

FIPRONIL EFFECTS ON ESTUARINE COPEPOD (*AMPHIASCUS TENUIREMIS*) DEVELOPMENT, FERTILITY, AND REPRODUCTION: A RAPID LIFE-CYCLE ASSAY IN 96-WELL MICROPLATE FORMAT

G. THOMAS CHANDLER,\*† TAWNYA L. CARY,† DAVID C. VOLZ,† SPENCER S. WALSE,‡ JOHN L. FERRY,‡ and SUSAN L. KLOSTERHAUS§

†Department of Environmental Health Sciences, Arnold School of Public Health,

‡Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, USA

§Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, Solomons, Maryland 20688, USA

(Received 28 February 2003; Accepted 23 May 2003)

**Abstract**—Fipronil is a novel  $\gamma$ -aminobutyric acid receptor-specific phenylpyrazole insecticide commonly used near estuarine environments for rice production, turf-grass management, and residential insect control. In this study, we evaluated the acute, developmental, and reproductive toxicity of fipronil to the estuarine harpacticoid copepod *Amphiascus tenuiremis*. Fipronil was highly toxic to *A. tenuiremis* (adult 96-h median lethal concentration [LC50] = 6.8  $\mu\text{g/L}$ ) and was more toxic to male copepods (96-h LC50 = 3.5  $\mu\text{g/L}$ ) than to non gravid female copepods (96-h LC50 = 13.0  $\mu\text{g/L}$ ). By using a newly developed 96-well microplate-based life-cycle toxicity test, we successfully reared single individuals of *A. tenuiremis* to adulthood in 200- $\mu\text{l}$  microwells and concurrently assessed developmental and reproductive effects (after paired virginal matings) of environmentally relevant aqueous fipronil concentrations (0.16, 0.22, and 0.42  $\mu\text{g/L}$  measured). Throughout the entire life cycle, copepod survival in all treatments was >90%. However, fipronil at 0.22  $\mu\text{g/L}$  and higher significantly delayed male and female development from stage 1 copepodite to adult by approximately 2 d. More importantly, fipronil significantly halted female egg extrusion by 71% in the 0.22- $\mu\text{g/L}$  fipronil treatment, and nearly eliminated reproduction (94% failure) in the 0.42- $\mu\text{g/L}$  fipronil treatment. A three-generation Leslie matrix-based population growth model of fipronil reproductive and life-cycle impacts predicted a 62% decline in population size of *A. tenuiremis* relative to controls at only 0.16  $\mu\text{g/L}$ .

**Keywords**—Fipronil Copepod *Amphiascus tenuiremis* Life-cycle toxicity test Population growth model

## INTRODUCTION

Fipronil—a phenylpyrazole insecticide developed by Rhone-Poulenc Agro (Rhone-Poulenc Agro Chemie, Lyon, France) in 1987—was registered for United States commercial rice production in 1998. Since then, fipronil-based products (with trade names Chipco® Choice, ICON 6.2 FS™, and Over n' Out!™, TechPac, Lexington, KY, USA) have rapidly gained popularity for pest management, including residential insect control, rice and cotton production, and turf-grass management. In insects, fipronil noncompetitively inhibits  $\gamma$ -aminobutyric acid (GABA)-induced ion influx by targeting the GABA-regulated chloride channel [1]. Consequently, fipronil binding blocks the inhibitory action of GABA, leading to hyperexcitation and, in appropriate concentrations, eventual death. Unlike  $\alpha$ -endosulfan, a similar GABA-disrupting insecticide [2], fipronil exhibits >500-fold selective toxicity to insects over mammals, primarily because of affinity differences in receptor binding between insect and mammalian receptors [3,4]. Fipronil's unique metabolic and environmental derivatives—an oxidative sulfone metabolite, a reductive sulfide degradate, and a desulfinyl photodegradation product—are equally toxic to insect GABA receptors [5]. Because GABA receptors are homologous among arthropod species [6], fipronil and its derivatives also are acutely toxic to nontarget crustaceans.

The most extensively studied environment for nontarget

fipronil effects are Louisiana rice and crawfish ponds, where fipronil is applied for control of the rice water weevil (*Lissorhoptrus oryzophilus*)—a pest that causes an annual economic loss of approximately \$10 million (Louisiana Department of Agriculture and Fisheries, Pineville, LA, USA, unpublished data). Fipronil-coated rice seeds are planted in the spring, yielding a late-summer rice harvest. For further economic gain, many harvested rice fields are subsequently cropped with crawfish—a practice that in 1998 caused large-scale, fipronil-induced crawfish kills due to aqueous or sediment-associated exposure.

