Quantitative Analysis of 2,4-Dichlorophenoxyacetic Acid in Water Samples by Two Immunosensing Methods

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Two sensitive methods based on a monoclonal anti-2,4-dichlorophenoxyacetic acid (2,4-D) antibody, a flow injection immunoanalysis (FIIA), and a fiber optic immunosensor are described and evaluated for the quantitative determination of 2,4-D in water samples. Detection limits for 2,4-D of ~0.03 μg/L with FIIA and 0.2 μg/L with the fiber optic immunosensor can be achieved, i.e. well below the maximum concentration permitted, in the case of FIIA, by the EC Drinking Water Directives of 0.1 μg/L. The midpoints of the 2,4-D test are found at concentrations of 0.3 μg/L with FIIA and 10 μg/L with the fiber optic immunosensor. Measurable concentrations range from 0.03 to 3 μg/L with FIIA and from 0.2 to 100 μg/L with the fiber optic immunosensor. The FIIA did not require concentration or cleanup steps for ground and surface water samples. In addition, validation experiments with GC/MS showed a good correlation of the data for the FIIA system. However, both tests can be applied as alarm systems for monitoring potential 2,4-D contamination in environmental water samples.

Keywords: *2,4-Dichlorophenoxyacetic acid (2,4-D); fiber optic immunosensor; flow injection immunoanalysis (FIIA); herbicide; immunoassay; immunosensor; monoclonal antibodies; pesticide; water samples*

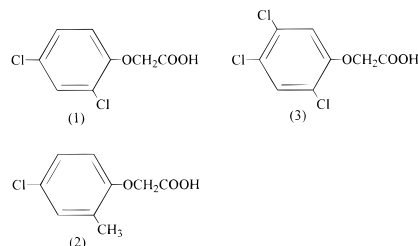
INTRODUCTION

Intensive agriculture with the associated use of a large number of different pesticides has led to a growing concern about the potential contamination of ground water. To guarantee a tight control over drinking water quality, the basic data set, i.e. the number of analyzed samples, has to be significantly enlarged. Unfortunately, classical analytical procedures that use solvent partitions are time-consuming and costly. Compounds containing a carboxylic group such as 2,4-D must usually be derivatized for gas chromatography (GC) because they are thermally unstable and lack volatility. Methylation with diazomethane is the traditional approach, but the response of the electron-capture detector (ECD) is sometimes weak and varies from one chemical to another. One problem associated with derivatization is that the reaction is usually time-consuming. Another problem is that the ECD requires rigorous cleanup procedures. As alternative methods, immunoassays and immunosensors are sensitive, specific, and precise, providing for rapid, cost-effective analyses. Immunochemical methods are proposed for pesticides that are difficult to analyze by standard techniques. In general, water samples require no cleanup or enrichment prior to the immunochemical analysis. Current concerns about potential health hazards connected with pesticide use have focused on 2,4-D as a suspected cancer-causing agent (Hoar et al., 1986). As a broadleaf weed killer, 2,4-D is used extensively on field crops, turf, and noncrop lands. The main advantages of acidic herbicides such as 2,4-D are spray efficacy and biodegradability. The widespread use of 2,4-D and associated health concerns have made monitoring of environmental samples for the presence of 2,4-D desirable. 2,4-D is occasionally detected in the Rhine River in concentrations of about 0.03 μg/L, i.e. well below the maximum limit of the EC Drinking Water Directives (Dr. U. Oehmichen, Wasserverband Hessisches Ried, Biebesheim, Germany, personal communication). Radioimmunoassays (RIA) for 2,4-D (Rinder and Fleeker, 1981; Knopp et al., 1985) and several enzyme-linked immunosorbent assays (ELISA) (Fleeker, 1987; Hall et al., 1989; Franek et al., 1994) as well as a polarization fluoroimmunoassay (Eremin, 1995) for the detection of 2,4-D in water and urine have been reported.

The aim of the present paper was the development and evaluation of two immunosensing methods [a flow injection immunoanalysis (FIIA) and a fiber optic immunosensor] for the automated analysis of 2,4-D in water samples.

MATERIALS AND METHODS

1. Materials. *1.1. Chemicals.* The monoclonal anti-2,4-D antibodies (ascitic fluid with 0.1% NaN_3, lyophilized, dissolved in distilled water; lot 4/E2/G2) were a generous gift from Dr. Milan Franek, Veterinary Research Institute, Brno, Czech Republic [preparation described in Franek et al. (1994)]. The following pesticide standards were obtained from Riedel de Haen AG (Seelze, Germany): 2,4-dichlorophenoxyacetic acid (2,4-D)(**1**); 2-(2,4-dichlorophenoxy)propionic acid (dichlorprop,



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2,4-DP); 4-chloro-*o*-tolyloxyacetic acid (MCPA) (**2**); *o*-chlorophenoxyacetic acid (2-CPA); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (**3**); 2,4-dichlorophenoxyacetic methyl ester (2,4-D methyl ester); 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB); 4-(4-chloro-2-methylphenoxy)butyric acid (MCPB); mecoprop; 2,4-dichlorophenol; 2-methyl-4-chlorophenol; 4-chlorophenoxyacetic acid (4-CPA); 2-methyl-6-chlorophenoxyacetic acid; 2-methyl-4,6-dichlorophenoxyacetic acid; 3,4-dimethylphenol. (**Warning**: Caution is advised in the handling of herbicides mentioned in this paper; avoid contact, wear protective clothing, avoid inhalation, and work under a fume hood.) In addition, the following reagents were used: (aminopropyl)-triethoxysilane (Aldrich, Steinheim, Germany); avidin (Sigma Chemie GmbH, Deisenhofen, Germany); bovine serum albumin (BSA, fraction V, Sigma); *N,N*-dimethyldodecylamine *N*-oxide (LDAO, 30% solution in water, Fluka Chemie, Buchs, Switzerland); fluorescein-labeled anti-mouse-IgG antibody (Sigma); goat anti-mouse IgG (Sigma); horseradish peroxidase (HRP, 1350 U/mg = 22 505 nkat, Serva, Heidelberg, Germany); hydrogen peroxide, 30% (Merck, Darmstadt, Germany); 3-(*p*-hydroxyphenyl)propionic acid (HPPA, Sigma); polyoxyethylenesorbitanmonolaurate (Tween 20, Merck); proteinase K from *Tritirachium album* (Serva); sulfosuccinimidyl 6-(biotinamido)-hexanoate (Pierce, Rockford, IL). All other chemicals used were of analytical grade.

