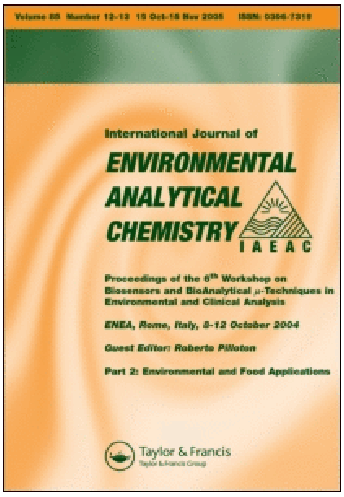

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Screening and Monitoring of Herbicides Behaviour in Soils by Enzyme Immunoassays

R. J. Schneider[a]; L. Weil[a]; R. Niessner[ab]
[a] Institute of Hydrochemistry, Technical University of Munich, Munich 70, FRG [b] Institut fuer Wasserchemie, Muenchen 70, Germany

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SCREENING AND MONITORING OF HERBICIDES BEHAVIOUR IN SOILS BY ENZYME IMMUNOASSAYS

R. J. SCHNEIDER, L. WEIL and R. NIESSNER*

Institute of Hydrochemistry, Technical University of Munich,
Marchioninistr. 17, W-8000 Munich 70, FRG.

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A simplified approach to screen cropland for residues of atrazine and its main metabolite deethylated atrazine has been presented. A time-saving and cost-effective screening method has been developed using an aqueous extraction step. The measurement of the extracts was done by immunoassay technique (ELISA). Antibodies against atrazine and deethylated atrazine were used in the study. The overall detection limit of the analysis of atrazine residues in soil is 1 $\mu g\,kg^{-1}$ and 2 $\mu g\,kg^{-1}$ for its deethylated metabolite, respectively. It is shown that this approach yields adequate information to judge the ground-water hazard potential of treated fields.

KEY WORDS: Immunoassay, soil, atrazine, aqueous extraction.

INTRODUCTION

Judging the ground-water hazard potential of pesticides requires detailed knowledge about the retention of the compounds in the soil as well as their transport down to lower aquifer levels into drinking water resources. South and east of Munich is situated in the western part of a large geological formation called the "Munich Gravel Plain". The quaternary fluvio-glacial gravel, the depth of which averages 12 m, is 80 to 90% of carbonate gravel and sand. The water-bearing strata (tertiary sands) are located at shallow depths[1–4]. The top soil cover in this region is only about 30 to 50 cm. Dairy farming is common in the region, thus corn is a major crop. Ground water is the main source of drinking water.

In the area studied drinking water is drawn off by a gallery of 8 ground-water wells the depths of which average 16.8 m. They were analyzed in July, 1990 giving concentrations of atrazine, a herbicide used for weed control in corn, from 0.10 to 0.28 $\mu g\,l^{-1}$ surpassing the EC limit for water for human use. In contrast to this, in waters from private wells that reach down to 104 m, the second ground-water storey, atrazine could not be detected at a detection limit of 5 $ng\,l^{-1}$.

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*Author to whom correspondence should be addressed. Prof. Dr. R. Niessner, Institut fuer Wasserchemie, Marchioninistr. 17, W-8000 Muenchen 70, Germany.

Another municipality in the same region of Bavaria obtained its drinking water from 6 wells. Concentrations of 0.09 to 0.25 $\mu g\, l^{-1}$ of atrazine and of 0.07 to 0.22 $\mu g\, l^{-1}$ of deethylated atrazine had been detected in samples taken at the end of August. Another sampling was to take place 3 months later, in autumn, so it was decided to analyze for metabolites, too.

Atrazine application in the ground-water protective zone is prohibited. The authorities responsible for water delivery suspected that the atrazine impact came or was still coming from this area. For the study it was one of the premises not to render it too expensive and to prepare the report as soon as possible. Therefore, a decision was taken to run a simplified screening first and to do an evaluation of the results with a few representative samples later, according to a definition by Rajagopal[5] that "... screening procedures are diagnostic tools used to determine the extent to which each sample should be analyzed."