Unlike many modern pesticides, fipronil and its derivatives are moderately persistent in the environment [7], and can be dispersed widely in high-sediment-resuspension systems such as wetlands and estuaries. For example, in the spring of 1999 through 2001, fipronil was consistently measured in the Louisiana Intracoastal Waterway at 0.3 to 0.8  $\mu\text{g/L}$  as far as 20 mi (32 km) downstream from rice-field sources (U.S. Geological Survey, Baton Rouge, LA, USA, unpublished data). In South Carolina, fipronil is applied by soil injection and granular broadcast on domestic, recreational, and commercial turf-grasses for fire ant control, and on coastal grasses and golf courses for mole cricket control. Presently, few data exist regarding the lethal and sublethal effects of fipronil on estuarine nontarget species despite the high probability of estuarine: fipronil contact. Fipronil is moderately toxic to freshwater daphnids (median lethal concentration [LC50] = 190  $\mu\text{g/L}$ ) [7] and crayfish (LC50 = 14–20  $\mu\text{g/L}$ ) [8], and is extremely toxic to estuarine mysids (LC50 = 0.14  $\mu\text{g/L}$ ) [7] and grass

\* To whom correspondence may be addressed  
(chandlgt@gwm.sc.edu).

shrimp ( $LC_{50} = 0.32 \mu\text{g/L}$ ) [9]. Fipronil also affects mysid reproduction and growth at nominal concentrations as low as  $0.005 \mu\text{g/L}$  [7], well below the limits of fipronil quantitation ( $0.05\text{--}0.10 \mu\text{g/L}$ ) [10]. Fipronil has high fugacity for sediments ( $\log K_{ow} = 4.01$ ) but sensitivities of sediment-dwelling estuarine fauna are unknown.

In this study, we used the meiobenthic copepod *Amphiascus tenuiremis* as a model benthic crustacean to investigate sublethal life-cycle effects of water-borne fipronil. Our objective was to exploit the rapid life cycle of *A. tenuiremis* (16–17 d at  $25^\circ\text{C}$ ) and the peculiar characteristic of being easily cultured in water-only and sediment:water matrices, to develop a 96-well microplate-based life-cycle assay, and test the developmental and reproductive response to environmentally relevant fipronil concentrations. In less than three weeks, this microplate method can evaluate teratogenesis, growth, development rates, sex ratio change, sex-specific fertility, and reproductive success with >90% control survival rates.

## MATERIALS AND METHODS

### Chemicals

Technical-grade fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4[(trifluoromethyl)-sulfinyl]-1H-pyrazole-3-carbonitrile; 98.0% pure) was obtained from ChemService (West Chester, PA, USA), and dissolved in 100% acetone to a stock concentration of 1 g/L. Diluted stock solutions of 10 and 100 mg/L were used to spike treatment solutions.

### Test organisms

*Amphiascus tenuiremis* (Mielke, 1974) is an easily cultured, diosaccid harpacticoid copepod that is amphi-Atlantic in distribution ranging from the North Sea/Baltic intertidal to the southern Gulf of Mexico [11]. *Amphiascus tenuiremis* is a surface to deep-burrowing (0–15 mm) species that preferentially dwells in oxidized muddy sediments and ingests sediments for diatoms and bacteria. Sexes are dimorphic with females and males reaching 0.40 mm and 0.25 to 0.30 mm in length, respectively. Males are streamlined in shape and have a swollen geniculate segment on their first antenna to clasp the female. After clasping, egg fertilization is internal with males extruding a membranous spermatophore sac onto the female genital pore. The life cycle of this species is diagrammed in Figure 1. In toxicant-free microplates at  $25^\circ\text{C}$  and 30‰ salinity, eggs develop in 2 to 3 d and hatch into non-swimming ellipsoid nauplii that grow through six molts in 6 to 7 d and emerge as swimming stage 1 juvenile copepodites. Males and females become sexually mature after the fifth and last copepodite molt (i.e., the 12th life stage), and are visibly sexually dimorphic by the fourth copepodite stage. In this microplate assay, separate males and females are reared individually in single wells (200  $\mu\text{l}$  of spiked seawater per well) either from hatching to adult, or from the first juvenile copepodite stage to adult. Egg-to-adult-to-egg microplate bioassays require 16 to 17 d at  $25^\circ\text{C}$ ; copepodite-to-adult-to-egg assays require only 8 to 10 d. Because copepod reproductive systems develop in the copepodite juvenile phase, the latter bioassay is often the most efficient for evaluating reproductive effects. This latter bioassay was used in our study because we were most interested in evaluating fipronil effects on reproductive success. Once sexually mature, isolated virgin males and females are paired, they mate, and about 80% of females

