 min.

Cromatographic Conditions. Liquid chromatography−atmospheric pressure chemical ionization−mass spectrometry (LC−APCI−MS) with positive ion mode of operation was used for the validation of the immunosensor measurements. A VG Platform from Micromass (Manchester, U.K.) equipped with an APCI interface was used. The cone voltage was set at 30 V, and the corona voltage was set at 3.5 kV. The ion source was set at 180 °C, and the probe temperature was 350 °C. The eluent was delivered by a gradient system from a Waters 616 pumps coupled to a Waters Model 600 S controller (Waters, Milford, MA). Gradient elution was performed as follows: from 25% acetonitrile and 75% HPLC

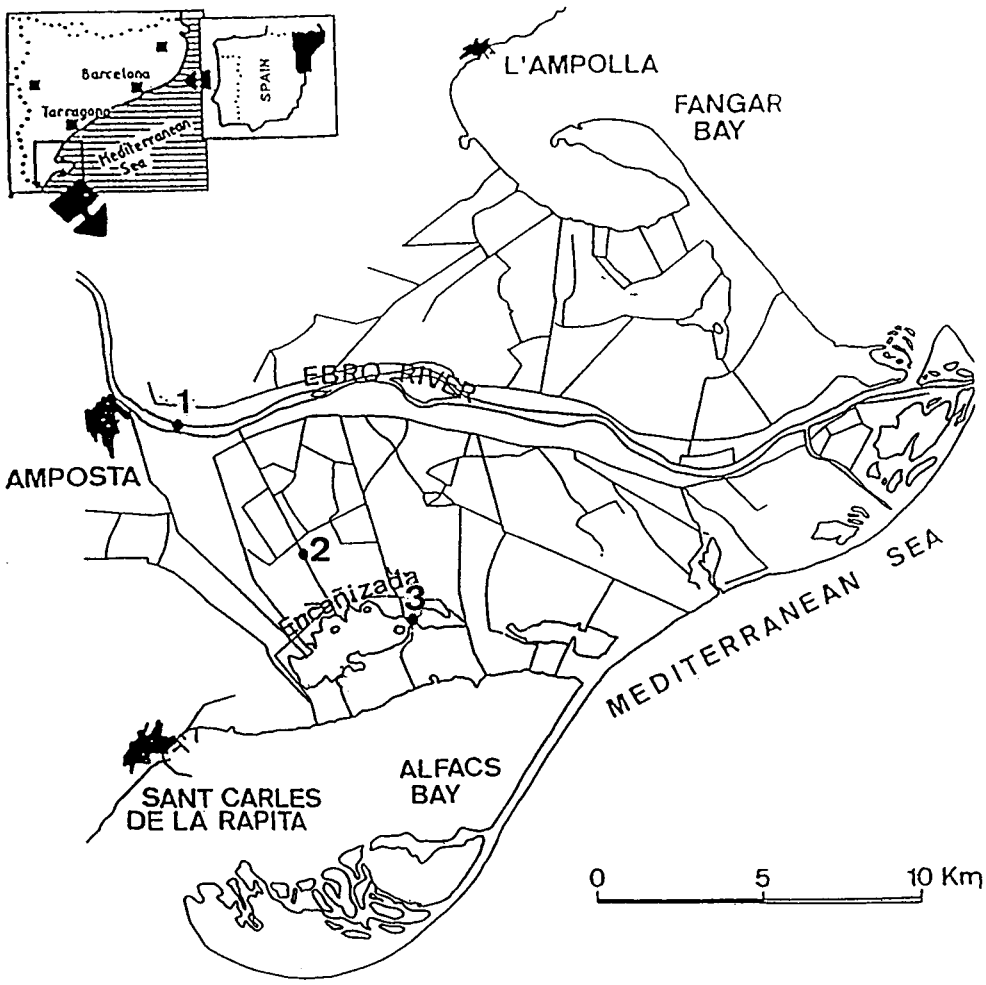

FIGURE 3. Map of the area where the monitoring took place.

water (0.5% acetic acid) to 100% acetonitrile in 30 min and back to the initial conditions in 10 min. Flow rate was set at 1 mL/min. The analytical column used was a 25 cm × 4.6 mm i.d. packed with 5 μm of octylsilica gel from Shandon (Cheshire, England). Samples were analyzed on-line, and the chromatograms were recorded under SIM conditions using positive ion mode. The typical fragments of deisopropylatrazine, deethylatrazine, simazine, atrazine, and deuterated atrazine were selected at m/z values of 174, 188, 202, 216, and 221, respectively. Deuterated atrazine was used as an internal standard for accurate determination of the triazines studied.

