Integration of Embryonic Zebrafish and Passive Sampling Device Extracts to Explore Effects of Pesticide Mixtures

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Abstract

Pesticides are ubiquitous, with more than one billion tons of pesticide products used in the United States annually. These compounds are characterized by their toxic effects to the target organism; however, pesticides are also well known for their deleterious effects to non-target species. Because pesticides have ecological and human health effects, it is important to investigate their prevalence in the environment, as well as their bioavailability and toxicity. Passive sampling devices (PSD) are commonly used to evaluate contaminants, including pesticides, found in water, air and soil. The PSD used for this research is an aquatic sampler that sequesters freely dissolved non-polar and semi-polar contaminants by diffusion into and adsorption to a lipid-free polyethylene membrane tubing (LFT). Thus, PSD can act reasonably as a biological surrogate, mimicking non-dietary bioavailability. Additionally, PSD extracts are proposed to be amenable to investigate toxicity of biologically available environmental mixtures utilizing the embryonic zebrafish (*Danio rerio*) model. To investigate this potential application, experiments were performed to evaluate the toxicity of non-deployed blank PSD extracts and non-deployed extracts spiked with individual pesticides or pesticide mixtures. For this proof of concept study, embryonic zebrafish were static waterborne exposed before the initiation of organogenesis. Two time points were monitored for mortality and alterations in development. The blank extracts did not result in any adverse developmental effects, relative to non-exposed controls. Embryos exposed to a comprehensive fourteen compound pesticide mixture extract produced an increase in adverse developmental responses as concentration increased. Experiments investigating the toxicity of individual compounds and partial pesticide mixtures were also performed. These preliminary studies indicate that the coupling of PSD extracts and *in vivo* toxicological assessments is realistic.

Introduction

Currently, there is great interest in finding a way to assess whether environmental concentrations of chemical compounds found in our rivers, lakes, and streams are at levels of ecological and human health concern. The purpose of this preliminary study was to test a method for investigating bioavailable environmental contaminants and evaluating toxicity that integrates two tools: passive sampling devices and the embryonic zebrafish model.

Passive sampling device (PSD) is a tool to evaluate contamination of water, air, and soil [1-3]. Aquatic PSDs are deployed beneath surface water and sequester only freely dissolved compounds (Fig. 1). The aquatic PSD developed by Anderson and colleagues employs lipid-free polyethylene membrane tubing (LFT) that sequesters and concentrates biologically available non-polar and semi-polar contaminants [2]. As seen in Figure 2, the tubing has a cavity size of approximately 10 Å [4], which is similar to the average pore size for a cell membrane of 9.8 Å [5]. Aquatic PSDs can be extracted and then analyzed using various gas chromatography techniques (Fig 3). The aquatic sampler is a valuable tool for assessing environmental concentration profiles for specific aquatic sites over a given time interval [2, 6]. These parameters permit the aquatic PSD to evaluate concentrations as water concentrations over time instead of at only a single sampling event. By incorporating extract concentrations and calculating temperature-dependent diffusion rates, the investigator can assess what concentrations of compounds are available to biota in the environment [7]. Understanding availability yields insights into hazard identification and exposure scenarios, two key components of risk assessment. Additionally, PSD extracts can be used to explore concentration

response relationships (Fig 3) and develop hazard characterization profiles for ecological or human risk assessments. The toxicity of deployed passive sampler extracts has been investigated, but methods and techniques are not yet robust enough for coupling the extracts with toxicity tests [6]. Many authors have suggested that further developing and improving environmental sampling techniques that integrate methods for toxicity would be advantageous for investigating risk [6, 8-12].

Toxicity is a result of harmful interaction between a chemical and the organism. Biological assays are frequently employed to investigate single compounds of environmental or human health interest. Zebrafish, are appealing for toxicity testing because they have high fecundity; one pair of adult spawning zebrafish can produce around 300 embryos per spawn approximately every week. The transparency of the embryo during development permits straightforward and consistent observation, allowing the observer to distinguish physical abnormalities during development [13]. The embryos develop rapidly, permitting assays to be high-throughput (Fig 4). Early development of vertebrate species is remarkably conserved amongst species [14]. Due to the shared physical, cellular, and anatomical characteristics among other vertebrates (Fig 5), the embryonic zebrafish model has advantages over other expensive and time consuming vertebrate whole body assessments, eg. mice, rat studies. This model has been used to investigate toxicity of legacy and emerging chemicals of concern, such as nanoparticles [14, 15], polyaromatic hydrocarbons (PAH) [16, 17], estrogen compounds including estradiol and ethylestradiol [18], metals [19] and a range of pesticides [20-23].

Many scientists recognize that there is a need for biological assessments to be efficient and sensitive to model risk [24]. Fifty percent lethal concentration (LC50) and lethal dose (LD50) bioassays are often employed to evaluate toxicity and extrapolate risk. but, it is difficult

to infer and/or represent environmental risk by evaluating the lethality of chemical concentrations that are above existing environmental concentrations [25]. There are efforts to improve risk assessments by improving and re-designing bioassays to evaluate more sensitive endpoints [26, 27]. Toxicity assays are valuable tools to examine sub-lethal effects of chemical compounds [28, 29]. Sub-lethal analysis using the embryonic zebrafish model has already been identified as advantageous for high-throughput screening of compounds that may be developmentally toxic [27, 30-32]. The embryonic zebrafish model is adaptable because it uses *in vivo* vertebrate assessments and additionally, the sequencing of the zebrafish genome yields further model applications [13, 30, 32-34].

An enhanced understanding of contaminant toxicity is desirable for all environmentally relevant compounds. One group of contaminants under constant surveillance is pesticides. Pesticides are known to be ubiquitous, with more than one billion tons of pesticide products used in the United States annually [35]. These compounds are characterized by their toxic effects to target organisms; however, pesticides are also well known for their deleterious effects to non-target species, including humans. Because pesticides have ecological and human health effects, it is important to investigate their prevalence in the environment, as well as their bioavailability and toxicity. The overall objective of this proof of concept study was to demonstrate that the plausibility of integrating the passive sampling device and the embryonic zebrafish model utilizing pesticides. However, it was outside the scope of the study to evaluate toxicity of pesticides based on environmentally relevant concentrations and mixes.

A total of fourteen compounds were utilized for this study. Insecticides classified as organochlorines, organophosphates, as well herbicides and pyrethroid complexes were among

the pesticides selected for investigation. Chemical structures, in addition to molecular weight, mode of action, chemical class and other relevant attributes are consolidated in Table 1.

Unlike most naturally occurring organic compounds, organochlorines (OC) are environmentally persistent as a result of chlorine substituent present in their molecular structures. In general, OCs are non-polar, resulting in high lipid solubility; this accounts their ability to bioaccumulate, bioconcentrate, and biomagnify. While many OCs can no longer legally be applied for agricultural pest prevention, due to detrimental ecological effects, they are still manufactured and used for certain vectors where the rate of diseases, continues to increase [25]. For these reasons, it is still important to investigate their prevalence in the environment and their bioavailability and toxicity to humans and other biota. Legacy OCs, like DDT, dieldrin, and to some degree lindane, are characterized by their tendency to bioaccumulate, and have well known ecological impacts for top predator species because they biomagnify [36]. Literature does support that dieldrin, DDT and lindane are developmentally toxic to zebrafish; however, not at environmental concentrations [37-39].

As OCs were phased out for ecological and political reasons, use of organophosphate pesticides (OP) increased primarily to meet the demands of the agricultural market. These pesticides are efficient and degrade quickly in the environment via hydrolysis of the ester bond. While these compounds are advantageous over other pesticides in some cases, OPs are characterized by their acute toxicity to both target and non-target species. The primary mode of action is inhibition of acetylcholine esterase activity, which alters neuronal function through the accumulation of acetylcholine. OP toxicity potential arises from acute exposure. Chlorpyrifos, fenitrothion and dimethoate were selected for investigation.

The widespread use of cypermethrin, fipronil, and propanil pesticides rationalizes the employment of the compounds for this study. Fipronil is a modern insecticide used to eradicate fleas and ticks and toxicity has been assessed using embryonic zebrafish [40, 41].

Cypermethrin is general use pyrethroid insecticide that has been detected in some streams at levels that pose are potentially harmful to aquatic invertebrates that are known to be sensitive to this compound [42]. Propanil, the final pesticide, is dissimilar from the other pesticides because the mode of action for this compound utilizes a photosynthetic pathway. Modest research was conducted to examine the compound's toxicity to fish. A recent publication established a lethal concentration for this herbicide thirty times greater than the concentration used for this study [43].

Because no single compounds are found in the environment, it is desirable to study how mixes of compounds are interacting. Understanding what methods are amendable to studying mixtures is important. Many authors have pointed out that there is a need for well-designed experiments to address mixture toxicity, because environmental systems contain a dynamic mixture of compounds. [23, 44-46]

This preliminary research puts forth a method to demonstrate how the two tools, one analytical, one biological, can be used together to investigate chemical mixtures bioanalytically. It is important to note that the concentrations used for the bioassays do not reflect environmental concentrations. Experiments were designed to investigate sub-lethal effects and exploit pesticide interactions and activities. To begin this study, experiments were performed to evaluate the potential toxicity of non-deployed blank PSD extracts and extracts spiked with individual pesticides or pesticide mixtures. The research tested five hypotheses: 1. Blank non-deployed PSD extracts will not induce adverse developmental responses in embryonic zebrafish. 2. PSD

extracts over-spiked with a comprehensive fourteen compound pesticide mixture will induce concentration-dependent increase of abnormal developmental responses in zebrafish embryos. 3. Mixture subsets of pesticide spiked PSD extracts will induce abnormal morphology in zebrafish embryos. 4. Individual pesticides spiked into PSD extracts will induce different sub-lethal effects in embryonic zebrafish. 5. Partial mixtures and individual pesticides exposures do not induce the same EZM scores as pesticide mixtures at similar molar concentrations. For this proof of concept study, embryonic zebrafish were static- waterborne, exposed pre-organogenesis, and monitored for mortality and alterations in development. During organogenesis different tissue types begin to develop in the embryo thus, it marks a sensitive time during development to introduce chemical insult. Exposure to the non-spiked extracts did not result in adverse developmental responses relative to non-exposed controls. Embryos exposed to a comprehensive fourteen compound pesticide mixture extract revealed a concentration dependent increase in multiple chemical-dependent adverse responses. Individual compounds, partial mixtures and a comprehensive mixture illustrated that various responses activities and interactions occurred between compounds. The primary biological responses observed were pericardial edema, yolk sac edema, and curved body axis, among others. These preliminary studies validate that the coupling the PSD extracts with *in vivo* toxicological assessment models is realistic.

