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Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day-old Japanese medaka (Oryzias latipes)

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Keywords
Japanese medaka, Oryzias latipes, Behavior, Acute toxicity

Comments
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ACUTE TOXICITY AND BEHAVIORAL EFFECTS OF CHLORPYRIFOS, PERMETHRIN, PHENOL, STRYCHNINE, AND 2,4-DINITROPHENOL TO 30-DAY-OLD JAPANESE MEDAKA (*ORYZIAS LATIPES*)

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Abstract—Five chemicals with different modes of action were evaluated in laboratory studies to determine their acute toxicity (48-h median lethal concentration [LC50]) and behavioral effects on 30-d-old Japanese medaka (*Oryzias latipes*). The order of toxicity for these xenobiotics was permethrin > chlorpyrifos > 2,4-dinitrophenol (2,4-DNP) > strychnine > phenol. The 48-h LC50s were significantly different and ranged from 0.011 to 24.1 mg/L. In addition, chlorpyrifos and permethrin accumulated in the tissues of juvenile *O. latipes*. Observations of five behavioral/morphological responses, including changes in equilibrium, general activity, startle response, and morphology (e.g., hemorrhage and deformities) were used as indicators of sublethal toxicity. Each chemical, with the exception of 2,4-DNP, elicited a distinct behavior or set of behavioral responses. The behavioral toxicology bioassay may be valuable in comparing and predicting the mode of action of new or unknown toxicants in this species of fish.

Keywords—Japanese medaka *Oryzias latipes* Behavior Acute toxicity

INTRODUCTION

Environmental pollutants are known to elicit adverse effects to aquatic organisms. Low levels of contaminants in aquatic ecosystems may affect an animal's behavior, physiology, growth, reproduction, and survival. Behavioral and morphological abnormalities, as a result of sublethal toxicity, can reduce an aquatic organism's health and fitness.

Conventional endpoints in acute toxicity testing have primarily assessed lethality, whereas some more recent studies have focused on sublethal effects [1,2]. Behavioral responses are effective indicators of contamination and reflect sublethal toxicity [1,2]. Drummond et al. [2] evaluated the use of behavioral and morphological changes in fish as a diagnostic endpoint for screening and differentiating chemicals according to their mode of action. After exposing 30-d old fathead minnows (*Pimephales promelas*) to different chemicals, Drummond et al. [2] observed unique morphological and behavioral signs of stress. The authors concluded that select abnormal responses are promising for predicting the mode of action of unknown xenobiotics. Drummond and Russom [3] further categorized chemicals corresponding to three general mode of action response syndromes: hyperactivity, hypoactivity, and physical deformity. Each syndrome or sign of stress was indicative of a different mode of action. Overall, toxicological studies have become more versatile and sensitive with the development and implementation of behavioral endpoints.

The present studies were conducted to determine the acute toxicity and the behavioral or morphological effects of five compounds with different modes of action on one fish species. Chlorpyrifos, permethrin, 2,4-dinitrophenol (2,4-DNP), phenol, and strychnine, which represent an organophosphate (an acetylcholinesterase inhibitor), a pyrethroid insecticide (a neurotoxicant), an uncoupler of oxidative phosphorylation, a polar narcotic, and an alkaloid convulsant, were studied because of these known modes of action. We selected the Japanese medaka (*Oryzias latipes*) as the test organism for the following reasons: This species has been previously used for toxicity testing [4,5]; *O. latipes* can be reared in the laboratory; spawning can be controlled by manipulating temperature, light, and food; and eggs hatch within 10 d and the fish reach maturity within 4 to 6 months [6].

This article compares the onset of abnormal behavioral responses, morphological changes, and mortality in 30-d-old *O. latipes* that were exposed to five chemicals with different modes of action. Results from this study may contribute to the understanding of the relationship between lethality and abnormal sublethal responses. In addition, this information should further prove that abnormal behavioral responses can be used as diagnostic endpoints for determining the source and mode of action of unknown compounds.

MATERIALS AND METHODS

Test animals

Oryzias latipes were reared at Iowa State University from original breeding stock obtained from the U.S. Environmental Protection Agency (U.S. EPA), Mid-Continent Ecology Division (Duluth, MN, USA). Fish were maintained in aquaria with a 16:8 h light:dark photoperiod at a temperature of 22 to 28°C. *Oryzias latipes* were fed twice daily with newly hatched brine shrimp (*Artemia franciscana*) (San Francisco Bay Brand,

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Newark, CA, USA) and/or Wardley Tropical Fish Food (Wardley Laboratories, Secaucus, NJ, USA). Fertilized eggs were collected and maintained in 60 × 5-mm petri dishes containing rearing solution previously described by Kirchen and West [6]. Newly hatched fry were removed from the petri dishes and placed into 38-L aquaria containing charcoal-filtered municipal water. Fry were fed Hatchfry Encapsulan grade II (Argent Chemical Laboratory, Redmond, WA, USA) and/or newly hatched *A. franciscana* twice daily until they reached the appropriate age for testing. *Oryzias latipes* fry and juveniles were maintained at the same environmental conditions as the adults. Thirty-day-old *O. latipes* were selected for this study, based on the results from the preliminary toxicity tests that indicated the 30-d-old fish were more sensitive to the test chemicals than were adults.