1.2. Buffers and Solutions. a. For Fiber Optics. The buffers used were 0.15 M phosphate-buffered saline (PBS), pH 8.0, as the carrier and dilution buffer, and 0.1 M glycine/HCl, pH 1.5, as the elution buffer.

b. For Enzyme Immunoassay Performance. The following were used: (1) carbonate buffer, 50 mM, pH 9.6, for coating; (2) PBS, 40 mM, pH 7.2 (containing 8.5 g/L NaCl), for the preparation of standards and the peroxidase tracer; (3) PBS washing buffer, 4 mM, pH 7.2 (containing 0.85 g/L NaCl and 0.5 mL/L Tween 20), for washing the microtiter plates; (4) substrate buffer for peroxidase [0.1 M sodium acetate (the pH was adjusted to 5.5 by adding 1 M citric acid)]; (5) substrate for peroxidase [400 μL of tetramethylbenzidine (TMB; 6 mg of TMB was dissolved in 1 mL of dimethyl sulfoxide) + 100 μL of 1% H_2O_2 filled to 25 mL with substrate buffer]; (6) stopping reagent for peroxidase (2 M H_2SO_4).

c. For FIIA Measurement. PBS, 40 mM, pH 7.2 (containing 8.5 g/L NaCl), was employed as the substrate buffer for peroxidase (carrier buffer). The following substrates for peroxidase were each dissolved separately in this buffer: (1) 5 mM HPPA and (2) 2 mM hydrogen peroxide. For the 1/1000 dilution of the 2,4-D peroxidase tracer 40 mM PBS buffer, pH 7.2 (containing 0.5 mL/L Tween 20), was used. For the regeneration of the immobilized antibodies 0.01 M glycine/HCl buffer, pH 2.0, was used.

1.3. Preparation of Standards. Five milligrams of 2,4-D or the related compound was dissolved in 50 mL of absolute ethanol with the aid of an ultrasonic bath (20 min). Starting with this solution, a stock solution was prepared consisting of 1 mg/L 2,4-D (= excess). A standard series was prepared by making several dilutions of the stock solution containing the following 2,4-D concentrations: 0.01, 0.03, 0.1, 0.3, 1, 10, and 100 μg/L. The stock solution and the standard series were made up in distilled water.

1.4. Equipment. The following laboratory equipment was used.

a. For Optical Fiber Measurement: a 3/2-way valve (Lee); a multichannel valve (Latek, Heidelberg, Germany); a peristaltic pump (minipuls, Gilson, Villiers, France); a xenon flash lamp [EG&G, Electro-Optics, MA, (flashtube Model FX-800)]; a photomultiplier (PMT, R 928, Hamamatsu); a fluorescence photometer (Oriel Type 3090, Oriel Scientific Ltd., Leatherhead, U.K.); and a flow-through cell (GBF, Braunschweig, Germany). The system was controlled using a microprocessor (PS3, Klöckner-Möller, Bonn, Germany).

b. For Enzyme Immunoassay Measurement: in addition to the equipment given under (*a*), a photometer for 96-well microtiter plates (Molecular Devices); a microtiter plate washer with eight channels (Nunc Intermed GmbH, Roskilde, Denmark); and an ultrasonic bath (RK 514, Sonorex Bandelin).

c. For FIA Measurement: five peristaltic pumps (Meredos GmbH, Bovenden, Germany), two 3/2-way valves (Lee, Westbrook, CT); two injection valves (Fiastar 5102-002 injector V-100, Tecator, Höganäs, Sweden); a fluorometer with a flow-through cell (Merck Hitachi, Darmstadt, Germany); an integrator (Shimadzu C-R6A Chromatopack), a relay station (Keithey, Metrabyte); and a special column reactor (GBF). The system was controlled using a personal computer and the Q-FIA programm (GBF).

1.5. Further Materials. Further materials used were hard-clad silica fibers from Ensign-Bickford Optics Co. (Simsbury, CT) with a core diameter of 400 μm for the optical fiber measuring device. For performing the enzyme immunoassays (in the form of random spot checks for comparison with FIIA and the fiber optic immunosensor) microtiter plates (96-well, type F-form, high binding capacity, MaxiSorp, Nunc) were used. Porous microglass beads surface-modified with hydrophilic amino groups or carboxylic groups for covalent coupling were a generous gift from Schuller GmbH, Wertheim/Main, Germany, and used as support material for the immobilization of the antibodies. The beads had a diameter of 50−100 μm and a pore size of 31.6 nm. Both the carboxyl and the amino surface-modified beads exhibited a high density of carboxylic acid and amino groups for covalent coupling, according to the manufacturer.

1.6. Water Samples. An important step for any validation study is the analysis of samples spiked with the analyte and comparison of the results with data from an established analysis method, i.e. in our case GC measurements.