Total atrazine levels in soil may be determined by solvent extraction or, as recently reported, by supercritical fluid extraction (SFE)[6]. Soxhlet extraction is often inefficient for complete recovery of pesticides from natural samples, the lack of agitation difficults extraction. Time-dependent nonequilibrium sorptive processes may render some of the analyte more resistant to extraction[7] forming "nonextractable residues"[8]. Extreme pH and elevated temperatures during extraction may enhance the naturally occurring process of atrazine degradation to its dealkylated or hydroxy-metabolites. Studies using ^{14}C-labeled species mostly fail speciation of residues which is utterly important concerning mobility, phytotoxicity, toxicity, degradation and extractability. Frequently, determination of total atrazine levels is of limited value as it has been shown for the prediction of phytotoxicity in some reports[9] when an aqueous extraction procedure had been developed to measure plant-available residues, i.e. residues that are dissolved in pore water of the soils. A modified DIN-method[10] to determine the leachability of atrazine residues by eluting repeatedly the soil, shaking it for 24 h with water, had been described[11]. Yet it was still unknown if an aqueous extraction step would be sufficient for comparison of contamination of a series of fields.

Determination of pesticides in extracts is usually done by gas-liquid chromatography (GC)[12], high performance liquid chromatography (HPLC) and thin-layer chromatography[13,14]. Recently, environmental analysts have been looking for powerful methods that allow for analyzing hundreds of samples daily. In fact, in clinical chemistry such methods exist. A great number of samples a day are analyzed for hormone levels, drugs and other substances in samples like urine or blood. They benefit from the extraordinary selective antibody/antigen-interaction in contrast to the low selective interaction with stationary phases in chromatography. These methods are all called immunoassays and have in common the use of a high-molecular reagent, a protein which is able to bind substances selectively, the antibody. Antibodies for analytical use are produced either in mammals like rabbits or sheep (polyclonal antibodies) or by hybridoma technique (monoclonal antibodies) which yields any quantity of antibodies needed. Furthermore, in some years tailor-made antibodies against almost any substance will be made available through genetic engineering.

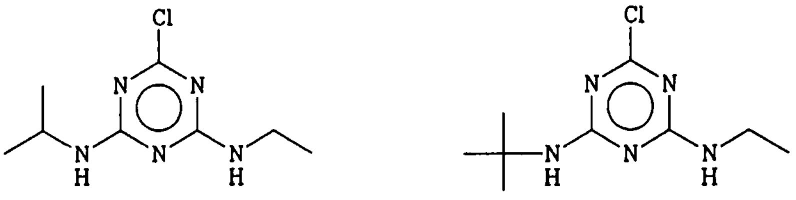
MATERIALS AND METHODS

Locality

The area of the study covered about 4 km^2 comprising 167 fields. A map had been drawn to show the line delimiting the so-called "inner water protective zone" (zone II) referred to in German ground-water protective legislation. It marks the area from which the water takes less than 50 days to get to the wells of the waterworks. Within this margin nearly half of the fields present were corn plots. Furthermore, the "outer water protective zone" (drainage boundary) and the well zone had been marked.

Data on pesticides

In Southern Germany atrazine is the most important herbicide[15]. It is applied at a rate of 1 kg ha^{-1} y^{-1}. The chemical structure of atrazine and its principal metabolites, deethylated atrazine and deisopropylated atrazine and a derivative called terbuthylazine are shown in Figure 1. Terbuthylazine is being proposed by plant protection services as an alternative to atrazine. According to local distributors up to the date when the sampling took place atrazine had been sold almost exclusively for this purpose.



Atrazine

Terbuthylazine

deethylated Atrazine

deisopropylated Atrazine

Figure 1 Chemical structure of some important triazines and their main metabolites.

Sampling

Sampling took place on July 24, 1990 from 33 corn plots in the outer and 24 in the inner protection zone. Samples were taken at a depth of 0 to 20 cm using a small spade. Each field was sampled at five positions on the diagonals of the plot, yet not closer than 5 m to its fringes. The weight of samples did not exceed 500 grams. Control samples were taken from grassland (3 fields) and one rapeseed plot to allow for comparison with background contamination caused by drift or former application. Blanks to test the performance of the analytical method were obtained by drilling two cores at 0 to 50 cm with a hollow punch at sites that were not accessible to pesticide spraying, in a little wood surrounding the well zone and at two sports grounds. This gave a total of 66 samples.

