APPLICATION OF IMMUNOASSAY TECHNIQUES (ELISA) FOR MONITORING ATRAZINE AND ITS METABOLITES IN THE UNSATURATED ZONE

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Abstract - The objective of the study was to develop analytical and sampling techniques for the detection of atrazine and their transformation products (DEA, DIA, DAA and HAY) in the subsurface aquatic environment at low concentrations. To validate the sampling and analytical protocol of immunoassay techniques, El Emporda area, where atrazine has been used during the last 20 years, was selected. El Emporda is located 200 Km North of Barcelona. Materials are of detritical nature (sand, silt, conglomerates) and the aquifer is multilayered. Atrazine concentrations in soil of 17 ng/g and 0.1 µg/l in water were found during a previous monitoring campaign, values suitable to be used with ELISA test. The Envirogard High Sensivity Triazine Plate Kit (Millipore ®) immunoassay kit was selected for the exercise. Water results from test application have evidenced the efficiency of the kit to semi-quantify Atrazine-DEA concentrations, and no false negative was detected. However, lack of accuracy for soil samples show the weakness of this test to detect atrazine residues. An analysis to evaluate the atrazina-DEA crossreaction showed the possibility to calculate both concentrations when both triazines are present.

Key words - ELISA, atrazine, unsaturated zone, groundwater

1. INTRODUCTION

The use of pesticides, both for increasing the yields of agricultural or horticultural products and for general pest control has a long tradition behind it. However, due to the compound characteristics, harm to water resources is to be feared anywhere were these

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substances are manufactured, stored, processed and used One major cause of the surface and groundwater pollution by pesticides can be traced back to large-scale agricultural use (i.e.cereals) or irrigated intensive agriculture and their mobility (Green and Hartley, 1987; Walker et alt., 1995). In this context, horticulture in particular must be classified as ecologically hazardous, since this sector uses approximately ten times more pesticides per year -in number of compounds and quantity- than are normally used in arable farming.

Due to the complex behaviour of pesticides and partly to the lack of validation of sampling technology, little is known about the mechanisms of pollution (Jury et alt., 1987). However, under appropriate hydrogeological conditions, active substances or their degradation products can enter into the aquifer. In regions with varied agricultural land use (i. e. horticulture) it is extremely expensive and difficult to determine and differentiate in detail direct relationships between the presence of pesticides in the water system and its origin. This specially applies to groundwater pollution, which may be the result of pesticide application years before, and where the crops being cultivated and the range of substances used have changed in the meantime. The reaction times for the overall system differ depending on the types of soil, type of aquifer, land use and climatic conditions (Ware, 1993).

The methods generally used to measure pesticides are High Performance Liquid Chromatography (HPLC) and Gas chromatography/Mass spectrometry (GC/MS) involving extraction of large volumes of water, extensive purification and other derivatization and expensive equipment. As a consequence, attention has been directed to newer methods, and Enzyme Linked ImmunoSorbent Assay (ELISA) appears to be a good alternative, at least for screening purposes (Bushway et alt., 1988; Wittmann and Hock, 1989; Ferguson et alt. 1993; Meulenberg et alt, 1995). Good selectivity, sensitivity, precision, and easy measuring many samples in one run (simultaneous samples analysis), makes immunoassay a cost-effective method for routine analysis.

Antibodies are the key components of immunoassay methods. They are used for the detection of compounds that have structural affinity to a specific region of the antibody molecule. The degree to which particular antibody selectively binds the analyte of choice determines its applicability. After addition of an enzyme substrate containing chromogen, the bound enzyme causes a color change which is inversely related to the amount of analyte present.

According to literature, ELISA has proved to be a relatively simple, fast analytical method especially effective when a small volume of water samples has to be analyzed for residues. Sampling and analytical protocols for atrazine and DEA in water from the aquifer

and the unsaturated zone was validated in an experimental area located in L'Emporda (Girona, Spain). DEA was the only metabolite found in natural waters.