As representative water samples, we chose to use a raw water sample derived from a river by bank filtration and the raw water sample after ozonization and purification by filtration over activated charcoal as starting matrices. The water samples collected on two different dates (January 22 and February 19, 1993) and at two different stages (raw water prior to and after purification) were provided by Dr. U. Oehmichen (Wasserverband Hessisches Ried, Biebesheim, Germany) together with the results of the conventional analyses for residues of pesticides, phenols, chlorine- and nitrogen-containing aromatic compounds, less volatile hydrocarbons, and anilines. The four water samples were measured with FIIA and the fiber optic immunosensor for a potential 2,4-D pollution and then two of them from the same date (February 19, 1993) were spiked with four different 2,4-D concentrations (0.05, 0.10, 1.00, and 5.00 μg/L). These eight spiked water samples were then analyzed with FIIA and the fiber optic immunosensor.

2. Methods. *2.1. Development of the Fiber Optical Sensor. 2.1.1. Immobilization of the Haptens on Optical Fibers.* Preparation and silanization of the fibers with (aminopropyl)-triethoxysilane were carried out as described by Bier et al. (1992).

2,4-D had to be activated prior to immobilization. The reaction described by Neises and Steglich (1978) was modified as follows: 10 μmol of 2,4-D, 50 μmol of *N*-hydroxysuccinimide, and 100 μmol of *N,N*-dicyclohexylcarbodiimide were stirred in 200 μL of dry *N,N*-dimethylformamide overnight at room temperature. Precipitated urea was pelleted by centrifugation, and the supernatant was incubated with amino-silanized optical fibers overnight at room temperature. Finally, the fibers were washed with ethanol and distilled water and stored in PBS at 4 °C.

2.1.2. Fiber Optical Sensor: Experimental Setup and Assay Procedure. The experimental setup of the fiber optical sensor and the assay procedure were as described earlier (Bier et al., 1992); a sketch is given in Figure 1. All buffers were supplemented by 0.08% LDAO. Briefly, the fiber is inserted into a flow-through glass cell with a volume of 100 μL. The cuvette was fixed in an aluminum block for thermal stabilization and the exclusion of ambient light. External excitation light was directed perpendicular to the fiber and cuvette axis by a Xe flash lamp. The light passed through an interference filter (480 nm, bandwidth 10 nm) and was guided to the cuvette by a fiber bundle. The emitted light was collected through the fiber and guided directly through a cutoff glass filter (510 nm longpass) to a photomultiplier (PMT). Both light

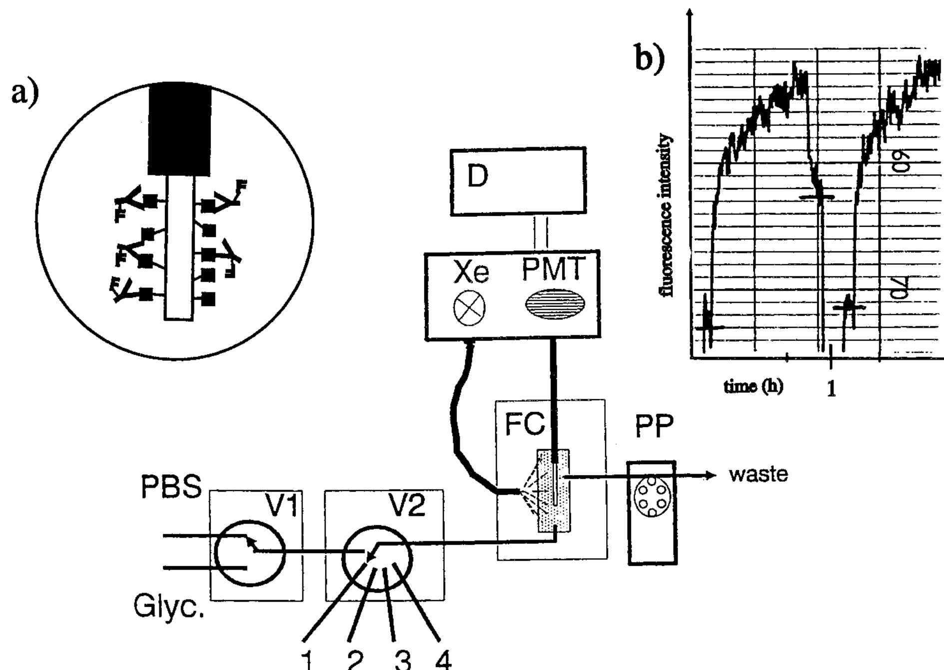

Figure 1. Experimental setup of fiber optic immunosensor. The fiber core with immobilized 2,4-D (■), visualized in insert a, was introduced in a flow-through cell (FC). The samples (1–4) previously supplemented with indirectly fluorescence-labeled anti-2,4-D antibody were assayed successively by a multichannel valve (V2). V1, 3/2-way valve; PP, peristaltic pump; Xe, xenon flash lamp; PMT, photomultiplier tube; D, data collection unit (recorder). A typical readout of the photomultiplier current out of a series of several consecutive measurements is shown in insert b. The assay steps are explained in detail under Materials and Methods.

source and detection unit were part of a fluorescence photometer; PMT voltage was adjusted to 1025 mV. All experiments were performed at room temperature.

The following steps in the measuring cycle were performed: (1) a washing step with 0.15 M PBS, pH 8.0, (5 min, ground signal); (2) incubation of the fluorescein-labeled anti-mouse-IgG antibody with the monoclonal anti-2,4-D antibody supplemented with various concentrations of 2,4-D for 30 min; (3) washing with PBS (5 min, reference signal); (4) reactivation by incubation with proteinase K (10 μg/mL PBS for 10 min), then washing with elution buffer (0.1 M glycine/HCl, pH 1.5, 5 min). The difference in photomultiplier output before (step 1) and after (step 3) incubation is taken as the system response.

2.2. Development of FIIA. As the technique of FIIA is based on an enzyme immunoassay format, an enzyme tracer has to be prepared first which has been tested for its suitability for FIIA by an enzyme immunoassay.