The sampling in another region on October 22, 1990 comprised 26 samples. The inner protective zone there was mostly wood but some cropland and sports grounds downstream were sampled, too. One corn plot in the inner zone was municipal property and had not been treated with plant protection products. The other fields upstream in this area had been planted mostly to cereals. 4 blanks were obtained from drilling in the well zone. A total of 6 corn plots in the outer zone were sampled.

Soil samples were transported in plastic bags to the laboratory where they were spread for air drying. Samples were thoroughly mixed and sieved through a 2.000 μm screen to remove stones, roots and lumps and then reduced to the laboratory sample (10 g). Representative samples can be obtained in this way by using amounts greater than 4 g as has been shown in a previous study[16]. Samples were stored at room temperature until extraction which occurred at most 4 days later. The dry weight was determined by drying at 105°C for a few representative samples. Water contents were in the range of 10%. Results were not corrected and refer to air-dried soil.

Simplified Extraction

After mixing, the samples were allowed to soak 72 h with ultrapure water, i.e. water with a total organic carbon content (TOC) below 1 $\mu g\, l^{-1}$ (e.g. water for HPLC). To enhance desorption, each sample was sonicated for 5 min., followed by centrifugation for 10 min. at 5000 r.p.m.. The samples had to be diluted by a factor of 10 to adapt concentrations to the ELISA measuring range. In Figure 2 the procedure of the screening is summarized.

Suitable determination method: enzyme immunoassay (EIA)

The assay procedure, the apparatus and chemicals were according to the test scheme published previously[17–19] and shown in Figure 3. All determinations were done in quadruplicate, running 12 calibrators and 12 samples on each plate. The sigmoidal calibration curve is set up by plotting the means or the medians of the absorbances read out on an eight-channel photometer ("reader") against the logarithms of standard concentrations and interpolating by Rodbard's[20] logistic four-parameter equation.

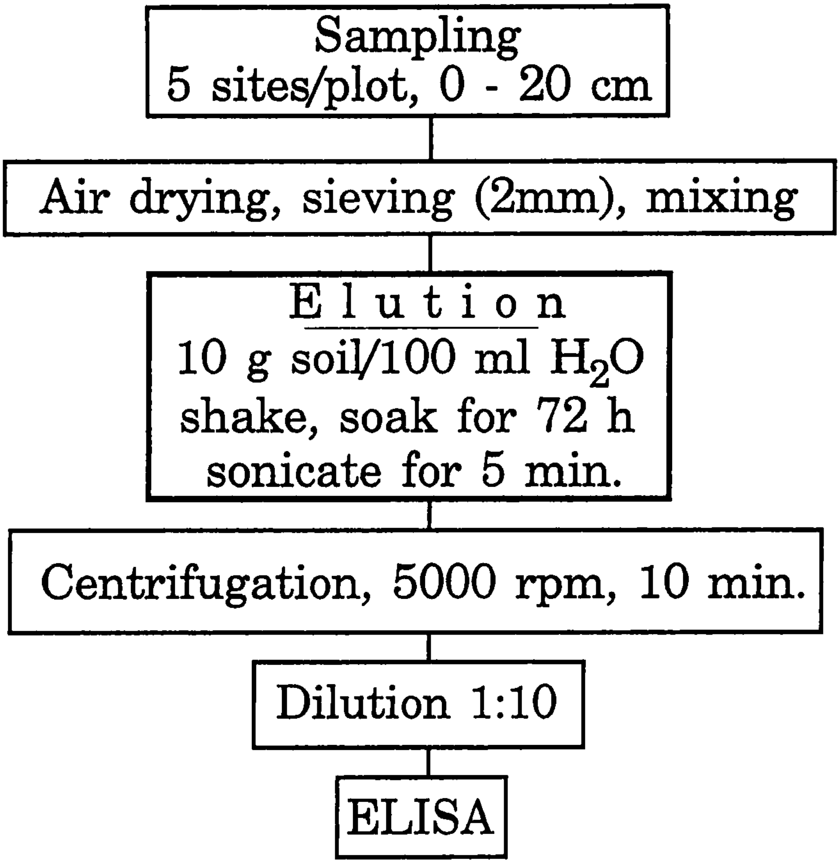


Figure 2 Scheme of sampling, sample preparation and analysis of fields.