Toxicant preparation

We selected the following chemicals due to their different modes of action. Permethrin (3-phenoxybenzyl-(1*R,S*)*cis,-trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) (88% pure) and chlorpyrifos (*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) (99% pure) were provided by the U.S. EPA (Triangle Park, NC, USA) and Dow Chemical Co. (Midland, MI, USA), respectively. The other chemicals included strychnine hemisulfate salt (>99% pure) (Sigma Chemical Co., St. Louis, MO, USA), phenol (>89% pure) (J.T. Baker Chemical Co., Milwaukee, WI, USA), and 2,4-dinitrophenol (2,4-DNP) (85% pure) (Aldrich Chemical Co., Milwaukee, WI, USA).

Toxicant stock solutions were prepared in either filtered deionized water (municipal water filtered with a Nanopure Ultrapure Water System, Barnstead Still and Sterilization Co., Boston, MA, USA) or an appropriate solvent before each test. Acetone (99.6% pure) was used as the carrier solvent for permethrin and chlorpyrifos. Strychnine, phenol, and 2,4-DNP were dissolved in filtered deionized water. The phenol stock solution was stabilized with 0.15% H_3PO_2 and the pH was adjusted to 6.8 to 7.5 with 1 N NaOH. 2,4-Dinitrophenol was dissolved in 1 N NaOH, then added to water and stirred for 30 min, and concentrated HCl was added to bring the pH of the stock solution back to 8.0.

Acute toxicity assay

Forty-eight-hour static acute toxicity tests were conducted with 30-d-old (range = 26–34 d) juvenile *O. latipes* that had a mean length of 12 mm. We selected a 48-h toxicity test for this study in order to compare these findings with results from our previous research. Fish were acclimated to the testing conditions for 24 h before beginning each experiment. Static 48-h tests were conducted according to the American Society for Testing and Materials (ASTM) guidelines [7]. Food was withheld 24 h preceding and during the 48-h exposure. Each static 48-h toxicity test consisted of five or more toxicant concentrations, controls, and blanks. Three or more replicates were performed for each treatment. The control and treatment solutions contained equivalent amounts of the carrier solvent. Carrier solvents were not added to the blank exposure chambers. Ten to 20 randomly selected fish were assigned to each glass test chamber (600-ml Pyrex beaker) containing 500 ml of test solution. Sharom and Solomon [8] reported that permethrin adsorbs to glass and plastic materials; therefore, steps were taken to maintain the appropriate chemical concentrations throughout the tests. Glass test chambers were filled with the appropriate aqueous permethrin solution and, after 24 h, the solutions were replaced prior to testing. Permethrin and chlorpyrifos test solutions were partially renewed to sustain the appropriate chemical concentrations. After 24 h of exposure, 300 ml of water was removed from each chamber and replaced with fresh aqueous solution. Exposures were conducted in an incubator (25 ± 1°C; 16:8 light : dark photoperiod). The water was aerated for at least 24 h before testing to insure oxygen saturation. At the initiation and completion of each test, dissolved oxygen (DO), hardness, alkalinity, and pH were measured by standard methods [9]. The overall means (±1 SD) for hardness and alkalinity were 136 ± 20 mg/L as CaCO, and 9.1 ± 4.1 mg/L as $CaCO_3$, respectively; DO was 7.1 ± 1.3 mg/L; pH was 7.3 ± 0.7; and the incubator temperature was 25 ± 1°C.

Observations to determine the mortality of *O. latipes* were made at 1 through 6, 8, 12, 24, 36, and 48 h, during the 48-h exposures. Juvenile *O. latipes* were considered dead when there was no sign of opercular movement or no response to external stimuli. Dead fish were recorded and removed from the testing chambers. If ≥10% mortality occurred in the blanks or controls, the test was terminated and the results were not included in the database. Fish that did not survive the permethrin and chlorpyrifos exposures were rinsed three successive times in acetone, weighed, and stored in hexane (99.9% pure) at −60°C until they could be analyzed for tissue residues on a whole-body wet-weight basis. At the termination of the permethrin and chlorpyrifos tests, the surviving fish were sacrificed and the procedures stated above were followed.

The median lethal concentration (LC50) values and 95% confidence limits (CLs) were calculated using probit analysis with the Statistical Analysis System (SAS®) [10]. The mortality count from each test chamber was considered as one replication. At least three replications were conducted in each treatment. Mean measured concentrations ($n \geq 4$) of the test solutions were used in conjunction with resulting fish mortality data to determine the LC50 values. The LC50s with nonoverlapping 95% CLs were considered significantly different.

Behavioral and morphological assays

Observations of behavioral and morphological response of 30-d-old *O. latipes* exposed to permethrin, chlorpyrifos, phenol, 2,4-DNP, and strychnine were conducted at 1 through 6, 8, 12, 24, 36, and 48 h during the acute toxicity tests. The methods developed by Drummond (R.A. Drummond, 1991, unpublished) and Drummond et al. [2] were followed. Controls and blanks were monitored, along with the toxicant concentrations, to provide a reference for assessing any behavioral and morphological changes. Responses were recorded if they differed from the controls and occurred in ≥10% of the fish within each test chamber. Five behavioral and morphological indicators were observed in this study: loss of equilibrium, general activity, startle response, hemorrhage, and deformity (including postural indicators). Operational definitions of symptoms of the five behavioral and morphological changes are listed in the Appendix. Each test chamber was observed for 10 to 15 min to allow sufficient time for an accurate evaluation of each fish. Startle responses were monitored by the following procedures in sequence: passing a hand over the test chamber (overhead moving visual stimulus), rapping on the chamber (vibrational stimulus), and lightly touching the fish with a wooden applicator stick (tactile stimulus). Several min-