Preconcentration of the samples was performed using an automated on-line solid-phase extraction (SPE) system (Prospekt). It consists of a cartridge exchange module, a solvent delivery unit (SDU) (Spark Holland), and a low-pressure six-port valve that is connected to the gradient pumps. Water samples were preconcentrated on 10 mm × 2 mm i.d. disposable precolumns of Prospekt (Spark, Emmen, The Netherlands) prepacked with 40 μm of polymeric sorbent (PRPL-S) (Baker, Deventer, The Netherlands). The precolumn was first conditioned with 10 mL of acetonitrile and 10 mL of water; then 50 mL of the natural water sample was percolated through the precolumn; and after switching the valve, the components were desorbed by the mobile phase and separated on the analytical column. Desorption of the pesticides from the precolumn holder was done in the back-flush mode in order to avoid dead volumes and chromatographic tailing.

Sampling. River water samples were collected monthly (from April to June 1998) from an estuarine area, the Ebre Delta, which is located in the south of Tarragona, Spain. The selected area for the monitoring is shown in Figure 3. The Ebre River is the third largest river flowing into the Mediterranean Sea. Its average annual flow is 550 $m^3\ s^{-1}$ and is only exceeded by the Rhone and Po Rivers. The large agricultural basin comprises 84×10^3 km^3 and involves rice, wheat, and corn cultivations. The Ebre River covers over 350 km^2. Sampling was carried out at three different points: (i) Ebre River, (ii) channel, and (iii) Encanyissada Lagoon. The first sampling point corresponds to the middle of the river (samples were collected from a bridge), and the second and third ones correspond to an irrigation channel and to a lagoon, respectively, from the large rice crop area of the estuary. Water sample pH ranged from 7.4 to 7.7 at all the sampling points studied. Conductivity showed differences regarding the water source; for instance, water samples from the lagoon showed higher conductivity values (5−30 mS) than the river and channel water samples (1−2 mS). Measurements of conductimetry were performed using a portable conductimeter from Crison (Alella, Barcelona, Spain). Samples were filtered through a 0.45-μm filter to eliminate particulate matters.

Cross-Reactivities. The performance of the immunosensor was evaluated by measuring the cross-reactivity (CR) of compounds structurally similar to atrazine, namely, simazine, deisopropylatrazine, and deethylatrazine. The most extended approach to calculate cross-reactivities is based on the determination of the cross-reactant concentration required to displace 50% of the labeled antigen (*12*). In this case, cross-reactivity is expressed as a measure of the horizontal separation of the standard and cross-reactant displacement

TABLE 1. Values of IC_{50}, Inhibition Concentration at 50% of Absorbance (in $\mu g\ L^{-1}$), Percentage Cross-Reactivities, % CR, and Limits of Detection[a] of Water Samples Spiked with Atrazine (Using Anti-Atrazine Antibody) as Standard and Simazine, Deisopropylatrazine, and Deethylatrazine as Cross-Reactants

		cross-reactants								
		simazine			deisopropylatrazine			deethylatrazine		
matrix	concn ($\mu g\ L^{-1}$)	IC_{50}	% CR	LOD	IC_{50}	% CR	LOD	IC_{50}	% CR	LOD
Milli-Q water	cd	0.6	100	0.06	0.6	100	0.06	0.6	100	0.06
	0.1	3.2	18.7	0.003	3.0	19.0	0.95	1.4	42.8	0.01
	1	5.0	12.0	1.01	3.4	17.6	0.57	5.3	11.3	0.56
	10	8.3	7.3	0.72	10.3	5.8	2.74	16.6	3.6	7.53
	100	14.4	4.2	1.45	2.7	22.2	0.003	1.0	60.0	1.03
river water	1	3.9	15.3	0.18	1.1	54.5	0.13	4.3	13.9	0.2

[a] LODs were calculated as the signal corresponding to three times the standard deviation of the blank signal.

curves. It can be calculated as the ratio between the IC_{50} values corresponding to the standard and the cross-reactant displacement curves. However, this approach does not take into account the effect of nonparallel cross-reactant displacement curves, which presents variation of the cross-reactivity value with the dose of cross-reactant. Moreover, this calculation determines cross-reactivities in absence of the target analyte, and it cannot be applied to evaluate the effect of cross-reactants in an environmental water sample. To evaluate the effect of the concentration on the measure of cross-reactivities, an extension of the Abraham's method (*13*) was used. The cross-reactivity can be defined as follows:

$$\text{cross-reactivity (\%)} = \frac{(\text{standard } IC_{50\ (\text{absence of cross-reactant})})}{(\text{standard } IC_{50\ (\text{presence of cross-reactant})})}$$

where IC_{50} is the inhibition concentration at 50% of the absorbance.

