

ABSORPTION OF *p,p'*-DICHLORODIPHENYLDICHLOROETHYLENE AND DIELDRIN IN LARGEMOUTH BASS FROM A 60-D SLOW-RELEASE PELLET AND DETECTION USING A NOVEL ENZYME-LINKED IMMUNOSORBENT ASSAY METHOD FOR BLOOD PLASMA

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Abstract—This work describes the uptake of two organochlorine pesticides from slow-release pellets by largemouth bass and the utility of a blood plasma enzyme-linked immunosorbent assay (ELISA) method for exposure verification. We measured blood and tissue levels by gas chromatography/mass spectrometry and by a novel ELISA method, and present a critical comparison of the results.

Keywords—Fish Exposure *p,p'*-Dichlorodiphenyldichloroethylene Dieldrin Enzyme-linked immunosorbent assay

INTRODUCTION

Evaluating dose–response relationships in toxicological research requires precise and reproducible methods for delivering the toxicant to the target organ of toxicity. Measuring the dose of toxicant delivered to a target organ can be impractical during in-life phases of a toxicity study, however, so blood levels are often used as surrogate measures of the internal dose. In order to validate a dose delivery method, it is desirable to verify that the target organ dose varies directly in proportion to the applied dose, but at a minimum, a clear and consistent relationship must be established between blood levels and the applied dose.

The original aims of this study were to verify the internal doses of *p,p'*-dichlorodiphenyldichloroethylene (DDE) and dieldrin delivered by slow-release pellets and to evaluate dose–response relationships for potential reproductive effects of these organochlorine pesticides (OCPs) in Florida largemouth bass (*Micropterus salmoides floridanus*). Dose delivery from the pellets was highly inconsistent; therefore, dose response was not interpretable from these data. Samples taken during the course of the study allowed correlation of DDE and dieldrin blood levels with target tissue levels and subsequent evaluation of a novel enzyme-linked immunosorbent assay (ELISA) method for quantifying these compounds in plasma from individual bass, a method much like those used in human drug screening and water or soil testing for the presence of various contaminants. These data indicate that the ELISA method was reliable and convenient for measuring plasma DDE and dieldrin concentrations as a surrogate dose estimate and have broader implications for interpreting dose–response data on organochlorine pesticides in largemouth bass.

MATERIALS AND METHODS

Experimental animals

Florida largemouth bass were obtained from a fish hatchery (American Sports Fish, AL, USA) in December 2001 and transferred to the United States Geological Survey-Florida Integrated Science Center-Center for Aquatic Resource Studies facility, where they were housed in 6,116-L concrete runs (366 × 183 × 91 cm) equipped with a flow-through system supplied by on-site pond water and aeration. On day 0 of the experiment (January 11, 2002), bass had a mean (± standard deviation) weight, length, and condition factor (K) of 141.9 ± 22.6 g, 213.1 ± 10 mm, and 1.46 ± 0.11, respectively. Water quality parameters were all within acceptable ranges for the duration of the experiment: dissolved oxygen (4.28–10.20 mg/L), temperature (12–24°C), and pH (7.5–8.6). Fish were fed Floating Finfish Silver feed (Zeigler Brothers, Gardners, PA, USA) ad libitum twice a week.

Experimental design

The organochlorine pesticides 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE lot 09020KU, 99.4% purity) and 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene (dieldrin, lot 077H3578, 91.2% purity) were obtained from Aldrich Chemical (Milwaukee, WI, USA) and shipped to Innovative Research of America (Sarasota, FL, USA), where they were incorporated into 60-d slow-release matrix pellets that contained cholesterol, lactose, celluloses, phosphates, and stearates as carriers and chemical binders.