Atrazine (2-chloro-4-(ethylamino)-6-i(sopropylamino)-s-triazine) is a triazine herbicide used at higher rates of application as a non selective herbicide, and may remain in the soil for several months after its application. The major transformation products or metabolites are deethylatrazine (DEA), deisopropylatrazine (DIA), dealkylatrazine (DAA) and hydroxiatrazine (HAY). Atrazine has been used in El Emporda agricultural area at locations overlying shallow water tables during the last twenty years so, there was a reasonable chance of detecting both the parent compound and its major metabolites. Moreover, there are several commercial immunoassay kits for its analysis, so these techniques for analysing atrazine and its metabolites in aquifer material could be validated.

2.3. STUDY AREA. ALT EMPORDA

The Alt Emporda is located in the Girona province (Fig. 1), approximately 200 km North of Barcelona (Spain). The plain is constituted by Neogene and Quaternary sediments. Neogene materials are of sedimentary origin with a few levels of volcanic rocks. Quaternary fluviodeltaic deposits constitute the sedimentary infilling of the basin. In detail, there is a broad alluvial plain constituted by the accumulation of sand and silt as a result of alluvial processes and discrete gravel deposits in a channeled pattern. In a hole, the environment of these marshy areas is known as *aiguamolls*: clay and silt sediments in the marshes and sand in the dune ridges.

Two different aquifers can be distinguished: the Neogen and the Quaternary; their hydraulic connection have not been clearly established yet. Neogen materials outcrop in the western part of the study area and generally and they are covered by Quaternary sediments. Geologic materials are of detrital origin -sand, silt and gravel- besides more cemented materials -marls, limestones, sandstones, etc. They show important lateral changes and varied lithological composition. From the hydrological point of view, main interest relies over the existing upper conglomeratic layer, which constitutes the first 10-15 m of the Neogene deposits.

The upper level of the Quaternary material consists of alluvial gravel, sand and silt with an upper layer of silt and clay of 2-8 m thick of marsh or floodplain deposits. Its maximum thickness is 20-30 m in the shoreline. Through the sea, the aquifer is conformed by two different continuous levels of gravel and sand separated by a bed of silt and clay. Near to the seashore the aquifer behaves as a multilayered system.

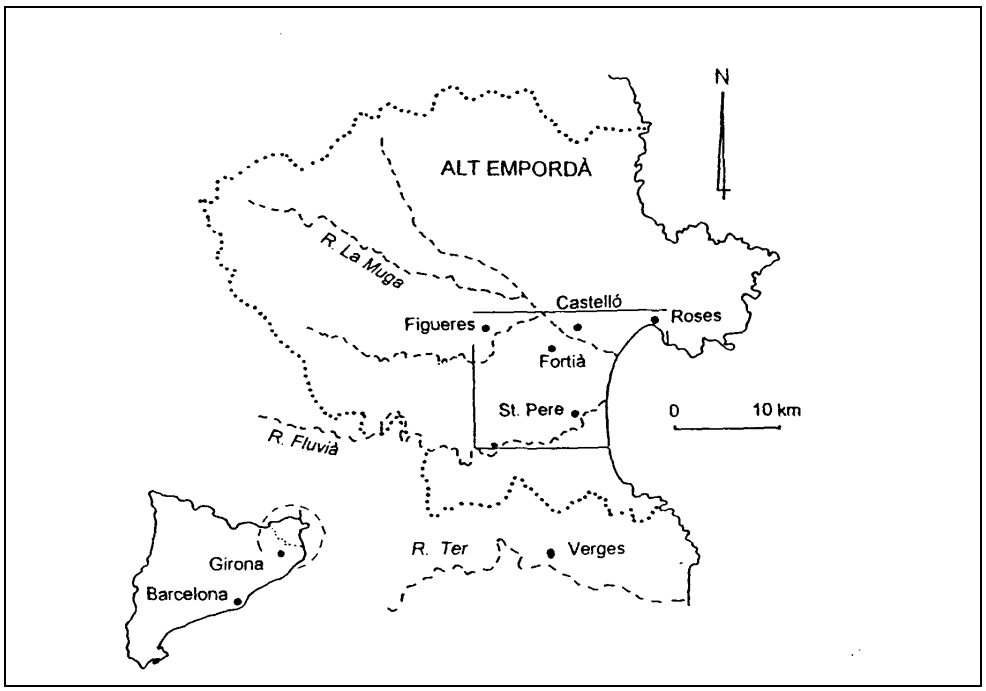

Figure 1. El Emporda Area.