Materials and Methods

Pesticides

A comprehensive fourteen compound pesticide mixture containing 100 µg/mL of each of the following pesticides: chlorpyrifos, cypermethrin, p,p-DDT, p,p-DDE, p,p-DDD, dieldrin, dicofol, dimethoate, α-endosulfan, β-endosulfan, fenirtothion, fipronil, and propanil, was obtained from Chem Service (West Chester, PA). Individual pesticides, including cypermethrin

(100 µg/mL),, p,p-DDT (100 µg/mL), dicofol (100 µg/mL), dimethoate (100 µg/mL),, α-endosulfan (100 µg/mL), β-endosulfan (100 µg/mL), fenirtothion (1000 and 100 µg/mL), fipronil (100 µg/mL), lindane (200 µg/mL) and propanil (100 µg/mL), were ordered from Chem Service or Accustandard (New Haven, CT) at 98 % (w/v) purity or greater. Dieldrin and chlorpyrifos stock solutions were made from neat in the laboratory to 1000 µg/mL in hexanes.

Two partial mixtures, an organochlorine (OC) mixture and an organophosphate (OP) mixture, were produced by mixing individual pesticides and spiking pesticide mixture into a PSD extract. The OC mixture was composed of 100 µg/mL p,p-DDT, 100 µg/mL dicofol, 100 µg/mL dieldrin, 100 µg/mL α-endosulfan, 100 µg/mL β-endosulfan and 100 µg/mL of lindane. The OP mixture was composed of 100 µg/mL chlorpyrifos, 100 µg/mL dimethoate, and 100 µg/mL fenitrothion. Individual compounds tested were cypermethrin, dieldrin, fenitrothion, fipronil, and propanil at concentrations between 0.9 and 8 µg/mL.

Construction and Preparation of Passive Sampling Device Tubing

Barefoot© low-density polyethylene lay-flat tubing (LFT) of approximately 75-95 nm thick by 2.54 cm wide was obtained from Brentwood Plastic, Inc. St. Louis, MO. Tubing was cleaned using a pre-extraction step with Optima grade hexanes from Fisher Scientific (Fairlawn, NJ). For cleaning, a roll of LFT was produced approximately 9 cm in diameter. The roll was placed on steel wire platform that was fitted to the bottom of a 1500 mL clear glass jar and covered in hexanes. The jar was placed on an orbital shaker for 48 h and hexanes exchanged twice. After cleaning, the tubing was dried at 20°C until solvent free. Pre-cleaned tubing was then stored at -20°C. For sample preparation, pre-cleaned tubing was looped at both ends and heat sealed following Standard Operating Procedures (SOP) for passive sampling device (PSD)

previously developed by members of the Anderson laboratory. Final measurement of sample tubing was 100 cm.

Passive Sampling Device Extracts

To prepare for extraction and mimic laboratory field sample dialysis, five constructed tubes were placed into 500 mL wide-mouth amber jars and stored at -20 °C. The tubes were removed from the jar and multiple cuts were made. First, each loop of the tubing was cut. Second, the tubing was cut in half and cut lengthwise along one seam. For the first dialysis, the cut tubes were rerolled together and placed at the bottom amber jar and then submerged in at least 200mL of hexanes for 4 hours. The hexanes were decanted into a 1000 mL round bottom flask (RBF) using a glass funnel. The funnel and the top of the RBF were rinsed with aliquots of hexanes using a glass Pasteur pipette. The RBF was temporarily sealed with aluminum foil and stored in the freezer. The funnel was then rinsed clean with hexanes and acetone. The tubing was again submerged in at least 200 mL of hexanes in the same amber jar for 2 hours.

After 2 hours, the hexanes were decanted to the RBF containing the first dialysate. The 400-500 mL dialysate extracts were concentrated on a Brinkmann rotovapor RE 120 (Brinkmann Instruments, Inc. Westbury, NY). The temperature of the rotovap water bath was set between 28 to 30° C. The extracts were concentrated to a volume approximately the size of a quarter. After concentration, the extract was quantitatively transferred (using 3 to 4 hexanes aliquots) to a centrifuge tube marked at increments between 0.5 and 2 mL. If the extract volume was greater than 2 mL, the extract was concentrated to the designated volume using a Zymark TurboVap LV evaporator (Gentech Arcade, NY).

Following the evaporation, the extract was vortexed, and brought up to the two mL volume with hexanes. The extract was then spiked with pesticide or left as a non-spiked extract.

Blank (blk) extracts were employed to determine if the LFT was developmentally toxic to zebrafish embryos. For quality control purposes, blk non-deployed LFT extracts selected from three different pre-cleaning and extraction sample production were used to for blk LFT exposures. For one of the extracts, a replicate was produced. For some samples, a portion of extract in hexanes was transferred to a 2 mL amber sample container and archived for future analysis by gas chromatography electron capture device (GC/ECD). The remaining extract was solvent exchanged in one of two ways. For one procedure the sample was blown down to dryness using evaporation techniques previously described. Once dry, the appropriate amount of dimethyl sulfoxide (DMSO) manufactured by J.T. Baker (Phillipsburg, NJ) was added. The sample was then vortexed and transferred to a 2 mL amber vial. The second procedure employed took the DMSO solvent, added it to the sample, votexed it, and evaporated off the hexanes. After solvent exchange was complete, the sample was transferred into a clean amber vial. Extract dilutions were prepared in 100% DMSO. All extracts were stored at - 20° C until the time of exposure.

Embryonic Zebrafish Exposures

Zebrafish (*Danio rerio*) embryos were cared for in compliance with the Institutional Animal Care and Use Committee guidelines and according to the Sinnhuber Aquatic Research Laboratory (SARL) criteria at Oregon State University (OSU). The embryos were spawned from the tropical 5D zebrafish strain. The fish water (FW) was filtered by reverse osmosis (RO). Instant Ocean (Aquarium Systems, Mentor, OH) was added to alter salinity. Ideal conductivity was optimum at 500µS. Adult spawning zebrafish were kept in 2.0 L polycarbonate tanks on a continuous water renewal system. The temperature of the tank water was maintained at 28°C with a pH of approximately 7.2. Embryos were collected from the spawning tanks at 0 to 4 hours

post fertilization (hpf), After collection, the embryos were cleaned using fish water rinses, screened for viability, and staged according to Kimmel et al. [29]. Screening involved estimating and recording the quantity and quality of the embryos and the removal of non-viable embryos and other debris. Viable embryos were placed in a 150 by 25 mm plastic petri dish and incubated at 28°C. Zebrafish embryos are enveloped in a chorion. The chorion is an acellular barrier that surrounds the embryos. To maximize contaminate uptake the the embryos were enzymatically dechorionated [14] with pronase (Fluka, a subsidiary company of Sigma Aldrich, St. Louis, MO). Two hundred to eight hundred embryos were placed in a glass petri dish filled with 25mL of fish water and 0.05 mL of 50 µg/mL pronase solution. The embryos were agitated gently for duration no longer than ten minutes and then rinsed thoroughly multiple times with FW. Following dechorionation the embryos were stored in an incubator at 28°C until the exposure process began. Exposure dilutions were prepared just prior to exposure to meet the needs of the experiment. To make the exposure solution, spiked PSD extracts, blank PSD extracts or pesticides treatment in DMSO was added to FW to obtain a final extract concentration of 1% (v/v) in FW. 100 µL of exposure solution was added to each well of a glass coated 96-well plate. One staged, viable, dechorionated embryo was transferred to each well containing solution. For all compounds except dieldrin and fenitrothion, 24 embryos (n) were used with one embryo per well at each concentration. All exposures began at six hpf. The exposures were timed to begin pre-organogenesis, because early embryonic development, gastrulation, and organogenesis are conserved across vertebrate species during this time. For the duration of the experiment, plated embryos were incubated at 28°C without light in exposure solution.

Toxicity evaluations measured deviations from normal development and atypical morphology, such as: curved body axis, pericardial edema, yolk sac edema, trunk, and tail

development, notochord defects, and mortality. Biological assessments occurred at 32 and 126 hpf and monitored for the presence or absence of developmental effects. An EZ (embryonic zebrafish) metric developed by the Tanguay laboratory was modified and applied to each pesticide concentration to assess overall toxicity [47].

All exposures were terminated at 126 hpf of continuous still water exposure using a low concentration of tricaine methanesulfonate (TMS) as anesthetic, followed by a high concentration of TMS appropriate for euthanasia. The euthanized larvae bodies were rinsed out of the well plate and disposed of in the biohazard waste. For some compounds and concentrations, embryos were digitally photographed using a Nikon Coolpix 5000 camera.

Range finding exposures were used to obtain an appropriate range for the exposure dilutions. Exposures dilutions were optimized to investigate sub-lethal abnormal morphology but were limited to a highest concentration that was a hundred fold diluted from the stock pesticide solution obtained. Control exposures were observed in parallel. All controls consisted of pure fish water only. A vehicle control DMSO was not performed, because previous 1% DMSO (v/v) exposures performed by the Anderson lab (data to be published) validated use of DMSO as an appropriate vehicle at this concentration when compared to fish water controls. Additionally, the literature establishes that DMSO at 1% or less is non-developmentally toxic to zebrafish embryos [14, 15, 48].