2.2.1. Preparation of Peroxidase Tracers. Several peroxidase tracers with diverse haptens (2,4-D; MCPA; 2,4-DB; 2,4-DP; 2-CPA; 2,4,5-T) were prepared. Peroxidase from horseradish was coupled to the hapten according to the carbodiimide/*N*-hydroxysuccinimide procedure [modified from that of Märtlbauer and Terplan (1988)].

One milligram of the hapten together with 1.7 mg (15 μmol) of *N*-hydroxysuccinimide and 6.2 mg (30 μmol) of dicyclohexylcarbodiimide was dissolved in 130 μL of dry dioxane and incubated for 18 h at room temperature. Then the mixture was slowly added to a solution of 2 mg (0.05 μM) of peroxidase (1350 U/mg = 22 505 nkat) in 3 mL of sodium hydrogen carbonate (0.13 M), incubated for another 3 h, and dialyzed for 3 days against 40 mM PBS, pH 7.2, or desalted with a Sephadex G-25 PD 10 column (Pharmacia).

The peroxidase tracer was stored at 4 °C after sterile filtration.

The hapten density of the peroxidase conjugate could be determined by comparing the absorption at 403 nm, at which only HRP absorbs, with that at 280 nm, at which both HRP and the tracer absorb.

2.2.2. Development of an Enzyme Immunoassay. Polystyrene microtiter plates were precoated overnight with 300 μL/well of goat-anti-mouse IgG (5 μg/mL carbonate buffer, pH 9.6) at 4 °C. The plates were drained and stored frozen at −24 °C or used immediately as follows. After a washing step with PBS washing buffer, the monoclonal antibodies (200 μL/well) were added in a dilution of 1:20000. After incubation at room temperature for 2 h or at 4 °C overnight, unbound antibodies were washed off with PBS washing buffer. Aliquots of 200 μL of standard or sample were added in four replicates to the monoclonal antibody-coated wells. This was followed by the addition of 50 μL of HRP-labeled hapten in PBS. After a 60-min incubation, the plates were washed and 200 μL/well substrate was added. The substrate reaction was stopped after 30 min with 50 μL of 2 M H_2SO_4 and the absorption was measured at 450 nm with an ELISA reader. Data analysis was performed with the aid of a commercial ELISA software package (SoftMax, Molecular Devices) using a four-parameter logistic equation for curve fitting and calculating the 2,4-D concentrations of the samples.

2.2.3. Development of a FIIA System. Starting from the optimized enzyme immunoassay, the format of a competitive enzyme immunoassay was transferred to a flow injection analysis system. The FIIA system was optimized according to the incubation times, i.e. incubation with the 2,4-D–peroxidase tracer and substrate incubation in the antibody column reactor, especially with respect to a maximum possible signal height or area combined with a high displacement of the 2,4-D–peroxidase tracer by very low 2,4-D concentrations.

The stability of the antibody column was checked, especially with regard to the number of measuring cycles (each assay takes 15 min to complete, including antibody regeneration) that can be performed with a newly packed antibody column reactor. In addition, the antibody columns were stored at 4 °C in the refrigerator, inserted into the FIIA system every 4 weeks, and checked for their binding properties as well as the amount of protein still immobilized on the support.

2.2.4. Performance of the Optimized FIIA. a. Preparation of the Immunoreactor Column. The antibodies were immobilized on porous microglass beads with activated surfaces (containing either hydrophilic amino or carboxylic groups) via carbodiimide activation. The amino groups at the surface of the porous glass beads are first reacted with succinic anhydride at pH 6 to form a derivative to which the amino groups of avidin can be coupled by a carbodiimide reaction. This

reaction synthesizes a spacer arm allowing the protein to be immobilized at a certain distance from the glass surface. For comparison purposes, avidin was coupled to the carboxylic acid surface modified beads via its amino groups, with the active ester formation procedure like that for the other beads containing the spacer arm. Thus, in the first case, avidin is immobilized via a spacer to the support, and in the second case, it is directly coupled to the surface.

b. Immobilization of Avidin to Activated Glass Beads Exposed Amino Groups for Covalent Coupling. The following succinylation procedure [modified from that of Cuatrecasas (1970) and Weetall and Lee (1989)] was used. One gram of amino group support material was suspended in 100 mL of 0.05 M sodium phosphate buffer, pH 6.0, and 0.3 g of succinic anhydride was added. The pH was maintained at 6.0 by titrating with 20% NaOH. After 15 h of shaking at room temperature, the solid was washed three times with 100 mL of water, twice with 100 mL of methanol, and once with 100 mL of dioxane. Complete reaction of the amino groups occurred as shown by the 2,4,6-trinitrobenzenesulfonate (TNBS) color reaction [cf. Habeeb (1966)]. The particles were then suspended in a solution of 1.2 g of *N*-hydroxysuccinimide (NHS) and 1.9 g of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) in 100 mL of dioxane and washed twice with 100 mL of methanol. For coupling to protein, 1 mL of dioxane suspension containing 100 mg of active ester particles was added to 15 mg of the protein dissolved in 10 mL of 0.1 M phosphate buffer, pH 8.0. The mixture was agitated gently for 1−2 h at room temperature. The solid support was then washed three times with 0.01 M PBS [containing 1% (v/v) BSA] and stored in the same buffer.

