Development and Application of Laboratory and Field Immunoassays for Chlorpyrifos in Water and Soil Matrices

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Two sensitive tests were developed for monitoring the environmental dissipation of chlorpyrifos, using a high-affinity polyclonal antibody immobilized onto microwells and polystyrene tubes. The microwell assay, which required 90–120 min to perform, was designed for laboratory use and could detect 0.05–0.07 ppb of chlorpyrifos (85% B_0), with 50% inhibition of antibody binding (50% B_0) at 0.2–0.6 ppb of chlorpyrifos. The tube assay was designed for field use, required 15–20 minutes to perform, and had a lower limit of detection for chlorpyrifos of 0.15 ppb and 50% inhibition of antibody binding at 0.8–1.6 ppb. The assays were thus sufficiently sensitive for direct analysis of chlorpyrifos in irrigation drainage water samples, after addition of phosphate buffer to 0.025 M to the sample to slow its hydrolysis. The assay was unaffected by several cations and anions that may be found in surface water. Performance of the assays was examined for three situations representing a wide range of incurred chlorpyrifos residue concentrations: trace levels in irrigation drainage water, moderate levels in runoff from horticultural application, and high levels in soil following application as a termiticide. These assays were applied to the analysis of surface water and soil. No cleanup was required for any matrix. Good correlations were obtained between chlorpyrifos levels determined using the immunoassays and chlorpyrifos levels determined by gas chromatography–mass spectroscopy (GC–MS) or HPLC.

Keywords: *Chlorpyrifos; ELISA; immunoassay; determination; water*

INTRODUCTION

Chlorpyrifos (Dursban, Lorsban; Dow Elanco) is used to prevent insect damage to growing crops of rice, sugarcane, potatoes, and fruits and vegetables (Worthing and Walker, 1987). Its activity against soil organisms and comparatively low mammalian toxicity have made it a common replacement for the organochlorine insecticides used for domestic termite control (Wright et al., 1988). With additional use in flea and tick control on domestic pets, chlorpyrifos is one of the most commonly used insecticides in domestic situations in Australia and the United States. An important use of chlorpyrifos in Australia and some other countries is for control of bloodworm, *Chironomus tepperi*, since the larvae feed on the endosperm and developing roots of the germinating plant, especially after aerial sowing (Way and Wallace, 1989). Chlorpyrifos is commonly applied aerially, in conjunction with selected herbicides, 1–2 weeks after sowing (Stevens, 1991).

Determination of chlorpyrifos at parts-per-billion levels in water requires either solvent extraction or solid-phase extraction followed by concentration before instrumental determination. Instability of the pesticide in stored water samples could mean that the levels determined some days later may be somewhat lower than those actually present in the environment at the time of sampling. Similarly, the requirement for extraction and cleanup of soil samples prior to instrumental analysis limits the number of samples able to be analyzed in this manner.

Immunoassay methods have been developed both as screening tools and as quantitative analytical methods for pesticide residues in the environment [reviewed in Jung et al. (1989), Wratten and Feng (1990), Ferguson et al. (1993a), and Skerritt (1994)]. The methods offer the potential for direct analysis of pesticides in unconcentrated water samples and have already been used in rapid tests for detection of other insecticides such as chlordane (Bushway et al., 1988), carbofuran (Bushway et al., 1992), and aldicarb (Brady et al., 1989). Analysis of irrigation drainage water for chlorpyrifos is important as the pesticide has high toxicity to certain fish species (e.g., trout LC_{50} = 3 ppb), mammals, and especially aquatic invertebrates. Persistence is hard to predict because it is highly dependent on water pH, turbidity, and temperature (Racke, 1993). Better data on residue levels would enable appropriate management practices such as release of uncontaminated water to multiuse storages or river systems and storage and reuse of contaminated water on-farm. Currently, analysis of chlorpyrifos at parts-per-billion levels in water matrices requires solvent or solid-phase extraction, followed by the use and evaporation of organic solvents (Felsot and Dahm, 1979; Brooks et al., 1990; Welling and de Vries, 1992).

Chlorpyrifos is also finding increasing use as a termiticide treatment for soil under or around domestic

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or commercial premises, replacing organochlorine insecticides that are now either discouraged or banned. A problem with the use of chlorpyrifos in this situation is that its persistence and thus insecticidal efficacy are variable, being influenced by temperature, weather, soil moisture, and soil type. We have also adapted the tube immunoassay to the determination of chlorpyrifos in soils.

MATERIALS AND METHODS

Reagents Used. Solvents used for residue extraction and immunoassay development were of analytical grade and from the following sources: methanol, acetone (Mallinckrodt, Paris, KY); acetone, acetonitrile, *N,N'*-dimethylformamide, dichloromethane, sulfuric acid (Merck, Kilsyth, Australia). Hexane, acetonitrile, and tetrahydrofuran for HPLC were of chromatographic grade (Mallinckrodt). Buffers and salts were obtained from Ajax Chemicals (Clyde, Australia). Other suppliers were as follows: bovine serum albumin and horseradish peroxidase (Boehringher, Mannheim, Germany); ovalbumin, 3,3',5,5'-tetramethylbenzidine, hydrogen peroxide (Sigma, St. Louis, MO). Pesticide standards used in cross-reaction studies were obtained from ChemService (West Chester, PA).

