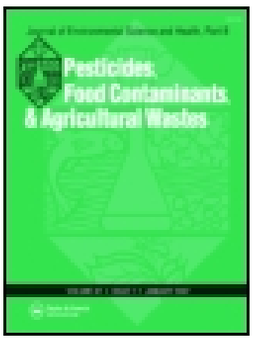

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# Toxicity of fipronil and its enantiomers to marine and freshwater non-targets

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Fipronil is a phenylpyrazole insecticide used in agricultural and domestic settings for controlling various insect pests in crops, lawns, and residential structures. Fipronil is chiral; however, it is released into the environment as a racemic mixture of two enantiomers. In this study, the acute toxicity of the (S,+) and (R,−) enantiomers and the racemic mixture of fipronil were assessed using *Simulium vittatum* IS-7 (black fly), *Xenopus laevis* (African clawed frog), *Procambarus clarkii* (crayfish), *Palaemonetes pugio* (grass shrimp), *Mercenaria mercenaria* (hardshell clam), and *Dunaliella tertiolecta* (phytoplankton). Results showed that *S. vittatum* IS-7 was the most sensitive freshwater species to the racemic mixture of fipronil (LC50 = 0.65 $\mu$g/L) while *P. pugio* was the most sensitive marine species (LC50 = 0.32 $\mu$g/L). *Procambarus clarkii* were significantly more sensitive to the (S,+) enantiomer while larval *P. pugio* were significantly more sensitive to the (R,−) enantiomer. Enantioselective toxicity was not observed in the other organisms tested. Increased mortality and minimal recovery was observed in all species tested for recovery from fipronil exposure. These results indicate that the most toxic isomer of fipronil is organism-specific and that enantioselective toxicity may be more common in crustaceans than in other aquatic organisms.

**Keywords:** Enantiomers; fipronil; freshwater; marine; non-targets; recovery; toxicity.

## Introduction

Fipronil is a phenylpyrazole insecticide marketed in the United States for control of insect pests in both agricultural and domestic settings. It is highly selective in its toxicity, having greater affinity for arthropod $\gamma$-aminobutyric acid (GABA) receptors than mammalian receptors.[1–2] Binding at the GABA receptor disrupts the chloride-gated channels, resulting in loss of neuronal signaling, hyperexcitation, and ultimately, death.[3–4]

Fipronil is chiral, occurring as two nonsuperimposable mirror images called enantiomers. Chiral compounds can be designated as (+) or (−), based on their rotation of plane polarized light, or as (R) or (S), based on the three dimensional structure of the molecule.[5] By comparison of the enantiomer separation technique in this study to that of Tiecher et al. [6], it was determined that the absolute configuration of fipronil is (R,−), (S,+).

Fipronil is manufactured as a racemate, containing 50% of each enantiomer. Thus, an equal amount of each enantiomer is released into the environment during application. Although enantiomers have identical physical and chemical properties and abiotic degradation rates [7], their individual toxicity, biological activity, and microbial degradation rates have been shown to differ.[7–13] Thus, fate, exposure and effects data for each enantiomer should be evaluated when assessing the risk of chiral pesticides to human and wildlife populations.[14]

As mentioned, enantioselective toxicity has been demonstrated with several chiral pesticides [12] including fipronil [13] with water fleas (*Ceriodaphnia dubia* and *Daphnia magna*). However, these results might not be consistent for all classes of organisms. For example, Teicher et al. [6] observed no statistical difference in the toxicity of fipronil and its enantiomers to three pest species of the class Hexapoda. With this variation in toxicity of fipronil and its enantiomers among organisms, it was of interest to further investigate the

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enantioselective toxicity of this insecticide across a wider range of non-target aquatic species.

In this study, the toxicity of fipronil and its enantiomers was assessed using three freshwater organisms, black fly larvae (*Simulium vittatum* IS-7), tadpoles (*Xenopus laevis*), and crayfish (*Procambarus clarkii*) and three marine organisms, grass shrimp (*Palaemonetes pugio*), clams (*Mercenaria mercenaria*), and phytoplankton (*Dunaliella tertiolecta*). These organisms represent two water conditions (freshwater and marine) and five classes of organisms (Hexapoda, Amphibia, Malacostraca, Bivalvia, and Chlorophyceae). Differences in toxicity among the enantiomers and the racemic mixture were assessed through comparison of median lethal concentration (LC50) values generated in acute (48 or 96 h) toxicity tests. Recovery of impaired organisms from exposure to the enantiomers and the racemate was also assessed.



## Materials and Methods

### *Organisms*

#### *Black flies*

Fourth-fifth instar black fly larvae, *S. vittatum* Zetterstedt cytospecies IS-7, were obtained from the University of Georgia (Athens, GA, USA) colony and were reared following the protocols of Gray and Noblet. [15] This population of black flies has been in colony since 1981 and is still genetically similar to the wild type.[16]

#### *Crayfish*

Crayfish, *P. Clarkii,* were obtained from the Louisiana State University Agricultural Center (Baton Rouge, LA, USA). Crayfish were shipped to the University of Georgia and immediately transferred to Nalgene® holding tanks containing 170 L of aerated, moderately hard water.[17] Sixty crayfish were placed in each of two tanks and fed 13.5 g of Advanced Nutrition Rabbit Chow (Purina Mills, St. Louis, MO USA). A 50% water change was conducted at approximately 30 h into the holding period. Crayfish were fed an additional 13.5 g of food at 48 h. Experiments were initiated after a 72 h holding period. Crayfish used in the experiments were between 7.1 and 10.5 cm in length (rostrum to telson).