c. Immobilization of Avidin to Activated Glass Beads Exhibiting Carboxylic Groups for Covalent Coupling. Avidin was coupled via its amino groups to the carboxyl-containing support at pH 6.3 in the presence of the water-soluble carbodiimide EDC. The coupling reaction is performed at a pH between 4.5 and 6.5 to promote the acid-catalyzed condensation reaction. This was performed according to the following procedure: 1 g of the beads was centrifuged and the supernatant discarded. Two milliliters of a 1 mg/mL avidin solution (= 15 μM) and 50 mL of 0.003 M phosphate buffer (coupling buffer), pH 6.3, were added to the bead pellet. After resuspension, the mixture was incubated at 4 °C for 1 h. Then 400 mg of EDC was added, and after vigorous mixing, the solution was maintained at 4 °C for at least 4 h. Then several washing steps followed to completely remove noncovalently bound avidin. The beads were rinsed two times with 0.01 M PBS, pH 7.2, once with 1.4 M NaCl/PBS, pH 7.2, and another two times with PBS, after which the beads were allowed to stand on ice (4 °C) for at least 4 h to allow the complete regeneration of the bound avidin. This was followed by two washing steps with PBS. The centrifuged beads were resuspended in 0.005 M phosphate buffer, pH 7.2 [containing 1% (v/v) BSA]. The beads could then be stored in the refrigerator after the addition of 5 mL of 2% (w/v) sodium azide solution as a preservative. Under these conditions the beads are generally stable for at least 90 days at 4 °C.

Biotinylation of anti-2,4-D antibody was achieved by incubating the antibody in PBS buffer, pH 7.2, with a 5-fold molar excess of sulfosuccinimidyl 6-(biotinamido)hexanoate at room temperature for 3 h. The reaction mixture was dialyzed extensively (for 48 h with a 2-fold exchange of buffer) against 0.01 M PBS, pH 7.2. Biotinyl antibody was added to avidin-derivatized beads at a 2-fold molar excess of antibody binding sites (as determined with [^{3}H]biotin). The beads were incubated while shaking with biotinyl antibody for 30 min at room temperature and then washed three times with 4 volumes of PBS, pH 7.2, alternated with 4 volumes of a citrate buffer containing NaCl, pH 3, followed by a final rinse with PBS, pH 7.2.

The antibody-coated beads were either filled into the column reactor for direct use or stored at 4 °C in solution after the addition of 2 mL of 0.01% thimerosal or 2 mL of 2% NaN_3. The beads were packed into a 3 mm (inner diameter) × 6 cm plexiglass column. The total number of active antibody binding sites per column was about 10^{-12} mol for both kinds of porous glass beads with immobilized antibodies. Nylon membranes (10 μm) were used to retain the beads in the column.

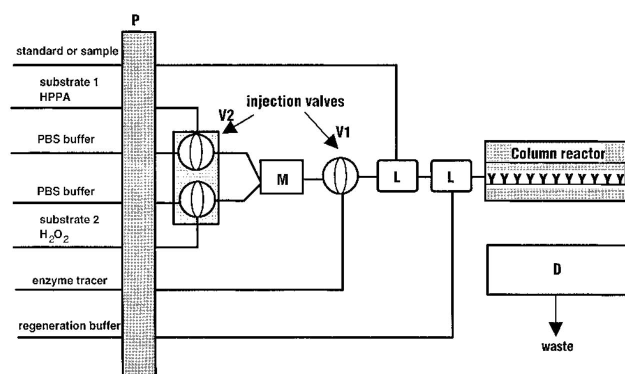

Figure 2. Instrumentation setup of FIIA. Five pumps with different reagents are working in a time-controlled sequence. All reagents have to pass the antibody reactor where the specific antibodies are located. These antibodies are immobilized after biotinylation on avidin-derivatized glass beads, and the antibody-coated beads are filled in a specially constructed column reactor which is regenerated during each measuring cycle. The fluorescence of the enzyme reaction product is measured with a fluorometer, and the peak height and area are registered with an integrator or by computer (Q-FIA program). P, pump; M, mixing chamber; L, Lee valve (3/2-way valve); D, detector (fluorometer combined with an integrator and/or computer).

2.2.5. Assay Format for the Optimized FIIA. Figure 2 shows the instrumentation set up for FIIA. Flow injection immunoanalysis is a sequential saturation assay in which the hapten (2,4-D) and the corresponding enzyme-labeled hapten (a 2,4-D-peroxidase conjugate) compete for a limited number of antibody binding sites. All reagents were moved in a cross-flow over the column reactor in a time-controlled cycle of pumping and injection. The fluorescence of the enzyme-generated product is measured downstream in a fluorometer flow-through cell (excitation wavelength, 320 nm; emission wavelength, 404 nm). Fluorescence intensity was registered as peak height or peak area either by an integrator or by a special computer program (Q-FIA). The peak height or peak area is inversely proportional to the 2,4-D concentration in the sample. Each assay took 15 min to complete, including the regeneration step. The regeneration step was performed by alternating rinses with 0.01 M glycine/HCl buffer, pH 2.0, for 1.5 min (flow rate, 0.72 mL/min) and rinsing steps with carrier buffer for 1.5 min to readjust the pH to 7.2. Background signals caused by unspecific binding were determined using glass beads on which only avidin was immobilized. To calculate 2,4-D concentrations in unknown samples, the background signal was subtracted and the data were converted to % B/B_0 values according to the formula

$$\% B/B_0 = (\text{rel } F - \text{rel } F_{\text{excess}})/(\text{rel } F_0 - F_{\text{excess}}) \times 100$$

where rel F = relative fluorescence minus background, rel F_{excess} = relative fluorescence at the excess concentration of 2,4-D (= 1 mg/L), and rel F_0 = relative fluorescence at the zero concentration of 2,4-D.

The detection limits were calculated according to the method of Funk et al. (1985) from 20 calibration curves.

2.3. Measurement of Water Samples. For measuring real (spiked) samples with the fiber optical sensor, the water samples were supplemented by concentrated buffer solution containing the antibody system. If the pH of a sample was lower than 4.0 or exceeded 9.0, the sample was adjusted to a pH between 7.0 and 7.5 [usually with 1 part of PBS buffer, pH 7.2, to 9 parts of sample (v/v)] for the measurement of the synthetic and environmental water samples with FIIA. If the 2,4-D concentration of a sample exceeded 1 μg/L with FIIA, dilutions of the samples were carried out until the 2,4-D concentration was in the measuring range between 0.03 and 3 μg/L.