Synthesis of Haptens. The preparation of the bifunctional reagent used in the syntheses of immunogens and the chlorpyrifos– and fenchlorphos–peroxidase (HRP) conjugates used in the work has been described elsewhere (McAdam and Skerritt, 1993). The haptens were coupled to carrier proteins and HRP by derivatization through the phosphate ester (McAdam et al., 1992). The immunogen used to prepare the antiserum in rabbits (McAdam et al., 1992) was actually a derivative of chlorpyrifos-methyl (rather than chlorpyrifos) coupled to ovalbumin [OA, conjugation ratio 8.3 mol of hapten/mol of ovalbumin, determined using reaction with trinitrobenzenesulfonate (Plapp et al., 1971)]. This was because none of six antisera prepared to either chlorpyrifos–OA or chlorpyrifos–IgY conjugates in different rabbits provided as sensitive an assay for chlorpyrifos (Edward et al., 1993). The conjugation ratios for the peroxidase conjugates used were 0.9 mol/mol for chlorpyrifos–peroxidase and ≥2 mol/mol for fenchlorphos–peroxidase. A conjugate of fenchlorphos was assessed in the tube ("field") assay only, because it is more stable than chlorpyrifos to hydrolysis [being the benzene analog of chlorpyrifos-methyl; see Eto (1974) and Worthing and Walker (1987)].

Immunoassays. Both the microwell (laboratory) and tube (field) assay formats used immobilized antibody, precoated for 16 h at 20 °C with diluted antiserum [either 200 μL diluted 1/50000 in 50 mM sodium carbonate, pH 9.6, onto polystyrene microwells or 500 μL diluted 1/5000 in 50 mM sodium carbonate, pH 9.6, onto 12 mm × 75 mm polystyrene tubes (Sarstedt, Numbrecht, Germany)], washed twice for 5 min with PBS (50 mM sodium phosphate–140 mM sodium chloride, pH 7.2) containing 0.05% Tween 20, blocked for 60 min at 20 °C with 1% (w/v) bovine serum albumin in PBS, and air-dried, followed by the simultaneous incubation of test sample/standard and HRP-labeled pesticide. Various dilutions of each conjugate were assessed in a checkerboard assay with a range of concentrations of chlorpyrifos; the assay conditions reported are those using the lowest conjugate concentration providing an OD over 1.0 at 450 nm. Plates or tubes coated with air-dried antibodies were stored desiccated at 4 °C for up to 6 months before use. Neither real-time data nor accelerated stability trials showed a significant change in assay color development or sensitivity compared with controls. For example, microwell plates and tubes stressed by storage at 37 °C for 3 or 10 days produced the following color development in the assay, compared with controls prepared at the same time and stored at 4 °C: microwell plates (OD 99 ± 3% and 92 ± 3%) and tubes (OD 108 ± 10% and 92 ± 7%) of control at 3 and 10 days, respectively in each case.

The microwell immunoassay was performed as follows: 1. Pipet 100 μL of standard/sample into antibody-precoated microwells. 2. Add 100 μL of enzyme conjugate (chlorpyrifos–peroxidase, 11 ng/mL, diluted in 1% bovine serum albumin–0.05% Tween 20 in PBS), mix, and incubate for 60 min at 18–23 °C. 3. Drain well contents, wash three times with purified water, tap dry. 4. Add 120 μL of substrate/chromogen (3,3',5,5'-tetramethylbenzidine/hydrogen peroxide, in acetate buffer, pH 5.5; Hill et al., 1991) mix, and incubate for 30 min at 18–23 °C. 5. Add 40 μL of stopping solution (1.25 M sulfuric acid) and mix. Read OD (450 nm).

Standards were prepared by dilution from a 0.1 mg/mL solution of chlorpyrifos in methanol–1% (v/v) acetic acid stored at 4 °C and diluted in reverse osmosis purified water using borosilicate glass tubes.

The tube assay was performed by addition of 160 μL of water sample [or in the case of soil extracts, an extract containing 10% (v/v) methanol in water] followed by 160 μL of conjugate [a conjugate of either chlorpyrifos–peroxidase (180 ng of peroxidase/mL) or fenchlorphos–peroxidase (225 ng of peroxidase/mL)] and mixed gently. After 10 min of incubation at room temperature, the contents were discarded and tubes washed five times with cold tap water. Then 160 μL of hydrogen peroxide substrate in acetate buffer, pH 5.5, and 160 μL of chromogen (containing 3,3',5,5'-tetramethylbenzidine; Skerritt et al., 1992) were added, and color was allowed to develop for 5 min at room temperature. Color development was terminated by addition of 40 μL of 1.25 M sulfuric acid, and following addition of 0.5 mL of water, OD at 450 nm was read on a colorimeter (either Artel Inc., Windham, ME, or EnviroQuant, Millipore, Bedford, MA).

Standard curves were prepared using five to seven concentrations of chlorpyrifos in the range 0.105–6.75 ppb, either in purified water (for cranberry bog water analysis), in 50 mM phosphate (for rice irrigation drainage water analysis), or in 10% (v/v) methanol in water (for analysis of soil extracts). Chlorpyrifos determination at higher levels (in bog water or soil) was performed using the fenchlorphos–enzyme conjugate, while analyses of the rice drainage water samples were performed using the chlorpyrifos–enzyme conjugate which provided a more sensitive assay (see below).