#### *Tadpoles*

*Xenopus laevis* tadpoles were obtained from Carolina Biological (Burlington, NC, USA) and housed in a plastic container of dechlorinated water in an environmental chamber at 24–26°C, with a 16:8 h light:dark photoperiod. Tadpoles were fed NASCO Tadpole Food (NASCO, Fort Atkinson, WI) upon arrival and allowed 6 h to adjust to their new environment.

#### *Grass Shrimp*

Adult grass shrimp (*Palaemonetes pugio*) (approximate length 25 mm) were collected from Leadenwah Creek (32° 38.930′ N, 80° 13.340′ W), a relatively uncontaminated tidal tributary of the North Edisto River estuary, SC. Shrimp were acclimated for 7–14 days in 76-L tanks at 25°C, 20 ppt salinity, and a 16:8 h light:dark photoperiod. Shrimp were fed a mixture of Tetramin® Fish Flakes and newly hatched *Artemia*. For larval testing, gravid females were placed in brooding traps to allow larvae to hatch and escape without interference. Larvae from at least 10 females were pooled for all tests.

#### *Clams*

Juvenile clams, *Mercenaria mercenaria* (size range 212–350 $\mu$m), were acquired from Atlantic Farm Inc., a commercial aquaculture facility located on James Island, SC. Typically at this life-history stage, the animal has developed a functional foot and settled to the benthos (one week post-settlement). Juvenile clams were acclimated for 48 h at 20°C, 30‰ salinity, and a 12:12 h light:dark photoperiod. Clams were fed 50 mL daily of *Isochrysis galbana* (6–8 million cells per mL) initially obtained from Atlantic Farms Inc.

#### *Phytoplankton*

A parent culture of *Dunaliella tertiolecta* was obtained from the University of Texas Culture Collection and then sterile transferred into F/2 marine media (20 ppt salinity).[18] Stock cultures of *D. tertiolecta* were kept in an environmental chamber at 25°C under cool-white fluorescent lighting ($86+/-$ 8.6 $\mu E/m^2/s$), with a 16:8 h light:dark photoperiod, and continual mixing using an orbital shaker. Cultures were sterile-transferred as needed to maintain log phase growth.

### *Test Chemical*

Fipronil (± 5-amino-1-[2,6-dichloro-4-[trifluoromethyl]-phenyl]-4[[trifluoromethyl]-sulfinyl]-1 *H* pyrazole-3-carbonitrile; 98% pure) was obtained from ChemService (West Chester, PA, USA). Enantiomers of fipronil were separated by Chiral Technologies (Exton, PA, USA) as described by Konwick et al. [13] Stock solutions of fipronil and its enantiomers were prepared by dissolving a weighed amount of the dry material in pesticide grade acetone and stored at 4°C.

### *Chemical Analysis*

Chemical analysis was conducted on exposure water from the black fly, crayfish, tadpole, clam, and grass shrimp tests. There was not enough volume to quantify concentrations in the phytoplankton test. Fipronil and its enantiomers were extracted from water using solid-phase extraction (SPE) as described by Konwick et al. [13] and analyzed by gas

**Table 1.** Comparision of test methods for all species used in this study.

| *Species* | *Life stage* | *Exposure duration* | *Test endpoint* | *Test media* | *Photoperiod* | *Temp.* |
|---|---|---|---|---|---|---|
| Black fly (*S. vittatum* IS-7) | $4^{th}$-$5^{th}$ instar larvae | 48h non-renewal | mortality | moderately hard water | 16h:8h light:dark | 20°C |
| Crayfish (*P. clarkii*) | Adult, 7.1–10.5 cm length | 96h static, non-renewal | mortality | moderately hard water | 16h:8h light:dark | 20°C |
| Frog (*X. laevis*) | tadpole | 96h static, renewed after 48h. | mortality | dechlorinated water | 16h:8h light:dark | 25°C |
| Grass shrimp (*P. pugio*) | adult | 96h static, renewed every 24h. | mortality | 20 ppt seawater | 16h:8h light:dark | 25°C |
| Grass shrimp (*P. pugio*) | 1–2 day old larvae | 96h static, renewed every 24h. | mortality | 20 ppt seawater | 16h:8h light:dark | 25°C |
| Grass shrimp (*P. pugio*) | stag VI embryo | 96h static, renewed every 24h. | mortality | 20 ppt seawater (filtered to (20 $\mu$m) | 24h dark | 27°C |
| Clam (*M. mercenaria*) | juvenile (212–350 $\mu$m) | 96h static, renewed every 24h. | mortality | 30 ppt seawater | 12h:12h light:dark | 20°C |
| Phytoplankton (*D. tertiolecta*) | log-phase growth | 96h static, non-renewal | cell density, biovolume | Guillard's f/2 media, 20ppt | 16h:8h light:dark | 25°C |



chromatography/mass spectroscopy (HP 6890/5973, Palo Alto, CA, USA) in selected ion mode using a BGB-172 chiral column (GB Analytik, AG, Anwil, Switzerland). Spike and recovery experiments resulted in 99% recovery of fipronil from water.