Piezometric levels measured in the inner part of the study area are above of the ground level or very close to it. Near the coast, head levels are usually about 1-2 m below the surface. Some wells tend to behave as flowing artesian wells during the fall and winter seasons, and at the end of the pumping period most of them recover their heads to the regional level, approximately 1 m below the ground surface. Transmissivity values range between 100-1,100 m^2 day for the upper level (up to 20 m approximately) and 500-1,500 m^2/day for the middle aquifer (30-50 m depth).

The soils of the area correspond to weakly developed soils with low organic matter contents and sandy texture.

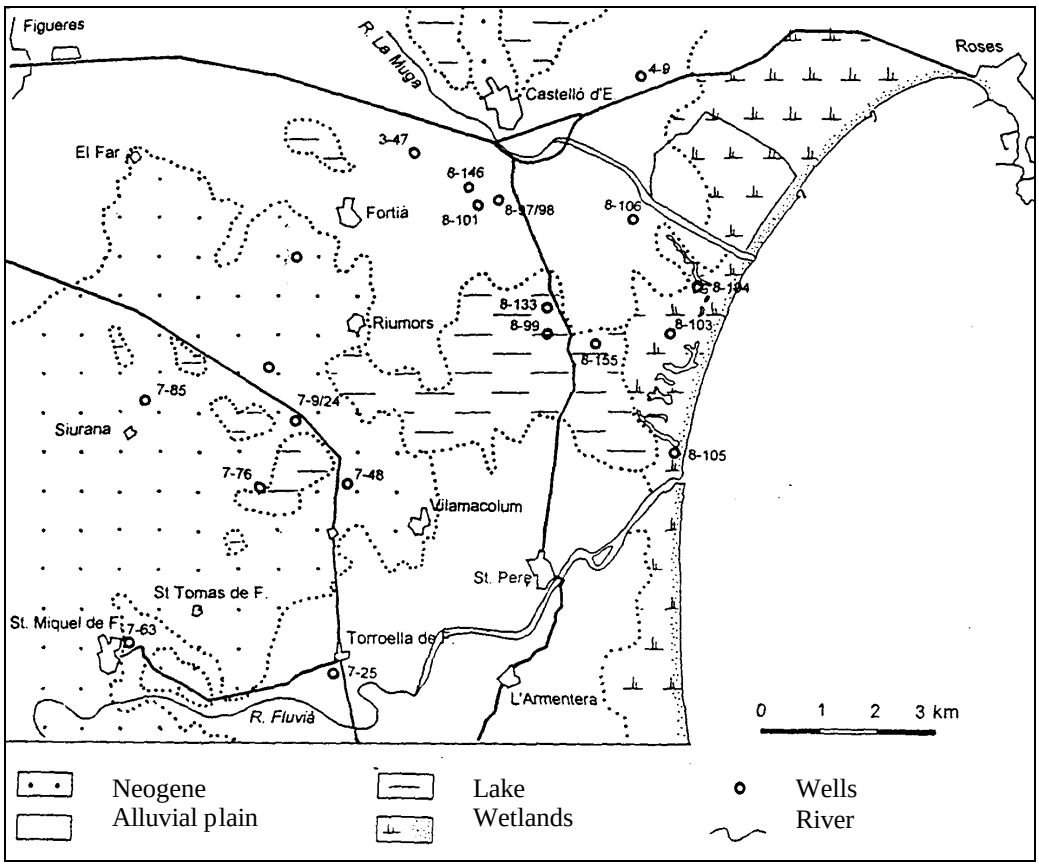

Fig 2. Geological framework.

3. MATERIALS AND METHODS

Total extension of the experimental field was 1364 ha; a pumping well for irrigation purposes, and a drainage canal were also present in the area. Water table variation ranged between 0.5 and 1.5 above sea level in the Quaternary aquifer, the one concerned in the study. Tensiometers and suction cups were installed and destructive drilling up to 2 m was realized for monitoring atrazine and metabolites in soil. Water samples were collected from suction cups and groundwater from the existing well.