Water Samples. Two types of samples were studied:

1. Irrigation water samples were collected between October and December in 1991 and 1992 from the Griffith area of southwestern New South Wales, Australia. These were from floodwater within rice paddies and in drainage water from the paddies, 1–60 days following aerial application of chlorpyrifos as Lorsban 500 EC. They were buffered by addition of 10 mL/L 5 M sodium phosphate (pH 6.9 upon dilution) and stored at 4 °C in amber glass bottles until analysis (up to 2 weeks). Buffering was not necessary if samples were frozen or analyzed immediately.

2. Bog drainage water was collected in August 1991 from cranberry cultivation in eastern Massachusetts. The water was collected prior to chlorpyrifos application in the field and treated in the laboratory using a rate equivalent to 1.25 kg of active ingredient per hectare. Samples were retained for 24 h at room temperature prior to analysis by GC or immunoassay.

Where stated, turbidities were determined using a nephelometric turbidimeter (McVan Instruments, Melbourne, Australia). The first set of water samples contained 0–5 ppb of chlorpyrifos, and these were analyzed in the immunoassay after buffering, as they were generally collected under warmer conditions than the second set. The second series, which typically contained 25–140 ppb of chlorpyrifos, were stored unbuffered at 4 °C and diluted either 1/10 or 1/100 with distilled water before analysis.

Soil Samples. Soil samples were from outside residential dwellings throughout Indiana where chlorpyrifos, as Dursban PC, had been applied 2–6 months earlier. It was applied to trenches dug into the soil at the rate of 8.5 kg of chlorpyrifos per linear meter per 10 cm of trench depth. Soil varied in composition from sandy loams to clay types; samples were stored at 4 °C until analysis. For extraction, 20 g of dried soil was wetted with 4 mL of water in a 100-mL glass bottle and chlorpyrifos extracted for 30 min by shaking on a wrist-action shaker with either 50 mL of acetone or 40 mL of methanol. The mixture was allowed to settle and then (in the case of

methanol) the extract diluted 1/10 in water and analyzed directly (Wetters, 1977). Alternatively, 0.1 mL of the acetone extracts was evaporated to dryness under nitrogen and the residue redissolved in 1 mL of 10% methanol in water.

Spiking, Ion Effect, and Freeze–Thaw Experiments. In one set of experiments, chlorpyrifos was spiked from a 1 ppm solution in methanol to a range of final concentrations (0.5–5 ppb) into reverse osmosis purified water and into surface water of low turbidity (5 NTU). After mixing, samples (in amber glass bottles) were incubated for 4 h in the dark at 20 °C before analysis. Other spiking experiments were also performed with buffered water and water of higher turbidities (to 56 NTU). To assess the effects of freeze–thawing on the chlorpyrifos determination, a set of 15 samples (range 0–30 NTU) were buffered and stored at 4 °C for 2 weeks. Two sets of aliquots were taken: one was frozen at −18 °C a single time for 4 weeks, while the other set was subjected to five cycles of freezing for 3–7 days, with thawing to 18–23 °C for 4–6 h between freezing cycles. The effects of ions on the performance of the microwell assay were studied by assessing three to five concentrations of the following salts, dissolved in purified water on two parameters: B_0 optical density and percentage inhibition by 0.5 ppb of chlorpyrifos. The salts tested were NaCl (up to 100 mM), $MgCl_2$ (up to 2 mM), $CaCl_2$ (up to 5 mM), $FeCl_2$ (up to 2 mM), $NaHCO_3$ (up to 50 mM), and Na_2SO_4 (up to 2 mM). Solvent effects on the microwell assay were assessed by adding solvent to pure water to the concentration indicated.

Instrumental Analyses. Rice irrigation drainage water samples were analyzed by gas chromatography–mass spectrometry (GC–MS) on a Varian 3300 chromatograph using a Hewlett-Packard 5970 mass selective detector (operated in the single ion monitoring mode), using a 5% phenylmethyl silicone column (25 m long, 0.2 mm diameter, Hewlett-Packard). For these analyses, 500-mL water samples were extracted using three extractions with 50 mL of dichloromethane in a separatory funnel; the extracts were combined and concentrated to 0.5 mL under nitrogen. One microliter was directly injected using a splitless injection (splitter off 1 min) and an injector temperature of 200 °C. The column temperature was ramped from 50 (isocratic 1 min) to 160 °C at 20 °C/min; the temperature was held at 160 °C for 4 min and then ramped at 5 °C/min to a final temperature of 250 °C. Chlorpyrifos was quantitated using fenchlorphos as an internal standard. The ions monitored were 197 (quantifying ion), 199, 314, and 316 for fenchlorphos and 109, 125, 283, and 285 (quantifying ion) for chlorpyrifos (dwell times 60 ms/ion). Chlorpyrifos had a limit of detection of 0.05 ppb; 0.50 ppb was determined with a coefficient of variation (CV) of 7.8% (n = 5). Recovery of a 0.50 ppb spike from river water (pH 6.9, turbidity 17 NTU, conductivity 0.25 mS/cm) was 90%, with a CV of 8.0%. Fenchlorphos (2.4 ppb) was recovered at 94% with a CV of 5.5% (n = 5).