### *Toxicity Tests*

Standard toxicity testing methods were applied for the six aquatic species studied. Given the diversity of habitats and taxa encompassed, there were considerable differences in test parameters (See Table 1 for summary of methods).

#### *Black flies*

The toxicity of fipronil and its enantiomers to *S. vittatum* IS-7 was assessed using a 48-h orbital shaker toxicity test [19] with slight modifications [20]. In addition, larval mortality was assessed at 20× magnification due to observations made during previous testing with fipronil [20] in which slight twitching movements of the labral fans were difficult to observe with the naked eye. Six insecticide concentrations were prepared in 100-mL volumetric flasks by spiking test water (moderately hard water) with insecticide stock solution. The maximum volume of stock solution used for preparation of the treatment solutions was 100 $\mu$L (0.07%). The contents were then emptied into 250-mL amber bottles until treatment. During treatment, 5 mL of the prepared solutions were added to the appropriate flasks creating test concentrations of 2.0, 1.0, 0.5, 0.25, 0.125, and 0.06 $\mu$g/L inside the flasks. Approximately 300 mL of spiked water was collected before and after each test from the highest and lowest concentrations tested and stored in amber bottles with Teflon lids at −20°C for analysis of fipronil concentrations.

Six insecticide concentrations and two controls, (a test-water and a carrier (acetone) control), were tested on one shaker with five flasks per concentration and control, bringing the final totals to 40 flasks and 600 larvae. The carrier control had an acetone content equivalent to the maximum volume of stock insecticide used in preparation of the treatment solutions. Each of the enantiomers or racemate was tested on a separate shaker on the same day and replicated three times over the course of three weeks. Tests were conducted with an ambient air temperature of ∼20°C and a 16:8 h light:dark photoperiod. Tests were considered valid if control mortality was <10%. Water quality (temperature, dissolved oxygen, conductivity, and pH) was measured in the controls before and after each experiment.

#### *Crayfish*

*Procambarus clarkii* were exposed to fipronil and its enantiomers in a 96-h static, non-renewal toxicity test. Crayfish were randomly selected from the holding tanks and five were placed into each of twenty, 40-L glass aquariums (6 aquariums/enantiomer or racemate; 2 carrier controls) containing 6 L of insecticide-treated moderately hard water. All tanks were aerated to keep dissolved oxygen elevated. Serial dilutions of the stock material were prepared in 400 mL of moderately hard water. The maximum amount of stock material used in preparation of the high dose was 9.6 mL. Two-hundred mL of the treatment solution were added to the appropriate aquarium creating concentrations of 400, 200, 100, 50, 25, and 12.5 $\mu$g/L. The carrier controls had an acetone content equivalent to the maximum volume of stock insecticide used in preparation of the treatment solutions (0.16%). Initial exposure concentrations were determined by analyzing a 25 mL aliquot from each of the aquariums containing the highest concentration of the respective enantiomer or racemate. For the low concentration, a 300 mL surrogate was treated in equal proportion to the 6 L of test water. Approximately 300 mL of water was collected for fipronil analysis after each test

from the highest and lowest concentrations tested for chemical analysis. All samples were stored in amber bottles with Teflon lids at −20°C. Each of the enantiomers or racemate was tested on the same day and replicated three times over the course of three weeks. Tests were conducted with an ambient air temperature of ∼20°C and a 16:8 h light:dark photoperiod. Tests were considered valid if control mortality was <10%. Mortality (lack of movement after prodding) was assessed at 24-h intervals over the course of the experiment. Any dead crayfish were removed from the aquarium at this time. Water quality (temperature, dissolved oxygen, conductivity, and pH) was measured in the controls before and after each experiment.

*Tadpoles*

The protocol used for acute toxicity testing was adapted from Mann and Bidwell.[21] Stock solutions of 2 mg/mL fipronil were prepared in 99.6% High-Performance Liquid Chromatography (HPLC) grade acetone for each enantiomer, as well as the racemate, and stored under refrigeration in amber glass vials. Stock solutions were diluted to 2, 1, 0.5, and 0.25 mg/L in soft water (SW; 10% v/v Perrier® in Milli-Q water) giving a total of 12 test solutions (4 concentrations each of 2 enantiomers and the racemate). A 0.5% acetone stock solution was created by diluting 5 mL of HPLC grade acetone to 1 L. All of the solutions except that of the highest concentration were mixed with the appropriate amount of this stock to obtain 0.1% acetone overall, including the carrier control. Approximately 325 mL of each of the 12 test solutions were poured into 600 mL glass beakers, with three replicates each. Five tadpoles were added to each beaker, for a total of 15 test organisms exposed to each solution. Similarly, 5 tadpoles were placed in nine carrier-control beakers to give a total of 45 control organisms. All beakers were returned to the same incubator. Fresh solutions were made after 48 h and the tadpoles transferred to these for another 48 h, for a total of 96 h of exposure. Dissolved oxygen (DO), temperature, and pH were monitored throughout all experiments. Fifteen mL samples of each solution were frozen in amber glass jars for fipronil analysis.