Table 1. Acute toxicity of five xenobiotics to 30-d-old *Oryzias latipes*[a]

Chemical	Type of toxicant	24-h LC50 (95% CL) (mg/L)	48-h LC50 (95% CL) (mg/L)
Permethrin	Pyrethroid	0.024 (0.023–0.025)	0.011 (0.010–0.012)
Chlorpyrifos	Organophosphorus	0.30 (0.28–0.33)	0.25 (023–0.27)
2,4-Dinitrophenol	Uncoupler of ATP	1.48 (1.35–1.61)	1.33 (1.23–1.45)
Strychnine	Alkaloid convulsant	>7	5.7 (4.7–6.2)
Phenol	Polar narcotic	>44.2	24.1 (21.8–26.6)

[a] LC50 = median lethal concentration, CL = confidence limit.

utes elapsed between each evoked startle response to allow *O. latipes* to avoid habituation.

Water analysis

Approximately 3 L of each exposure concentration was made and distributed into the appropriate test chambers (550 ml/chamber). Fifty milliliters of test water was sampled from the mid-depth of each test chamber. Water samples from the same concentration were pooled together into one sample and analyzed. Each water concentration was analyzed using two replicates at 0 and 24 h and one replicate at 48 h.

Permethrin and chlorpyrifos water samples were prepared on ENV-18 solid phase extraction (SPE) tubes (Supelco, Belefonte, PA, USA). The SPE tubes were first conditioned with 2 ml methanol, followed by 2 ml deionized water. Twenty milliliters and 2 ml of sample were run through the ENV-18 SPE tubes for permethrin and chlorpyrifos, respectively. The tubes were vacuum dried and the chemicals were eluted with 40 ml hexane. The initial 10 ml of eluate was collected as one fraction, followed by six 5-ml fractions. Concentrations of permethrin and chlorpyrifos were determined using a Varian model 3740 gas chromatograph (GC) (Varian Associates, Sunnyvale, CA, USA), equipped with a ^{63}Ni electron-capture detector and 31 cm × 2 mm (i.d.) columns containing 5% OV-101 and 3% Dexsil 300 on 80/100 mesh Chromosorb W-HP (Supelco), respectively. Ultrapure nitrogen (99.9%) was used as the carrier gas at a flow rate of 25 ml/min. Oven temperatures were 290°C and 230°C for permethrin and chlorpyrifos, respectively. The injector port and detector temperatures were 300°C and 350°C. Permethrin and chlorpyrifos detection limits were 0.005 and 0.001 mg/L. Recoveries from permethrin- and chlorpyrifos-spiked water samples were 95.0 ± 6.3% (n = 12) and 92.0 ± 5.5% (n = 15), respectively.

Phenol, 2,4-DNP, and strychnine water samples were filtered with a 0.5-mm filter and analyzed by direct aqueous injection using isocratic, reverse-phase, high-performance liquid chromatography (HPLC) with a UV variable-wavelength detector at 280 nm, 275 nm, and 254 nm, respectively. Instrumentation consisted of a Waters U6K injector and 6000A solvent delivery system (Waters Associated, Milford, MA, USA), C_{18} microshort (3.3 cm × 6 mm) column (Perkin Elmer, Norwalk, CT, USA), and Kratos Spectroflow 757 variable-wavelength detector (Kratos Analytical Instruments, Ramsey, NJ, USA). The mobile phase consisted of methanol : water (1:1, v/v) with a flow rate of 0.5 and 0.8 ml/min for phenol and 2,4-DNP, respectively. A 0.02 M KH_2PO_4 : acetonitrile (3:1, v/v) mobile phase at 3.0 ml/min was utilized for the strychnine samples. Recoveries from phenol-, 2,4-DNP-, and strychnine-spiked water samples were 97.1 ± 4.8% (n = 10), 96.4 ± 3.5% (n = 10), and 93.0 ± 6.3% (n = 4) recovery, respectively. Detection limits were 0.5 mg/L for the phenolic compounds and 0.1 mg/L for strychnine.

Peak heights were used to construct each calibration curve and to quantitate the samples. All the standard curves had correlation coefficients exceeding 0.990. Water spikes, as well as known and unknown standards, were analyzed concurrently with the samples to ensure accuracy.

Residue analysis

Oryzias latipes from each test chamber were pooled (dead and surviving fish were pooled separately) together and analyzed as one replication, providing at least three replications of each water concentration per toxicity test. Permethrin and chlorpyrifos fish tissue analysis was modified from methods described by Bradbury and Coats [11]. The tissue extraction procedure was followed with modifications in the sample cleanup. Tissue samples were cleaned with LC-Si SPE tubes (Supelco) that were previously conditioned with 2 ml hexane. The full contents of the sample and an additional 60 ml of hexane were vacuum-filtered through the SPE tubes. Filtered samples and hexane eluates were rotary evaporated to 2 ml and diluted with hexane to a final volume of 5 ml. Tissue samples were analyzed by GC as described in the methods for the water analysis. Extraction and cleanup of permethrin- and chlorpyrifos-spiked *O. latipes* tissue samples averaged 91.2 ± 7.0% (n = 10) and 93.5 ± 5.3% (n = 12) recovery, respectively. One-way analysis of variance (ANOVA) and the least squared means were used to test for significant differences among the treatments and between the tissue analysis for dead and surviving fish tissues at the p = 0.05 level of significance [12].