Soil samples were extracted for both GC and HPLC instrumental analysis using the method described for the immunoassay (Wetters, 1977). Briefly, 20 g of air-dried soil was wetted with 2 mL of water and mixed, then 50 mL of acetone was added and the sample mechanically shaken for 30 min at 280 excursions per minute. An aliquot of the extract (2 mL) was filtered (0.45-μm pore size nylon syringe filter) and 2 μL injected into the gas chromatograph (splitless mode). Cranberry bog water and soil samples were analyzed by either GC or HPLC. GC with electron capture detection was performed using a Hewlett-Packard 5890 chromatograph with a Model 3396A integrator. Separations were performed on a DB-1 30 m × 0.53 mm column with confirmatory analyses performed on a DB-1701 column (both from Alltech, Deerfield, IL). The temperature program ramped from 190 to 214 °C at 4 °C/ min and then at 10 °C/ min to a final temperature of 250 °C, where it was held for 7 min. Water samples (250 mL) were each passed onto a C_{18} Sep-pak (Waters, Milford, MA) which had been preconditioned using 5 mL of methanol and then 10 mL of water. Chlorpyrifos was eluted using 2 mL of ethyl acetate.

HPLC was performed using a Valco injector (Vicci Instruments, Houston, TX) with a 25-μL loop, a Waters 510 pump (Milford, MA), and a Hewlett-Packard 1040A photodiode array detector (Avondale, PA). Extracts of soil samples (5 μL) were analyzed using a 5-μm Spherex amino-derivatized silica column (Phenomenex, Torrance, CA), which was eluted isocratically at 1.5 mL/min using hexane/tetrahydrofuran (97:3), and detection was at 290 nm. Water samples (25 μL) were injected directly onto an Ultremex C_{18} column (Phenomenex), which was eluted isocratically at 1.1 mL/min using acetonitrile/water (80:20); detection was at 224 nm.

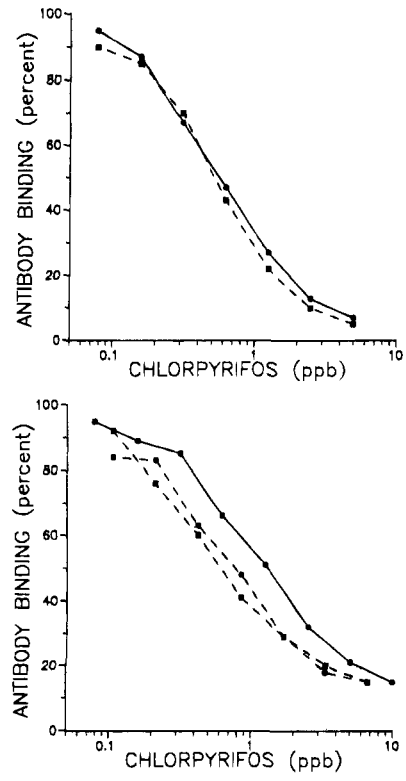

Figure 1. Standard curves (A, top) in the laboratory assay for chlorpyrifos in unbuffered (●) and buffered (■) purified water samples using chlorpyrifos–peroxidase and (B, bottom) in the field test for chlorpyrifos in water (●) or 10% methanol (■, as in the assay for soil extracts) using FCP peroxidase (dashed lines) or chlorpyrifos–peroxidase (solid lines). Data are means of three to six determinations performed on separate days. Error bars are omitted for clarity; the ranges of coefficients of variance of percentage inhibition for each point are for (A) 3–7 % and 2–10%, respectively, and for (B) 6–13% and 4–10%, respectively. Antibody binding in the absence of free chlorpyrifos is defined as "100% antibody binding".

RESULTS AND DISCUSSION

Sensitivity of Assays. The microwell assay using chlorpyrifos–peroxidase detected chlorpyrifos sensitively (Figure 1A), with 50% inhibition of antibody binding (50% B_0 values) in the range 0.2–0.6 ppb, depending upon the ambient temperature and conjugate dilution. The limit of detection (90% B_0) was 0.03–0.09 ppb (Figures 1A and 2 show examples near the lower end of the sensitivity range). The assay was dynamic enough in the center of the standard curve for quantitative analysis, since a 4-fold difference in chlorpyrifos concentration gave on average a 48% difference in inhibition. Our experience is that at least a 25% difference in inhibition for a 4-fold difference in concentration is needed for good assay precision. The assay is thus sufficiently sensitive to measure chlorpyrifos at the threshold levels for toxicity to various species (Racke, 1993) without the need for sample concentration. The percentage antibody binding versus chlorpy-

rifos concentration standard curves prepared in the 50 mM phosphate buffer used for field samples were not significantly different from those prepared in purified water (Figure 1A). However, the standard curves require preparation in buffer if buffered samples are used, as the actual OD values obtained for standards prepared in water were about 10% higher than in buffer.

The tube assay using chlorpyrifos–peroxidase was slightly less sensitive than the microwell assay (Figure 1B), in keeping with comparisons for immunoassays for other organophosphates (Beasley et al., 1993; Skerritt, 1994). When the fenchlorphos conjugate was used, the B_0 OD was 0.7–1.0, and the assay gave a 50% B_0 range of 0.6–0.8 ppb. At the dilution used in these studies, the chlorpyrifos conjugate usually gave a B_0 OD of 1.1–1.5 and a 50% B_0 range of 1.0–2.0 ppb. The corresponding limits of detection were between 0.1 and 0.2 ppb. Preliminary experiments also demonstrated that this assay had specificity similar to that of the assay using chlorpyrifos–peroxidase.