*Grass shrimp*

Aqueous static renewal 96-h bioassays [22] were conducted with the racemic fipronil using three age groups (Stage VI embryos, one to two day hatched larvae, and adults) (results previously reported in Key et al.[23]). The (S,+) and (R,−) enantiomers were tested with larval and adult shrimp only. Larval and adult shrimp tests were conducted in a Revco® environmental chamber at 25°C, 20 ppt salinity, and a 16:8 h light:dark photoperiod. Before each daily media change (24 h), water quality parameters (dissolved oxygen, pH, temperature, and salinity) were measured in the seawater controls. Pesticide grade acetone was used as the carrier in all tests. The final acetone concentration (0.1%) was equivalent in the control and pesticide treatments.[24]

Larvae used for all tests were 1–2 days old and exposed in 600 mL glass beakers containing 400 mL of media with 10 larvae/beaker and three replicates/treatment. The concentrations tested were 0, 0.125, 0.25, 0.5, 1.0, 2.0 $\mu$g/L for both enantiomers. Adult shrimp were exposed in 4L wide-mouth glass jars containing 2L of media and 10 shrimp/jar with three replicates/treatment. Mortality was assessed daily. Larvae were fed newly hatched *Artemia* after each daily media change. Adults were not fed during the exposure.[25]

*Clams*

Aqueous toxicity was assessed using 96-h static renewal bioassays. All tests were conducted as serial dilutions and received the same carrier concentration as the controls. Nominal concentrations tested with racemic fipronil were 1.9, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 $\mu$g/L. Nominal concentrations tested with the individual enantiomers were 37.5, 75, 150, 300, and 600 $\mu$g/L.

The aqueous toxicity bioassays were conducted in 16 oz. glass jars each containing 180 mL of test solution, with five replicates for each test concentration. Thirty clams were added to each jar, and the jars were placed in a Revco® Environmental Chamber under aeration. The test was conducted under culture conditions described above. Clams were not fed during the exposures. Water quality parameters (temperature, dissolved oxygen, salinity, and pH) were recorded daily during the exposure period from each control replicate. The test was renewed daily, and mortality was assessed at that time. Clam mortality was determined by visible inspection using an Olympus SZH10 Microscope under 7.0× magnification. Clams were determined to be alive if locomotion was exhibited within five minutes of observation on the scope. Dead organisms were obvious, based on a gaping shell.

*Phytoplankton*

Standard 96-h static algal toxicity bioassay protocols [26] were employed to determine the effective pesticide concentrations that reduce population growth rate by 50% (EC50). Fipronil stock solutions were prepared in 100% acetone and the doses were administered to obtain a final acetone concentration of 0.1% in each treatment and control. Nominal concentrations of the racemate tested were 63, 125, 250, 500, and 1000 $\mu$g/L. Nominal concentrations of the (S,+) and (R,−) enantiomers tested were 100, 200, 400, 800, and 1600 $\mu$g/L.

Three replicate test tubes were used for each treatment, each containing 25 mL of media. Each tube was inoculated from a sterile culture flask to provide an initial cell density of approximately 50,000 cells/mL. The test was conducted under culture conditions described above, and tubes were repositioned within the environmental chamber each day to minimize possible spatial differences in illumination and temperature on growth rate.

Cell density was assessed at approximately the same time each day (0, 24, 48, 72, and 96 h) via direct counts. A minimum of 18 grids or 400 cells was counted from 100 $\mu$L aliquots on an improved Neubauer hemacytometer. Two homogenized samples were counted per tube per time period. Cellular biovolume was estimated at 96 h microscopically according to Wetzel and Likens. [27] Dimensions of 25 randomly selected cells from each control and treatment replicate were measured using an ocular micrometer. Cellular biovolume ($\mu m^3$ $cell^{-1}$) was then calculated using measured cell dimensions and the equation for cylindrical volume.

### *Recovery Tests*

Additional tests were conducted to determine if organisms could recover from exposure to the enantiomers or the racemate. After assessment at 48 or 96 h (see test durations for individual organisms), organisms alive or impaired from the lowest treatment level producing mortality or impairment were transferred to clean test water for an additional 48 h (24 h for grass shrimp). Due to the inconsistency of impaired organisms among enantiomers and the racemate in the crayfish experiments at the lower treatment levels, impaired crayfish from all treatment levels were used. Control organisms were transferred into similar water for comparison. Individuals were then reassessed after the designated time using similar techniques as in the toxicity tests to determine recovery (behavior similar to controls), impairment (abnormal behavior), or mortality. Recovery experiments were not conducted for *M. mercenaria* or *D. tertiolecta* due to difficulty in determining impairment in the organisms or for *X. laevis* due to lack of impaired organisms observed.

### *Data Analysis*

For each species, enantioselective toxicity was determined through comparisons of LC50 values generated using the Trimmed Spearman-Karber method.[28] Median lethal concentration values with non-overlapping 95% confidence intervals were considered to be significantly different. If LC50 values from the individual repetitions were not significantly different, data were combined and analyzed as one data set. Otherwise, the median of the three repetitions was reported as the actual LC50.