A total of 15 replicates (n=15), each replicate representing 24 zebrafish embryos, of blk non-deployed PSD extracts were evaluated for morphological effects to verify if PSD exposure resulted in toxicity. To exhaust plausibility of PSD toxicity the comprehensive mixture without PSD background was also tested.

For all concentrations of pesticide mixtures and individual compounds, 24 embryos were exposed per repetition, except for the compounds fenitrothion and dieldrin. For all compounds except fenitrothion and dieldrin, three repetitions (n =3) were performed. For fish water control 17 replicate assessments were performed (n=17). Two repetitions were performed for fenitrothion and dieldrin (n=2) with 16 embryos per repetition. Concentration of pesticides exposed ranged from (2.4 to 43 µM).

Integration of Methods

A clear synthesis of the methods employed for this study is depicted in Figure 6.

Percent Incidence and EZ Metric

Percent incidence was determined at126 hpf for multiple effects. Embryos were assessed for an array of abnormal morphological effects. Differences among treatments were tested, as well as differences between treatment and control.

To evaluate the overall effect of the treatment on the embryonic zebrafish a scoring system was developed. The method used was adapted from embryonic zebrafish (EZ) metrics values developed by Harper, S.L., et al. using EZ Metric values for nano fullerenes, was used [47, 49]. The scoring evaluates a group response to any given treatment for a given lethal or sub-lethal endpoint. The adapted EZ metric took the number of animals that exhibited an effect out of a group of 24 embryos and multiplied it by the non-biologically based ranking given for that effect and assigned it a group score or EZM Score. For example, the sum of 12 effects for a group of 24 embryos at 126 hpf would score 21.6 on EZM scale (Table 2). The ranking of all sub-lethal effects is the same for the non-biological scale because, while some effects may limit the life span of an animal, others might hinder that animal's ability to survive. Severity and variety of effects yield different EZM scores where a group score between 0 and 4 would

indicate a low effect level. An EZM score between 5 and 8 would indicate a moderate effect and a score between 9 and 24 would indicate a serious outcome for the group.

Statistics

A Mann-Whitney Rank Sum Test was performed to determine differences in EZM score and incidence between control and treated groups where significance was determined when $p<0.05$. The Fisher Exact statistical analysis was used to compare blk PSD extract treatments to FW controls; significance was assigned to values p values less than 0.05. To determine differences between treatments, exposures were paired and the Mann-Whitney Sum Test was performed with the previously mentioned parameters. Statistics were completed using Sigmaplot version 8 (Systat Software Inc., San Jose, CA).

Results

Zebrafish were assessed for a total of eighteen endpoints. Sixteen of the eighteen were sub-lethal endpoints and focused on abnormal morphology. The maximum number of endpoints observed for a live embryo in this study was nine. Due to the poor quality of life of an embryo exhibiting this number of endpoints, it is reasonable to assume that the survival into the larval stage is limited, as the embryo often is immobilized by the deformations that it has sustained and that it is nearly impossible for an embryo to exhibit all sixteen effects simultaneously. The embryonic zebrafish metric (EZM) score was calculated employing similar parameters to designate ranking. EZM scoring is helpful for making quick assumptions about the toxicity of a treatment; however, it does not reflect in the score what is driving the high score EZM scores were generated for all treatments and controls to generate an integrative assessment or burden of toxicity to the embryo. If mortality was high for a group of embryos EZM Score was also high. Thus, if numerous sub-lethal effects are observed in live embryos EZM Score will also be high.

Analysis of endpoint incidence revealed what was or was not contributing to EZM scores that were significantly different from FW controls.

Zebrafish embryos exposed to blank PSD extracts showed did not have different EZM scores relative to FW controls ($p = 0.832$). No significant increase was seen in incidence of mortality, heart edema, yolk sac defect, curved axis above fish water control for,($p = 1.00, 1.00, 0.235, 0.371$).

Control (FW) EZM scores when compared to exposures of Pest mix PSD concentrations: 6.4, 7.2, 13, 19, 26, 39 µM were significantly different ($p= 0.047$, and <0.001 for the remaining five concentrations). EZM score was not significantly different for the 39 µM Pest mix PSD treatment and 39 µM or 43 µM Pest mix ($p= 0.277, 0.136$). Percent incidence was assessed for nine sub-lethal effects at four concentrations, 4.3, 7.7, 19, 39 µM. Heart edema (HE), snout deformation (Snt), curved axis (Ax), eye deformation (E) and mortality were all observed at a significantly higher frequency at 126 hpf than control for embryos exposed to the 19 and 39 µM Pest mix (HE, $p= <0.001, <0.001$; Snt, $p= <0.001, <0.001$; Ax, $p= <0.001, <0.001$; E, $p= <0.001, 0.046$; M126, $p= <0.001, <0.001$). Yolk sac defect (YSD) frequency was different than control for 7.7, 19 and 39 µM exposures ($p= <0.001, <0.001, <0.001$). Incidence of YSD was not different than control for only the 4.3 exposure ($p= 0.153$). Exposure to the two lower concentrations of Pest mix exhibited no significant difference than control for HE, Snt, Ax, E or M126 (HE, $p= 0.894, 0.201$; Snt, $p= 0.974, 0.712$; M126, $p= 0.692, 0.140$;) Incidence of mortality (30 hpf), tail deformation (T), and body proportion (stubby; St) were not significantly different than FW controls at the four concentrations (M30, $p= 0.868, 0.862, 0.841, 0.893$; T, $p= 0.873, 0.568, 0.162, 0.819$; St, $p= 0.974, 0.867, 0.656, 0.628$).

Exposure to OC mix produced an EZM score that was different than control (p= <0.001). The OC mix induced three endpoints, of nine evaluated, were different from FW at 126 hpf: mortality, yolk sac defect and heart edema (p= <0.001, <0.001, 0.003). EZM scores for OC mix and dieldrin at 21 µM were significantly different (p= <0.001); EZM score was higher for OC mix. Of the concentrations tested, increasing concentration of dieldrin spiked PSD did induce an increase in EZM score. EZM scores for dieldrin at both 13 and 21 µM were significantly different than FW controls (p= 0.004, 0.002) while the two lower concentrations were not significantly different from FW controls (p= 0.571, 0.259). When comparing incidence of effects for the two highest dieldrin exposures, YSD was the only endpoint different than control (p= 0.020, 0.031), indicating that YSD is responsible for the EZM score for the two highest dieldrin concentrations.

There was a significant difference was found between OP Mix and FW EZM score (p= 0.001). The OP mix induced only one endpoint, yolk sac defect, that was different from control at 126 hpf (p = 0.007). Increasing concentrations of fenitrothion spiked PSD did not induce an increase in EZM scores. EZM scores for fenitrothion at the lowest and highest concentrations were not significantly different than FW controls (p= 0.914, 0.145). Exposures to all fenitrothion concentrations tested induced no difference in HE, Snt, Ax, E, YSD, T, St, M30 or M126.

Cypermethrin and fipronil exposures produced EZM scores that were different than control (p= <0.001, <0.001). Fipronil exposures yielded four different endpoints were different from control: St, Ax, YSD and HE (p = 0.001, <0.001, <0.001, 0.005). Incidence of HE and YSD were different from FW for cypermethrin assessments (p= 0.031, <0.001).

Multiple analyses were run comparing EZM scores of partial mixtures and individual pesticides to the comprehensive mixture. Exposures to propanil and OC mix do not produce a

different EZM scores when compared to their respective molar concentration of the comprehensive pesticide mixture (p= 0.203, 0.492). Exposures to cypermethrin and fipronil resulted in a different, higher, EZM scores than their respective pesticide mixture concentration (p= 0.010, <0.001). Exposures to fenitrothion and OP mix resulted in different, lower, EZM scoring than the pesticide mixture at the same molar concentration (p= <0.001, <0.001).

Discussion

This research investigated integrating the aquatic PSD and embryonic zebrafish to eventually study developmental toxicity of environmental mixtures. Coupling these methodologies to investigate the toxicity of environmentally relevant, bioavailable mixtures *in vivo* would exceed current methods that investigate individual compound toxicity or laboratory produced mixtures. This study demonstrates that utility of integrating the embryonic zebrafish model with passive sampling devices. For this research, environmental risk scenarios were not addressed nor were environmentally relevant concentrations; however, the potential exists.

Certain pesticides or chemical concentrations are not known to induce abnormal morphology. In this study we saw that OC pesticides, fipronil, and cypermethrin contributed to the toxicity of the mixture, while the OP pesticides and propanil were not likely to be contributing to the morphology of the zebrafish embryo. Thus, observational biological assessment model such as the one employed for this study should be used when the compound or concentration has been identified to induce a certain endpoint. Zebrafish have been be used to evaluate sensitive more endpoints like behavior to investigate toxicity of nominal chemical concentrations [41]. Studies using salmon have associated behavioral effects with neuronal inhibition caused by chemical insult [46]. The embryonic zebrafish model has the potential to be modified for an investigation looking at more sensitive endpoints.

The PSDs are advantageous tools for many reasons. First they have the ability to uptake only freely dissolved compounds in water and potentially aqueous sediments. Other studies have investigated developmental toxicity using contaminated sediment contact assays. While these test are valuable because they assess real exposure scenarios, bound particles or compounds typically filtered out of environmental extracts may conceal or contribute to toxicity in unfamiliar ways [50].

The ultimate goal of toxicity testing is to understand if risk exists for humans as well as ecosystems. The chorion was removed for the pesticide exposures to maximize contaminant uptake; however, when integrating the PSD and zebrafish model the goals of the study should determine whether or not to remove the chorion. It may be advantageous to run assessments with and without chorion in parallel to determine if differences exist for the compounds/mixture of interest.