RESULTS AND DISCUSSION

Acute toxicity assay

The 48-h LC50s for each of the five chemicals tested were significantly different (Table 1). The most toxic chemical to 30-d-old *O. latipes* was permethrin, followed in decreasing order by chlorpyrifos, 2,4-DNP, strychnine, and phenol. The 48-h LC50s ranged from 0.011 mg/L for permethrin to 24.1 mg/L for phenol. Comparison of the LC50 values indicated that permethrin was 2,190, 518, 120, and 23 times more toxic than phenol, strychnine, 2,4-DNP, and chlorpyrifos, respectively. The lipophilic insecticides, permethrin and chlorpyrifos, were more toxic to *O. latipes* than the more polar compounds. Review of the literature indicates a similar order of toxicity for these compounds to juvenile *P. promelas*. The 96-h flow-through LC50s for 30-d-old *P. promelas* were permethrin > chlorpyrifos > 2,4-DNP > phenol [13–15].

Permethrin. Permethrin acute toxicity to 30-d-old *O. latipes* was comparable to the literature values for other fish

species. Mulla et al. [16] reported static 48-h LC50s of 0.005, 0.006, and 0.097 mg/L for adult desert pupfish (*Cyprinodon macularius*), rainbow trout (*Oncorhynchus mykiss*), and western mosquitofish (*Gambusia affinis*), respectively. Holcombe et al. [17] determined 0.0174 and 0.0321 mg/L permethrin 48-h LC50s for *O. mykiss* and *P. promelas.* The permethrin 48-h static LC50 determined for juvenile *O. latipes* in this study was lower than the value recorded in the literature for adults. Kikuchi et al. [18] observed a permethrin 48-h static LC50 of 0.06 mg/L for adult *O. latipes.*

Permethrin and other synthetic pyrethroids are highly toxic to fish (96-h LC50 usually < 0.01 mg/L) [19], but have comparatively low toxicity to other nontarget vertebrates such as birds and mammals (male rat acute oral LC50 = 2,000 mg/kg) [19,20]. In contrast to birds and mammals, fish do not quickly hydrolyze permethrin, which may be attributed to a low level of esterases. Slower biotransformation and elimination rates result in increased toxicity. The toxicity, elimination, and metabolism of permethrin in *O. mykiss* and mice have been documented [19,21,22]. Fish primarily metabolize pyrethroids by oxidative degradation, with ester hydrolysis being a secondary reaction. Glickman et al. [21] and Glickman and Lech [22] reported that the oxidation and hydrolysis of permethrin in *O. mykiss* tissues was comparatively slower than in mammalian tissues. In addition, Glickman and Lech [22] noted that permethrin's site of action, along with its slower biotransformation and elimination rate, may contribute to the difference in its potency to fish and mammals. The basis for the selectivity of the pyrethroids has been reviewed [19].

Chlorpyrifos. The LC50 of chlorpyrifos to 30-d-old *O. latipes,* based on nonoverlapping confidence limits, was lower at increased exposure times (Table 1). Similar results were reported in the literature with other fish species. Mayer and Ellersieck [13] observed 24-h and 96-h static LC50s of 0.110 mg/L and 0.015 mg/L for *O. mykiss.* Comparison of the 24-h static LC50 values for chlorpyrifos indicated that 30-d-old *O. latipes* were three times less sensitive than *O. mykiss.* Adult longnose killifish (*Fundulus similis*), Atlantic silverside (*Menidia menidia*), and striped mullet (*Mugil cephalus*) had 96-h LC50s of 0.0041 mg/L, 0.0017 mg/L, and 0.0054 mg/L, respectively [23]. Jarvinen et al. [14] reported a 96-h LC50 value of 0.12 mg/L for *P. promelas* larvae during a continuous-exposure chlorpyrifos test. Holcombe et al. [17] determined 24-h and 48-h LC50 values of 0.020 mg/L and 0.248 mg/L for *P. promelas.*

In contrast to permethrin, chlorpyrifos is readily metabolized by fish [24]. Chlorpyrifos, like other phosphorothionate ester acetylcholinesterase (AChE) inhibitors, is more toxic after it is metabolically activated to the P=O ester by the cytochrome P_{450} monooxygenase (CP450) enzyme systems [24,25]. Schell et al. [26] studied hepatic CP450 activity in adult *O. latipes.* They reported that the *O. latipes* CP450 system was induced by beta-naphtholflavone as seen in other fish species. The inhibition of AChE in fish by chlorpyrifos has been observed by Jarvinen et al. [27]

2,4-Dinitrophenol. The acute toxicity of 2,4-DNP to *O. latipes* (48-h LC50 of 1.33 mg/L) was in the same range as reported in other fish species. A 2,4-DNP flow-through 96-h LC50 of 2,4-DNP for 30-d-old *P. promelas* was reported to be 17 mg/L [15]. McKim et al. [28] reported an LC50 value for 2,4-DNP of 2.07 mg/L for juvenile *O. mykiss* following a 48-h flow-through exposure. Howe et al. [29] observed 24- and 48-h LC50s for 2,4-DNP of 13.2 and 10.36 mg/L for *O. mykiss.* Comparison of the 48-h LC50s (Table 1) indicates that 30-d-old *O. latipes* were more sensitive to 2,4-DNP than were either juvenile *P. promelas* or *O. mykiss.*

Strychnine. In this study, the strychnine static 48-h LC50 for 30-d-old *O. latipes* was 5.7 mg/L. Most of the mortality occurred after 24 h of exposure. Bradbury et al. [30] reported a mean survival time of 12.8 h for *O. mykiss* exposed to 4.75 mg/L of strychnine.