For phytoplankton, cell count data were log transformed ($\log_{10}+ 1$), the slope of the converted values over time was calculated, and population growth rate (divisions/day) was determined. [29] The data were then analyzed using the linear interpolation method for sublethal toxicity [30], whereby a 96-h EC50 ($\mu$g/L) value was generated for cell density, growth rate, and biovolume. Statistical differences among treatments were determined using analysis of variance (ANOVA). Where ANOVA revealed a significant difference among treatments ($P < 0.05$), Dunnett's procedure for multiple comparisons was used to determine which treatments differed significantly from the control.[31]

## Results

### *Chemical Analysis*

Analytical data for the fipronil analysis is summarized in Table 2. Nominal concentrations of fipronil were not significantly different from measured concentrations in all experiments except with the black flies, where concentrations at 2.0 $\mu$g/L were significantly higher with the (R,−) enantiomer and lower with the racemate (paired t-test, $P < 0.05$). Percent deviations from the nominal were 25% for the (R,−) and 20% for the racemate. Concentrations for the black flies LC50 analysis were adjusted by multiplying the nominal concentration by the percent deviation of the measured concentration and adding the product to the nominal [(R,−) enantiomer] or subtracting the product from the nominal (racemate). Nominal concentrations were used in the LC50 analyses for all the other organisms. No significant difference was detected in concentrations of samples collected at the start compared to those collected at the end of the experiments (paired t-test, $P > 0.05$).

#### *Black flies*

The toxicity of the individual enantiomers to *S. vittatum* IS-7 was similar with 48-h LC50 values of 0.72 $\mu$g/L (95% CI: 0.66–0.78 $\mu$g/L) for the (S,+) enantiomer and 0.74 $\mu$g/L (95% CI: 0.69-0.81 $\mu$g/L) for the (R,−) enantiomer (Table 3). The racemate was slightly more toxic with a 48-h LC50 value of 0.65 $\mu$g/L (95% CI: 0.60–0.70). However, since the 95% confidence intervals overlapped, this difference was not significant.

#### *Crayfish*

Enantioselective toxicity was observed with *P. clarkii* (Table 3). The (S,+) enantiomer was the most toxic with a 96-h LC50 of 81.70 $\mu$g/L (95% CI: 62.90–106.10 $\mu$g/L). The racemate had an LC50 of 124.89 $\mu$g/L (95% CI: 87.20–179.24) and the (R,−) enantiomer had an LC50 of 163.50 $\mu$g/L (95% CI: 124.37–214.94 $\mu$g/L). The (S,+) enantiomer was determined to be significantly more toxic than the (R,−) enantiomer since the 95% confidence intervals did not overlap.

#### *Tadpoles*

The LC50 values for the two enantiomers and the racemate were not significantly different after 96 h of exposure (Table 3). The (R,+) LC50 was 910.00 $\mu$g/L (95% CI: 650–1280 $\mu$g/L), while the racemate had an LC50 value of 850.00 $\mu$g/L (95% CI: 660-1090 $\mu$g/L). The (R,−) enantiomer appeared to have a slightly higher LC50 value than the other two (1140.00 $\mu$g/L; 890.00–1450.00 $\mu$g/L), but the difference was not statistically significant. The (S,+)

**Table 2.** Analytical concentrations of fipronil and its enantiomers from acute toxicity tests*.

| *Organism* | *Test* | *Fipronil* | *Nominal (μg/L)* | *Initial (μg/L)* | *Final (μg/L)* |
|---|---|---|---|---|---|
| Black fly (*S. vittatum* IS-7) | Non-renewal | (S,+) | 2.00 | 2.50 ± 0.23 | 2.19 ± 0.43 |
| | | | 0.06 | 0.07 ± 0.01 | 0.07 ± 0.01 |
| | | (R,−) | 2.00 | 2.49 ± 0.11 | 2.39 ± 0.13 |
| | | | 0.06 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| | | (S,+/R,−) | 2.00 | 1.62 ± 0.08 | 1.79 ± 0.31 |
| | | | 0.06 | 0.06 ± 0.01 | 0.05 ± 0.01 |
| Crayfish (*P. clarkii*) | Non-renewal | (S,+) | 400.00 | 341.80 ± 3.07 | 386.63 ± 22.70 |
| | | | 12.50 | 11.55 ± 0.31 | 14.72 ± 3.57 |
| | | (R,−) | 400.00 | 359.70 ± 8.61 | 378.40 ± 23.39 |
| | | | 12.50 | 11.10 ± 0.80 | 11.32 ± 1.88 |
| | | (S,+/R,−) | 400.00 | 371.20 ± 12.87 | 370.53 ± 15.79 |
| | | | 12.50 | 12.15 ± 1.23 | 11.45 ± 0.35 |
| Frog (*X. laevis*)† | Renewal | (S,+) | 2000.00 | 2030.00 | 1750.00 |
| | | | 250.00 | 250.00 | 240.00 |
| | | (R,−) | 2000.00 | 2140.00 | 1810.00 |
| | | | 250.00 | 215.00 | 210.00 |
| | | (S,+/R,−) | 2000.00 | 2590.00 | 1010.00 |
| | | | 250.00 | 200.00 | 100.00 |
| Grass shrimp (*P. pugio*) | Renewal | (S,+) | 10.00 | 9.23 ± 0.49 | 8.50 ± 0.23 |
| | | | 0.02 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| | | (R,−) | 10.00 | 9.83 ± 0.31 | 11.95 ± 0.93 |
| | | | 0.02 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| | | (S,+/R,−) | NA** | NA | NA |
| Clam (*M. mercenaria*) | Renewal | (S,+) | 600.00 | 638.00 ± 21.87 | 542.00 ± 0.67 |
| | | | 37.5 | 35.60 ± 0.31 | 31.60 ± 0.97 |
| | | (R,−) | 600.00 | 617.00 ± 16.07 | 503.00 ± 70.29 |
| | | | 37.5 | 34.50 ± 1.20 | 31.10 ± 2.33 |
| | | (S,+/R,−) | NA** | NA | NA |