Fipronil was the only compound to induce a stubby body endpoint incidence different than control. Curved body axis was only different in pesticide mixture and fipronil treatments. Incidence of heart edema was different than control for pesticide mixture, organochlorine mixture, cypermethrin and fipronil. Given that certain pesticides, induce both specific (stubby-body, curved body axis) and non-specific endpoints (yolk sac defect, heart edema) the embryonic zebrafish model does demonstrate the ability to expose what compounds may be inducing the effect and/or generally assess toxicity of a mixture.

Toxicity of dieldrin, DDT, fipronil among other pesticides has been assessed using embryonic zebrafish; however, differences in materials and methods used for the assessments were not consistent with the methods used for this study. Standardization of the model standardizes the outcome, but limits the potential of the model to develop and adapt. Comparing

differences in technique is valuable way to help identify the sensitivities in a method like timing of exposure or duration of exposure. This can reveal how to optimize the study to the objectives of the study.

Mixture behavior is confounding; very few studies have undertaken mixture toxicity because there are too many variables to account for when attempting to model an environmental exposure. This study was a modest attempt to generate and analyze laboratory generated mixtures; however, the research addresses why it might be important to consider mixtures even for laboratory experimentation. It would be valuable to follow up with studies aimed at investigating mixtures based on mode of action and individual compounds from those mixtures.

Coupling methodologies to investigate the toxicity of environmentally relevant, bioavailable mixtures *in vivo* would advance current methods that evaluate environmental risk, but it is important to ask how coupling would maximize both models to suit this purpose.

Conclusions

Zebrafish embryos exposed to blank PSD extracts showed no significant abnormal responses above fish water control. PSDs are amendable for use in embryonic zebrafish bioassays. EZM score was for control (FW) and all concentrations of Pest mix greater than 6.4 µM were significantly different. EZM score was not significantly different for the 40 µM Pest mixture with PSD when compared to the Pest mix at 40 µM and 43 µM. Therefore PSDs can be used in toxicity assessments with embryonic zebrafish. The organochlorine mixture did induce toxicity above control and above equivalent molar concentrations of dieldrin. The dieldrin is not as toxic as other compounds in the organochlorine mixture. Analysis of multiple endpoints showed that incidence of mortality, heart edema, and yolk sac defect were different for OC mix when compared to control. Increasing concentration of dieldrin spiked PSD did induce an

increase in EZM score in exposed zebrafish embryos relative to control. Analysis of multiple endpoints confirmed that the incidence of yolk sac defect at 126 hpf was higher than control for dieldrin at the two concentrations. OP mix EZM score was different than control EZM score; however, OP Mix exposures produced only an increased incidence of yolk sac defect. Increasing concentration of fenitrothion spiked PSD did not induce an increase in EZM score for exposed embryos; there was found to be no difference between control and highest and lowest concentrations. Analysis of multiple endpoints revealed no difference in % incidence among three fenitrothion treatments 4, 7, 18 μM confirming that the compound was not developmentally toxic to the embryonic zebrafish using the current biological assessment and exposure concentrations. Toxicity (EZM scores) of cypermethrin and fipronil exposures was different than control. EZM Score of propanil was not different when compared to control. Cypermethrin, fipronil, and propanil did not produce a difference in the incidence of mortality for 30 and 126 hpf, eye, tail, snout deformations when compared to control. Fipronil exposures had an increased incidence of heart edema, yolk sac defect, stubby body, curved axis when compared to control. Propanil exposures were not different than control for nine endpoints analyzed. Cypermethrin exposures had an increased incidence of heart edema and yolk sac defect when compared to control. Additionally, exposures to propanil and OC mix do not produce a different EZM scores when compared to their respective molar concentration of the comprehensive pesticide mixture; however, exposures to cypermethrin and fipronil resulted in a higher EZM scores relative to their respective pesticide mixture concentration. Exposures to fenitrothion and OP mix resulted in lower EZM scoring than the pesticide mixture at the same molar concentration.

We conclude that PSD extracts are amendable for developmental biological assays with embryonic zebrafish to explore mixture toxicity. This study indicates that the coupling of PSD

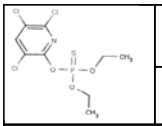
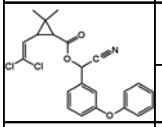
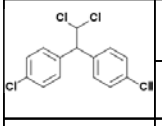
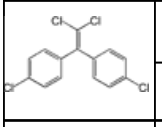
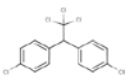
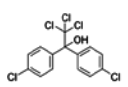
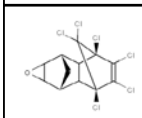
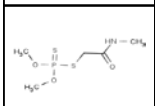
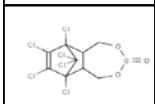
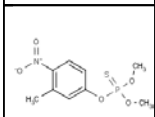
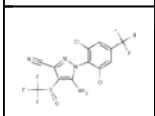
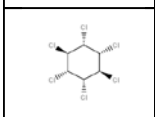
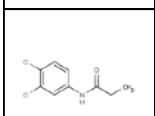
extracts and *in vivo* toxicological assessments is realistic. Mixture toxicity is complex. Mixes can produce synergistic, additive or antagonist effects. The integration of PSD and embryonic zebrafish is advantageous for evaluating mixture scenarios. Individual pesticides, partial mixtures and a comprehensive mixture demonstrated that various responses activities and interactions occurred between compounds. Currently a knowledge void exists for mixture toxicity; this proof of concept study proposes a method for attack.

Acknowledgements

This project was funded generously by BioResource Research mentors Dr. Kim Anderson and Dr. Robert Tanguay of the Environmental and Molecular Toxicology department at OSU. This study would not have been complete without valuable insights given by the both mentors and laboratory group members: Sarah Allan, Wendy Hillwalker, Jane La Du, Lucas Quarles and Lisa Truong. Data analysis was enhanced by employing the original Embryonic Zebrafish Metrics method developed by Stacy Harper.

Tables and Figures

Table 1. Pesticide properties such as physical characteristics and toxicity related information included in the following table. Mode of action identifies the mechanism that induces toxicity to the target organism (see source number 4). (next page)

	Name: Chlorpyrifos	Chemical Class: Organophosphate (Insecticide)	Molecular Weight: 350.59
	LC50: 0.01 (Bluegill sunfish)[1]		Mode of Action: Cholinesterase inhibitor
	Name: Cypermethrin	Chemical Class: Pyrethroid (Insecticide)	Molecular Weight: 416.30
	LC50: 0.0018 (Bluegill sunfish)[1]		Mode of Action: Na+ channels inhibits closing
	Name:DDD	Chemical Class: Organophclorine (Insecticide)	Molecular Weight: 320.04
	LC50: literature value for aquatic vertebrate not found		Mode of Action: Na+ channels inhibits closing
	Name:DDE	Chemical Class: Organophchlorine (Insecticide)	Molecular Weight: 318.02
	LC50: literature value for aquatic vertebrate not found		Mode of Action: Na+ channels inhibits closing
	Name: DDT	Chemical Class: Organophchlorine (Insecticide)	Molecular Weight: 354.49
	LC50: 33.7 (Zebrafish embryo)[2]		Mode of Action: Na+ channels inhibits closing
	Name: Dicofol	Chemical Class: Organochlorine (Insecticide)	Molecular Weight: 370.48566
	LC50: 0.51(Bluegill sunfish)[1]		Mode of Action: Site II e-transport inhibitor
	Name: Dieldrin	Chemical Class: Chlorinated Hydrocarbon (Insecticide)	Molecular Weight: 380.91
	LC50: 8.00 (Zebrafish embryo)[2]		Mode of Action: Antagonist GABA channels
	Name: Dimethoate	Chemical Class: Organophosphate (Insecticide)	Molecular Weight: 229.26
	LC50: 17.6 (Bluegill sunfish)[1]		Mode of Action: Cholinesterase inhibitor
	Name: Endosulfan	Chemical Class: Organochlorine (Insecticide)	Molecular Weight: 406.95
	LC50: 1.20 (Bluegill sunfish)[3]		Mode of Action: Antagonist GABA channels
	Name: Fenitrothion	Chemical Class: Organophosphate (Insecticide)	Molecular Weight: 277.23
	LC50: 2.50 (Bluegill sunfish)[1]		Mode of Action: Cholinesterase inhibitor
	Name: Fipronil	Chemical Class: Carbonitrile (Insecticide)	Molecular Weight: 437.15
	LC50: 0.0852 (Bluegill sunfish)[3]		Mode of Action: Antagonist GABA channels
	Name: Lindane	Chemical Class: Organochlorine (Insecticide)	Molecular Weight: 290.83
	LC50: 0.06 (Bluegill sunfish)[1]		Mode of Action: Antagonist GABA channels
	Name: Propanil	Chemical Class: (Herbicide)	Molecular Weight: 218.08
	LC50: 5.40 (Bluegill sunfish)[1]		Mode of Action: Photosynthetic e- inhibitor

1. Tomlin, CDS. *The Pesticide Manual* (2003)
2. Ton, C. et al. "Zebrafish as a Model for Developmental Neurotoxicity Testing" (2006)
3. Pesticide Information Profiles EXOTOXNET <http://extoxnet.orst.edu> (1996)
4. Stenersen, J. *Chemical Pesticides, Mode of Action and Toxicity* CRC (2004)

Table 2. The embryonic zebrafish metric (EZM) Score quantifies the toxic endpoints of a treatment for a group of 24 embryos. The toxic endpoints are defined as deviations from normal zebrafish development.