Phenol. Phenol was the least toxic of the five xenobiotics studied (48-h LC50 of 24.1 mg/L). Holcombe et al. [31] observed a 96-h phenol LC50 of 38.3 mg/L for 28- to 43-d-old *O. latipes.* Fogels and Sprague [32] reported phenol flow-through 48-h LC50s of 11.6, 30.9, and 36.3 mg/L for *O. mykiss,* zebrafish (*Brachydanio rerio*), and flagfish (*Jordanella floridae*), respectively. Phenol flow-through 96-h LC50 values were 28 and 29 mg/L for 30-d-old *P. promelas* [15]. A static 96-h LC50 of 40 mg/L was determined for male guppies (*Poecilia reticulata*) [33].

Phenol may be eliminated from the body of higher vertebrates as either free phenol or conjugation products, such as phenyl sulfate or phenyl glucuronide. Kobayashi and Akitake [34] reported that goldfish (*Carassius auratus*) rapidly absorbed and excreted phenol. The *C. auratus* that were transferred to phenol-free water had a 25% decrease in the phenol content of their bodies, after 1 h. Free and bound forms of phenol were excreted. Layiwola and Linnecar [35] reported the biotransformation of ^{14}C-phenol in several freshwater fish. Four of the eight species tested excreted phenyl sulfate and phenyl glucuronide conjugates. Phenol may be less toxic to *O. latipes* because of rapid biotransformation and elimination from the body, thus reducing the accumulation of phenol at the target site.

Behavioral and morphological assays

Behavioral and morphological symptoms of intoxication, as described in the Appendix, were monitored following exposure to permethrin, chlorpyrifos, phenol, 2,4-DNP, and strychnine. One symptom that occurred with all five chemicals was the loss of equilibrium; therefore, this effect alone is not diagnostic for any chemical or mode of action. Distinct behavioral and morphological abnormalities were observed for each of the chemicals tested, except for 2,4-DNP (Table 2).

Pollutants may affect virtually all aspects of behavior in aquatic organisms. Behavioral monitoring is a promising diagnostic tool for screening and differentiating chemicals according to their mode of action [2]. Drummond et al. [2] proposed that chemicals with different modes of action will evoke a distinct behavior or set of behavioral symptoms. In the present study, effects of permethrin, chlorpyrifos, 2,4-DNP, and strychnine were similar to those reported in the literature for other fish species. Drummond et al. [2] monitored the behavior and morphological changes of juvenile *P. promelas* exposed to 139 different chemicals. They noted that 84% of these compounds resulted in a loss of equilibrium in fish. This corresponds with the loss of equilibrium that was observed in *O. latipes* after the exposure of each of the five chemicals tested in this study.

Permethrin. Permethrin, a synthetic pyrethroid insecticide, elicited abnormal swimming. Juvenile *O. latipes* exposed to >0.009 mg/L swam hyperactively with an excessive lateral flexure in the caudal area. Some of the fish exhibited a temporary 90° bend at midbody. After 24 h of exposure, severely intoxicated fish became hypoactive, underreactive to startle

Table 2. Diagnostic behavioral effects

Behavioral and morphological symptom	Chemical				
	Permethrin	Chlorpyrifos	2,4-Dinitrophenol	Strychnine	Phenol
Loss of equilibrium	Yes	Yes	Yes (occasional)[a]	Yes	Yes
General activity	Hyperactive to hypoactive, excessive lateral flexure[b,c]	Hypoactive	Unchanged	Hypoactive, convulsions and tetany[b,c]	Agitated,[b,d] hyperactive to hypoactive
Startle response	Underreactive[e]	Underreactive[e]	Unchanged	Underreactive[e]	Overreactive to underreactive[e]
Hemorrhage	None	Caudal area[b]	None	None	None
Deformities	None	Scoliosis and/or lordosis,[b,c] pectoral fins (forward)[b,f]	None	None	None

[a] Just before death.
[b] Distinguishing behavior, morphological change or sign of stress.
[c] In caudal area.
[d] Initial agitated swimming response that occurred within 2 h of phenol exposure.
[e] Severely intoxicated fish ceased to respond.
[f] Pectoral fins postured at 45° to 90° angle from the head of the fish, little or no movement of pectoral fins.

stimuli, and mortality eventually resulted. *Oryzias latipes* exposed to ≤0.005 mg/L permethrin showed no obvious behavioral changes within 48 h. The onset of abnormal lateral flexure in *O. latipes* was dose dependent: as the permethrin exposure concentration increased, time of onset decreased. Signs of intoxication occurred within 24 h for concentrations below the 48-h LC50.

Permethrin, like other synthetic pyrethroids, is a neurotoxicant [21,36]. Permethrin, classified as a type I pyrethroid, characteristically causes hyperactivity, prostration, fine tremors, lack of coordination, and paralysis in vertebrates and insects [36].

Behavioral symptoms observed in 30-d-old *O. latipes* exposed to permethrin were similar to the effects described in other fish species [17,21,37,38]. Hansen et al. [37] exposed sheepshead minnow (*Cyprinodon variegatus*) fry to permethrin and observed an abnormal lateral flexure of the caudal area, with the tail appearing to almost touch the head. Glickman et al. [21] reported hyperactivity and loss of equilibrium in *O. mykiss* exposed to >0.01 mg/L permethrin.