Sampling period extended from November 1994 until June 1995 while current agricultural practices were developed. Dose of application was 1.44 kg/ha. Some of the principal characteristics of atrazine are shown in table 1.

Table 1. Chemical characteristics of atrazine (after Wauchope et alt., 1991)

Common Name	Atrazine
Chemical Name	2-chloro-4-ethylamino-6-isopropylamino-s-triazine
Empirical Formula	$C_8H_{14}ClN_5$
Molar Mass	215.7
Melting Point (^{0}C)	173-175
Vapour Pressure (mbar at 20^{0}C)	4.0×10^{-7}
Solubility at 25^{0}C)	33 mg/l
pK_a Value	1.7
Half-life (d)	60
Soil Sorption (K_{oc})	100 (average)
Vapour Pressure (mm Hg)	2.89×10^{-7}

3.1. PESTICIDE SOIL AND WATER SAMPLING

Best methodology to detect the presence of a contaminant in the saturated zone is by monitoring the infiltration water through the unsaturated soil. From this point of view, field soil and porous water sampling are the most critical parameters.

Current monitoring techniques of aquifer material in the vadose zone are based on the installation of suction cups or soil sampling through drilling. While the first one allows a temporal record of the quality of the water being sampled, destructive sampling -soil sampling- only allows a point record of the parameter being measured.

Suction cups were used to sample porous water in the most surficial part of the soil. Centrifugation of soil to obtain pore water for chemical analysis was unsuccessful. Characteristics of aquifer material did not provide enough volume of water for this purpose. Continuous soil sampling was made by auger drilling, and undisturbed samples at a constant depth were taken. Soil samples were carefully stored in aluminium foil and frozen until being analyzed in the laboratory, while replicates were used for the determination of physical parameters (volumetric water content, saturated and unsaturated hydraulic conductivity, bulk density), clay content analysis (through XR) and organic matter. Standard techniques being described elsewhere have been used (Wilson, 1990).

Monitoring soil-water potential, a measure of the equilibrium water pressure relative to that of the soil atmosphere and the flux direction (upward-downward flux) was measured by tensiometers at different depth. Monitoring the direction of flux is important to assess when the leaching is produced.

Water from the aquifer was sampled from the existing well.

4. ANALYTICAL METHODS

Different analytical methods were developed for the residue analysis of atrazine and their metabolites in water and soil samples. Techniques being used were GC, HPLC and immunoassay.

4.1. ATRAZINE AND METABOLITE ANALYSIS. GAS CHROMATOGRAPHY

Gas chromatography was applied to the analysis of Atrazine in soil and water samples using an HP 5890 series II gas chromatograph, provided with two detectors ECD and NPD, an HP ultra 2 capillary column (25 mm x 0.20 mm id, 0.33 μm film thickness) and a HP 7630A autosampler. An injection of 2 μl was made by the autosampler in splitless mode (Hernández et al., 1993)..

Liquid-liquid extraction with dichloromethane was applied to water samples in order to obtain the recoveries for spiked samples. Moreover, feasibility of SPE using C-18 cartridges was also checked following a systematic approach. Soil extraction was carried out for wet samples by sonication with acetone. Water/acetone was removed by partition with dichloromethane and the applicability of SPE to the water-acetone extract was also checked for the isolation of pesticides.

4.2. ATRAZINE ANALYSIS. IMMUNOASSAY TECHNIQUES

The ELISA test was applied to water from suction cups, groundwater from the aquifer and soil samples. Water samples were assayed with the Envirogard High Sensitivity Triazine Plate Kit (ENVR P0048, Millipore®). According to the kit characteristics, least detectable dose (LDD) is 0.01 ppb of atrazine and it may show cross-reactivity with DEA, DIA and DAA. Final concentrations of pesticide were determined through absorbance measurement by using a spectrophotometer at 450 nm of wavelength; being the reference wavelength 620 nm.

The absorbance of samples divided by the absorbance of a negative control (0 μg/L of atrazine) in a percentage form (%Bo) was used to standarize the difference in optical density (OD) caused by the external variables of time and temperature among the sample runs. The linear regression line between %Bo and the log concentration for a series of standard solutions was used to predict atrazine concentration (named atrazine equivalents) in water samples. Dose-response curves showed linear behavior between 0.01 and 0.05 μg/L.