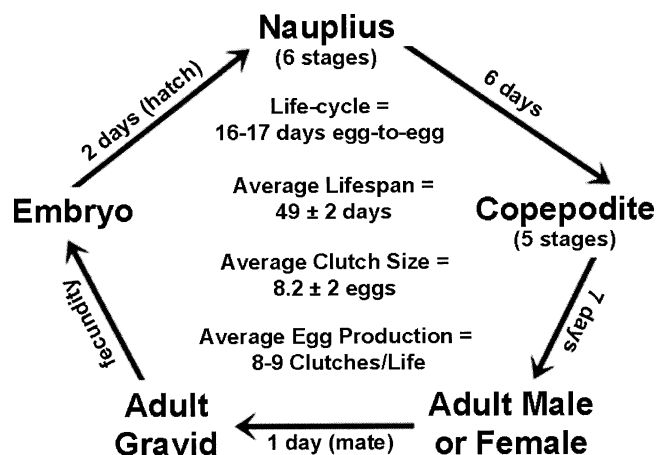


Fig. 1. Life cycle (16–17 d egg-to-egg) of the estuarine harpacticoid copepod *Amphiascus tenuiremis*. In microplates at  $25^\circ\text{C}$  and 30‰ seawater, eggs develop in 2 to 3 d and hatch into nonswimming ellipsoid nauplii that grow through six molts in 6 to 9 d and emerge as swimming stage 1 juvenile copepodites. Adults are sexually mature after the fifth and last copepodite molt. After mating, female egg-clutch extrusion typically occurs in 1 to 2 d.

produce their first egg sacs in 1 to 2 d. Fertilization of isolated virgin females can be successfully postponed by up to 14 d after reaching adulthood, and females normally will not extrude eggs unless paired with a reproductively functional mature male.

### Collection of copepods for toxicity tests

Study copepods were obtained from among 15 flow-through sediment monocultures of *A. tenuiremis* [12,13]. Copepod stock sediment cultures had the following characteristics: toxicant-free muddy sediment substrate consisting of clay, silt, and fine sand particles <0.07 mm in size (median grain diameter  $\sim 0.01\text{--}0.02$  mm); a highly filtered, carbon-polished seawater source saturated with oxygen and relatively free of ammonia ( $<30 \mu\text{g NH}_4/\text{L}$ ;  $\text{pH} = 8.0\text{--}8.3$ ); and a varied diet of a chlorophyte (*Dunaliella tertiolecta*), a chrysophyte (*Isochrysis galbana*), and a diatom (*Phaeodactylum tricorutum*) fed ad libitum twice per week. All copepods were obtained from sediments by aspirating 50 to 100 ml of the upper 0- to 5-mm sediment layer from each culture and separating various life stages by size-selective sieving. Females, males, and copepodites largely retained on the 0.125-mm, 0.090-mm, and 0.075-mm sieves, respectively. Because *A. tenuiremis* is phototactic, sieve fractions were transferred to seawater-filled  $100 \times 20\text{-mm}$  plastic petri dishes and illuminated with fiber optic light pipes, and respective copepod classes collected clean near the light pipe ends using pasteur pipets.

### Acute toxicity test

A 96-h acute toxicity test with adult male and female *A. tenuiremis* was performed to determine median lethal toxicity of fipronil. Four nominal fipronil concentrations (4.3, 7.2, 12.0, and  $20.0 \mu\text{g/L}$ ) and an acetone control ( $<1\text{-ml/L}$ ) were tested. All test glassware was washed with 10% HCl, rinsed three times with deionized water, and rinsed with 100% pesticide-grade acetone before the preparation and addition of control or fipronil treatment solutions. Artificial seawater (30‰ salinity; Instant Ocean®, Aquarium Systems, Mentor, OH, USA) was aerated until dissolved oxygen exceeded >90% saturation and then filtered at  $0.45 \mu\text{m}$ . Seawater was spiked with fipronil

(100 mg/L stock) in a 100-ml volumetric flask with an analytical-grade Wire-Trol® glass capillary ( $\pm 0.2 \mu\text{l}$ ) pipette (Drummond Scientific, Broomall, PA, USA). The control and treatment solutions were transferred to 250-ml glass beakers and homogenized for 1 h in the dark with polytetrafluoroethylene-coated magnetic stirring bars. After mixing, 30 ml of control or treatment solution was added to 50-ml glass crystallizing dishes. Each treatment, including the carrier control, employed four replicates for a total of 20 crystallizing dishes. Ten adult males and 10 nongravid females were transferred into each crystallizing dish in  $<0.2 \text{ ml}$  of seawater by using a pasteur pipet. The chambers were incubated static at  $20^\circ\text{C}$  for 96 h under 12:12 h light:dark conditions. Dissolved oxygen (8.3 mg/L), salinity (30‰), and pH (8.3) were measured at test initiation. At the end of the exposure period, the number of surviving male and female copepods in each replicate was counted.