Cross-reactivities were studied at four concentration levels (0.1, 1, 10, and 100 $\mu g\ L^{-1}$) of cross-reactant, and two different matrixes were studied, Milli-Q and river water.

Results and Discussion

Determination of Cross-Reactivities. The first attempt of this study was to determine the cross-reactant concentration effect on the analytical response, and for this purpose a clean water sample, such as Milli-Q water, was selected to reduce any other contribution deriving from the water matrix. Table 1 shows the parameters of the calibration curves of atrazine at a fixed dose of cross-reactant in a range of standard concentrations, from 0.001 to 100 $\mu g\ L^{-1}$. The percentages of CR of samples containing atrazine as standard and deisopropylatrazine, simazine, and deethylatrazine as cross-reactants at different concentrations were calculated. For a fixed concentration of cross-reactant, both series corresponding to the displacement curves of simazine and deisopropylatrazine showed values of CR and IC_{50} in the same order because of the similarity of their structures to that of atrazine. For instance, values of IC_{50} for a fixed dose concentration of 0.1 $\mu g\ L^{-1}$ ranged from 3.2 $\mu g\ L^{-1}$ for simazine to 3.0 $\mu g\ L^{-1}$ for deisopropylatrazine. As it was expected, the displacement of the sigmoidal curve when increasing the concentration of cross-reactants led to higher values of IC_{50}. On the other hand, the high cross-reactivity values, up to 42.8%, concerning the displacement curve of deethylatrazine are remarkable. The binding is strongest for compounds that have structures most closely resembling atrazine. In this way, the antibody will recognize and bind with high specificity compounds that exhibit an isopropyl group, such as atrazine and deethylatrazine (see Figure 2 for the structures of the hapten derivatives). These results are in agreement with previous studies from our group (*14, 15*); reported cross-reactivity values ranged from 80.8% for deethylatrazine to 14.6 and 17.1% for deisopropylatrazine and deethylatrazine in Milli-Q water, respectively, when applying commercial atrazine RaPID ELISA. A previous study involving the use of the same antibodies applied to an ELISA format (results not shown here) (*16*) showed similar values of cross-reactivity. Data are plotted in Figure 4a, it can be noticed that the