The activity of the fenchlorphos conjugate was also more stable than that of the chlorpyrifos conjugate. Sterile storage of the chlorpyrifos conjugate (diluted to 1 μg/mL) for 3 months at 4 °C resulted in an average 33% loss of color development in the assay, while storage for 1 month at 20 °C resulted in an average 52% loss in color development, although in neither case was the sensitivity of the assay reduced. In contrast, no significant loss of color development was observed when the fenchlorphos conjugate was stored under these trial conditions.

Behavior of the Microwell Assay. The following ions, potentially found in surface water, did not affect either the sensitivity or color development of the assay: Na^+ (up to 100 mM), Mg^{2+} (up to 2 mM), Ca^{2+} (up to 5 mM), Fe^{2+} (up to 1 mM), Cl^- (up to 100 mM), HCO_3^- (up to 50 mM), SO_4^{2-} (up to 2 mM). Although for water analysis samples are analyzed directly in the assay, the effects of a number of miscible solvents were evaluated in the assay as their use will be required when soil or foods are studied (Bushway, unpublished results). Of the solvents evaluated, methanol did not influence B_0 OD at up to about 15% (v/v, final concentration). At 5% (v/v), other solvents inhibited color development to varying degrees: acetonitrile (28% inhibition), acetone (65% inhibition), and *N,N'*-dimethylformamide (82% inhibition).

It was important that glass rather than plastic tubes were used for dilution of standards. Contact of diluted chlorpyrifos with plastic tubes (polypropylene, Bio-Rad, Hercules, CA) resulted in apparent adsorption of chlorpyrifos in standards (especially below 1 ppb), with a resultant shift of the standard curve (Figure 2). The shift increased with time. In contrast, it was not seen with some other organophosphates, such as diazinon, when similar concentrations were analyzed using a sensitive immunoassay specific for diazinon (Ferguson et al., 1993b). However, similar adsorption behavior to polypropylene but not glass tubes has been found for low concentrations of chlordane in aqueous systems (Johnson-Logan et al., 1992). In addition, it was important that samples to be analyzed were not subjected to repeated freeze–thawing. Spiking experiments (Figure 3) and analyses of field samples (Figure 4) demonstrated that providing the samples were not high in turbidity, good correlations were obtained between data from either GC or immunoassay analyses of fresh samples and immunoassay data from samples

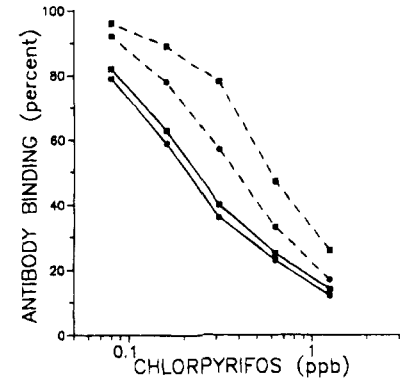

Figure 2. Comparison of chlorpyrifos dilutions made into glass (solid lines) and plastic tubes (dashed lines) and analyzed in the microwell assay for 5 (●) and 60 min (■) after preparation. Data shown are from three separate determinations performed on separate days.

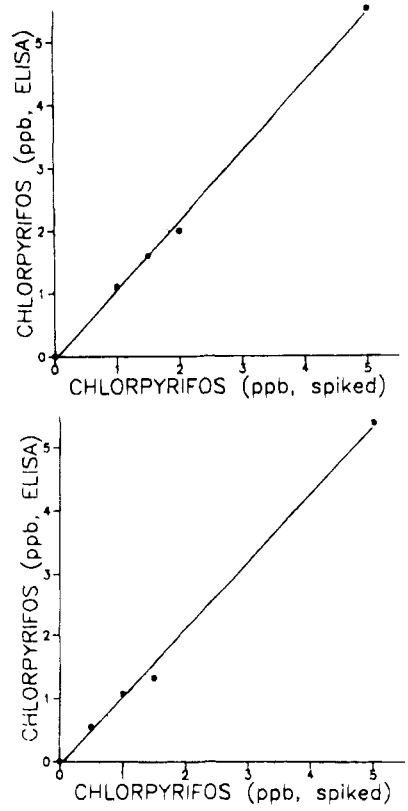

Figure 3. Spike and recovery of chlorpyrifos using the laboratory assay for (A, top) unbuffered purified water and (B, bottom) unbuffered surface water. Water samples were not frozen before analysis; data shown are from one experiment, which was replicated with similar results.

frozen a single time. However, two aliquots of a set of 15 samples (range 0–30 NTU, mean 8 NTU) were analyzed in the same experiment; one after a single freeze–thaw cycle and the other after five cycles. Chlorpyrifos data from the repeatedly frozen samples averaged only 48% of those in the samples frozen once.