#### 96-Well microplate life-cycle toxicity test

**Preparation of treatment solutions and microplate wells.** An acetone control and three nominal fipronil concentrations (0.22, 0.36, and  $0.60 \mu\text{g/L}$ ) were prepared in seawater as above. The highest fipronil concentration ( $0.60 \mu\text{g/L}$ ) was targeted to the approximate LC<sub>5</sub> for adult copepods. A 10 mg/L fipronil stock (in acetone) was used to spike all fipronil seawater solutions. All treatment solutions were spiked and homogenized as above. Twelve ultra-low-attachment (i.e., hydrophilic) polystyrene 96-well ( $<300\text{-}\mu\text{l}$  well volume) microplates (Corning Costar, Corning, NY, USA) were hydrated with deionized water for 1 h and allowed to air dry before test initiation. Stage 1 copepodites were size-selectively sieved from sediment monocultures by using a 70- to  $63\text{-}\mu\text{m}$  size inclusion. Randomly selected individual stage 1 copepodites were gently transferred into designated microwells by using glass capillary pipettes silanized with an air-dried solution of 80, 3, and 1.5% ethyl alcohol, isopropyl alcohol, and ethyl sulfate, respectively. This solution coated the inside of the glass pipette with a nontoxic clear polymer that promoted non-sticky transfer of copepodites of *A. tenuiremis*. After copepodite loading, overlying transferred seawater was aspirated under a stereomicroscope by using an analytical-grade 500- $\mu\text{l}$  Hamilton® glass syringe (Hamilton, Reno, NV, USA) so that  $\leq 5 \mu\text{l}$  of seawater remained. This standardized the starting test volume in each well, and allowed for minimal dilution of the treatment solutions by the initial copepod transfer itself. Control and treatment solutions ( $200 \mu\text{l}/\text{microwell}$ ) were then added to the appropriate wells by using an analytical-grade Wire-Trol glass capillary ( $\pm 0.2 \mu\text{l}$ ) pipette. After treatment solutions were added, each well received  $2 \mu\text{l}$  of a fresh, centrifuged,  $10^7$  cells/ml, 1:1 mixed algal cell suspension of *I. galbana* and *D. tertiolecta* by using a Finnpiptette® multi-channel analytical pipettor (Thermo Labsystems, Vantaa, Finland). Each microplate was then covered and placed in a temperature-regulated incubator at  $25^\circ\text{C}$  with an indirect 12:12 h light:dark photoperiod.

**Experimental design.** One hundred forty-four test microwells for each treatment or control were distributed over three microplates per treatment or control—for a total of 12 microplates—to prevent fipronil cross-contamination, to meet statistical assumptions of sample independence, and to minimize concerns of pseudoreplication [14]. For each replicate microplate within a treatment, every other 12-well row was alternated for copepod rearing, and then for female:male pairing

and mating, respectively. This design allowed copepod rearing and mating for each treatment replicate to occur within the same single microplate. Alternating row usage also aided daily copepod well examination by inverted stereomicroscope.

**Renewing treatment solutions.** By using a 500- $\mu\text{l}$  Hamilton glass syringe, treatment solutions in each microwell were aspirated under microscopic observation to approximately  $20 \mu\text{l}$  every 3 d. Care was taken to ensure that copepods were not aspirated into the syringe. If copepods were aspirated, the affected test microwell was disqualified from the test. Fresh treatment solution was prepared as above and then added to aspirated microwells within each microplate. Each microwell containing copepod(s) were fed every 6 d with  $2 \mu\text{l}$  of  $10^7$  cells/ml mixed algal cell suspension as above.

**Copepod rearing, pairing, and mating.** Copepodite survival and development were checked and recorded at the same time daily in each test microwell by using an Olympus CK2 inverted stereomicroscope under  $\times 4$  magnification (Opelco USA, Dulles, VA, USA). The sex of virgin copepods was recorded at reproductive maturity. By 7 or 8 d at  $25^\circ\text{C}$ ,  $>90\%$  of control copepodites had molted into sexually mature adults, but treatment copepodites exhibited delayed development with fipronil concentration. Because matings cannot succeed until reproductive maturity, and it is statistically important that control and treatment matings are contemporaneous, all matings were delayed until  $>80\%$  of treatment copepods had reached sexual maturity. Sexually mature males and females were removed from each control and treatment test microwell by using a silanized glass capillary pipette, and placed briefly into separate male- or female-specific 50-ml crystallizing dishes containing fresh control or respective treatment solution; this ensured a continuous treatment solution exposure. One single male and female copepod were then randomly removed from the appropriate crystallizing dishes and placed into a previously unused microwell from the same microplate as used for rearing. This was performed for the control and fipronil treatments until all wells of the four previously unused alternating rows were successfully filled with a mating pair. All microwells containing mating pairs were then aspirated down to  $\leq 5 \mu\text{l}$  seawater and loaded with  $200 \mu\text{l}$  of fresh control or fipronil treatment solution. Each well received  $2 \mu\text{l}$  of fresh algal mixture prepared as above. Each microplate was covered and placed in a temperature-regulated incubator at  $25^\circ\text{C}$  as above. Each microwell containing a mating pair received fresh treatment solution and algal food every 3 and 6 d, respectively. Each mating pair was checked daily for the following endpoints: male and female survival, days to first female clutch extrusion, and fertility success expressed as percentage of females extruding at least one viable clutch. The test was terminated after 9 d after mating to accommodate any fipronil-induced delays in first clutch release.