A typical calibration curve for the ELISA using the antibody designated internally as AK C193 is shown in Figure 4. The test reaches a detection limit of 5 ng l^{-1} in water (3 SD). The measuring range is over about three decades. The enzyme immunoassay using AK C193 shows significant cross-reactivity only against propazine[19]. Cross-reactivity (CR%) usually is defined as the ratio of the concentrations of the concerned substances giving 50% of the absorbance of the blank, yet it is a function of the concentration. As previously communicated[19], terbuthylazine (CR 7%) will not lead to additional atrazine concentration equivalents above 70 ng l^{-1} even if present at the 1 $\mu g\, l^{-1}$-level. The ELISA test using an antibody AK C10 (Figure 5), that detects deethylated atrazine and deisopropylated atrazine and shows negligible cross-reactivity against atrazine[21], has a detection limit of 0.02 $\mu g\, l^{-1}$, that means 2 $\mu g\, kg^{-1}$ of these metabolites in soil. The results were calculated as deethylated atrazine. In literature some more triazine immunoassays and their application to soil residue analysis have been described[22–24].

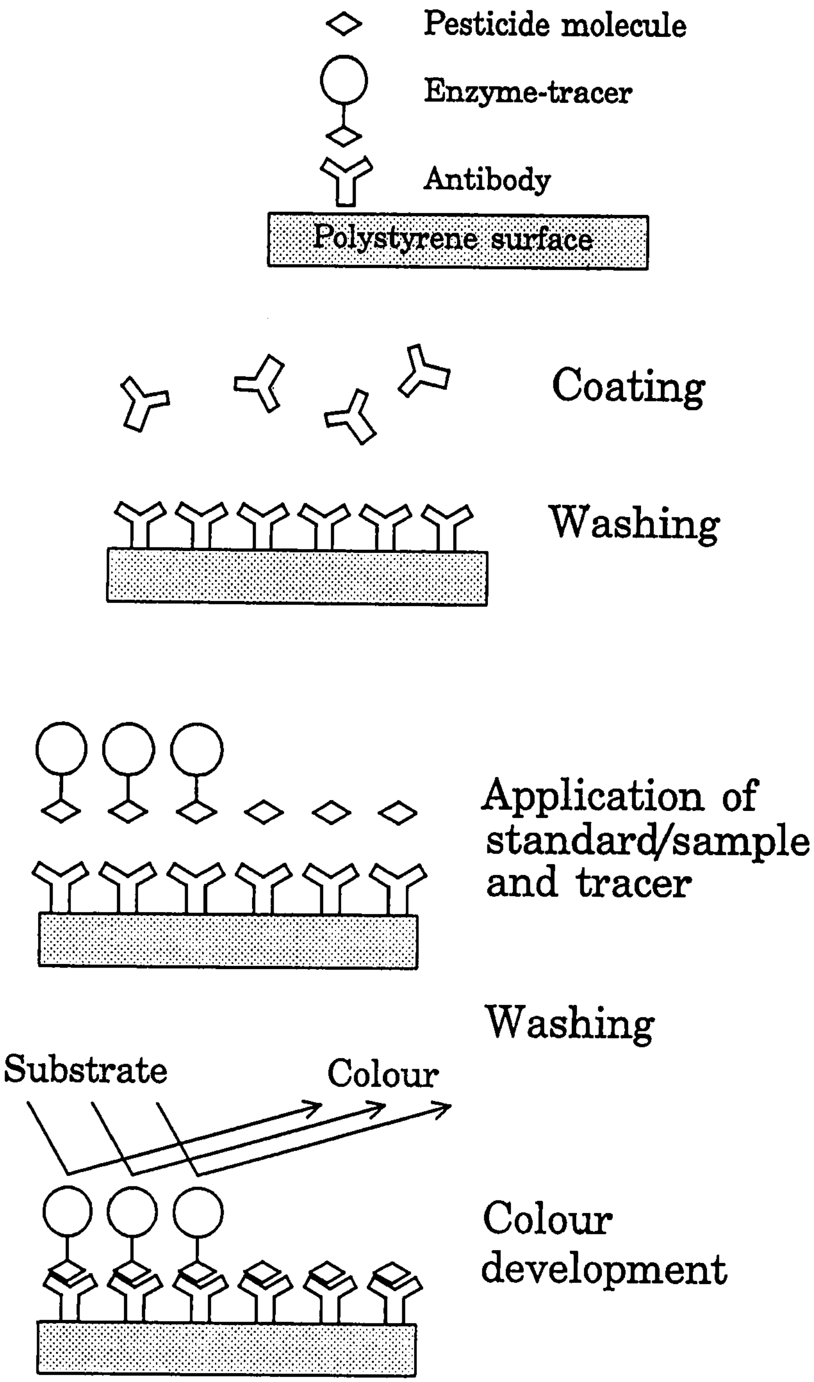


Figure 3 ELISA protocol.

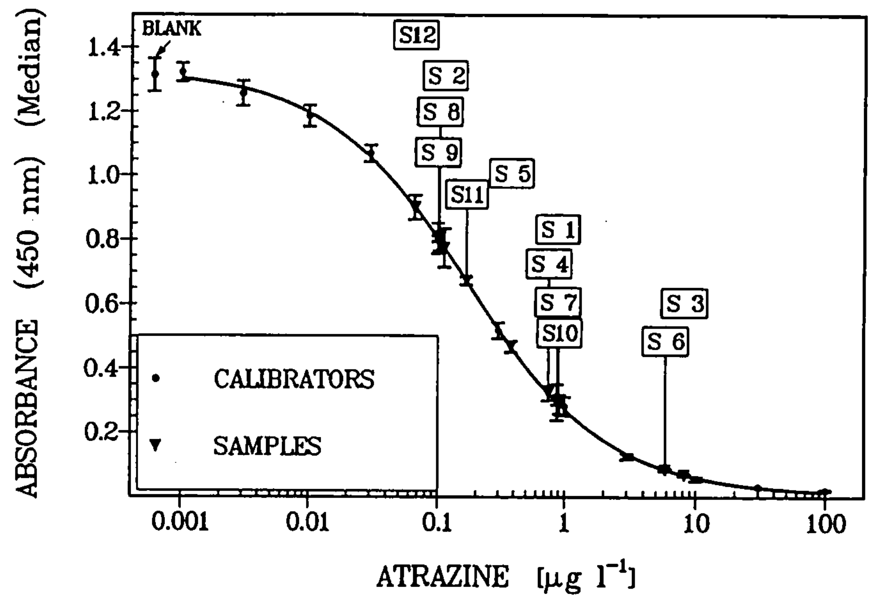


Figure 4 Calibration graph of the atrazine ELISA used. Antibody dilution 1:20000 of AK C193, Tracer dilution 1:10000. Pre-incubation 30 min., tracer incubation 10 min., colour development 20 min., medians and ranges (correspond to 2 standard deviations) of quadruplicates; indicated are the positions of 12 samples S1 through S12. Detection limit: 10 ng l^{-1}.