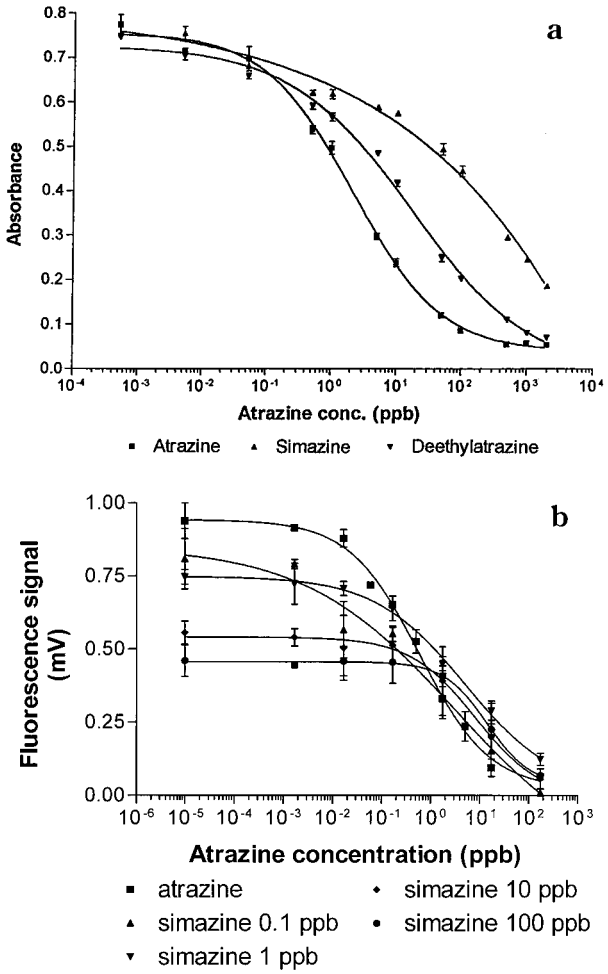

FIGURE 4. (a) Standard calibration curve of atrazine and their common cross-reactants, simazine and deethylatrazine, obtained by ELISA analysis. Limit of detection (LOD) (90% of absorbance) of 0.1 $\mu g\ L^{-1}$. (b) Displacement curves for atrazine (standard) and a series of different concentrations of simazine (cross-reactant). LODs were 0.06 $\mu g\ L^{-1}$ for atrazine, and for the series of cross-reactants, LODs were 0.003, 1.01, 0.72, and 1.45 $\mu g\ L^{-1}$ for atrazine in the presence of 0.1, 1, 10, and 100 $\mu g\ L^{-1}$ simazine, respectively. LODs were calculated as the signal corresponding to three times the standard deviation of the blank signal.

TABLE 2. Values of IC_{50}, Inhibition Concentration at 50% of Absorbance (in $\mu g\ L^{-1}$), Percentage Cross-Reactivities, % CR, and Limits of Detection[a] of Water Samples Spiked with Simazine (Using Anti-Simazine Antibody) as Standard and Atrazine, Deisopropylatrazine, and Deethylatrazine as Cross-Reactants

		cross-reactants								
		atrazine			deisopropylatrazine			deethylatrazine		
matrix	concn ($\mu g\ L^{-1}$)	IC_{50}	% CR	LOD	IC_{50}	% CR	LOD	IC_{50}	% CR	LOD
Milli-Q water	0	0.6	100	0.08	0.6	100	0.08	0.6	100	0.08
	0.1	2.0	30	0.004	1.1	54.5	0.05	2.6	23.1	0.46
	1	15.5	4.0	0.1	1.1	54.5	1.06	1.4	42.8	0.63
	10	12.3	4.8	3.6	1.2	50.0	0.03	–[b]	–	–
	100	19.8	3.0	8.5	1.4	42.9	0.1	5.0	12.0	0.33

[a] LODs were calculated as the signal corresponding to three times the standard deviation of the blank signal. [b] (–) nonparallelism.

calibration curves of deethylatrazine presented lower horizontal displacement as the simazine calibration, thus leading to higher values of cross-reactivity. LOD values obtained in the biosensor are also in good agreement with those published in the literature for ELISA methods; reported LODs for atrazine and simazine in Milli-Q water were 0.051 and 0.349 $\mu g\ L^{-1}$, respectively (*14*). Another important factor to take into account is the diminution of the cross-reactivity as a function of the increasing concentration of cross-reactant. This indicates that the highest cross-reactivity is at low doses of cross-reactant, as expected from polyclonal antibodies (*12*).

Figure 4b shows the shapes of the atrazine standard dose–response curves in the presence and absence of simazine. As the concentration of the cross-reactant increases, the slope of the sigmoidal curve increases and the dynamic range of the curve decreases, leading to a loss of sensitivity that can be confirmed by the LOD values obtained at each concentration of cross-reactant.

Table 2 shows the calculated values of IC_{50}, the percentage of CR, and LODs of water samples spiked with simazine as standard and atrazine, deisopropylatrazine, and deethylatrazine as cross-reactants. The highest values of cross-reactivity are observed when deisopropylatrazine is present in the sample as cross-reactant. Deisopropylatrazine presents the same substituent as simazine (ethylamino group); therefore, the binding of this compound to the simazine antibody is higher than those that exhibit atrazine and deethylatrazine (see Figure 2 where the simazine hapten derivative is shown). For instance, we obtained values about 50% of cross-reactivity for concentrations of deisopropylatrazine ranging from 0.1 to 100 $\mu g\ L^{-1}$. As expected, calibrations showed less sensitivity when increasing the concentration of cross-reactant.