Sixty-day release pellets were inserted intraperitoneally with a steel trochar into 200 fish, 25 per treatment. Treatments included three doses of DDE (2.5, 5.0, and 10.0 mg/pellet), three doses of dieldrin (0.25, 0.5, and 1.0 mg/pellet), placebo (matrix only), and sham (no pellet inserted). Each fish was

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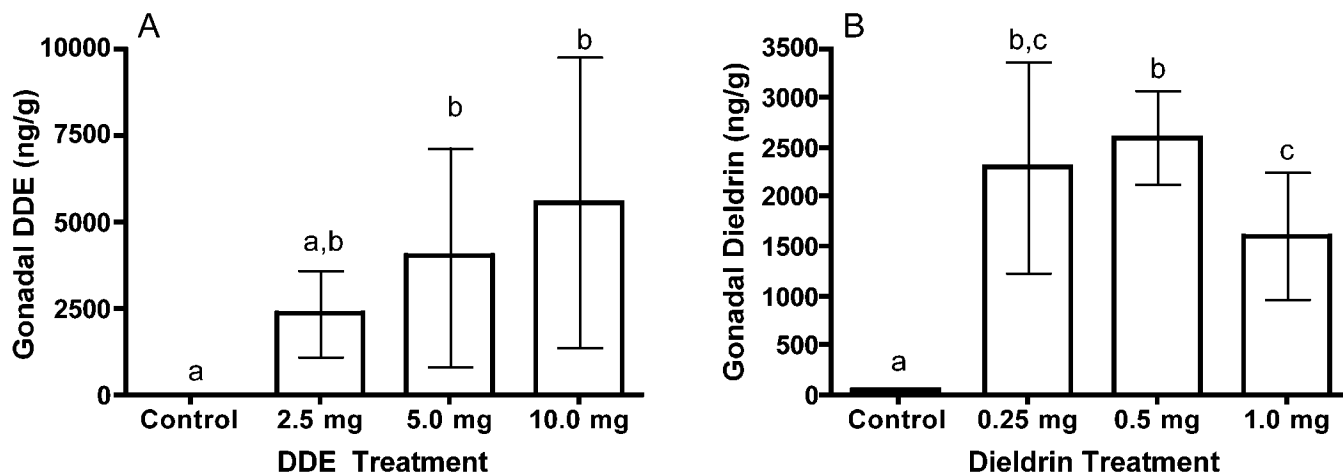


Fig. 1. Gonadal concentrations (mean \pm standard deviation) of (A) *p,p'*-dichlorodiphenyldichloroethylene (DDE) and (B) dieldrin after 60 d of treatment with a 60-d slow-release matrix pellet containing the specified dose. The control treatment is the combination of sham and placebo groups. Bars with different letter designations are significantly different ($p < 0.05$).

also implanted with an intraperitoneal pit tag (Trovan, Bel Air, MD, USA) to allow identification of individual fish for repeat measurement.

Blood and tissue collection

Fish were measured (weight and length) and bled at days 0 (January 11, 2002), 30 (February 10, 2002), and 60 (March 12, 2002). Approximately 1 ml of blood was obtained from the caudal vein using a heparinized 20-gauge needle, dispensed into 3 ml heparinized vacutainers, labeled, and stored on ice until centrifuged. Blood samples were centrifuged at 1,000 g , 4°C, for 20 min to separate red blood cells from plasma. Plasma was removed with a transfer pipette, placed in a cryovial, and stored at -80°C . Liver, gonad, muscle, and blood samples were also collected for contaminant analysis from two fish per treatment after 30 and 60 d of exposure. Gonads were collected from a subset of five females per treatment at day 60. Tissues were wrapped in tin foil, and blood remained in the glass vacutainer; all were frozen at -20°C until OCP analysis by gas chromatography/mass spectrometry (GC-MS).