Samples of water stored and frozen in glass bottles were buffered with a PBS.T solution prior to their analysis (Millipore, 1991). To establish the standard curve in a more accurate way, four replicates of 9 different atrazine concentrations, were obtained through the standard calibrators provided by the kit. Replicates were used prior to the development of the test, and in order to assess the variability of the method, reproducibility and consistency of the dose-response curve of atrazine was checked by calculation of 4 standard curves at different periods of time. To obtain the DEA standard curve water solutions of DEA with acetone at different concentrations were obtained.

For soil samples, and following the kit instructions, 5 g of sieved soil were mixed with 5 mg of metanol were mixed and diluted with water due to the high concentration level of atrazine in soil (Millipore, 1991). To asses and validate this technique, two standard curves were prepared: water-metanol and negative soils foritfied with known concentrations of atrazine.

4.2.1. Atrazine-DEA cross-reaction

To study the performance of immunoassay techniques for simultaneous presence of Atrazine and DEA, 4 replicates of water solution with all possible Atrazine-DEA combinations at known concentrations were analyzed. Fixed concentrations, prepared from previous standards, were the following:

Atrazine (ppb): 0.025, 0.075, 0.125, 0.225
DEA (ppb): 0.5, 1, 1.75, 2

The standard curves final concentration were the sum of all possible combinations of atrazine-DEA concentrations. Theoretical concentration of Atrazine ('read Atrazine') was estimated from OD readings, after calculation of the Atrazine standard curve following the steps explained in the previous paragraph.

6. RESULTS

Standard curves obtained at different periods of time are shown in figure 3. A detailed observation of this figure leads to the conclusion that some inconsistencies of the method might appear depending on the curve being used; dispersion is more important at low concentrations. Atrazine calculations from the highest (C3) curve and the lowest curve (C1) may differ by about 0.03 μg/L. Results of a statistical analysis (Candela et al., 1998) the clearly confirmed the lack of consistence. In order to avoid false readings it is advisable to calculate the standard curve simultaneously to the test application.

6.2.1. Water samples

Table 1 shows atrazine concentration in soil solution from suction cups and groundwater analyzed with GC and ELISA. Although there is not a clear trend immunoassay technique always overestimates concentration of atrazine by approximately 0.05 ppb when comparing with GC. Similar results have been obtained in literature (Thurman et alt., 1990; Brady et alt., 1995). However, ELISA did not detect any false negative reading, if the positive limit to be considered is 0.1 μg/L (EU Directive). Attempts to quantify both relationships did not show any significant correlation.