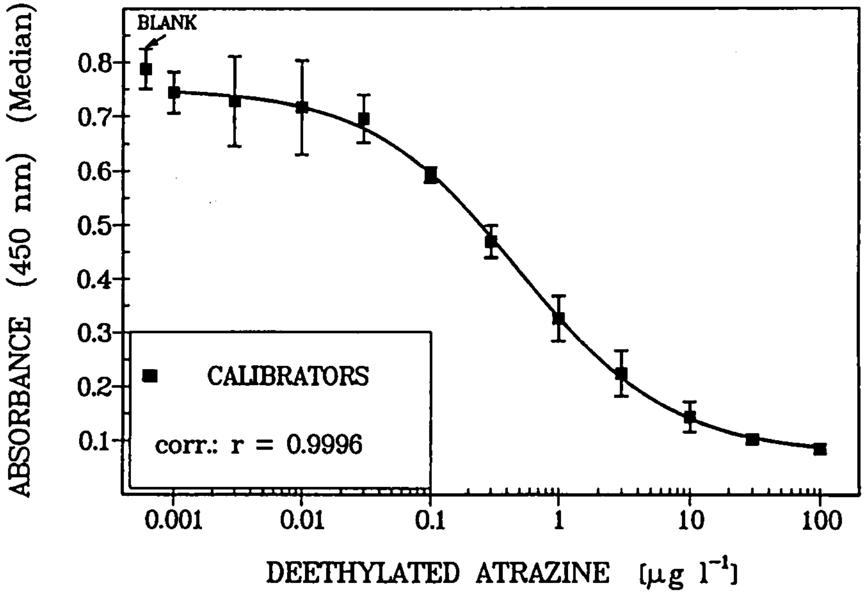


Figure 5 Calibration graph of the deethylated atrazine ELISA used. Antibody dilution 1:20000 of AK C10, Tracer dilution 1:10000. Pre-incubation 30 min., tracer incubation 10 min., colour development 20 min., medians and ranges (corresponding 2 SD) of quadruplicates of 12 standards; coefficients of correlation of the 4-parameter-fit is 0.9996. Detection limit: 20 ng l^{-1}.

Confirmatory studies

The soil under concern is of the Rendzina type. The high percentage of clay and organic carbon might not only increase retention in the soil but renders residues more resistant to extraction. To evaluate the results of the study the extraction behaviour of triazine herbicides from the soils screened has been tested. Four rapid extraction methods for five model soil samples, obtained by mixing soil from exemplary plots, were performed. Values of GC determination were compared with results obtained by ELISA.

RESULTS AND DISCUSSION

Classification of fields

The screening comprising 66 soil samples took less than one week. Sampling was done in one day, sample preparation was done by one person in 3 days and the measuring work on another day. Concentrations determined ranged from 3 $\mu g\,kg^{-1}$ to 1.9 $mg\,kg^{-1}$ of atrazine in soil. The drillings (4 samples) showed all to be below 10 $\mu g\,kg^{-1}$ as they were situated in non-accessible areas. Control plots planted to grass or rapeseed ranged from 3 to 14 $\mu g\,kg^{-1}$ (6 samples). Drift and preceeding crops seemed to be of little influence, since rapeseed and greenland fields gave 8 and 10 $\mu g\,kg^{-1}$ although they were surrounded by corn plots with 86, 130, 160 and 500 $\mu g\,kg^{-1}$. One rapeseed plot had even been planted to corn the year before. From the total of the 57 corn plots only 6 fields gave results below 15 $\mu g\,kg^{-1}$. A field that was known to be planted to corn for 5 subsequent years was contaminated with 190 $\mu g\,kg^{-1}$. To get a better insight the number of the samples were plotted against the logarithm of the concentrations found (Figure 6). The frequency distribution appeared to be bimodal. This allowed classifying the samples: soils with contaminations below 0.05 $mg\,kg^{-1}$ have been defined to be from "clean" fields, atrazine residues being from earlier applications, drift or matrix effects. 20 samples of this category have been found with its maximum between 0.006 and 0.1 $mg\,kg^{-1}$ mass concentration. Above this level the plots were classified as "contaminated" by atrazine applied that year or before. There was another maximum in this class made of 17 plots with concentrations between 0.1 and 0.2 $mg\,kg^{-1}$. The nine fields with more than 0.32 $mg\,kg^{-1}$ of atrazine residues should have received atrazine in the year of the sampling. The amounts found justified to judge these plots as a risk to ground water in an area with that specific geological background.

Concerning the screening in autumn, only 3 plots were ranked to be contaminated with atrazine (0.020–0.025 $mg\,kg^{-1}$), all three being corn plots. None of the 17 fields without residues above the detection limit of atrazine was a corn plot. The corn field within the inner zone that had not been sprayed appeared to contain no atrazine nor deethylated atrazine. As can be seen in Figure 7 concentrations at this time were much lower perhaps due to leaching of non-aged residues down to deeper levels, degradation or forming of bound residues.