Assay Specificity. In both the microwell and tube assays, chlorpyrifos-methyl (Reldan), fenchlorphos, and bromophos-methyl and -ethyl were the only other pesticides detected in the assay with sensitivity comparable to that of chlorpyrifos (Table 1). The two formats had similar specificities, as did the assay with either chlorpyrifos–peroxidase or fenchlorphos–peroxidase. The assay detected chlorpyrifos-methyl more sensitively than the ethyl form (chlorpyrifos), an ex-

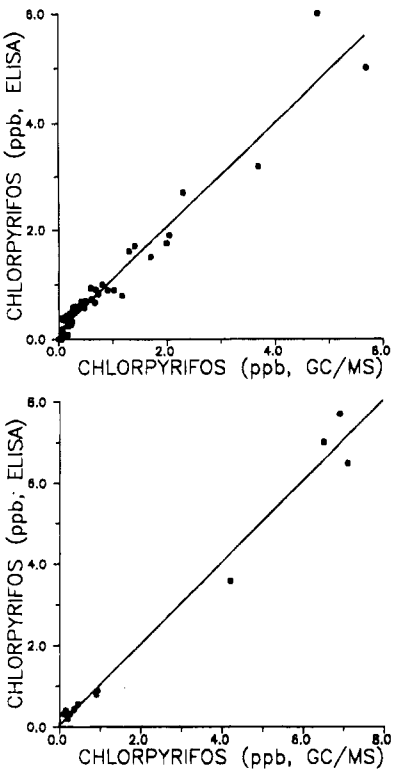

Figure 4. Relationship between chlorpyrifos levels in surface water (rice irrigation drainage) assessed by immunoassay and by GC–MS using (A, top) microwell assay (n = 32, ELISA = 0.918 × GC + 0.11, r = 0.978, P < 0.001) and (B, bottom) tube assay (n = 12, ELISA = 1.005 × GC + 0.05, r = 0.999, P < 0.001). Data are from single determinations; samples were frozen once before immunoassay analysis.

Table 1. Specificity of Laboratory and Field Immunoassays for Chlorpyrifos[a]

	laboratory assay			field assay
	50% B_0	90% B_0	x-rn[b]	% x-rn
chlorpyrifos	0.3	0.05	100	100
analogs and metabolites				
chlorpyrifos-methyl	0.1	0.02	330	300
chlorpyrifos oxon	1	0.4	30	55
mono-dechloro-CP	4	0.8	8	nt[c]
3,5,6-trichloro-2-pyridinol	>5000	1000	–[d]	–
fenchlorphos	0.4	0.02	75	35
other organophosphates				
bromophos-methyl	0.7	0.05	45	110
bromophos-ethyl	1.1	50	25	nt
dicapthon	5	0.1	6	5
dichlofenthion	110	5	0.30	nt
azinphos-ethyl	120	1	0.25	nt
azinphos-methyl	190	10	0.15	nt
tetrachlorvinphos	200	10	0.15	0.35
fenitrothion	340	21	0.10	nt
parathion	1600		–	nt
methyl-parathion	4500		–	0.1
fenthion	>5000	350	–	nt
diazinon	>5000	650	–	nt
herbicides				
triclopyr	70	3	4	2
picloram	180	30	1.6	nt
2,4,5-T	>1000		–	–
2,4-D	>1000		–	–

[a] The following compounds gave 50% B_0 values over 20 ppm: organophosphates cythioate, dichlorvos, dimethoate, fensulfothion, malathion, pirimphos-methyl, pirimiphos-ethyl, temephos; carbamates carbaryl, carbofuran, aldicarb, aldicarb sulfone; pyrethroids phenothrin, fenvalerate, permethrin, bioresmethrin; organochlorines chlordane (no inhibition at 100 ppb). [b] % x-rn = percentage cross-reaction [50% B_0 (chlorpyrifos)/50% B_0 (test compound) × 100]. [c] nt, not tested. [d] –, <0.01% cross reaction.

pected finding given that the immunogen hapten was actually based upon the former. The other compounds are also closely related to chlorpyrifos; fenchlorphos is the benzene analog of chlorpyrifos-methyl, while bromophos-methyl is identical to fenchlorphos, but with a bromine replacing the chlorine para to the phosphate ester. As the use of these pesticides is usually restricted to stored products, the cross-reaction is not of practical consequence. The biologically active oxon of chlorpyrifos was also detected but not the inactive trichloropyridinol metabolite. Some other organophosphorus insecticides were detected at ppb concentrations; in most cases they were chlorine-substituted compounds that are not used widely in agriculture. A number of major herbicides have two and three chlorines on a benzene or pyridine ring; for example, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), triclopyr (the pyridine analog of 2,4,5-T), and picloram. Of these compounds, only triclopyr showed cross-reaction that could be of practical importance, with a limit of detection (90% B_0) of 3 ppb. Thus, caution should be exercised in interpreting the results of the assay with water or soil samples from sites which may have been treated with triclopyr (Garlon) for woody weed control.

The specificity pattern of the (polyclonal) antibody used in this assay differed from that of a monoclonal antibody raised to the same hapten (Skerritt et al., 1992). Both antibodies detected chlorpyrifos-methyl more sensitively than chlorpyrifos, but the monoclonal antibody required three ring substituents and did not recognize the oxon form of chlorpyrifos. Unlike the monoclonal antibody, where the concentration–inhibition curves of the cross-reacting compounds were reasonably parallel, the slopes of the inhibition curves for the polyclonal antibody used in this study differed for different compounds. This could be seen by comparison of the 50% B_0 and 90% B_0 values. For chlorpyrifos and several other compounds, the latter value was usually about 5-fold lower than the 50% B_0 concentration, indicating a reasonably steep standard curve. However, some other compounds (e.g., dicapthon and triclopyr) had shallow standard curves. This was not related in an obvious manner to the chemical structure of the inhibiting pesticide, nor was the slope of the inhibition curve related directly to the particular 50% B_0 concentration for the compound.