Analysis of tissues for OCPs

Chemical analysis by GC-MS was conducted at the Center for Environmental and Human Toxicology, University of Florida (Gainesville, FL, USA). Briefly, whole blood or tissues were first homogenized to eliminate any concentration variability within the tissue due to differences in lipid content. A portion of the sample (2–5 g) was then extracted into ethyl acetate. Samples were purified using C18 and NH₂ solid phase extraction cartridges. Total OCP content was determined by GC-MS, according to U.S. Environmental Protection Agency method 8270 [1]. Samples were analyzed multiple times in full scan mode for analyte identification and in selected ion mode for quantitation to improve sensitivity. Percent recovery ranged between 75 and 100% with a limit of detection of 0.75 to 1.5 ng/g (ppb).

Analysis of blood plasma for OCPs

Blood plasma concentrations of DDE and dieldrin were determined by ELISA (Abraxis LLC, Warmister, PA, USA; DDE kit, lot 3C06437; Cyclodiene kit, lot 3C0643). These kits were developed to test soil and water samples in the ranges

of 1.25 to 75 ng/ml (ppb) DDE and 0.25 to 25 ng/ml dieldrin. Abraxis determined that dilution of plasma samples 1:50 in 10% methanol for DDE and 1:100 in 25% methanol for dieldrin gave detection ranges of 62.5 to 3,750 ng/ml and 25 to 2,500 ng/ml, respectively. Preparation of standard solutions in the same methanol concentrations with 10 μl of clean largemouth bass plasma (the same volume as the samples) reduced matrix interference in reading sample concentrations. Standards for DDE were prepared by adding DDE stock solution (10,000 ng/ml, included in kit) to a 1:50 dilution (10% methanol) of plasma from untreated bass. This solution was then serially diluted to create a standard curve of 0, 1.25, 2.5, 5, 10, 25, and 75 ng/ml as per kit instructions. Standards for dieldrin (0, 0.25, 0.5, 1, 2.5, 5, 10, and 25 ng/ml) were included in the kit prepared in 25% methanol and required only addition of 10 μl of clean largemouth bass plasma. Parafilm was placed over each standard tube and vortexed to mix.

Twenty-five microliters of each standard or sample was pipetted in duplicate into a 96-well plate. Next, 100 μl of rabbit anti-DDE/anti-cyclodiene antibody was added to each well. Wells were covered with tape, and contents of the plate were mixed by rotating the plate horizontally on the benchtop for approximately 30 s. The plate was then incubated at room temperature for 60 min. Following incubation, the covering was removed, and the contents were dumped by vigorous shaking. Wells were then washed three times with 250 μl 1 \times wash solution, followed by blotting off excess wash with a paper towel. Anti-rabbit-horseradish peroxidase secondary antibody (100 μl) was added to each well, covered, and allowed to incubate at room temperature for 30 min. The covering was then removed, the contents were dumped, and the wells were washed three times in 1 \times wash solution. Hydrogen peroxide color solution (100 μl) was then added to each well and incubated at room temperature for 20 min. Acidic stopping solution (50 μl) was added to each well. The plate was read at 450 nm on a MRX microplate reader (Dynex Technologies, West Sussex, UK) within 15 min of application of the stopping solution. Results are given as ng/ml.

The limit of detection for DDE was 62.5 ng/ml. Cross-reactivities of the *p,p'*-DDE antibody were 46% *p,p'*-DDD, 16% *o,p'*-DDD, 10% *p,p'*-DDT, and 3.2% *o,p*-DDE. The limit of detection for dieldrin was 25 ng/ml. Cross-reactivities of

Table 1. Linear regression parameters for blood to tissue correlations

Tissue level (y [ng/g])	Chemical	Sex	Slope (m)	Intercept (b)	n	r ²	p
Liver	DDE ^a	Female	1.5	-8	9	0.84	<0.05
		Male	1.4	-46	6	0.88	<0.05
Gonad	DDE	Female	10	120	9	0.95	<0.05
		Male	10.5	2	6	0.75	<0.05
Muscle	DDE	Female	0.25	36	9	0.59	<0.05
		Male	0.5	9	6	0.40	>0.05
Liver	Dieldrin	Female	0.6	-5	4	1.00	<0.05
		Male	0.3	52.5	6	0.97	<0.05
Gonad	Dieldrin	Female	5.2	-51	4	0.93	<0.05
		Male	1.5	412	6	0.68	<0.05
Muscle	Dieldrin	Female	0.3	-9	4	0.85	>0.05
		Male	0.13	48	6	0.49	>0.05