Matrix Effect. Immunoassays are based on competitive interactions between antibodies, analytes, and analyte derivatives. These interactions can be affected by the pH or the ionic strength of natural water matrix. It has also been reported that the presence of other natural substances, such as dissolved organic carbon that interact weakly with the antibody, can induce an overestimation of the immunoassay response (*17*). In this work, the influence of groundwater and river water types on the immunosensor response was evaluated. Water matrixes were studied by adding phosphate buffer solution to accomplish an standard ionic strength. Table 3 shows the calculated values of IC_{50} and LODs for the standard calibrations of atrazine and simazine using different water types. The calibration curve of atrazine showed an increase on the IC_{50} parameter as a consequence of the complexity of the matrix, i.e., the inhibition concentration at 50% of absorbance ranged from 0.56 $\mu g\ L^{-1}$ when using Milli-Q water to 3.2 $\mu g\ L^{-1}$ for river water samples. The calibration curves corresponding to the different waters are shown in Figure 5. It is important to mention the horizontal displacement of the calibration curve performed in river water, which is confirmed by the IC_{50} value of 3.2 $\mu g\ L^{-1}$. Interactions between atrazine and humic substances have been already reported (*18*), and it is an important factor to take into account to explain such displacement. Cation exchange, hydrogen bonding, and charge-transfer interactions were proposed to be responsible for the binding of atrazine to humic substances. As it can be seen from the data reported in Table 3, LODs of 0.26 and 0.18 $\mu g\ L^{-1}$ are achieved for atrazine in groundwater and river water samples, respectively. On the other hand, simazine seems to present less dependence on the water matrix; in this sense, we obtained nearly the same LOD values for all the matrixes studied.

Table 1 also shows the values of the calibrations performed in river water for a fixed dose of cross-reactant, 1 $\mu g\ L^{-1}$. In all cases, an increase on the CR value is observed, thus indicating the influence of humic substances on the response, as it has been already mentioned in this section.

TABLE 3. Matrix Effect on the Calibration Curve of Water Samples Spiked with Atrazine and Simazine (from 0.001 to 100 $\mu g\ L^{-1}$)

	atrazine		simazine	
	IC_{50}	LOD[a]	IC_{50}	LOD[a]
Milli-Q water	0.56	0.06	0.33	0.08
groundwater	1.25	0.26	1.21	0.12
river water	3.22	0.18	2.79	0.10

[a] LODs were calculated as the signal corresponding to three times the standard deviation of the blank signal.

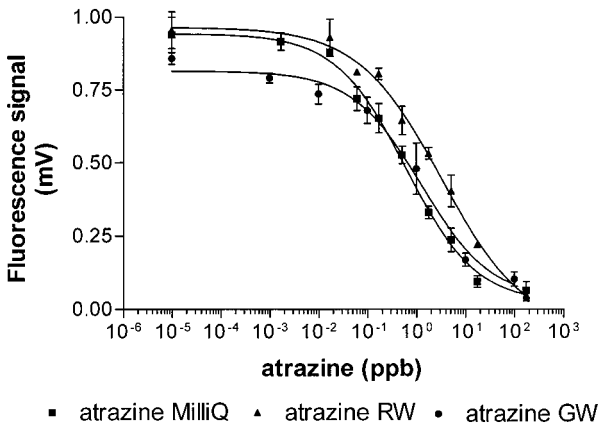

FIGURE 5. Standard calibration curves of atrazine in Milli-Q water, groundwater, and river water. Concentration range from 0.001 to 100 $\mu g\ L^{-1}$ atrazine. IC_{50} values obtained were 0.56, 1.25 and 3.22 $\mu g\ L^{-1}$, for Milli-Q water, groundwater (GW), and river water (RW), respectively.

TABLE 4. Calibration Data and Limits of Detection Obtained by On-line Preconcentration of 50 mL of River Water Followed by Liquid Chromatography−Mass Spectrometry Detection[a]

compound	calibration equation	r^2	LODs
deisopropylatrazine	$y = 0.2x + 0.028$	0.9986	0.047
deethylatrazine	$y = 4.4x + 0.014$	0.994	0.010
simazine	$y = 7.0x + 0.022$	0.9992	0.003
atrazine	$y = 10.8x + 0.012$	0.9994	0.003

[a] Water samples were spiked at levels of atrazine, simazine, deisopropylatrazine, and deethylatrazine ranging from 0.005 to 1 μg L^{-1}.