Endpoint	EZM Score
Mortality (M) 30 hpf	24
Notochord (Not)	1.8
Mortality 126 hpf	21.6
Notochord (Not)	1.8
Heart (HE)	1.8
Brain (B)	1.8
Yolk Sac (YSD)	1.8
Body (St)	1.8
Circulation (C)	1.8
Eye (E)	1.8
Jaw (J)	1.8
Tail (T)	1.8
Somites (S)	1.8
Caudal Fin (FC)	1.8
Pectoral Fin (FP)	1.8
Snout (Snt)	1.8
Body Axis (Ax)	1.8
Otic (Ot)	1.8

Figure 1. Schematic of aquatic PSD field deployment.

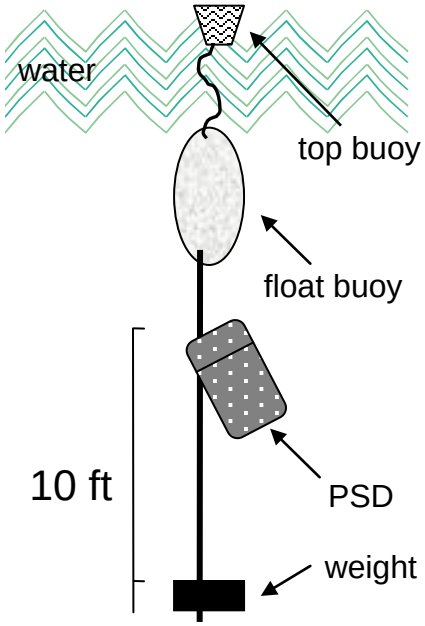


Figure 2. This Figure illustrates contaminant partitioning between organism or surrogate and the surrounding medium. The cell membrane has pore size has been recorded to be similar to the cavity size of PSD tubing [4, 5].

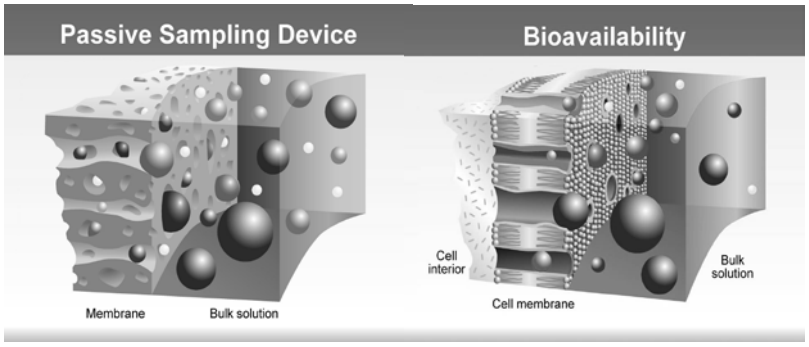


Figure 3. This diagram is an overview of how field deployed PSD extracts are collected and illustrates the applications of quantitative analysis as well as exploitation for biological assessments.

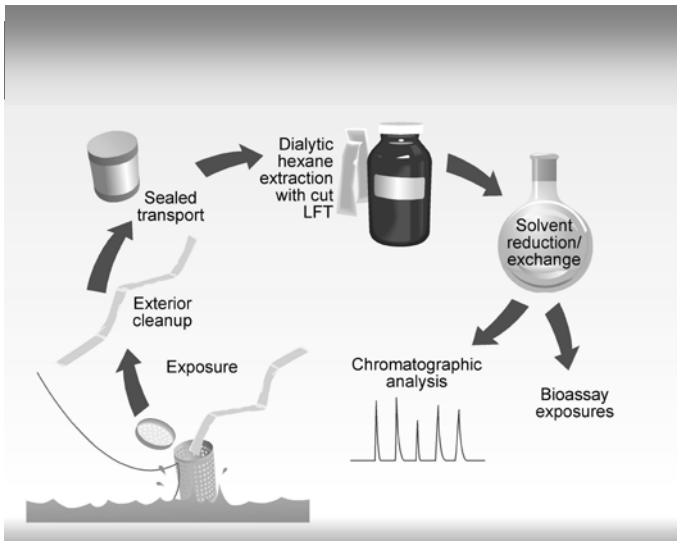


Figure 4. Rapid development of the zebrafish embryo makes biological assessments for developmental toxicity in desirable and is unique to the model. The drawing shows within 72 hours post fertilization (hpf) maturation is nearly complete.

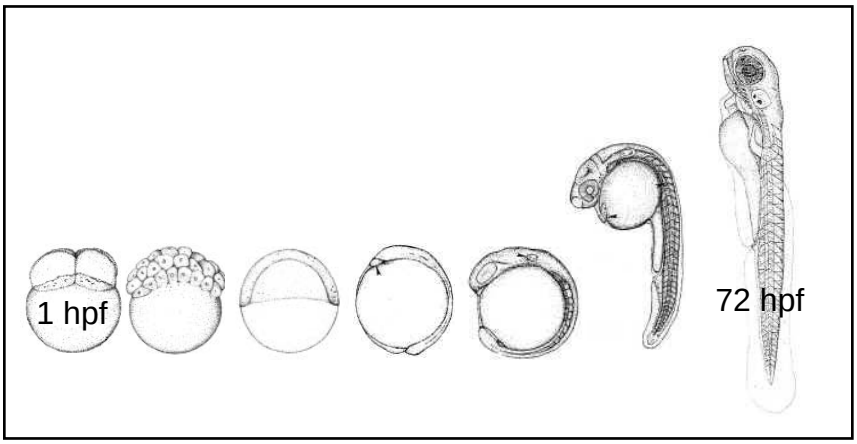


Figure 5. Early embryonic development (shown) is largely conserved among vertebrate species.

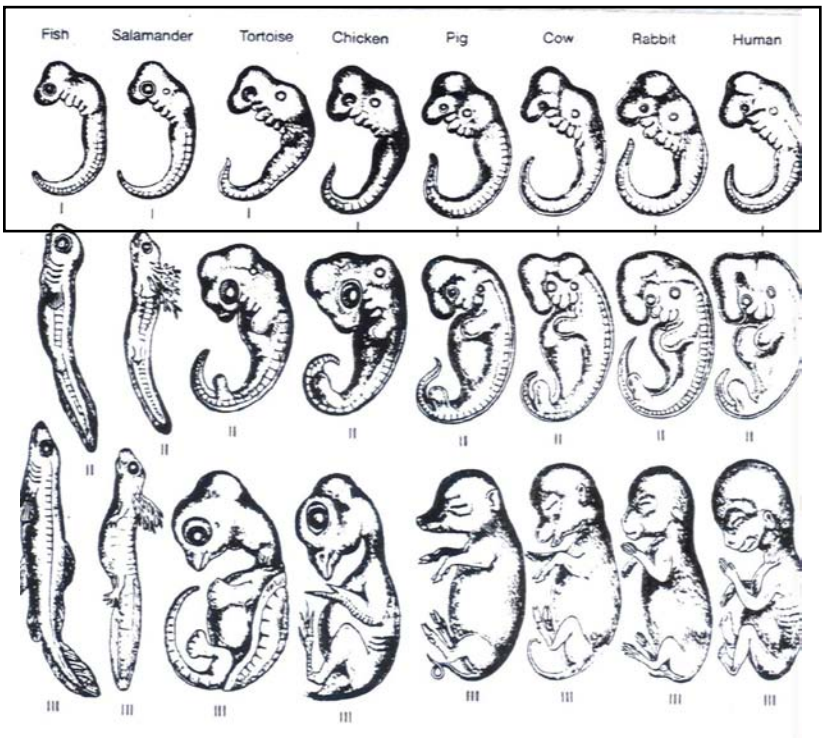


Figure 6. At 126 hours post fertilization (hpf) numerous sub-lethal endpoints were observed. Arrows direct attention to overt endpoints observed. The abbreviated labels Ax, E, HE, J, and YSD define each effect respectively, curved body axis, eye deformation, heart edema, jaw deformation, and yolk sac defect (where both discoloration and edema were observed.)

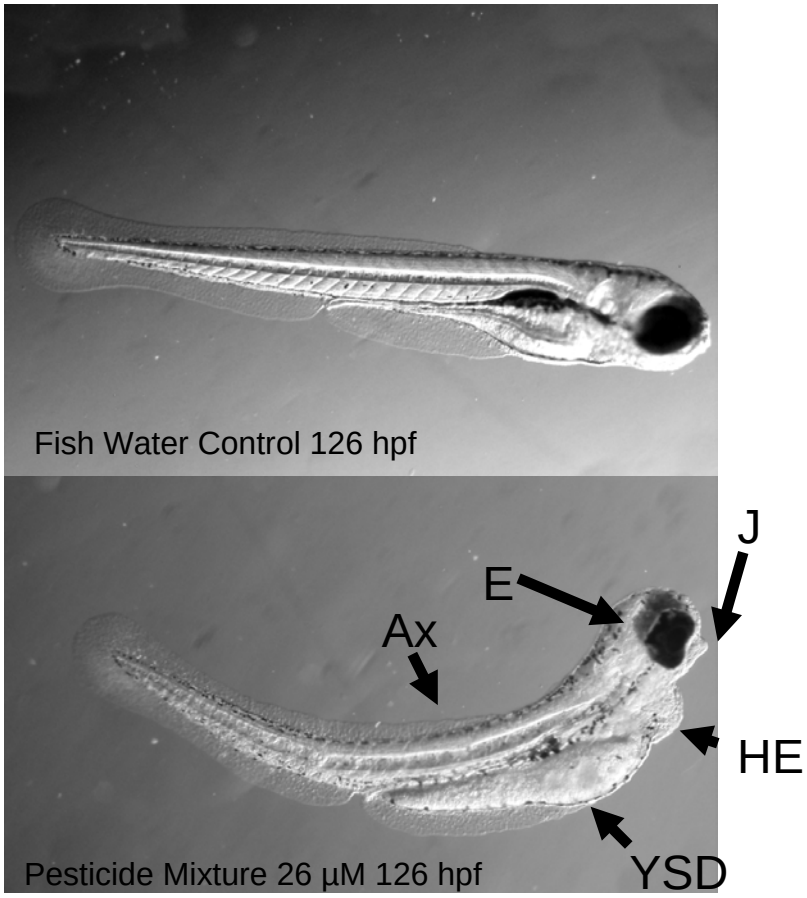
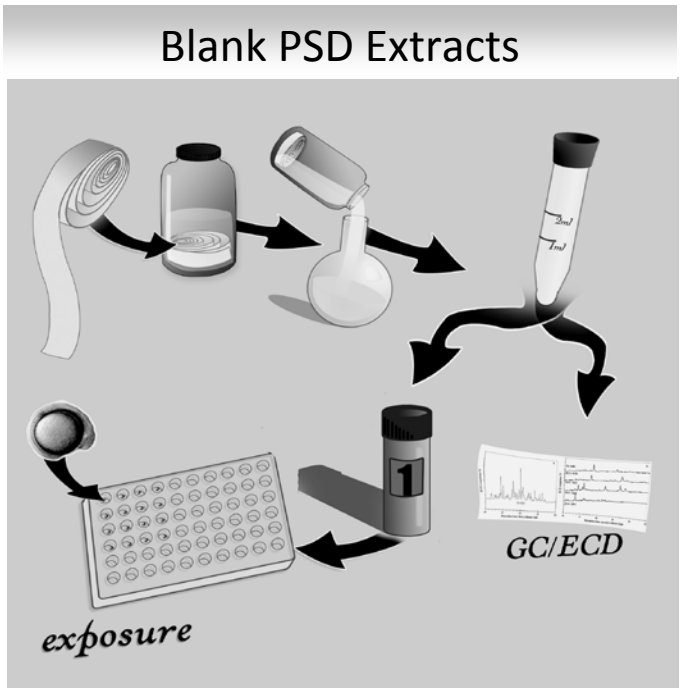