^a DDE = *p,p'*-dichlorodiphenyldichloroethylene.

the cyclodiene antibody were 150% endosulfan, 58% heptachlor, 26% aldrin, 26% chlordane, and 8.2% toxaphene.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS[®]) software, Version 9 (Cary, NC, USA). Analysis of variance followed by Duncan's multiple range tests was performed to determine differences in internal dose between treatment groups. Linear regression was used to compare GC-MS and ELISA results.

RESULTS AND DISCUSSION

Largemouth bass

Survivorship over the course of exposure was 88%. No long-term adverse effects were observed in surviving fish. Weight, length, and K steadily increased over the course of the 60-d experiment for males and females and did not vary between treatments.

Dosing consistency

High variability in DDE and dieldrin concentrations was observed in gonad, liver, muscle, and blood concentrations

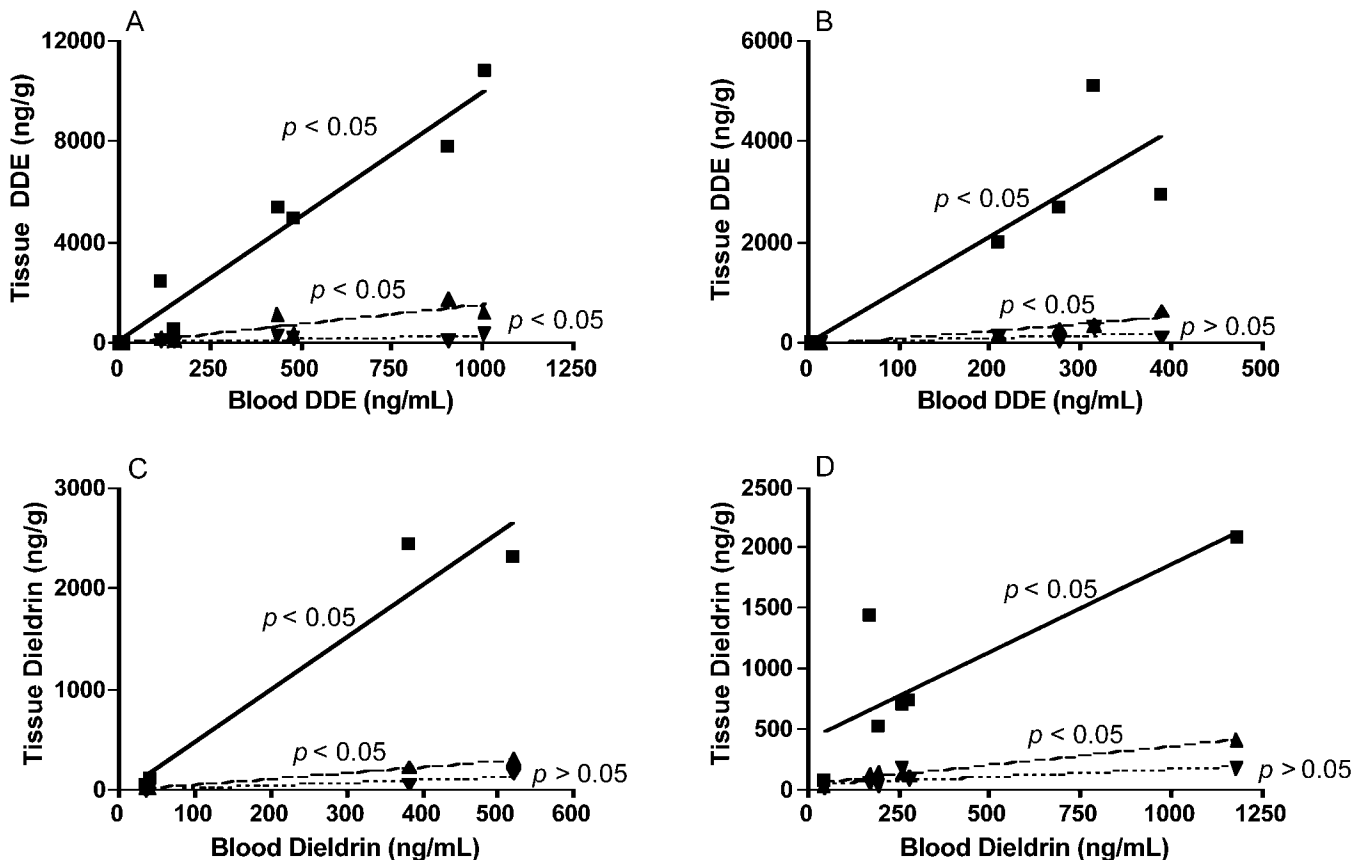


Fig. 2. Blood-to-tissue correlations of *p,p'*-dichlorodiphenyldichloroethylene (DDE) in (A) females and (B) males; dieldrin in (C) females and (D) males. ■ gonad; ▲ liver; ▼ muscle.

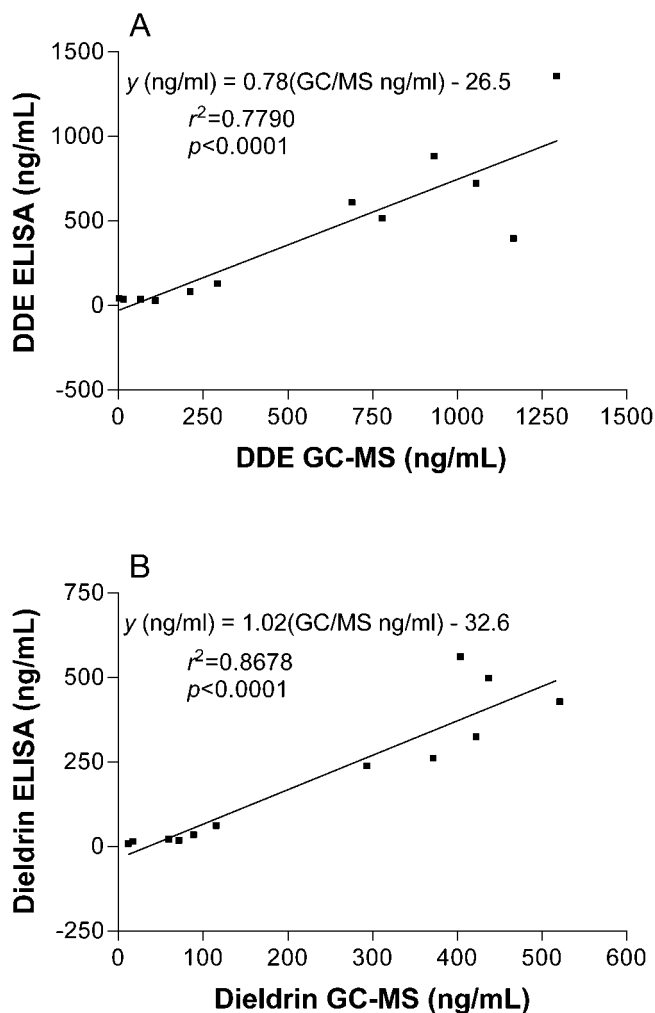


Fig. 3. Linear regression of (A) *p,p'*-dichlorodiphenyldichloroethylene (DDE) or (B) dieldrin concentrations from enzyme-linked immunosorbent assay (ELISA) versus gas chromatography/mass spectrometry (GC-MS).