Analysis of Surface Water Samples in the Microwell Assay. Standard curves prepared in clear irrigation supply water (19 NTU) and turbid drainage water (111 NTU) were superimposable (data not shown), indicating that clarification was not necessary. However, due to binding of chlorpyrifos to particulate matter, there is the possibility of underestimates of total chlorpyrifos in field samples, using either the immunoassay or GC methods. This effect may be more pronounced if solvent extraction is used and at levels of chlorpyrifos somewhat higher (100–500 ppb) than those found in the irrigation water samples examined in this study (Saner and Gilbert, 1980).

Good correlations were obtained for chlorpyrifos spiked at four levels (1–5 ppb) and recoveries by immunoassay for spikes into purified water (prepared with buffering (r = 0.999, recovery = 106%) and without buffering (r = 0.998, recovery = 108%; Figure 3A) and unbuffered turbid (111 NTU) river water (regression r = 0.999, recovery = 110%; Figure 3B). The method was tested on water samples collected from rice bays at various times after the aerial application of chlorpyrifos

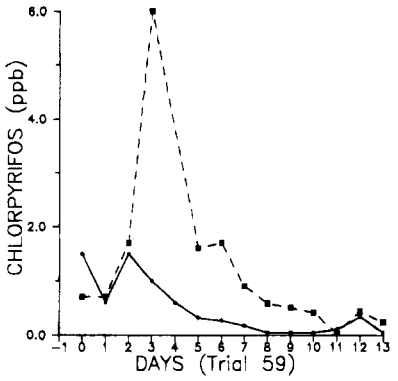

Figure 5. Field dissipation of chlorpyrifos following a spraying trial (trial 59) in rice floodwater measured by ELISA. Data shown are for two sites within one paddy: near inflow, where dilution with chemical-free water occurs (solid lines), and at a downstream part of the bay (dashed lines). Data are means of two determinations.

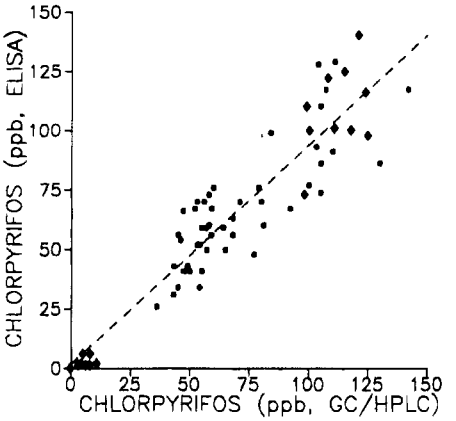

Figure 6. Comparison of chromatographic [either HPLC (●) or GC (♦)] and immunoassay methods for the determination of chlorpyrifos in cranberry bog water. Data are from single GC, HPLC, and immunoassay determinations. Immunoassay data (ppb) = 0.92 × GC/HPLC data + 1.7, $r = 0.903$, $n = 68$, $P < 0.0001$.

and also from drainage water of a number of farms that include rice crops (Figure 4A). There were good correlations between GC–MS and immunoassay data. The set of samples analyzed in Figure 4 had turbidities ranging from 0 to 54 NTU, with (in the case of Figure 4A) all but five samples being below 20 NTU. Within the range of turbidities studied, there did not seem to be an obvious effect of turbidity on the relationship between GC–MS data and immunoassay data, although overestimates were seen in the immunoassay with two samples of high turbidity and very low (0.1–0.2 ppb by GC–MS) chlorpyrifos. The precision of the immunoassay was high: a set of 18 unbuffered irrigation drainage water samples containing 0–8 ppb of chlorpyrifos were aliquoted into glass vials and frozen. Separate aliquots were defrosted and analyzed in immunoassays performed 3 months apart. The correlation between the first assay and the second assay was excellent ($r = 0.993$), with values in the second assay slightly lower (average 17%) than the first.

One aim of this work (Figure 5) was to use the assay to investigate the dissipation of chlorpyrifos in the field and carry-over from rice floodwater into irrigation drains. Data from two sites obtained over 14 days in a dissipation study are shown in Figure 5. At the downstream end of the rice paddy, peak chlorpyrifos levels (almost 6 ppb) were reached 3 days after aerial application. The other site tested was nearer to the actual spray site. While levels peak earlier, they were somewhat lower due to continuous dilution with "clean" water. Further details of location and spraying methods are given in Bowmer et al. (1994).

Analysis of Water Samples in the Tube Assay. Two sets of water samples were analyzed in the tube assay; a set of irrigation drainage water samples containing low levels of chlorpyrifos (0–8 ppb) were analyzed in the assay using chlorpyrifos–peroxidase (Figure 4B), and a set of cranberry bog water samples containing higher concentrations were analyzed in the assay using fenchlorphos–peroxidase (Figure 6). Similar to the microwell assays, no matrix effects were seen in the tube assay using either conjugate. With both sets of samples, good correlations between chlorpyrifos determined by immunoassay and the instrumental methods were obtained.

Analyses of Soil in the Tube Assay. The set comprised 23 soil samples, of which three were negative for chlorpyrifos by either GC, HPLC, or immunoassay. Six of the samples had relatively low levels of chlorpyrifos (2–27 ppb by GC), while much higher levels (up to 333 ppm by HPLC) were found in the remainder. Acetone and methanol were compared as chlorpyrifos extractants, although, because of solvent inhibition, the acetone was evaporated from extracts and the residue redissolved in methanol. The correlation between chlorpyrifos determined by GC and immunoassay (ELISA) in the acetone extracts of the 20 soils that contained chlorpyrifos was high ($r = 0.978$, $P < 0.001$). Good correlations were seen between immunoassay and HPLC data for acetone extracts of soils that contained above 50 ppm of chlorpyrifos (the lower limit of quantitation for the HPLC method used; Figure 7A). The slope of the regression when plotted on linear rather than logarithmic axes was 0.86. Precision of the assay was investigated through repeated analyses of a set of eight soil samples, containing up to 290 ppm of chlorpyrifos (Table 2).