NA= not applicable

*Values reported are the mean of three repetitions ± standard error. Samples were collected from the highest and lowest concentrations tested. In non-renewal tests, samples were taken at the start and end of the test. For renewal tests, samples were taken at the start and prior to the first renewal.

** Analytical data for the racemate (S,+/R,−) were not obtained due to use of mortality data from previously published work.

† Samples from the three repetitions were combined to accommodate the volume needed for the analytical method.



enantiomer did show significant overall toxicity earlier than either the racemate or the (R,−) enantiomer. Enough mortality occurred at 24 h to calculate an LC50 for the (S,+) enantiomer (data not reported), while an LC50 could not be calculated until 72 h and 96 h for the racemate and (R,−) enantiomer respectively.

### *Grass shrimp*

For adult grass shrimp, racemic fipronil previously yielded a 96-h LC50 of 0.32 μg/L (95% CI: 0.24–0.41 μg/L).[23] In this study the 96-h LC50 for adult shrimp and the (R,−) enantiomer was 0.32 μg/L (95% CI: 0.22–0.48 μg/L). For the (S,+) enantiomer, the 96-h LC50 was 0.37 μg/L (95% CI: 0.25–0.57) (Table 3). The overlapping confidence intervals indicate no significant difference among the racemate and the enantiomers.

Larval grass shrimp were less sensitive to racemic fipronil than adult shrimp, with a 96-h LC50 of 0.68 μg/L (95% CI: 0.57–0.80 μg/L).[23] Larval shrimp sensitivity to the (S,+) enantiomer was similar to that of the racemate, with a 96-h LC50 of 0.54 μg/L (95% CI: 0.45–0.64 μg/L), whereas the (R,−) enantiomer yielded a significantly lower 96-h LC50 of 0.35 μg/L (95% CI: 0.29–0.43 μg/L) (Table 3). Grass shrimp embryos were relatively insensitive to fipronil in terms of lethal toxicity, with a 96-h $LC_{50}$ greater than 512 μg/L,[23] and embryo testing with the enantiomers was not conducted.

### *Clams*

*Mercenaria mercenaria* exposed to racemic fipronil had a 96-h LC50 of 177 μg/L (95% CI: 46–674 μg/L). The 96 h LC50 determined for the (S,+) enantiomer was 208 μg/L (95% CI: 137–318 μg/L), and for the (R,−) enantiomer, it was 187 μg/L (95% CI: 124–281 μg/L) (Table 3). The overlapping confidence intervals indicate no significant difference between the racemic mixture and the enantiomers.

### *Phytoplankton*

There was no significant effect of racemic fipronil on cell density or growth rate of *D. tertiolecta* at concentrations up

**Table 3.** Median lethal concentration (LC50) values and 95% confidence intervals (CI) for test organisms exposed to racemic fipronil and its individual enantiomers*.

| *Organism* | *Fipronil* | *LC50 (μg/L)* | *95% CI* |
|---|---|---|---|
| Black fly (*S. vittatum* IS-7) | (S,+) | 0.72[a] | 0.66–0.78 |
| | (R,−) | 0.74[a] | 0.69–0.81 |
| | (S,+/R,−) | 0.65[a] | 0.60–0.70 |
| Crayfish (*P. clarkii*) | (S,+) | 81.70[a] | 62.90–106.10 |
| | (R,−) | 163.50[b] | 124.37–214.94 |
| | (S,+/R,−) | 124.89[ab] | 87.20–179.24 |
| Frog (*X. laevis*) | (S,+) | 910.00[a] | 650.00–1280.00 |
| | (R,−) | 1140.00[a] | 890.00–1450.00 |
| | (S,+/R,−) | 850.00[a] | 660.00–1090.00 |
| Grass Shrimp (*P. pugio*) (Adult) | (S,+) | 0.37[a] | 0.25–0.57 |
| | (R,−) | 0.32[a] | 0.22–0.48 |
| | (S,+/R,−) | 0.32[a] | 0.24–0.41 |
| Grass Shrimp (*P. pugio*) (Larvae) | (S,+) | 0.54[a] | 0.45–0.64 |
| | (R,−) | 0.35[b] | 0.29–0.43 |
| | (S,+/R,−) | 0.68[a] | 0.57–0.80 |
| Clam (*M. mercenaria*) | (S,+) | 208.00[a] | 137.00–318.00 |
| | (R,−) | 187.00[a] | 124.00–281.00 |
| | (S,+/R,−) | 177.00[a] | 46.00–674.00 |
| Phytoplankton (*D. tertiolecta*) | (S,+) | NA | NA |
| | (R,−) | NA | NA |
| | (S,+/R,−) | 631.20** | NA |