**Stage-structured population growth model.** Fipronil effects on population growth of *A. tenuiremis* were estimated by using microplate life-cycle data fitted to a matriarchal stage-structured three-generation Leslie matrix model (RAMAS® EcoLab 2.0, Applied Biomathematics, Setauket, NY, USA) [15–17]. The three-stage (copepodite-to-virgin female-to-gravid female) matrix model determined projected population sizes based on stage-specific survival rates, the proportion of copepodites developing into virgin females, the proportion of females producing at least one viable clutch, and fecundity (i.e., hatched nauplii per female). Our use of this model here assumes that all nauplii developed into stage 1 copepodites,

Table 1. Adult fipronil 96-h median lethal concentration (LC50) values ( $\mu\text{g/L}$ ) with confidence intervals for various freshwater and estuarine crustaceans

Species	Formulation	Measured 96-h LC50	95% Confidence interval	Source
<i>Americamysis bahia</i> (mysid)	Fipronil <sup>a</sup>	0.14	— <sup>b</sup>	[7]
<i>Palaemonetes pugio</i> (grass shrimp)	Fipronil	0.32	0.24–0.41	[9]
<i>Leptocheirus plumulosus</i> (amphipod)	Fipronil	0.72	0.64–0.81	— <sup>c</sup>
<i>Amphiascus tenuiremis</i> (male copepod)	Fipronil	3.5	2.5–5.0	Current study
<i>Amphiascus tenuiremis</i> (adult copepod)	Fipronil	6.8	5.4–8.7	Current study
<i>Amphiascus tenuiremis</i> (female copepod)	Fipronil	13.0	9.6–17.6	Current study
<i>Procambarus clarkii</i> (red swamp crayfish)	Fipronil	14.3	5.2–23.4	[8]
<i>Procambarus zonangulus</i> (white river crayfish)	Fipronil	19.5	11.1–27.9	[8]
<i>Procambarus clarkii</i> (red swamp crayfish)	ICON 6.2 FS <sup>®d</sup>	180.0	—	[26]
<i>Daphnia magna</i> (water flea)	Fipronil	190.0	—	[7]

<sup>a</sup> Technical-grade fipronil (Rhone-Poulenc Agro Chemie, Lyon, France).

<sup>b</sup> 95% confidence interval not reported.

<sup>c</sup> D.S. Block (University of South Carolina, Columbia, SC, unpublished data).

<sup>d</sup> ICON (TechPac, Lexington, KY, USA).

thus final population projections should be compared relative to the control rather than as absolute abundances under fipronil exposure. Life-cycle toxicity test data were used to derive treatment-specific instantaneous rates of increase ( $\lambda$ ) for population growth. Treatment-specific population growth was modeled through three generations beginning with 60 stage 1 copepodites. Model constraints included logistic density dependence, demographic stochasticity, an environmental carrying capacity of 10,000 individuals, and 50 replications of the Leslie matrix model [18].

#### Analytical chemistry

For the acute toxicity test, triplicate seawater samples (1.5 ml) from control and treatment solutions were collected into 20-ml amber vials at test initiation ( $t = 0$  h) and termination ( $t = 96$  h), and processed by a liquid 1:1 methyl *tert*-butyl ether extraction. For the life-cycle toxicity test, triplicate seawater samples (3 ml) from fresh control and treatment solutions were collected into 20-ml amber vials at test initiation and every 3 d immediately before solution renewal, and processed by a liquid 2:1 methyl *tert*-butyl ether extraction. A liquid 2:1 methyl *tert*-butyl ether extraction was used because of the low fipronil concentrations tested in the life-cycle bioassay. Fipronil could not be analyzed from treatment solutions after addition to microplate test chambers (i.e., after 3 d) because of limiting solution volumes (0.2 ml/well) and analytical detection limits. However, for pooled replicate samples of 8 ultra-low-attachment microwells (total vol = 1.6 ml), recovery efficiencies for fipronil at 72 h were approximately 80% (T.L. Cary, unpublished data). For all seawater samples tested, an internal standard ( $2.5 \times 10^{-5}$  % 4-bromoanisole) was added to the seawater: methyl *tert*-butyl ether mixture, vortexed for 1.5 min, and sonicated for 5 min. The methyl *tert*-butyl ether layers containing extractable analytes were transferred to 9-mm-i.d. amber crimp-top gas chromatography vials and analyzed for fipronil analytes with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a <sup>63</sup>Ni electron capture detector (Hewlett-Packard, Palo Alto, CA, USA) operated in splitless mode. The carrier gas was helium with injector and detector temperatures at 230 and 310°C, respectively. A DB-5ms 30-m  $\times$  0.25-mm i.d. 0.25- $\mu\text{m}$  film thickness column (Agilent Technologies, Palo Alto, CA, USA) was operated at an initial temperature of 100°C for 1 min with a 10°C/min