For better comparison of data from FIIA and fiber optics, spot checks were performed with several water samples using the respective enzyme immunoassay.

RESULTS

Our main goal was to establish two immunosensing formats, FIIA and the fiber optic immunosensor, as alarm devices for the routine monitoring of 2,4-D traces in water samples. Legislative requirements such as the EC Directives for Drinking Water and the German Drinking Water Ordinance, which prescribe an upper limit for pesticide contamination in drinking water of 0.1 μg/L, must be taken into account during method development. The development and successful application of a flow injection system for the analysis of triazine residues (Wittmann and Schmid, 1994) and a fiber optic immunosensor for triazine detection (Bier et al., 1992) have been described earlier.

Detection of 2,4-D with the fiber optic immunosensor requires two steps: first, preincubation of the sample containing the hapten 2,4-D with the labeled antibody, followed by the incubation of the analyte/labeled antibody mixture with the immobilized hapten. The difference between the fluorescence readings prior to and after incubation of the fiber is a measure of the amount of analyte in the sample. The regeneration of the fiber was achieved by incubation of proteinase K for 10 min. Acidic elution steps were applied prior to and after the proteinase incubation. With this regeneration protocol the baseline is stable for more than 8 weeks and more than 500 measurement cycles.

The flow injection system was based on an antibody-supported column reactor. In a first step toward FIIA development, a suitable hapten−peroxidase tracer had to be selected. From the different hapten−peroxidase conjugates prepared, the best tracer (in terms of a maximum signal height and a replacement of the tracer at very low 2,4-D concentrations) turned out to be the one in which the hapten 2,4-D was conjugated to peroxidase. Except for the 2,4-DB−HRP conjugate, none of the other hapten−HRP tracers showed binding to the antibodies. The 2,4-DB−HRP tracer could only be applied at high concentrations and showed a rather poor displacement of 2,4-D only at higher concentrations. The 2,4-D−HRP conjugate exhibited a coupling rate of three molecules of 2,4-D per molecule of peroxidase as determined via spectra. The 2,4-D tracer showed 98% enzyme activity remaining after the coupling procedure.

The established enzyme immunoassay was then transferred to the FIIA principle. There was no difference in the immunoreaction observed between the antibodies immobilized via the system avidin/biotin directly to the carboxylic groups of the glass beads and those using a spacer between the support and avidin. Former experiments showed that antibody coupling via avidin/biotin is superior to direct covalent coupling of the antibodies to the support (Wittmann and Schmid, 1994). The antibody columns turned out to be stable in antibody activity and amount of immobilized protein for a minimum of 500 measuring cycles and could be stored for at least 6 months at 4 °C without any significant loss of antibody activity or amount of protein bound to the support.

In a first step, the methods developed have to be characterized according to detection limit, sensitivity, accuracy, and precision. Figure 3 shows the calibration curves obtained with the optimized FIIA and the fiber optic immunosensor. (The curve of the enzyme immunoassay is not shown because it is so similar to that of the optimized FIIA.) The midpoint of the test is located at that point on the curve where 50% of the antibody binding sites are occupied by 2,4-D and 50% are bound by the 2,4-D−enzyme tracer. The midpoint of the FIIA was found at ~0.3 μg/L and that of the fiber optic immunosensor at ~10 μg/L, i.e. higher than that of the FIIA by a factor of 33. With FIIA a detection limit of about 0.03 μg/L could be reached; the range of measurement lies between 0.03 and 3 μg/L 2,4-D. A detection limit of approximately 0.2 μg/L for 2,4-D could be reached with the fiber optic immunosensor. The range of measurement was from 0.2 to 100 μg/L 2,4-D. A 2,4-D standard series consisting of a minimum of three concentrations between zero and a pesticide excess concentration (= 1 mg/L) has to be run before measurement of up to a maximum of 20 samples prior to new calibration with FIIA. Each standard concentration or sample was measured in quadruplicate. In addition, an average coefficient of variation of 4% was achieved with FIIA and one of 10% with the fiber optic immunosensor. Some important assay parameters of the FIIA such as the midpoint of the assay, the lower detection limit, and the coefficients of variation are similar to those of the corresponding enzyme immunoassay, although the measuring ranges of FIIA and the fiber optic immunosensor are always narrower than that of the respective enzyme immunoassay. This may be due to the fact that, in contrast to the enzyme immunoassay, neither system works under equilibrium conditions. Total assay times are 15 min with FIIA and 55 min with the fiber optic immunosensor, in comparison to the enzyme immunoassay, which takes 2 h to complete.

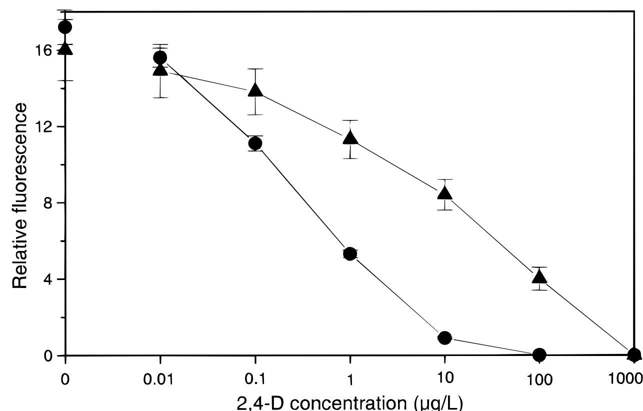

Figure 3. Representative standard curves obtained with the optimized FIIA (●) and the improved fiber optic immunosensor (▲) for 2,4-D determination. The tests were run in quadruplicate with FIIA and the fiber optic immunosensor. The standard deviations are indicated as error bars.