*Values for all organisms are based on a 96-h exposure except for *Simulium vittatum* IS-7 which was a 48-h exposure.
Entries with NA indicate that the values were not able to be obtained with the described methods. Statistical differences in LC50 values are denoted by different letters.
**96-h median effective concentration (EC50).

to 1000 μg/L. A 96-h EC50 (using cellular biovolume as an endpoint) was 631.20 μg/L (confidence limits could not be determined) (Table 3). The no-observable-effect (NOEC) and lowest-observable-effect (LOEC) concentrations were 250 μg/L and 500 μg/L, respectively. No significant effect of either fipronil enantiomer on cell density, growth rate, or biovolume was observed at concentrations up to 1600 μg/L, thus 96-h EC50 values could not be determined. Toxicity at concentrations above 1600 μg/L could not be assessed because the fipronil precipitated out of solution.

### *Recovery*

Black fly larvae exposed to 0.06 μg/L of the (S,+), (R,−), and racemate had percent mortalities of 2.3, 3.5, and 1.8%, respectively. The majority of the larvae still alive in each treatment were impaired such that the larvae could not attach to the bottom of the Petri dish during assessment and showed minimal movement. After 48 h in clean water with food, additional mortality was observed. Percent mortality in larvae previously exposed to the (S,+), (R,−), and racemate was 46.3, 65, and 57.1%, respectively (Figure 1). Control mortality was < 1%. Few larvae recovered from the exposures. Percent recovery for the (S,+), (R,−), and racemate was 20.4, 4.3, and 18.6%, respectively.

In the crayfish experiment, no recovery was observed in impaired crayfish; however, an increase in mortality was observed. Percent mortality in crayfish previously exposed to the (S,+), (R,−), and racemate was 50, 57, and 60%, respectively (Figure 1). No control mortality was observed.

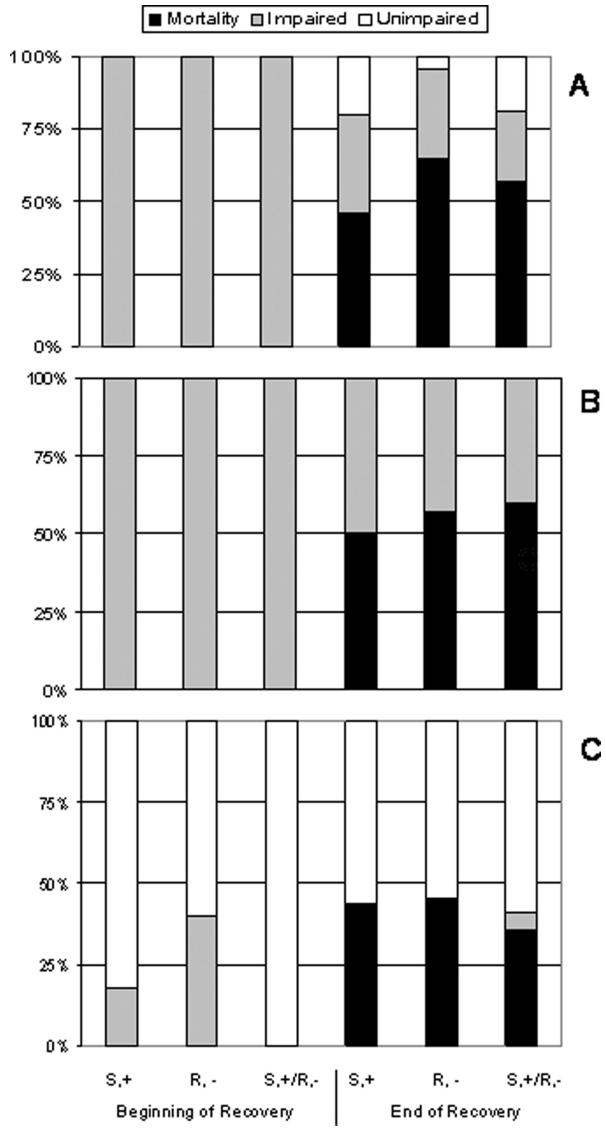


**Fig. 1.** Percent mortality, impaired, and unimpaired organisms at the beginning and end of the recovery experiment; a) *Simulium vittatum* IS-7; b) *Procambarus clarkii*; c) Adult *Palaemonetes pugio*. Impaired organisms showed abnormal behavior or movements. Unimpaired organisms behaved similar to controls.

For adult grass shrimp, the percent mortality resulting from the (S,+), (R,−), and racemate exposures at 2.0 $\mu$g/L were 23%, 33%, and 43%, respectively. Shrimp in the racemate exposure were either dead or alive, whereas in the (R,−) and (S,+) exposures there was 40% and 17.4% shrimp that exhibited impaired swimming ability. Mortality continued to occur in all fipronil exposures after the shrimp were moved to clean water. There was no recovery of impaired shrimp, and the total mortality rates for racemic fipronil and the enantiomers was similar after the recovery phase (Figure 1). Neither recovery nor mortality in the recovery experiment was significant among the enantiomers and the racemate for the three species tested (paired t-test, $P > 0.05$)



## Discussion

### *Enantioselective Toxicity*

Enantioselective toxicity of current-use pesticides has only recently been studied for environmental safety issues. [12,14] In theory, if one enantiomer in a racemic pesticide formulation degrades more rapidly and is less toxic to non-target organisms than the other, formulations enriched in the former enantiomer potentially would cause less of an impact on the environment. Likewise, chiral pesticides in which only one enantiomer is active against the target species could be modified such that only the toxic enantiomer is produced in a single enantiomer or an enriched formulation, decreasing the amount of the inactive isomer that could be active in non-target organisms. These theories cannot be put into practice unless non-target toxicity data exist.