increase to 270°C and a 10-min hold. Retention time for fipronil under these conditions was 12.8 min; extraction efficiencies for fipronil were  $96.7 \pm 1.5\%$ .

#### Statistical analysis

All statistical procedures were performed with SAS<sup>®</sup> System Version 8.02 software (SAS Institute, Cary, NC, USA). Adult- and sex-specific mortality data by fipronil exposure concentration were analyzed by using the trimmed Spearman-Kärber method to determine LC50 [19]. Adult- and sex-specific LC50 values were estimated by using measured fipronil concentrations. For the life-cycle toxicity test, all proportional data were arcsine-square root transformed (percent survival, percent reaching adulthood, and percent gravid females) or square root transformed (days to adult and days to first female clutch extrusion) before analysis of variance (ANOVA) procedures. General linear model (GLM)-nested ANOVA (within treatment and microplate) and Tukey multiple comparison procedures were used to test for differences in copepod survival, development, and reproductive success among controls and fipronil treatments ( $\alpha = 0.05$ ). A GLM-MIXED procedure was used to test for treatment interactions for copepod survival at 12 and 21 d, and development times to adult male or female ( $\alpha = 0.05$ ). Within treatments, if no significant plate-to-plate, row-to-row, or column-to-column differences were found, data for percent survival, percent adult, or percent gravid were pooled within each replicate plate, and treatment significance was tested by GLM ANOVA ( $\alpha = 0.05$ ).

## RESULTS

Fipronil was not detected (detection limit = 0.05  $\mu\text{g/L}$ ) in control treatments of the acute and life-cycle toxicity tests. Measured fipronil concentrations ( $2.77 \pm 0.21$ ,  $5.44 \pm 0.83$ ,  $10.84 \pm 1.63$ , and  $19.64 \pm 0.97$   $\mu\text{g/L}$ ) in the acute toxicity test were  $\geq 64\%$  and averaged 82% of nominal concentrations. Measured fipronil concentrations ( $0.16 \pm 0.15$ ,  $0.22 \pm 0.17$ , and  $0.42 \pm 0.25$   $\mu\text{g/L}$ ) in the life-cycle toxicity test were  $\geq 61\%$  and averaged 68% of nominal concentrations. All water-quality parameters (salinity, pH, and dissolved oxygen) met American Society for Testing and Materials guidelines [20]. Fipronil was highly toxic to *A. tenuiremis* generally (adult 96-h LC50 = 6.8  $\mu\text{g/L}$ ; Table 1), with much higher toxicity to male copepods (96-h LC50 = 3.5  $\mu\text{g/L}$ ) than to nongravid

Table 2. *Amphiascus tenuiremis* survival (mean  $\pm$  1 standard deviation) at 12 and 21 d during the microplate life-cycle fipronil bioassay<sup>a</sup>

Fipronil <sup>b</sup> ( $\mu\text{g/L}$ )	Percent survival	
	Day 12	Day 21
0	93.1 $\pm$ 3.0 <sup>c</sup>	91.1 $\pm$ 5.1
0.16	95.1 $\pm$ 1.2	93.7 $\pm$ 2.0
0.22	97.2 $\pm$ 2.4	96.5 $\pm$ 3.1
0.42	97.2 $\pm$ 1.2	95.8 $\pm$ 3.6

<sup>a</sup> No significant differences detected ( $\alpha = 0.05$ ).

<sup>b</sup> Fipronil (Rhone-Poulenc Agro Chemie, Lyon, France).

<sup>c</sup> Each treatment mean represents three replicate microplates initiated with 48 stage 1 copepodites per replicate.

female copepods (96-h LC50 = 13.0  $\mu\text{g/L}$ ; Table 1). Overall, estuarine crustaceans—including *A. tenuiremis*—appear more acutely sensitive to fipronil than are freshwater crustaceans, particularly daphnids (Table 1).