Chlorpyrifos. Several behavioral and morphological responses were observed in 30-d-old *O. latipes* during sublethal exposure to chlorpyrifos (Table 2). Distinguishing symptoms included a continuous forward posturing of the pectoral fins, hemorrhage (vertebral area), and scoliosis in the caudal region. The time until initial onset of morphological responses and mortality was shorter at higher chlorpyrifos concentrations (Fig. 1). These adverse sublethal effects occurred within 24 h in concentrations below the 48-h LC50. Fish exposed to ≥0.10 mg/L chlorpyrifos held their pectoral fins, with little or no movement, at 45° to 90° angles from their heads. This sign of intoxication occurred at 8 and 24 h for concentrations of 0.4 mg/L and 0.2 mg/L, respectively. Generally, hemorrhages and scoliosis in the caudal area were noted before adverse effects in the pectoral fins. After 24 h of exposure, intoxicated *O. latipes* swam lethargically, and were underreactive to startle stimuli.

Chlorpyrifos is an organophosphorus insecticide that causes the inhibition of AChE. Excess acetylcholine causes continuous firing of muscle fibers [25]. Drummond et al. [2] reported that fish exposed to any one of several AChE inhibitors showed common tetany, lordosis, and scoliosis. This abnormal vertebral bending presumably led to hemorrhage and permanent disfigurement. Jarvinen et al. [14] recorded 50% deformities, which consisted of a lateral bend in the spine, in fathead minnows after 15 h of 0.122 mg/L chlorpyrifos exposure. Holcombe et al. [17] also noted spinal deformities in *P. promelas* and *O. mykiss.* The behavioral and morphological changes observed in 30-d-old *O. latipes* were consistent with the findings of Drummond et al. [2], Jarvinen et al. [14], and Holcombe et al. [17].

2,4-Dinitrophenol. 2,4-Dinitrophenol, at lethal concentrations, elicited very few behavioral effects before death. An occasional loss of equilibrium was observed just prior to death. Compared with the other four xenobiotics tested, 2,4-DNP displayed the fewest or no overt behavioral and morphological symptoms.

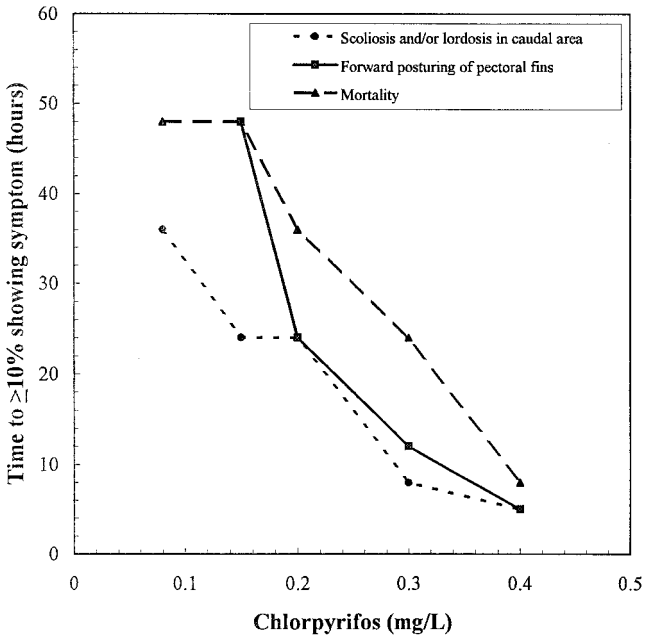

Fig. 1. Initial onset of morphological responses and mortality of 30-d-old *Oryzias latipes* exposed to chlorpyrifos. Data points plotted include additional chlorpyrifos exposure concentrations not shown in Table 5.

2,4-Dinitrophenol is a weakly acidic uncoupler of oxidative phosphorylation. Uncoupling occurs when target membranes become permeable to H^+, causing dissipation of the H^+ gradient [39]. Drummond et al. [2,3] monitored the behavioral effects of uncouplers to 30-d-old *P. promelas*. Chemical uncouplers were observed to cause a loss of equilibrium, an increased opercular rate, and hyperactive spontaneous swimming in *P. promelas.*

Strychnine. Strychnine-induced symptoms included a loss of equilibrium and convulsions, which eventually led to tetany (Table 2). Severely intoxicated fish ceased to swim and respond to external stimuli. Signs of stress occurred at 2 h and 24 h for ≥10 mg/L and ≥1 mg/L, respectively. Mortality occurred soon after the advanced signs (convulsions and tetany) of intoxication. The onset time of these behavioral responses decreased with greater exposure concentrations.

Strychnine is an alkaloid convulsant that blocks inhibitory pathways mediated by Renshaw cells in the spinal cord, resulting in hyperresponsiveness to sensory stimuli and convulsions [40]. Signs of stress observed in strychnine-exposed 30-d-old *O. latipes* were similar to responses reported for other fish species [2,3,30]. Drummond and Russom [3] monitored behavioral responses in juvenile *P. promelas* exposed to strychnine and they categorized strychnine hemisulfate salt under the physical deformity syndrome. Signs of acute toxicity for this syndrome included overreactive or underreactive response to external stimuli, body spasms, increased opercular rate, convulsions, lordosis/scoliosis, hemorrhages near the vertebral column, and tetany [3]. Fish in an extremely intoxicated state were often less responsive to external stimuli [2].