Toxicity assessments of chiral pesticides to non-target organisms to date have been limited.[12,13] In addition, these studies have only focused on one group of non-target organisms, cladocerans. Both Liu et al. [12] and Konwick et al. [13] showed enantioselective toxicity in the chiral pesticides assessed, possibly giving the impression that these pesticides will always show enantioselective toxicity no matter what organism is exposed. However, the data presented in this paper clearly show that enantioselective toxicity is organism specific and might not be as common as previous work has demonstrated.

Of the six species tested in this study, two, crayfish, *P. clarkii*, and larval grass shrimp, *P. pugio*, showed selective sensitivity to a specific enantiomer. Crayfish were significantly more sensitive to the (S,+) enantiomer. This is consistent with the results reported by Konwick et al. [13] in which another crustacean, *C. dubia,* were significantly more sensitive to the (S,+) enantiomer. Thus, GABA receptors in this subphylum could be conserved across lineages and more receptive to the three-dimensional orientation of the (S,+) enantiomer. Contradicting this theory, however, were the larval grass shrimp, which are also crustaceans, but showed significantly more sensitivity towards the (R,−) enantiomer. This is most interesting considering no enantioselective toxicity was observed with the adults. It has been demonstrated that specific subunits of the GABA receptor develop over time in rat brains.[32] Thus it may also be possible that the GABA receptors in the grass shrimp could change during development such that the receptors in the adult bind less selectively than those in the larvae.

Fipronil, formulated as Icon 6.2 FS$^{TM}$, had been used in rice culture to control the water rice weevil, *Lissorhoptrus oryzophilus*, until 2001 when adverse effects to crayfish cultured in rice-growing areas were linked to fipronil exposure.[33] In this situation, a formulation enriched in the (R,−) enantiomer might provide some protection to the crayfish, assuming *L. oryzophilus* was equally sensitive to both enantiomers. However, a great deal of rice and crayfish culturing takes place adjacent to the coast where marine species such as *P. pugio* reside. In addition, fipronil use on agricultural crops, home lawns and turf grass in the coastal zone presents significant potential for estuarine contamination. Because larval *P. pugio* are more sensitive to the (R,−) enantiomer, by attempting to protect one non-target species, another might be at greater risk, making decisions about potential benefits of enantiomer-enriched formulations difficult.

### *Marine vs. Freshwater*

It appears that select marine and freshwater organisms are highly sensitive to fipronil and its enantiomers. In this study, the most sensitive freshwater organism was the black fly, *S. vittatum* IS-7. This is not surprising considering that fipronil has been shown to be highly specific for insect GABA receptors.[1,2] A previous study with fipronil and black flies reported LC50 values ranging between 0.19 and 0.29 $\mu$g/L [20]; much lower than the LC50 reported in this study. However, larval mortality was not assessed with a microscope in the previous study. Thus, small movements by the cephalic fans (observed in this study) would not have been observed, leading to an overestimation of the lethal response. Although black flies were the most sensitive of the freshwater organisms tested in this study, enantioselective toxicity was not demonstrated. Lack of enantioselectivity was also demonstrated by Teicher et al. [6] in three pest species of insects. Apparently, insect GABA receptors are likely able to bind both enantiomers equally making this class of organisms (hexapods) non-responsive to enantiomeric effects.

The most sensitive marine species was the grass shrimp, *P. pugio*. Although only the racemate was shown to affect adults significantly more than the larvae (Table 2), larvae were generally less sensitive to the (S,+) enantiomer. As previously stated, this might be related to the development of the GABA receptor in this organism. Our data suggest that larval grass shrimp might have certain subunits developing at different rates or the receptor in general changes over

time, such that the (R,−) enantiomer binds more efficiently earlier in development than the (S,+) enantiomer.

### *Recovery*

The three species tested for recovery from exposure to fipronil and its enantiomers showed little to no recovery. However, additional mortality during the recovery phase was observed in all three species with approximately 50% of the affected individuals dying in the clean water. Thus, toxic doses were obtained by the organisms even though mortality had not set in. Consequently, the LC50 values reported in this study are likely underestimates of the actual toxicity of fipronil and its enantiomers to these non-target organisms.

## Conclusion

This study illustrates the importance of using multiple species of different phylogenies when assessing the toxicity of chiral chemicals. Although enantioselective toxicity was not observed with the majority of the organisms tested, crayfish and larval grass shrimp were significantly more sensitive to the (S,+) and (R,−) enantiomers, respectively. Thus, a formulation of fipronil enriched in either the (S,+) or the (R,−) would be detrimental to at least one, if not more, non-target organisms. Our results also indicate that life-stage might be important in assessing chiral chemicals if in fact target receptors change over the course of development. These issues should be addressed in future studies.

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