Copepodite (juvenile) *A. tenuiremis* were individually reared to sexual (reproductive) maturity in microvolumes (200  $\mu\text{L}$ ) of environmentally relevant fipronil concentrations (<1  $\mu\text{g/L}$ ) with >90% survival at all concentrations. Percent survival data at 12 d and 21 d from each plate within each treatment were pooled after GLM-nested ANOVA procedures showed no significant differences across microplates ( $p > 0.50$ ), rows ( $p > 0.35$ ), and columns ( $p > 0.25$ ) within each treatment. Likewise, percent reaching adult data at 12 d from each plate within each treatment were pooled after GLM-nested ANOVA procedures showed no significant differences across plates ( $p > 0.41$ ), rows ( $p > 0.41$ ), and columns ( $p > 0.05$ ) within each treatment. Copepod survival was unaffected by fipronil with no significant differences in survival at 12 or 21 d ( $p > 0.05$ ; Table 2). Fipronil at  $\geq 0.22$   $\mu\text{g/L}$  significantly delayed development from stage 1 copepodite to adult by approximately 2 d ( $p < 0.01$ ; Fig. 2), with female and male development specifically delayed by 2.1 to 2.4 or 1.5 to 1.7 d, respectively (Fig. 3). Interestingly, in the high fipronil treatment (0.42  $\mu\text{g/L}$ ), development of copepodites into adult females took approximately 1.5 d longer than development into adult males ( $p < 0.01$ ; Fig. 3).

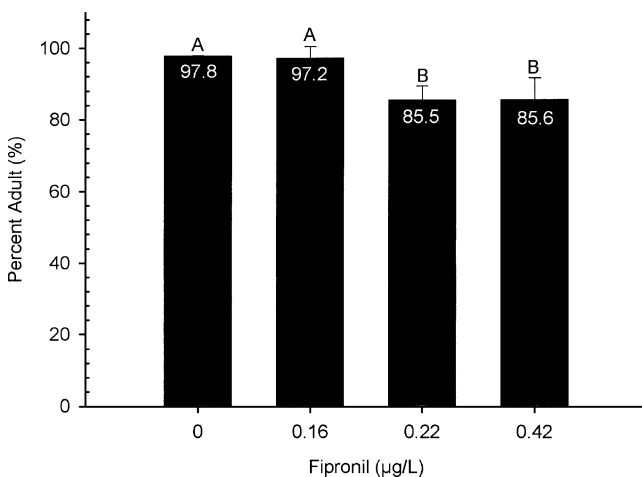


Fig. 2. Mean percent adult *Amphiascus tenuiremis* at day 12 versus aqueous fipronil concentrations. Each treatment bar represents the mean of three replicate microplates initiated with 48 stage 1 copepodites per replicate. Bars with dissimilar letters are significantly different ( $p < 0.05$ ). Error bar =  $\pm$  1 standard deviation.

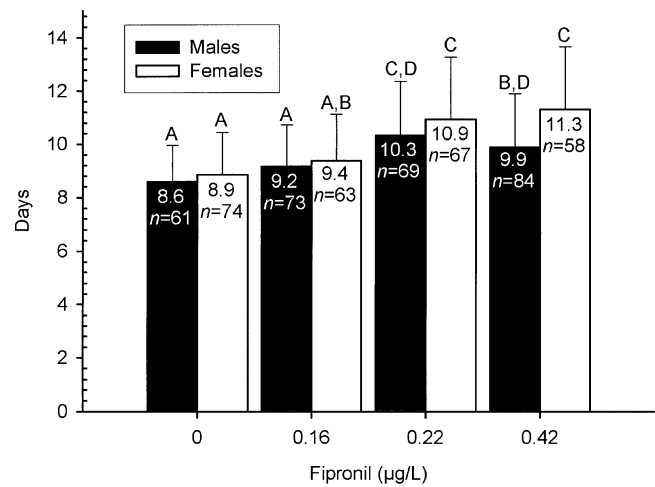


Fig. 3. Mean days to adult for male and female *Amphiascus tenuiremis* versus aqueous fipronil concentrations. Bars with dissimilar letters are significantly different ( $p < 0.05$ ). Error bar =  $\pm$  1 standard deviation.