Recoveries were lower (76%) for methanol extracts of soil in the immunoassay compared with acetone extracts (Figure 7B), although the results were highly linearly correlated. It was not clear whether this was due to poorer extraction of chlorpyrifos from the soils by methanol or due to matrix interference. Analyses of pesticide-free extracts of three different soil types did not reveal apparent matrix effects. While acetone has been used by several groups for extraction of chlorpyrifos from soils (Miles et al., 1979; Getzin, 1981), a wide variety of other extractants (Pike and Getzin, 1981; Fuhremann and Lichtenstein, 1980) including methanol have also been used for organophosphate extraction (Durand and Barcelo, 1991). However, use of methanol (which avoids the need to evaporate off solvent) would be satisfactory for screening purposes.

GENERAL DISCUSSION

We have described the development of an immunoassay for chlorpyrifos, which has been successfully adapted to both a standard microwell format for laboratory use and a rapid "field" format based on polystyrene tubes. In both cases, the assay requires only a single washing step. Although the assays cross-reacted with some analogs of chlorpyrifos, none of the other major chemicals used in irrigation agriculture or domestically were detected.

The sensitivity of the microplate assay was slightly higher than that of the tube assay, because the longer

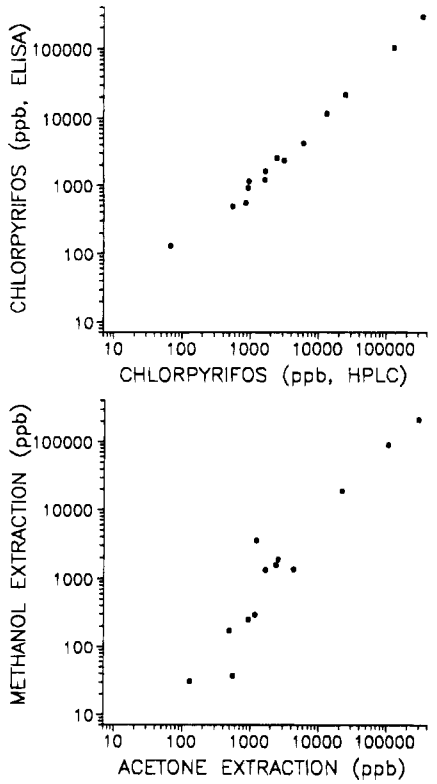

Figure 7. Analysis of chlorpyrifos in soil by immunoassay: (A, top) comparison of HPLC data with immunoassay data from acetone extraction, ELISA (ppm) = 0.86 × HPLC − 0.3, $r = 0.999$, $n = 14$, $P < 0.001$; (B, bottom) comparison of immunoassay data obtained using methanol and acetone extraction, chlorpyrifos (methanol extraction, ppm) = 0.76 × chlorpyrifos (acetone extraction, ppm) + 0.5, $r = 0.998$, $n = 14$, $P < 0.001$. Data are from single HPLC and immunoassay determinations.

Table 2. Precision of Chlorpyrifos Analysis by Immunoassay in Soils[a]

soil sample	chlorpyrifos (ppb)	intra-assay (% CV)	inter-assay (% CV)
1	nd	8.1	6.9
2	6.0	5.7	7.3
3	36	10	12
4	130	10	12
5	930	8.9	13
6	22000	5.5	12
7	100000	8.5	18
8	290000	6.8	18

[a] Data are from soils extracted using acetone. Data shown are percent coefficients of variation from six determinations based on one day (intra-assay) or from six determinations on six different days.

incubation period used allowed use of lower concentrations of chlorpyrifos−enzyme conjugate. However, in both cases, the assay was sensitive to concentrations of chlorpyrifos below 1 ppb, enabling for the first time the analysis of chlorpyrifos in drainage water without concentration. This sensitivity brings with it the need for caution in sample handling and storage. Initially, disposable polystyrene tubes were used for dilution of standards. However, somewhat greater sensitivity with standards containing under 1 of ppb chlorpyrifos was found when these were diluted in glass tubes. The loss of sensitivity was probably due to adsorption of the low levels of chlorpyrifos onto the walls of the plastic tubes. Chlorpyrifos levels in samples containing under 5 ppb also showed stepwise decrease upon repeated freezing and thawing. While good correlations were seen between chlorpyrifos levels determined in field water samples by the immunoassays and by GC−HPLC, the sets of samples were predominantly of only low-to-moderate turbidity. A greater understanding of the ability of the immunoassays to measure chlorpyrifos that is particle-bound is required before the assay could be applied to very turbid samples.

The tube assay was successfully coupled with the use of a single solvent extraction step for laboratory analysis of chlorpyrifos in soil. More rapid but less quantitative analysis of pesticides in soil can also be performed by extraction of the soil by hand-shaking soil and solvent in a screw-cap polyethylene bottle containing ball bearings (Skerritt, 1992).

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