Figure 7. Overviews of the methods employed for this study are depicted in the following diagrams. While the diagram for spiked PSD extracts shows multiple dilutions produced, not all spiked extracts were evaluated at multiple concentrations.



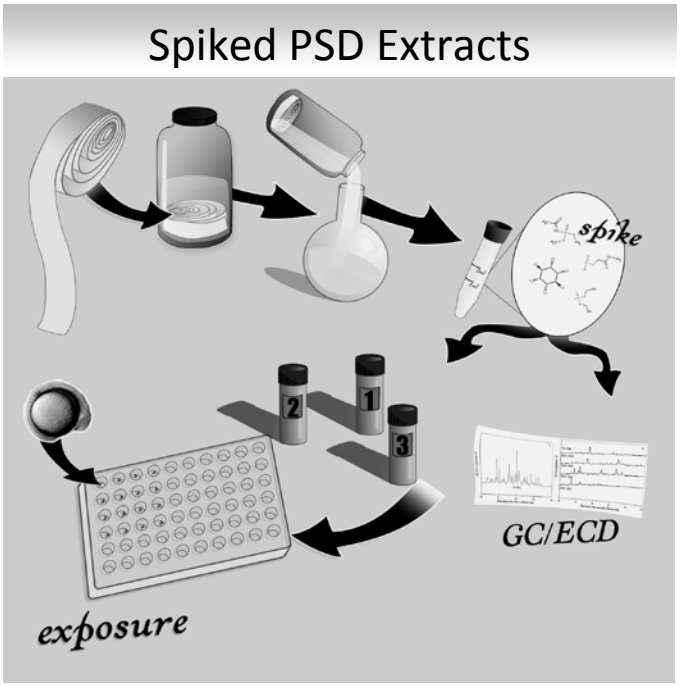


Figure 8 a. Embryonic zebrafish metric (EZM) scoring was used to compare control (FW) and 1% (v/v) blank PSD extracts treatments. b. Incidence of mortality, curved body axis, heart edema, and yolk sac defect were also assessed with no significant differences between FW and blank PSD extracts exposures.

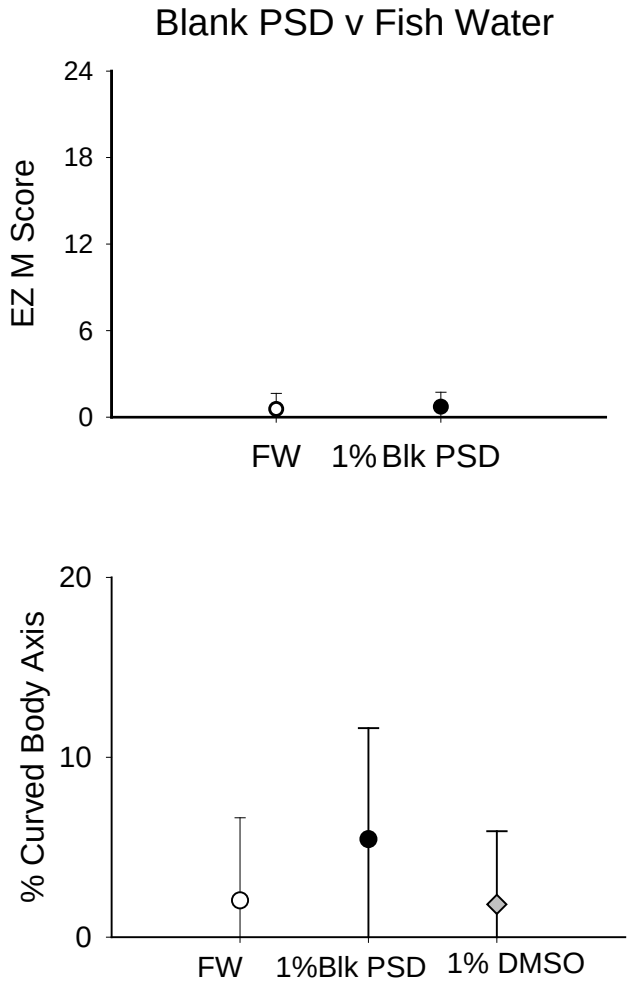


Figure 9. a. Zebrafish embryos were exposed to a comprehensive fourteen pesticide mixture. The pesticide mixture contained 100µg/mL of the following pesticides: chlorpyrifos, cypermethrin, p,p-DDT, p,p-DDE, p,p-DDD, dieldrin, dicofol, dimethoate, α-endosulfan, β-endosulfan, fenitrothion, fipronil, and propanil. EZM score was used to compare control to pesticide mixture treatments. b. To illustrate a similarity in toxicity, EZM score for the Pest mix with and with out PSD extract at the same concentration were graphed for overlapping concentrations. The small circle encloses the overlapping data points. The large circle encloses the same data points distinguished from one another. (*r*= significantly different compared to fish water control (FW) *t*= significantly different compared to the pesticide mixture)

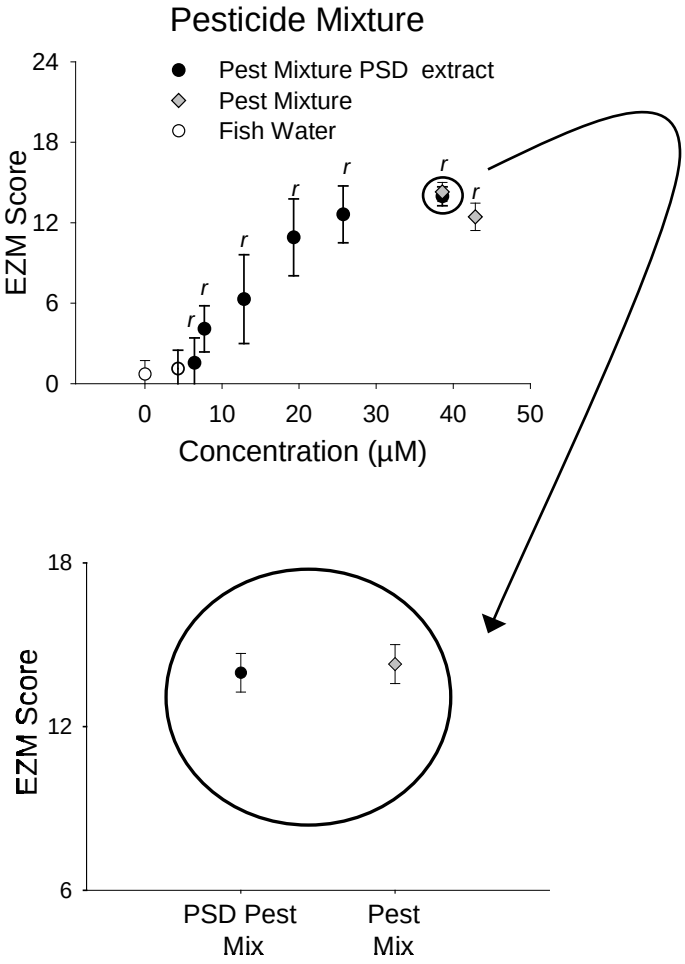


Figure 10. Because EZM score reflects the general toxicity of a treatment and not specifically what is driving the toxicity, the following graphs reveal a more in depth analysis of what endpoints contribute to the overall EZM score of pesticide mixture treatments. It appears that all four of the following effects contribute to the EZM score. (next page)