An important step for any validation study is the analysis and verification of samples supplemented with the analyte and comparison of the results with data from an established analysis method, i.e. in our case GC. As representative water sample, a raw water sample and the same water sample after purification (via ozonization and filtration over activated charcoal) was selected. The main reason for this was that, in general, the drinking water supply stations are dealing with raw water treatment to obtain drinking water. In Germany, either ground water (from deep wells) or surface water (via bank filtration) is taken as raw water for drinking water preparation. Therefore, two different matrices, ground water and surface water, have to be

Table 1. Environmental Water Samples Spiked with 2,4-D[a]

sample no.	2,4-D added (μg/L)	2,4-D amt determined with FIIA (μg/L ± SD)	2,4-D concn determined with the fiber optic immunosensor (μg/L)
raw water			
1		<0.03	<0.2
2	0.05	0.07 ± 0.015	<0.2
3	0.10	0.15 ± 0.008	0.25
4	1.00	1.08 ± 0.052	3.7
5	5.00	5.02 ± 0.090	9.9
purified water			
6		<0.03	<0.2
7	0.05	0.05 ± 0.020	<0.2
8	0.10	0.11 ± 0.010	<0.2
9	1.00	1.01 ± 0.067	2.2
10	5.00	4.98 ± 0.087	7.5

[a] Two different kinds of water, a raw water and a purified raw water, were obtained together with the analysis data of their pollution with pesticides, phenols, chlorine and nitrogen aromates, less volatile hydrocarbons, and anilines from Dr. U. Oehmichen, Wasserverband Hessisches Ried, Biebesheim, Germany. The two water samples were each fortified with four different 2,4-D concentrations (0.05, 0.10, 1.00, and 5.00 μg/L) and measured with the FIIA (in quadruplicate) and with the fiber optic immunosensor (in duplicate).

considered according to potential pesticide contamination with regard to the EC Drinking Water Directives. Table 1 shows that the 2,4-D concentrations of fortified raw water and the spiked raw water samples after purification can be precisely determined with FIIA. In the case of the fiber optic immunosensors overestimations were observed. In addition, the two original samples containing no 2,4-D pollution were analyzed as zero samples or blanks with both the FIIA and the fiber optic immunosensor methods with no false positive results. With regard to the time per assay performance of the fiber optic immunosensor (55 min), the samples were only measured in duplicates, thus increasing the relative standard deviation (RSD) to 20%. In light of this, it is remarkable that the German Drinking Water Ordinance allows for a standard error of ±0.05 μg/L at the 0.1 μg/L concentration. It is shown that the added 2,4-D concentrations could be precisely determined only by the FIIA. This can be derived to a certain extent from the assay variability. Intra-assay variabilities (assay performed on the same day by different persons) of 4% for the FIIA and 10% (4 replicates) or 20% (duplicates), respectively, for the fiber optic immunosensor were calculated. An interesting aspect was that the interassay variability (assay performed by the same person on different days) for the FIIA remained constant at 4%, whereas the variability of the fiber optic immunosensor turned out to reach values up to 30% at maximum, showing tolerances from 10 to 30%. No matrix effects causing overestimations or false positive results were observed using the FIIA. Due to the cross-reactivities of the antibody (cf. Figure 4) with FIIA, only 2,4-D from the different acidic herbicides was detected. As the raw water sample selected represented a surface water sample (probably containing humic acids), the matrix effects of the fiber optic immunosensor data could stem from interferences with sample ingredients (e.g. humic acids) in the spiked raw water sample, although overestimations were obtained even in the case of the spiked purified raw water samples. Several further water samples from different origins (ground water, rain water, and surface water) were measured with the FIIA and the fiber optic immunosensor (data

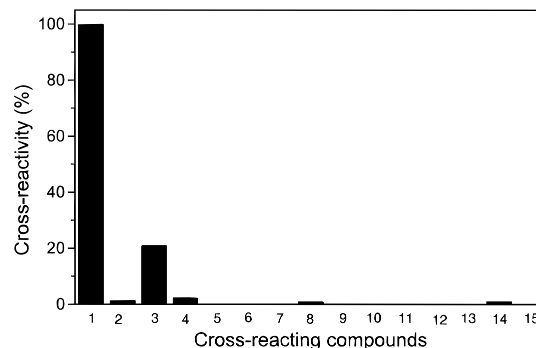

Figure 4. Cross-reactivities (in percent) based on 2,4-D (=100%), determined with the 2,4-D enzyme immunoassay and the 2,4-D-FIIA. Each standard concentration was analyzed in quadruplicate. (1) 2,4-D; (2) 2,4,5-T; (3) 2,4-D methyl ester; (4) MCPA; (5) 2,4-DB; (6) MCPB; (7) mecoprop, (8) 2,4-dichlorophenol; (9) 2-methyl-4-chlorophenol; (10) 1-chlorophenoxyacetic acid; (11) 1-methyl-5-chlorophenoxyacetic acid; (12) 1-methyl-4,5-dichlorophenoxyacetic acid; (13) 3,4-dimethylphenol; (14) dichlorprop; (15) 2-CPA.

not shown) yielding similar results as for the representative raw water and purified raw water sample selected.