Monitoring of River Water Samples and Validation of the Immunosensor. Chlorotriazine herbicides and their corresponding metabolites are usually found in surface river and estuarine waters at levels ranging from 0.05 to 1 μg L^{-1} (*4, 14, 19*). Monitoring of river water samples in the Ebre area was carried out to assess the performance of the biosensor, and the results obtained were compared with those obtained by on-line SPE−LC−APCI−MS. The working range obtained for LC−MS was from 0.005 to 1 μg L^{-1} for atrazine and simazine and from 0.01 to 1 μg L^{-1} for deethylatrazine, whereas deisopropylatrazine showed a linear range from 0.05 to 1 μg L^{-1}. No presence of deisopropylatrazine was detected in any of the samples analyzed, as also shown before in other studies when using ELISA and GC−MS methods (*14, 19*) Calibration graphs and limits of detection are shown in Table 4.

From Table 5, it can be mentioned that all the samples showed similar concentrations of atrazine (0.02−0.1 μg L^{-1}) during the studied period, while in the case of simazine a slight decrease of its concentration is observed from April to June. Simazine has not been considered as one of the most widely used herbicides for agricultural purposes in that area. Previous work (*19*) indicated that simazine is widely used in Spain for nonagricultural applications such as weed control in railroads and industrial areas. When considering the percentages of triazines detected in this area during the 3-month period, approximately 60% of the total triazines applied correspond to simazine, and the remaining 40% correspond to atrazine and deethylatrazine. Considering the total of atrazine and deethylatrazine, a slight increase on the percentage concentration of these compounds from April to June can be observed, which can be attributed to the beginning of atrazine application (May−June) in the Ebre River basin. No presence of triazines was detected in river water samples corresponding to sampling point 1. This can be explained if we take into account that the samples were collected in the middle of the river, which has a high flow rate (see Experimental Section); therefore, low contamination levels are expected. The total concentration of triazines in the Ebre River did not exceed 0.2 μg L^{-1}. Table 5 shows the comparative results obtained by LC−MS and immunosensor. Samples analyzed by the immunosensor showed relative standard deviations ranging from 1 to 9% ($n = 3$), thus indicating the good accuracy of the method. Out of 18 measurements, 7 carried out using anti-simazine antibody and 3 using anti-atrazine antibody could not be detected because the concentrations were below the detection limit. Detection of simazine was limited by the performance of the low affinity of the anti-simazine antibody. Although concentrations of simazine and atrazine were not so different during the monitoring study, the determination of simazine by the biosensor suffered from a lack of sensitivity since samples containing 0.1 μg L^{-1} of simazine, which corresponds to the LOD of the method, could not be detected. On the other hand, the determination of atrazine suffers from a high percentage of cross-reactivity due to the presence of deethylatrazine and in a less extension of simazine. As it was pointed out before, in the cross-reactivity studies, the cross-reactivity of deethylatrazine at 1 μg L^{-1} is about 40% and that of simazine is about 18%. This is reflected in the values obtained for atrazine in comparison to those obtained by LC−MS. The correlation between on-line SPE−LC−APCI−MS and the immunosensor was carried out for the Ebre water samples collected from April to June 1998. The obtained slope was 0.729, thus indicating the slightly higher values obtained by the biosensor in comparison to LC−MS. Figure 6 also shows an overestimation of RIANA prototype versus the LC−MS data, which was expected and is a common behavior when comparing biological methods like ELISA with chromatographic data (*14, 18*). The other remarkable fact is that no false positives were detected, and the only cases where chlorotriazines were not detected (see Table 5) are attributed not to a false negative of the RIANA but to the LODs. Figure 7 corresponds to the chromatographic traces of the preconcentration of a 50-mL Ebre River water sample followed by LC−APCI−MS. The selected ions allow the detection of the different triazines without problems of interfering peaks.

The immunosensor described in this paper can be a useful instrument for monitoring triazine pesticides in environmental waters. The high sensitivity achieved (with LOD in real river water samples of 0.2 and 0.1 μg L^{-1}), the capability to analyze complex water matrixes, and the fastness of the analysis time (15 min per sample) provide a useful tool in the biosensor field.