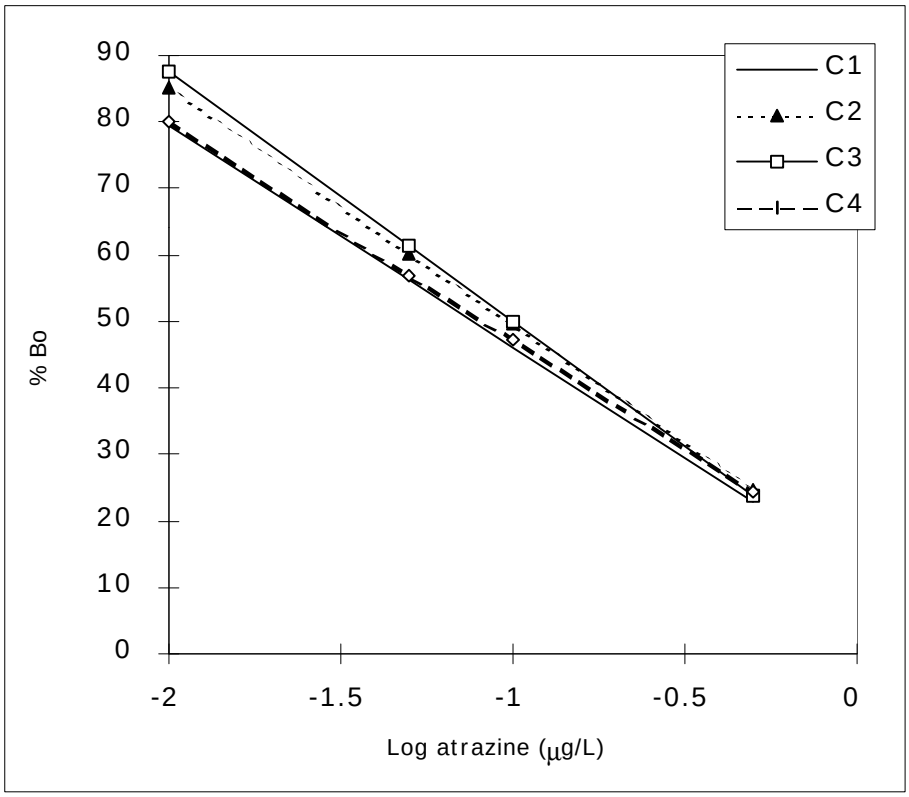

Figure 3. Graph of standard curves obtained at different periods of time (C1: May 12th; C2: June 6th; C3: July 4th; C4: July 4th of 1995) showing differences of atrazine concentration for high %Bo values.

6.2.2. Soil samples

Similarity of results among ELISA and GC was poor, probably caused by the DEA presence and matrix interferences (data are not presented here). However, no false negative was detected, and no trend appeared.

The extraction method of atrazine from soil, requires a previous a dilution procedure. For this reason, real concentration of samples being analyzed may vary between 1-50 ppb at the linear segment of the curve. Consequently, accuracy of the atrazine detection in soil is always lower than in water.

Table 1. Atrazine and DEA results from Gas Chromatography (GC) and ELISA. (Suction cups at 100, 150 and 200 cm depth; *missing values; n.d. under detection limits, C' duplicate values)

WATER SAMPLES ANALYSIS			
22/2/95	GC:Atracina (µg/L)	GC:DEA (µg/L)	ELISA (µg/L)
C100	0.08	0.26	0.10
C150	0.10	n.d.	0.08
C'150	0.09	0.10	0.06
C200	0.08	n.d.	0.14
C'200	n.d.	n.d.	n.d.
well	0.06	0.10	0.09
ATRAZINE APPLICATION			
22/5/95			
C100	12.57	n.d.	>10
C150	0.45	0.12	0.63
C'150	16.57	0.27	>10
C200	0.17	n.d.	0.53
C'200	n.d.	n.d.	*
29/5/95			
C100	n.d.	n.d.	*
C150	0.34	0.20	0.42
C'150	11.12	0.28	*
C200	1.23	n.d.	1.98
C'200	n.d.	n.d.	*
7/6/95			
C100	0.18	0.41	0.30
C150	0.13	0.10	0.50
C'150	8.16	0.25	*
C200	n.d.	n.d.	0.20
C'200	n.d.	n.d.	0.09
20/6/95			
C100	0.86	0.35	2.01
C150	0.14	n.d.	0.28
C'150	3.06	n.d.	4.13

C200	n.d.	n.d.	0.09
C'200	n.d.	n.d.	0.20
well	0.72	n.d.	*
26/6/95			
C100	0.75	0.72	0.95
C150	0.24	n.d.	0.35
C'150	3.15	n.d.	3.48
C200	n.d.	n.d.	0.15
C'200	n.d.	n.d.	n.d.
well	n.d.	n.d.	0.23

6.2.3. Atrazine-DEA cross-reaction

As stated in the kit characteristics, ELISA results do not differentiate among triazines present in soil and water. One of the objectives of this research was to evaluate, and quantify, possible presence of different triazines using immunoassay techniques. In this experiment, only Atrazine-DEA crossreaction has been accomplished. As previously mentioned, the only metabolite detected in natural waters from 'El Emporda' was DEA.

The analysis here presented only focuses in water sample results; the important lack of accuracy of comparison between ELISA and GC for soil samples suggested the exclusion from this exercise.