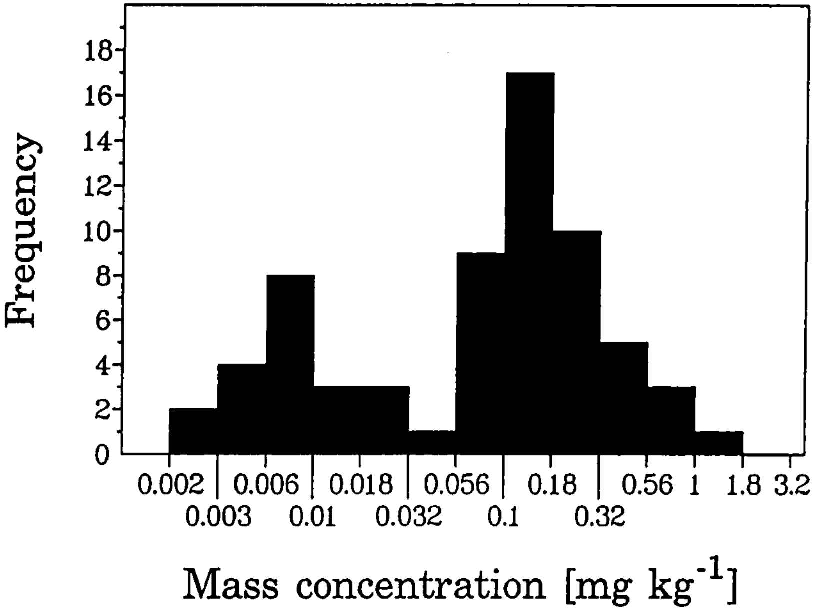


Figure 6 Frequency distribution of atrazine residues vs. concentrations. 66 samples, sampling end of July, logarithmic concentration scale showing a bimodal distribution of atrazine residues.

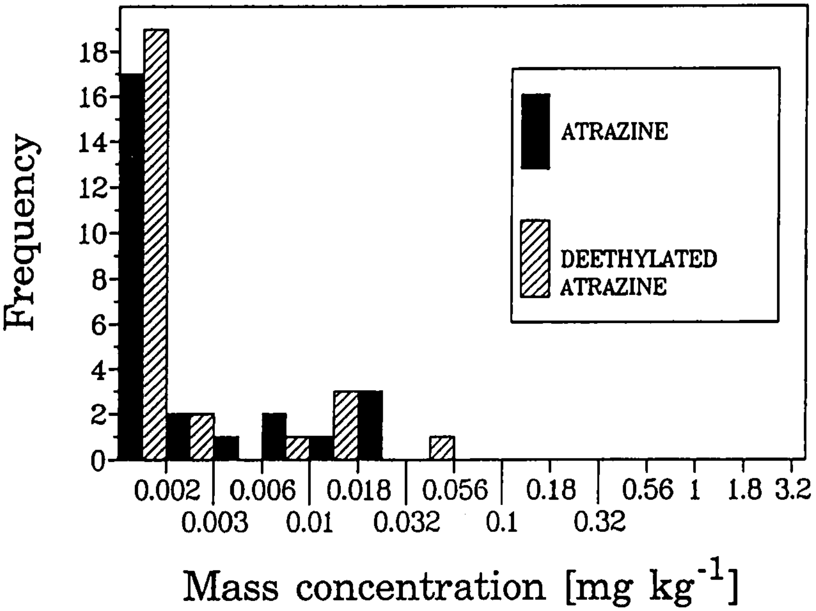


Figure 7 Frequency distribution of residues of atrazine and deethylated atrazine vs. concentrations. 26 samples, sampling end of October, logarithmic concentration scale, about 70% of the samples' mass concentrations of herbicides are below the detection limit.

Co-occurrence patterns

It is obvious that the frequencies of atrazine and deethylated atrazine were similar in each group of samples. Samples showing a high concentration of residue of atrazine also showed high amounts of deethylated atrazine. This is a co-occurrence pattern that results from the product/metabolite-relationship of these substances. It can be seen that in the case of screening for non-aged residues some months after application, the measuring of deethylated atrazine gives no additional information on the contamination level of the fields and the screening can be done without it.

Confirmation of results

This comparative work is part of a validation program of immunoassay determination of herbicide residues in soil extracts[25] and only the results will be summarized. When high concentrations of atrazine have been found, propazine and simazine were detectable, concentrations being usually around 3% for propazine and 2.5% for simazine. Probably, these residues originate from impurities in the formulation applied. Terbuthylazine and pendimethalin (N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine), which will replace atrazine in the future, as well as all the dealkylated metabolites of atrazine could be detected in soils from corn plots. Deethylated atrazine was the most abundant metabolite in soil according to literature[26].