Phenol. Behavioral effects observed in *O. latipes* exposed to phenol consisted of loss of equilibrium and initial agitated hyperactive spontaneous swimming, followed by hypoactive swimming (Table 2). Extremely hyperactive *O. latipes* did not respond to external stimuli. Signs of phenol-induced intoxication occurred at sublethal levels (48-h LC50 = 24.1 mg/L). Agitated swimming movements were observed within 1.5 h in *O. latipes* exposed to phenol concentrations ≥9 mg/L. The onset time for the loss of equilibrium decreased with higher phenol exposure concentrations (24 h for ≥10 mg/L versus 8 h for ≥32 mg/L).

Results from the present study were consistent with behavioral effects reported in other fish species. Phenol, a polar narcotic, causes anesthesia or narcosis. Drummond and Russom [3] classified phenol under the hyperactivity syndrome. Signs of acute toxicity for this syndrome include hyperactive spontaneous swimming, overreactive and/or no response (severely intoxicated fish) to external stimuli, abdominal edema, and mortality within 24 h. Colgan et al. [33] reported that adult *P. reticulata* were intoxicated at sublethal phenol concentrations of >20 mg/L (96-h LC50 was 40 mg/L). Adverse behavioral symptoms included a quick appearance of agitated swimming, loss of equilibrium, and eventually, slower swimming movements. An increase in body pigmentation was also noted [33]. Other effects of phenol include increased respiration along with rapid swimming and overreaction to external stimuli followed by depressed activity [41]. The initial rapid swimming and hyperactivity followed by hypoactivity in fish may be caused by transient chemical irritation. Long-term effects to lower phenol concentrations include necrotic changes in fish tissues including gills, kidney, liver, and skin [41].

Table 3. Comparisons of the initial onset (≥10%) of behavioral responses, morphological responses, and mortality, showing the greater sensitivity of the behavioral and morphological tests

Chemical	Water concn. (mg/L)	Time for ≥10% response (h)	
		Behavioral or morphological	Mortality
Permethrin	0.010	24	36
	0.040	<8	24
Chlorpyrifos	0.20	24	36
	0.30	8	24
2,4-Dinitrophenol	0.50	—[a]	12
	0.75	—[a]	12
Strychnine	1.0	24	48
	10.0	8	24
Phenol	15.0	5	36
	30.0	3	24

[a] Behavioral and morphological change or sign of stress was not evident before mortality.

Comparisons of behavioral effects and acute toxicity

Comparisons were made between the initial behavioral responses, morphological responses, and mortality for each of the chemicals tested (Table 3). The initial onset time was defined as the amount of time needed for ≥10% of the fish to show an abnormal response. We selected the ≥10% as the time of onset to prevent artifacts in the data.

The present study showed that behavioral and morphological responses were more sensitive endpoints of toxicity than was mortality (Table 3). Each of the chemicals tested, except for 2,4-DNP, elicited a distinct pattern of behavior or set of behavioral responses. These abnormal responses were consistently observed at concentration levels below the 48-h LC50. In addition, as the exposure concentrations increased the initial time of onset for the effect decreased. Behavioral and morphological changes can be used as important diagnostic tools for determining the source of an environmental contaminant and its mode of action.

Tissue residues

Permethrin. Tissue residue analysis for permethrin is presented in Table 4. Permethrin accumulated in the tissues of 30-d-old *O. latipes*, with residue levels for surviving fish at 48 h of 5 to 23 times greater than the water concentrations. Residues in all surviving fish were similar regardless of the permethrin exposure concentrations; means ranged from 0.113 to 0.121 μg/g. Fish that survived the 0.025-mg/L concentration had significantly greater accumulation of permethrin than was detected in fish that died during exposure to this concentration. Lower levels of permethrin in the deceased fish may result from the quick tissue decomposition and the release of oils following death. Residue levels associated with death increased at higher permethrin exposure concentrations. Permethrin residues in tissues of dead *O. latipes* exposed to ≥0.030 mg/L were significantly greater than those exposed to ≤0.025 mg/L.

Permethrin accumulates in the tissues of several fish species including adult *O. latipes* [18,37,38]. Spehar et al. [38] found that juvenile *P. promelas* accumulated permethrin after a 30-d exposure. The mean residue levels ranged from 0.19 to 4.51 mg/g. Hansen et al. [37] reported that accumulation in *C. variegatus* increased at higher permethrin water concentra-

Table 4. Accumulation of permethrin in 30-d-old *Oryzias latipes* tissue following a 48-h exposure

Mean water concentration[a,b] (mg/L)	Measured residue concentration[b,c,d] (μg/g)	
	Surviving	Dead
<0.005 ± 0.0004 (4)	0.113 ± 0.043 (4) A,B,C,D	—[e]
0.010 ± 0.002 (4)	0.114 ± 0.095 (4) A,B,C,D	0.044 ± 0.040 (5) A,B
0.025 ± 0.002 (3)	0.121 ± 0.066 (10) D	0.045 ± 0.017 (5) B
0.030 ± 0.002 (4)	—[e]	0.164 ± 0.123 (3) C,E
0.035 ± 0.004 (4)	—[e]	0.190 ± 0.057 (3) D,E