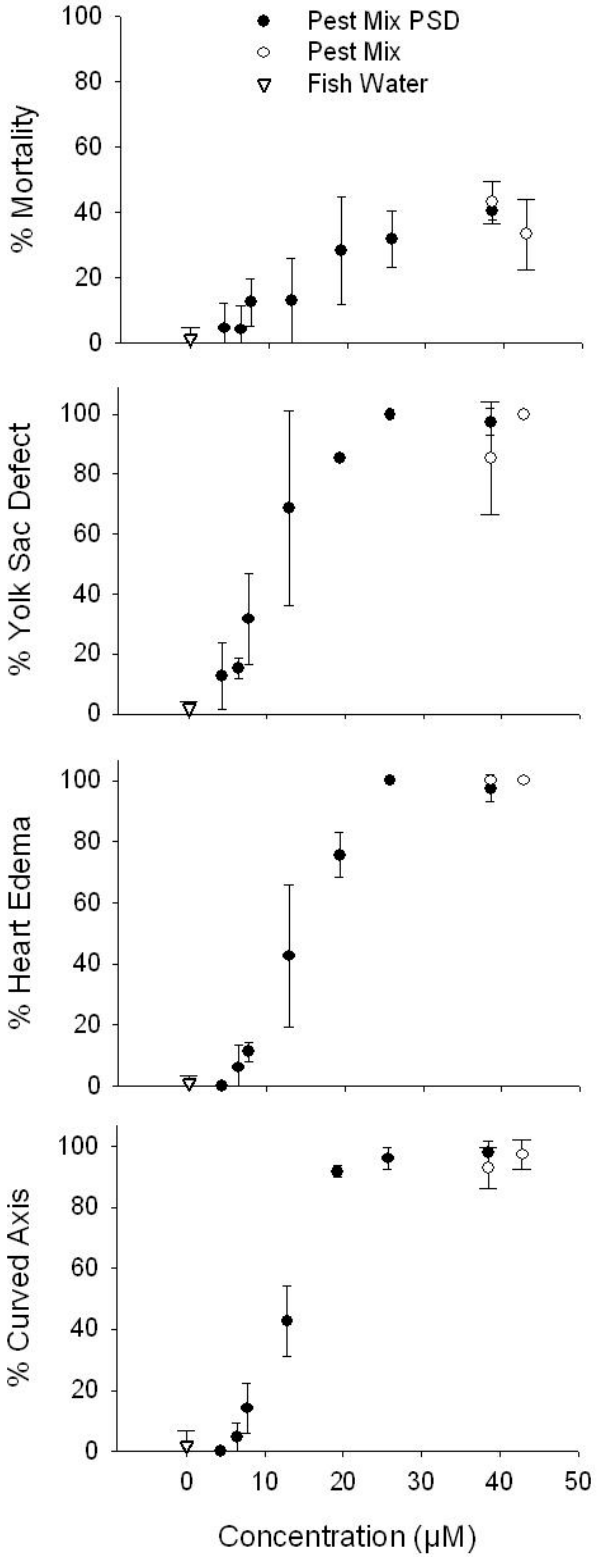

Pest Mix PSD
Pest Mix
Fish Water
% Mortality
% Yolk Sac Defect
% Heart Edema
% Curved Axis
0
20
40
60
80
100
0
10
20
30
40
50
Concentration (µM)

Figure 11. A partial pesticide mixture composed of organochlorines (OC) and an individual pesticide, dieldrin, were employed for embryonic zebrafish exposures at various molar concentrations. The graphs are stacked. The EZM scores for assessments appear to be related to the percent mortality. (*r*= significantly different compared to control. Control EZM score not included on this graph, see Figure 8. *t*= significantly different compared to the dieldrin at 21 µM)

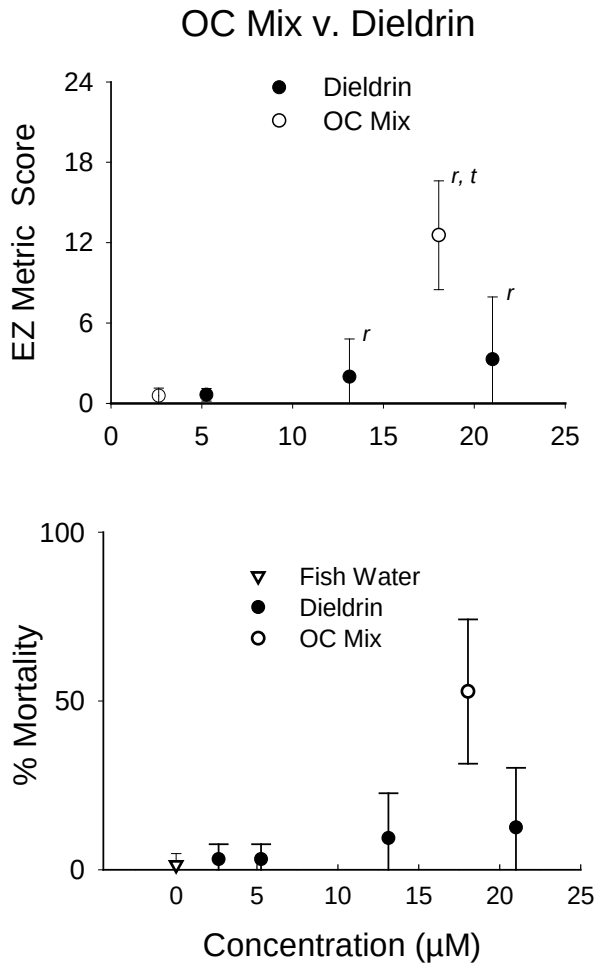


Figure 12. A partial pesticide mixture composed of three organophosphates (OP) and an individual pesticide, fenitrothion, were employed for embryonic zebrafish exposures at various molar concentrations. The graphs are stacked. EZM scores for OP mix and control assessments are different. The low incidence of mortality caused by the OP mix suggests that the mix is not driving the OP EZM score. Statistical analysis confirms that percent mortality for OP mix and FW are not significantly different and that yolk sac defect is different when comparing the mix and FW exposures (see Figure 14). (*r*= significantly different compared to fish water control (FW). Fish water EZM score not included on this graph, see Figure 8.)

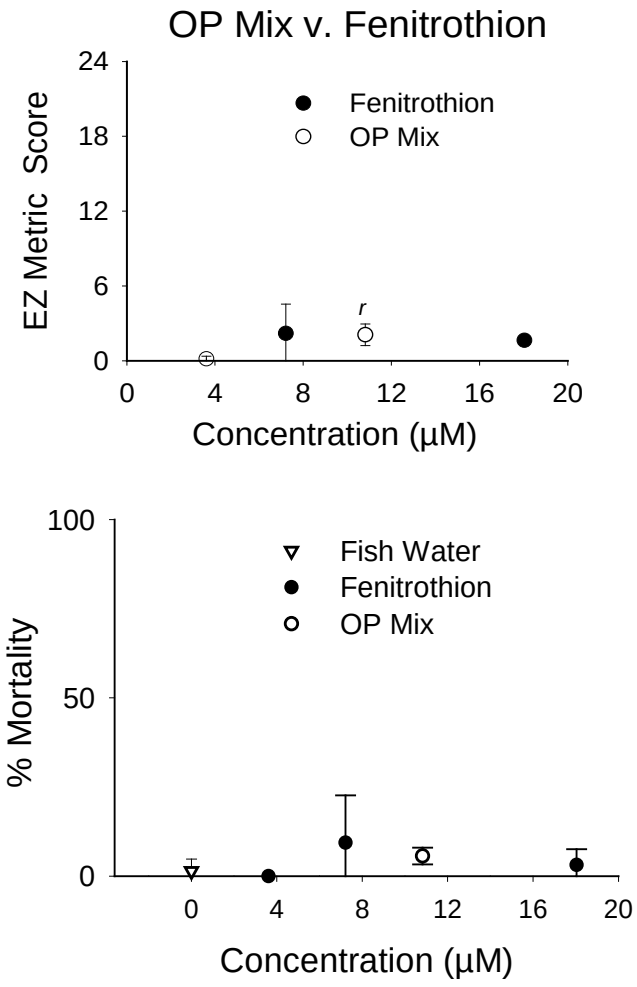


Figure 13. Individual pesticide toxicity was investigated graphically using EZM scores for three different pesticides: cypermethrin, propanil, and fipronil. (Statistical analysis has not been performed comparing pesticides to control.) The endpoints that induced the overall toxicity (EZM score) of cypermethrin and fipronil, that is the incidence of the endpoints different than FW, were: stubby body, curved axis, yolk sac defect, and heart edema as seen in Figure 14. (*r*= significantly different compared to control.