The immunoassay measurements showed to have higher standard deviations but were quite comparable to GC results. Usually ELISA yielded somewhat higher concentrations. It was found that the ratio of residues extracted by water to those extracted by more effective methods is about constant for these soils and is about 1:3 or 2:3, i.e. one or even two thirds of the residue extractable with methanol is extractable by sonication with water at about two months after application. This confirms results that organic solvents may be only slightly more efficient in the extraction of atrazine from soil[27]. In fact there are a lot of advantages with aqueous extracts: the residues extracted have more ground-water relevance, samples need little or no clean-up and with cleaner samples lower detection limits for the measurement can be attained.

Plausibility

In an earlier work[11] it has been shown that about 6 weeks after atrazine application on a sandy loam, 94% of the atrazine detected in a soil column of 0 to 60 cm is retained in the first 20 cm. The effect is almost the same for deethylated atrazine (91%). Below this level the concentrations were much lower showing almost no gradient[28]. If an input of 1 kg ha^{-1} y^{-1} is assumed (100 mg should fall on each m^2), a penetration to 20 cm of the top soil, with an average soil density of 1300 kg m^{-3}, would lead to mass concentrations of 0.385 mg kg^{-1}. Most of the contaminated samples ranged from 0.10 to 0.18 mg kg^{-1}, indicating that 30 to 40% of the atrazine applied is still in the soil after 2 months.

The 100 mg of atrazine that falls onto 1 m^2 of a field could be washed down to ground water by the 400 mm m^{-2} typical annual ground-water recharge in the region. This percolating water would reach a concentration of 250 $\mu g\,l^{-1}$. Water taken from the ground-water wells averaged a concentration of 0.22 $\mu g\,l^{-1}$ of atrazine which is lower by a factor of 1000. In fact, usually a factor of elimination of pesticides of 10.000 is thought to be efficient to prevent ground water surpassing 0.1 $\mu g\,l^{-1}$ of pesticide concentration. So it can be concluded that the results obtained are quite reasonable. In our earlier study it had been found that three and a half months after spraying only 6% of atrazine had been present in the top soil but still 40% of deethylated atrazine. This is possible because the processes of sorption, leaching and degradation of the metabolite are countercurrent to formation of more metabolite from atrazine[11]. Now on contaminated corn fields an average concentration of atrazine of 0.304 mg kg^{-1} in July and of 0.023 mg kg^{-1} in October has been found. Consequently, the amount of atrazine decreased to 7.5% of the value about 6 months after spraying. Furthermore, it has been reported that deethylated atrazine is the most rapidly formed soil metabolite of atrazine and the most mobile one, exhibiting a leachability even greater than that of atrazine. In this regard, the deethylated atrazine found in autumn in the ground water at the second sampling site may result from atrazine application in that year.

CONCLUSIONS

It has been shown that aqueous eluates were suitable for the screening of easily extractable triazine residues in the soils studied. In the evaluation of the ground-water hazard potential of these fields, i.e. the tendency of atrazine to desorb from the soils and percolate down to ground water, enables to identify fields to which atrazine has been applied during the last vegetational period. To get a better reliability screenings should be repeated in spring as the pre-spraying season, in summer and in autumn. This will also help to judge the potential of the proposed screening method. Control of the catchment area is necessary, especially because atrazine use has been banned in Germany since April 1, 1991 and authorities fear frequent violation of the regulation. These monitorings that would block the capacities of specialized laboratories for a long time and that are hardly affordable for universitary investigational groups, can be done rapidly and cost-effectively by ELISAs. Sampling programs could be made more efficient and the costs of "environmental zeros" minimized.

However, for using the ELISA in routine soil analysis much can be done in the field of novel extraction techniques, the scale-down enabling to use expensive extraction modifiers or solvents. Perhaps, immunoassays may help in the investigation of speciation of residues, because it is of increasing importance to distinguish between the different states of binding of pesticides onto soil organic matter or onto particles traveling in the subsurface. In the analysis of extracts containing great amounts of matrix compounds special regard will have to be paid to the recognition of matrix effects trying to overcome the problems or to establish simplified clean-up steps for ELISA. Array techniques, i.e. measurements of the same sample with antibody/tracer systems of different selectivity and chemometric evaluation of the results will enhance reliability of immunoassays enabling the analyst to distinguish between various compounds.

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