DISCUSSION

Our aim was to establish and evaluate two immunosensing formats, an FIIA and a fiber optic immunosensor, making the automated monitoring of water samples for potential pollution with the herbicide 2,4-D possible. An advantage of both immunosensing formats described in this paper is the easy handling of water samples in contrast to GC (Ahmed et al., 1989; de Beer et al., 1989; Hodgeson et al., 1994) and HPLC analysis (Bogus et al., 1990), for which sample pretreatment and an enrichment step are required. For GC analysis 2,4-D had to be extracted from water using either organic solvents or a solid-phase extraction, e.g. with an XAD-2 resin column. In addition, 2,4-D had to be derivatized to a volatile compound prior to GC analysis, e.g. with (2-cyanoethyl)dimethyl(diethyl)aminosilane. GC analysis allows a more sensitive determination of 2,4-D than HPLC. Detection limits of 0.05 μg/L can be achieved with 1−2 L of a water sample. The recoveries with GC analysis of water samples were in the range of 96−99% with an average coefficient of variation (CV) of 6%. GC analysis is a very sensitive method for determining 2,4-D traces, but it is rather complicated, laborious, and expensive. In contrast, HPLC analysis of 2,4-D requires no derivatization but shows higher detection limits of 6 ppb of 2,4-D with good recoveries of 93.8%. The detection limit of 6 ppb is not sufficient if the water samples are to be surveyed for a trace 2,4-D contamination below the maximum permitted value of the EC Directives for Drinking Water of 0.1 μg/L. In contrast, for FIIA measurement no concentration or cleanup steps are required for ground and surface water samples. With the fiber optic immunosensors, some problems occurred with the measurement of environmental water samples. Prior to further consideration of the fiber optic immunosensor for routine monitoring of environmental water samples, a sample pretreatment procedure has to be worked out to prevent matrix effects. The fiber optic immunosensor is suitable yet for a control toward the presence of 2,4-D in a water sample because the two original samples containing no 2,4-D pollution were analyzed as zero samples. Both immunosensing meth-

ods need only a few milliliters of the water sample in contrast to 1 L required in the case of GC or HPLC analysis.

Compared to the conventional methods GC and HPLC, the immunoassays described for 2,4-D analysis (Hall et al., 1989; Eremin, 1995; Fleeker, 1987; Knopp et al., 1985; Rinder and Fleeker, 1981) need no cleanup prior to the analysis of water and urine samples. Three different types of immunoassays are described: radioimmunoassay (RIA), ELISA, and polarization fluoroimmunoassay. Hall et al. (1989) described a polyclonal antibody based ELISA and a RIA with rather high working ranges of 100−10 000 and 50−10 000 μg/L, respectively. The time needed to perform the ELISA was ca. 4 h; the RIA takes approximately 3 h to complete. In addition, CVs of 7% with the ELISA and 9% with the RIA were described. Both tests exhibited cross-reactivities with other chlorophenoxyacetic herbicides such as MCPA (15%), 2,4,5-T (11%), dichlorprop (6%), and mecoprop and dicamba (<6%). Knopp et al. (1985) described a RIA based on polyclonal antibodies with a detection limit of 0.5 μg/L 2,4-D.

There are a number of commercially available immunoassay kits on the market. Immunosystems sells a 2,4-D ELISA which can be performed either in tubes (detection limit for 2,4-D, 0.5 μg/L) or in microtiter plates (detection limit for 2,4-D, 1.0 μg/L). A plate test with a detection limit of 10 ng/L 2,4-D and a dipstick assay with a detection limit of 500 ng/L 2,4-D are commercially available from the company Pab Production. An immunoassay based on magnetic particles and magnetic separation is commercially offered by Ohmicron. In addition, the Abicaps format is marketed by Abion.

It was shown that the two immunosensing methods presented in this paper can be used as fully automated alarm devices. With the FIIA, detection limits for 2,4-D in the same range as with the commercially available plate test by Pab Production were reached, whereas even the detection limit obtained with the fiber optic immunosensor compares favorably with the ones of all the other ELISAs described so far. From the immunosensors for pesticide analysis described so far, the optical devices dominate. Two direct measurement principles have also been described, namely the application of piezoelectric quartz crystals [e.g. Guilbault et al. (1992) and of grating couplers [e.g. Bier and Schmid (1994)]. The detection limits reached by all of these direct immunosensors are not sufficient for a direct measurement of drinking water samples for a routine control with regard to the EC Guidelines for Drinking Water. In addition, especially in the case of the optical sensors, the devices required are rather expensive and sophisticated with the need of a clean room.

A broad range of different areas in the United States and Australia have been analyzed for pollution with 2,4-D in water samples (Ang et al., 1989; Cohen, 1986; Cova, 1990). Cohen et al. (1990) reported on the 2,4-D concentrations in ground water samples in the United States. In a river water sample, 2,4-D was determined at a concentration of 0.10 μg/L; in all of the other water samples analyzed 2,4-D was absent. This is due to the low persistence of 2,4-D, although it has a high mobility. In Australia, a total of 659 water samples were analyzed between November 1986 and June 1987 in the North Coast region of New South Wales (Ang et al., 1989). No pesticide residues were detected in 482 of the samples (73.1%). Trace level residues (defined as 0.5 μg/L) were found in 147 samples (22.3%), and residues above trace levels were detected in 30 samples (4.6%). Actual 2,4-D concentrations of up to 9.0 μg/L were found. In comparison, the maximum residue level recommended by the Australian National Health and Medical Research Council is 100 μg/L.

In addition, Dogheim et al. (1990) surveyed raw buffalo milk and fish samples which were collected in two Egyptian governorates for their 2,4-D contents as well as for 14 organochlorine pesticides and 2 PCBs (Aroclors 1254 and 1260). The highest 2,4-D concentration they found in catfish (*Clarius lazero*) was 2.44 ppm, whereas in milk samples they could detect very low 2,4-D contents in the lower micrograms per liter range. For this reason, one possible further application of the FIIA could be the analysis of food with simpler extraction than the sophisticated multistep extraction and sample clean up necessary for GC analysis.

Another possible application range of the FIIA could be the monitoring of urine samples from farmers spraying 2,4-D on their fields; Knopp and Glass (1991) described a RIA to screen the urine of occupationally 2,4-D exposed sprayers. The data obtained proved that all sprayers showed detectable quantities of 2,4-D (with the highest 2,4-D urinary concentration of about 2.5 ppm) in morning urine samples over 4 or 6 days in the postspraying period.

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