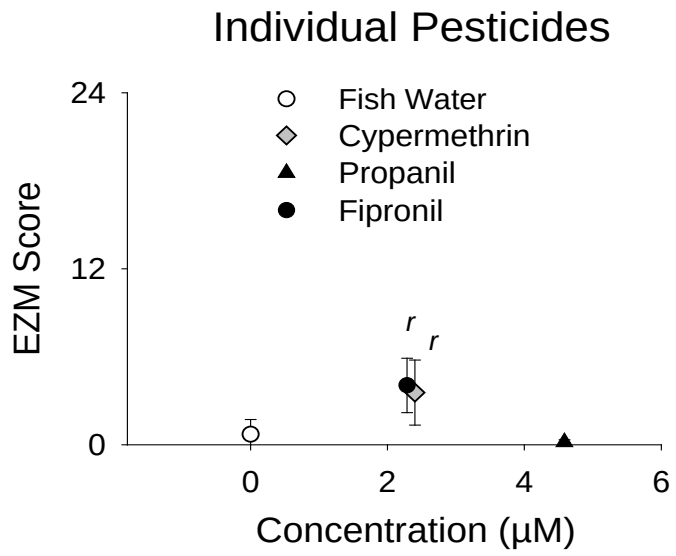
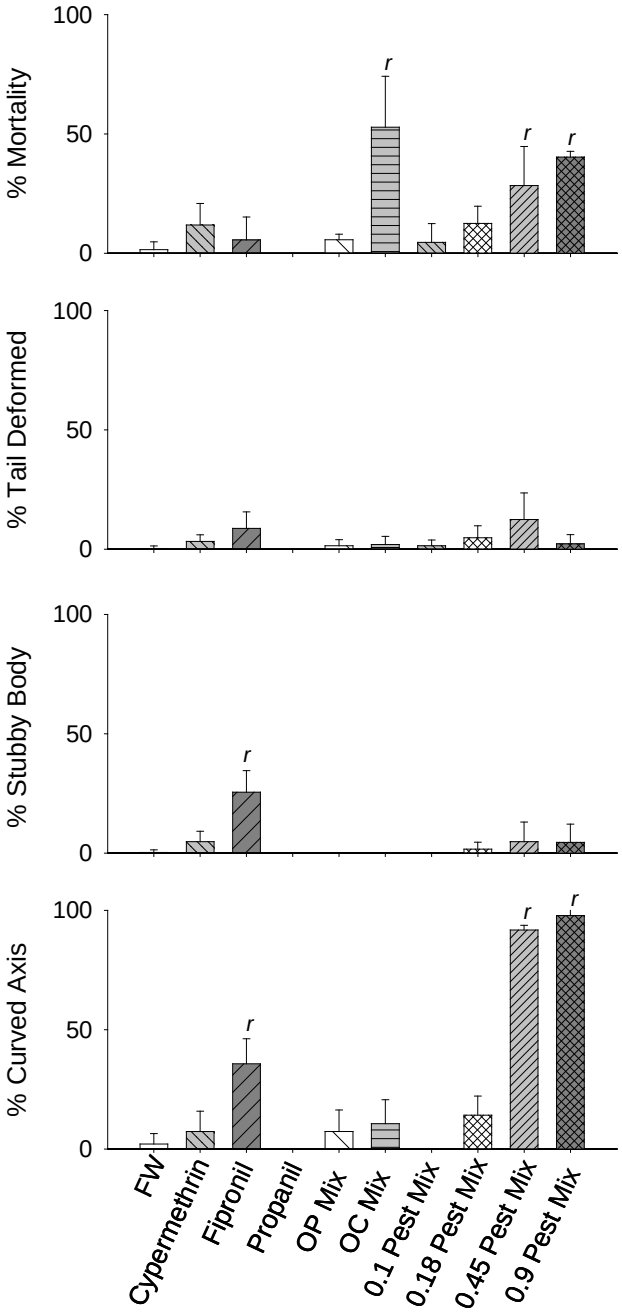


Figure 14. Eight graphs were produced to identify the endpoints that were responsible for producing a high EZM scores for various treatments. Furthermore, the graphs indicate what endpoints are specific to certain pesticides and what endpoints are non-specific. (next page)


% Mortality
% Tail Deformed
% Stubby Body
% Curved Axis
100
50
0
r
FW
Cypermethrin
Fipronil
Propanil
OP Mix
OC Mix
0.1 Pest Mix
0.18 Pest Mix
0.45 Pest Mix
0.9 Pest Mix

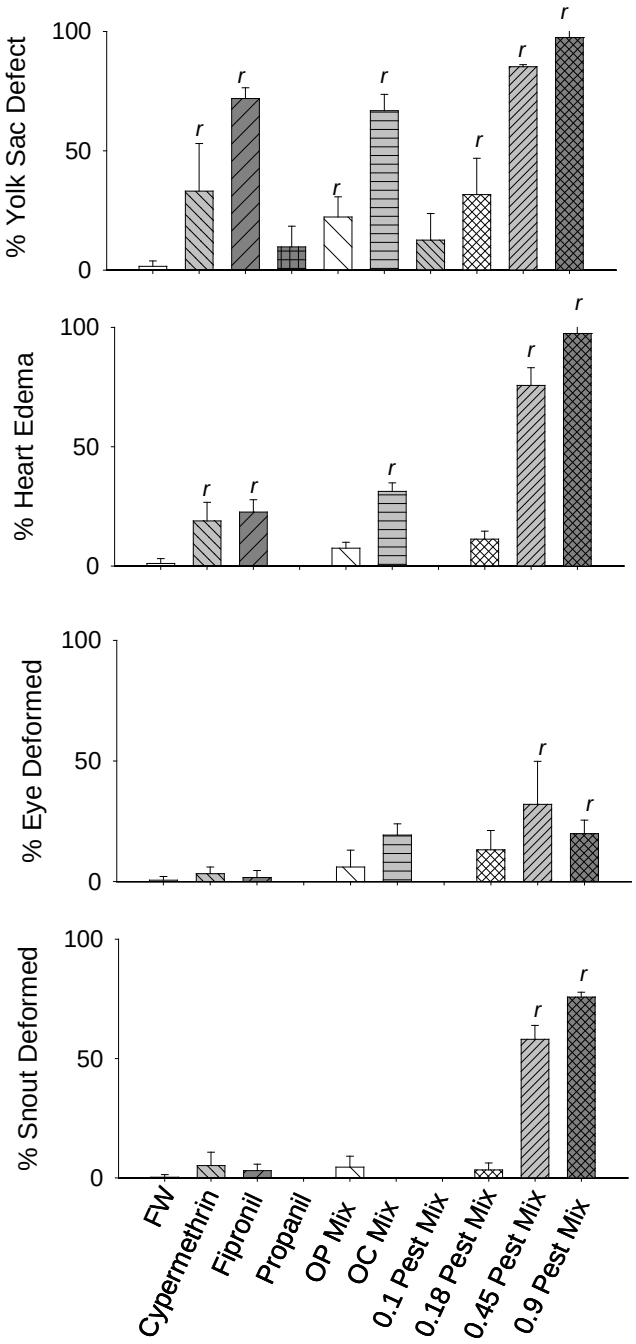
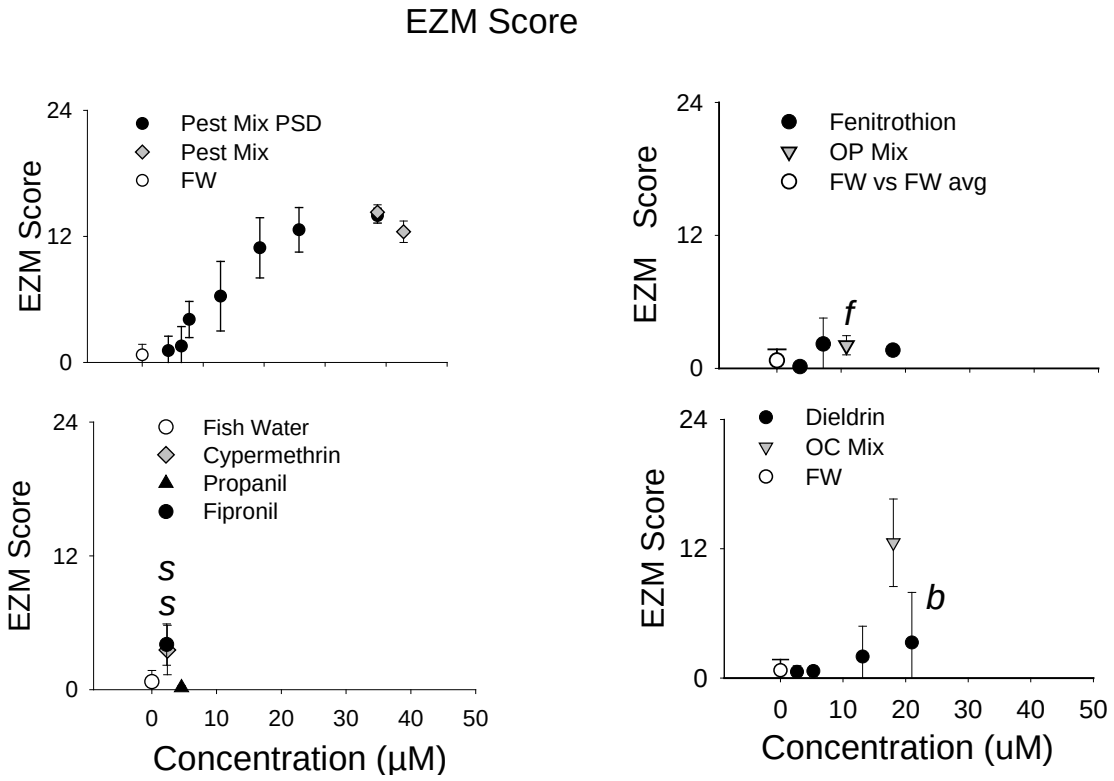

% Yolk Sac Defect
% Heart Edema
% Eye Deformed
% Snout Deformed
100
50
0
r
FW
Cypermethrin
Fipronil
Propanil
OP Mix
OC Mix
0.1 Pest Mix
0.18 Pest Mix
0.45 Pest Mix
0.9 Pest Mix

Figure 15. Comparison of EZM scores assisted in understanding what compounds or mixtures were key contributors to general toxicity (*s* = different than 4.2 µM Pest mix , *b* = different than 20 µM Pest mix, *f* = different than 13 µM Pest mix, not all concentrations of dieldrin, fenitrothion were analyzed).



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2. Anderson, K.A., et al., *Field Trial and Modeling of Uptake Rates of In Situ Lipid-Free Polyethylene Membrane Passive Sampler.* Environmental Science & Technology, 2008. **42**(12): p. 4486-4493.
3. Wells, J.B. and R.P. Lanno, *Passive sampling devices (PSDs) as biological surrogates for estimating the bioavailability of organic chemicals in soil,* in *Environmental toxicology and risk assessment: science, policy and standardization - implications for environmental descisions,* B.M. Greenberg, et al., Editors. 2001, American Society for Testing and Materials: West Conshohocken, PA. p. 253-270.
4. Comyn, J., *Polymer Permeability*. 1985, London, England: Elsevier Applied Science LTD.
5. Opperhuizen, A., et al., *Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals.* Chemosphere, 1985. **14**: p. 1871-1896.
6. Sabaliunas, D. and A. Södergren, *Use of semi-permeable membrane devices to monitor pollutants in water and assess their effects: A laboratory test and field verification.* Environmental Pollution, 1997. **96**(2): p. 195-205.
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