[a] Average of the 0-h, 24-h, and postexposure (48-h) water concentrations; detection limit was 0.005 mg/L.
[b] Mean ± SD (*n*).
[c] Reported on whole-body wet-weight basis; detection limit was <5 ng/g.
[d] Means with the same letter are not significantly different ($p = 0.05$).
[e] Insufficient sample size.

tions. They found levels of 0.46 and 5.7 mg/g in *C. variegatus* exposed to 0.001 and 0.01 mg/L permethrin, respectively. Kikuchi et al. [18] reported an accumulation of 1 to 6 mg/g permethrin in adult *O. latipes* following a 0.038- to 0.060-mg/L 48-h static exposure. They noted greater accumulation in the tissues of dead fish. The fish that survived had residue levels of 4 mg/g or less, in contrast to 3 to 6 mg/g in dead fish. Similarly, in this study, mean concentrations in surviving fish did not exceed 0.121 μg/g, whereas residues associated with mortality reached a mean of 0.190 μg/g.

Chlorpyrifos. Chlorpyrifos accumulated in *O. latipes* tissues (Table 5). Residue levels in the surviving fish at 48 h were 727 to 1,143 times greater than the water concentrations. The accumulation of chlorpyrifos in the surviving fish was greater at higher chlorpyrifos concentrations. Residue levels in surviving fish tissues were significantly greater than in dead fish at concentrations ≥0.301 mg/L. Lower chlorpyrifos residue levels in tissues of the dead *O. latipes* may be a result of: severe intoxication, reducing the uptake rate of chlorpyrifos; rapid death precluding substantial accumulation; fish being less equipped to metabolize chlorpyrifos to the active metabolite; and tissues decomposing, thus losing lipids and lipophilic compounds following death.

Chlorpyrifos is a lipophilic compound that concentrates somewhat in the tissues of aquatic organisms. Goodman et al. [42] observed an increase in chlorpyrifos whole-body residues in California grunion (*Leuresthes tenuis*) at higher chlorpyrifos water concentrations. They found that *L. tenuis* accumulated 0.58 mg/g of chlorpyrifos during a 26-d exposure to 0.0013 mg/L. Jarvinen et al. [27] reported bioconcentration factors (BCFs) of approximately 1,700 for *P. promelas*. Smith et al. [24] reported rapid accumulation of radiolabeled chlorpyrifos in *C. auratus* during the first 10 h of exposure, and the maximum levels were reached within 12 h.

Table 5. Accumulation of chlorpyrifos in 30-d-old *Oryzias latipes* tissue following a 48-h exposure.

Mean water concentration[a,b] (mg/L)	Measured residue concentration[b,c,d] (μg/g)	
	Surviving	Dead
0.052 ± 0.036 (4)	37.8 ± 21.5 (4) A	30.9 ± 17.3 (6) A
0.194 ± 0.022 (12)	145 ± 92.1 (7) B,C	51.8 ± 28.2 (8) A,B
0.301 ± 0.025 (6)	260 ± 169 (10) D	89.2 ± 55.9 (7) A,B
0.343 ± 0.029 (4)	358 ± 106 (4) D	119 ± 75.6 (3) A,C
0.405 ± 0.024 (4)	463 ± 184 (5) E	155 ± 65.5 (3) C

[a] Average of the 0-h, 24-h, and postexposure (48-h) water concentrations; detection limit was 0.001 mg/L.
[b] Mean ± SD (*n*).
[c] Reported on whole-body wet-weight basis; detection limit was 1 ng/g.
[d] Means with same letter are not significantly different ($p = 0.05$).

CONCLUSIONS

A distinguishing behavior or set of behavioral and morphological symptoms was observed for four of the five tested chemicals. Changes in behavior and morphology were proven to be more sensitive diagnostic endpoints than was mortality. Thirty-day-old *O. latipes* and these selected behavioral and morphological symptoms may be useful for monitoring effluents, for predicting the mode of action of unknown xenobiotics, and for testing water from wastewater treatment facilities before its discharge.

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APPENDIX

Operational definitions of behavioral/morphological responses caused by a toxicant (modified from R.A. Drummond [1991, unpublished] and Drummond et al. [2])

Behavioral and morphological symptom	Description
Loss of equilibrium	Fish roll over on side or back; fish may hang vertically (sporadic or sustained)
General activity	
Hyperactive	Fish swim faster than control fish; dart around tank without being provoked (nearly continuous)
Hypoactive	Fish swim slower than control fish; lethargic; may be almost inactive; motionless
Agitated	Abnormal, erratic swimming movements (usually sustained); frenzied swimming; exaggerated movements
Abnormal lateral flexure	Occasional, strong unilateral spasms during swimming; fish temporarily bend in a C-shape with the tail nearly touching the head
Convulsions	No swimming; continuous ataxia with intermittent body spasms; violent shaking
Startle response to experimental or applied stimuli (see methods)	
Overreactive	Fish are hyperexcitable; dart away from stimuli faster than the control fish
Underreactive	Fish dart away from stimuli slower than control fish; lethargic; may be unreactive to stimuli
Hemorrhage	Bleeding (subcutaneous or cutaneous) in the caudal area or around gills, fins, anus, or eyes
Deformities and postural indicators	
Lordosis	Sustained, abnormal spinal curvature; dorsal surface convex (no lateral bending)
Scoliosis	Sustained, abnormal lateral bending to either side
Pectoral fins (forward)	Pectoral fins are held perpendicular to the body or in a more forward posture toward the head (45° to 90° from the head); little or no movement of the pectoral fins