from all treatment groups (only gonad data shown; Fig. 1). Despite this high variability, mean gonadal DDE concentrations correlated with the amount of chemical purported to be contained in the pellets (Fig. 1a). Gonadal dieldrin doses, however, were not related to the amount of chemical purported to be in the pellets; that is, the low-dieldrin treatment group received a higher mean gonadal dose than the high-dieldrin treatment group (Fig. 1b). The high variability and lack of consistent correlation between applied and received doses suggests that the pellets were defective in either their content or their release kinetics. Indeed, it became clear at necropsy that the pellets had dissolved to widely varying degrees. Furthermore, a few fish had increased levels of both DDE and dieldrin in their tissues. Taken together, these findings indicate that the pellets were faulty with respect to both content and release kinetics.

Regardless of intragroup variation, individual whole-blood OCP concentrations varied in direct proportion to most tissue concentrations regardless of sex (Table 1 and Fig. 2). Tissues with higher lipid concentrations (gonad and liver) had greater concentrations of either chemical than the blood because of

the lipophilic nature of OCPs; however, the tissue-to-blood relationships were significant. The significant and linear correlation between blood and target tissue concentrations allowed a blood sample from each individual fish to be tested using an ELISA detection kit in order to obtain a surrogate measure of the absorbed dose.

The ELISA detection kits for DDE and dieldrin were validated by comparing ELISA to GC-MS results for several pools of blood plasma from control and treated fish. Correlation of GC-MS and ELISA results are presented in Figure 3, demonstrating the limited ability of the ELISA kit to detect concentrations below 100 ng/ml. All control fish and some treated fish registered OCP concentrations below 100 ng/ml, indicating they were at, near, or below the limit of detection of the ELISA kits. Nonetheless, the correlation between GC-MS and ELISA methods was highly significant and linear for both DDE and dieldrin. Therefore, the ELISA method proved useful for analyzing blood samples from every fish in the study and permitted a relative determination of each fish's absorbed dose of DDE or dieldrin. However, because of the small sample size studied here, further experimentation should be conducted to refine the relationship between the ELISA and GC-MS methods before blood OCP concentrations determined by ELISA could be used to calculate target tissue doses. We employed the ELISA method here only to obtain a surrogate measurement of the absorbed dose.

In summary, this study demonstrates that time-release pellets may be problematic as a dosing method for fish. The method was originally designed for implantation in endothermic animals (mostly laboratory rodents). In exothermic animals such as fish, changes in water temperature—and thus body temperature and associated metabolic rates—were likely an important source of differences in the absorbed dose. Additionally, the hydrophobicity of the OCPs may have in part hindered their release from the pellet.

As demonstrated here, exposure consistency represents a common source of error in ecotoxicological research. Indeed, many studies simply administer an applied dose but fail to perform the necessary analyses to determine how much of the applied dose was absorbed, if the applied dose reached the target tissue, or the extent of variability in the absorbed dose within treatment groups. Clearly, the data presented here demonstrate that slow-release pellets can produce wide variability in the absorbed dose of OCPs in fish. By testing the blood plasma using an ELISA detection kit for either OCP, surrogate dose levels were accurately determined for individual fish, and this data could be employed for analyzing dose response data.

Although this study demonstrated the utility of the ELISA assay for quantifying OCPs in fish plasma, it should be noted that the antibodies contained in the ELISA kits have relatively high cross-reactivity with similar compounds. Their use may be limited to laboratory settings where background contaminant levels can be controlled. Here, GC-MS analysis of control fish indicated that the background levels were low or not detectable, which allowed analysis of plasma OCP levels produced solely from the experimental exposure. Additional laboratory utility of this method arises, as collection of blood plasma is nonlethal, allowing researchers to monitor dose levels achieved over the duration of an exposure. However, kits may need to be developed for use in various species, as plasma matrix interference may differ across species.

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