TABLE 5. Concentrations (μg L^{-1}) of Atrazine, Simazine, Deethylatrazine, and Total Triazines in the Ebre Area after Their Analysis by SPE−LC−APCI−MS and Immunosensor[a]

	concentration (μg/L)						
	simazine		atrazine		deethylatrazine	total triazines	
sample	immunosensor anti-simazine antibody	LC−MS	immunosensor anti-atrazine antibody	LC−MS	LC−MS	immunosensor[b]	LC−MS[c]
April 1	bdl[d]	0.05	bdl	0.04	0.04	bdl	0.13
April 2	1.0 (9)	0.92	0.46 (8)	0.05	n.d.	1.46	0.97
April 3	bdl	0.04	0.18 (5)	0.03	0.03	0.18	0.09
May 1	bdl	0.09	bdl	0.09	0.04	bdl	0.22
May 2	0.34 (1)	0.37	0.41 (9)	0.07	0.03	0.75	0.47
May 3	bdl	0.05	0.37 (4)	0.08	0.05	0.37	0.18
June 1	bdl	0.10	bdl	0.07	0.03	bdl	0.20
June 2	bdl	0.10	0.51 (7)	0.05	0.04	0.51	0.19
June 3	bdl	0.05	0.33 (4)	0.02	0.03	0.33	0.10

[a] Sampling points were as follows: (1) Ebre River; (2) channel; and (3) Encanyissada Lagoon. LODs were calculated as the signal corresponding to three times the standard deviation of the blank signal. RSD (%) values for the immunosensor measurements are shown in parentheses. [b] Total triazine concentration corresponding to the sum of simazine and atrazine. [c] Total triazine concentration corresponding to the sum of simazine, atrazine, and deethylatrazine. [d] bdl, below detection limits.

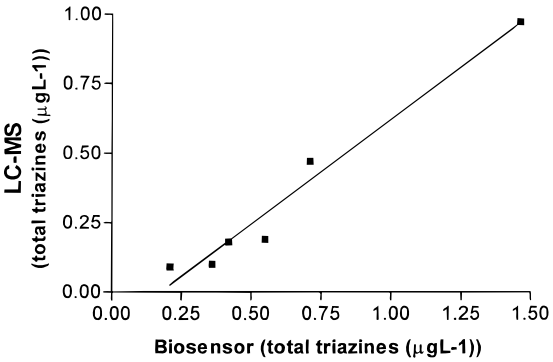

FIGURE 6. Correlation between total immunosensor values, using anti-atrazine and anti-simazine antibodies, and total triazines determined by on-line solid-phase extraction followed by LC−MS for all the Ebre River water samples studied in this work. Regression equation: $y = 0.729x - 0.104$, $r^2 = 0.98$.

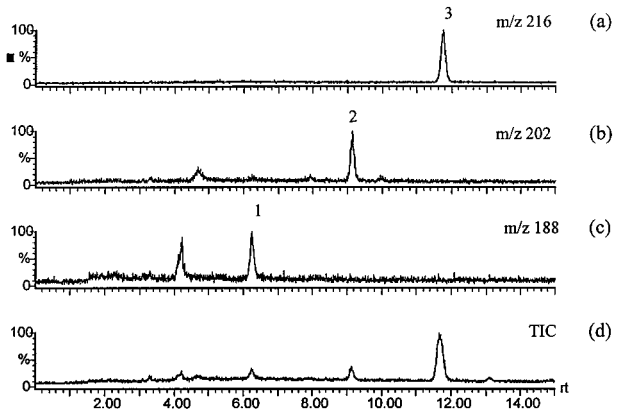

FIGURE 7. On-line solid-phase extraction of 50 mL of water from the Encanyissada Lagoon (sampling point 3) corresponding to the sampling carried out in May 1998. Chromatograms corresponding to the selected ions (a) *m/z* 216, (b) *m/z* 202, (c) *m/z* 188, and (d) total ion current chromatogram are shown. Peak numbers correspond to (1) deethylatrazine, (2) simazine, and (3) atrazine. Analytical conditions are described in the Experimental Section.

The advantage of the RIANA prototype is that it can be used to monitor total chlorotriazines in river water samples with some overestimation of the target values but without any false positive determination. During the monitoring study of the Ebre River, atrazine and simazine were detected at levels ranging from 0.1 to 1.0 μg L^{-1}. Since the European Community (EEC) Drinking Water Directive (DWD) establishes that individual pesticides and their transformation products (TPs) should be monitored at the 0.1 μg L^{-1} level and the total pesticide concentration cannot exceed 0.5 μg L^{-1}, this method can be applied to control total triazine levels in drinking waters. On the other hand, the biosensor could be useful for the compliance of the U.S. EPA legislation for drinking waters, which requires the monitoring of atrazine at the 2 μg L^{-1} level.

The RIANA prototype has been constructed by Perkin-Elmer (Uberlingen, Germany), and at present only three units are available at the laboratories of the different partners of the European Union Project. Commercialization of the prototype is envisaged soon.

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