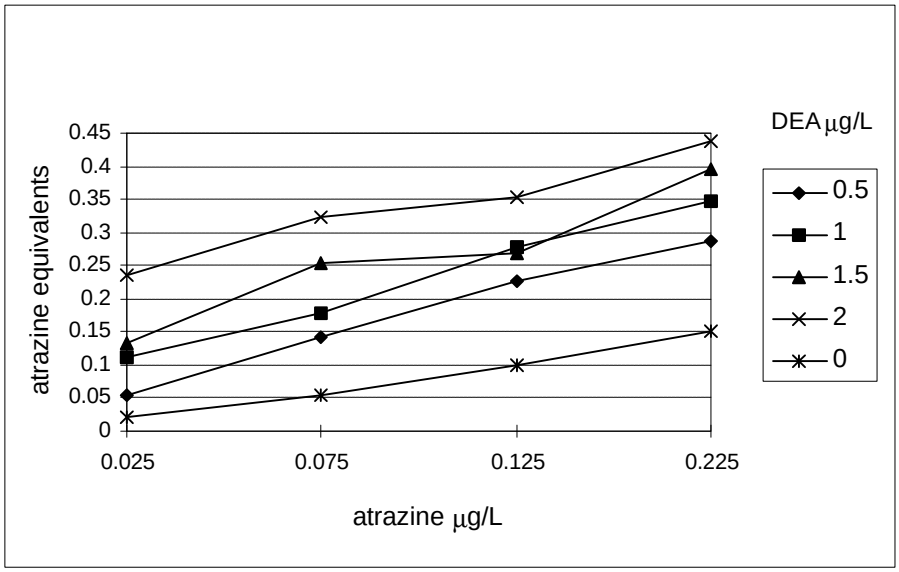

Fig. 4. Graph of atrazine equivalent versus atrazine concentration (µg/L) and the correspondence with several DEA concentrations (µg/L).

Figure 4 presents all possible combinations of atrazine-DEA at fixed concentrations previously selected for cross-reaction comparison. The graph shows O D values and atrazine concentrations (in XY coordinates) while the different lines represent atrazine

concentration at increasing levels with reference to a fixed value of DEA. A theoretical line with 0 ppb of DEA is also shown for comparison purposes.

As can be deduced from the graph, for a given value of 'read atrazine' (or atrazine equivalent) several atrazine-DEA combinations are obtained. For example, if the obtained value ‘read atrazine’ is 0.2', the reading might correspond to the following possible couple of concentrations

DEA (ppb)	1.5	1	0.5	0
Atrazine (ppb)	0.06	0.08	0.11	0.21

As a conclusion, the graph shows the impossibility to discriminate between the two selected triazines in water solution, through ELISA readings.

In order to quantify the atrazine-DEA cross-reaction an attempt was made to characterize the observed variable 'read atrazine' by trend analysis. Trend analysis is based on the computation of multiple regression of significant factors explaining relationships between the two triazines.

Atrazine: 0.025, 0.075, 0.125, 0.225
DEA: 0.5, 1, 1.75, 2

The statistical analysis evidenced that when both atrazine and DEA simultaneously appear in water, ELISA readings are a linear combination of both analytes (Candela et alt., 1998).

7. CONCLUSIONS

From the evaluation of the ELISA methodology developed during the course of the research project in the experimental field, the following conclusions can be obtained.

The Immunoassay technique constitutes a cheap, fast and efficient semi-quantitative technique for atrazine analysis, and a good alternative at least for screening purposes.

Atrazine and AMPA were sampled in soil and water from El Emporda area. Soil samples, porous water suction cups and water from the aquifer were used for the assessment of the ELISA test in soil and water respectively.

Water analysis of atrazine and DEA by ELISA test always show no false negative however, they normally overestimate results compared with Gas chromatography. Analysis

of soil samples by immunoassay techniques gave results no comparable with G.C; important dissimilarities were present although no false negative was detected.

Presence of DEA-atrazine cross-reaction in water not detected by the kit (ENVR P0048 from Millipore) was also assessed through the study of behavior of several Atrazine-DEA known solutions. The statistical analysis evidenced the existence of a linear multiple correlation between the value 'read atrazine' and the Atrazine-DEA concentrations.

ACKNOWLEDGEMENTS

Support for this study was partly provided by the Comisión Interministerial de Ciencia y Tecnología –CICYT, project AMB97-859. Their finantial suport is gratefully acknowledged.

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