After control and fipronil-exposed copepods reached adulthood, random male–female pairs from within the same treatment were mated at the same concentration(s) as rearing, and tracked up to 9 d for reproductive success. Fipronil significantly decreased the incidence of female egg production by 71% in the 0.22- $\mu\text{g/L}$  fipronil treatment, and nearly eliminated reproduction (94% decrease) in the 0.42- $\mu\text{g/L}$  fipronil treatment ( $p < 0.01$ ; Fig. 4). The time from mating to female egg extrusion also was significantly delayed. Reproductively successful females took 1.9 or 2.3 d longer to extrude eggs in the 0.16- and 0.22- $\mu\text{g/L}$  fipronil treatments, respectively ( $p < 0.05$ ; Fig. 5). At 0.42  $\mu\text{g/L}$ , female egg extrusion occurred in only 2 of 30 pairs, and interestingly was not delayed ( $2.0 \pm 1.4$  d) in those two pairs able to produce eggs. Estimated population growth rates ( $\lambda$ ) for *A. tenuiremis* in 0-, 0.16-, 0.22-, and 0.42- $\mu\text{g/L}$  fipronil treatments were 2.36, 1.89, 1.79, and 1.28, respectively. By three generations, the RAMAS model predicted a 62, 71, and 92% population size decrease (relative to the control) at 0.16, 0.22, and 0.42  $\mu\text{g/L}$ , respectively (Fig. 6). This population size decline was primarily driven by low reproductive success in the fipronil treatments (Fig. 6).

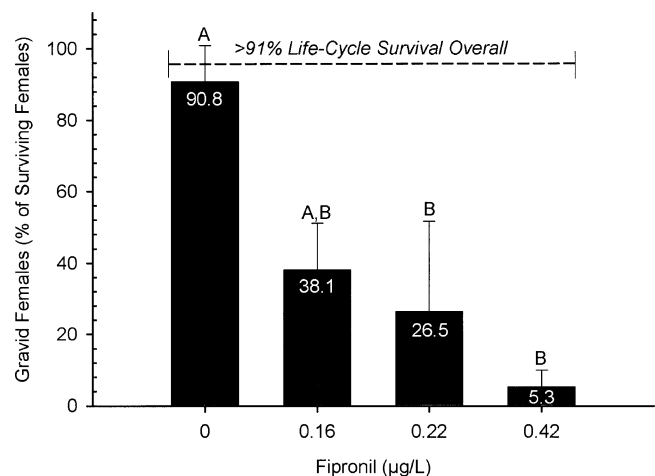


Fig. 4. Mean percent gravid female *Amphiascus tenuiremis* versus aqueous fipronil concentrations. Each treatment bar represents the mean of three replicate microplates. Bars with dissimilar letters are significantly different ( $p < 0.05$ ). Error bar =  $\pm$  1 standard deviation.

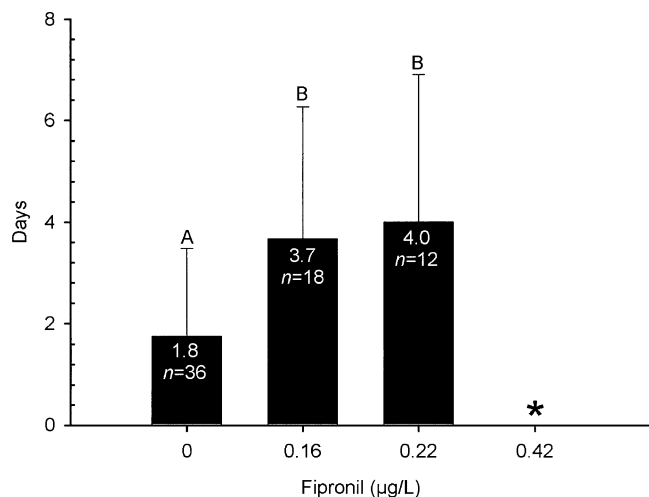


Fig. 5. Mean days from mating to egg extrusion by female *Amphiascus tenuiremis* versus aqueous fipronil concentrations. Error bar =  $\pm 1$  standard deviation. \* At 0.42  $\mu\text{g/L}$ , only 2 of 30 mating pairs were able to reproduce; thus, a nonrobust sample size ( $n = 2$ ) prohibited meaningful statistical analysis.

## DISCUSSION

Fipronil is a phenylpyrazole insecticide that targets the GABA-gated chloride channel in nerve-cell membranes and is highly toxic to arthropods [1]. In this study, fipronil had significant lethal and sublethal effects on the estuarine harpacticoid copepod *A. tenuiremis*. The overall adult 96-h LC50 for the sediment-dweller *A. tenuiremis* (6.8  $\mu\text{g/L}$ ) is 28 times less than for the freshwater planktonic cladoceran *Daphnia magna*, but approximately 50-fold higher than for the more sensitive demersal estuarine crustacean *Americamysis bahia* (Table 1). Based on current data, pelagic and benthic estuarine macrocrustaceans are more acutely sensitive to fipronil than is *A. tenuiremis*, whereas freshwater crayfish and daphnids are less sensitive. Among life stages of *A. tenuiremis* tested, adult male copepods were almost four times more sensitive to fipronil than were non gravid females (Table 1). Thus, differences in fipronil sensitivity are numerous, not only between

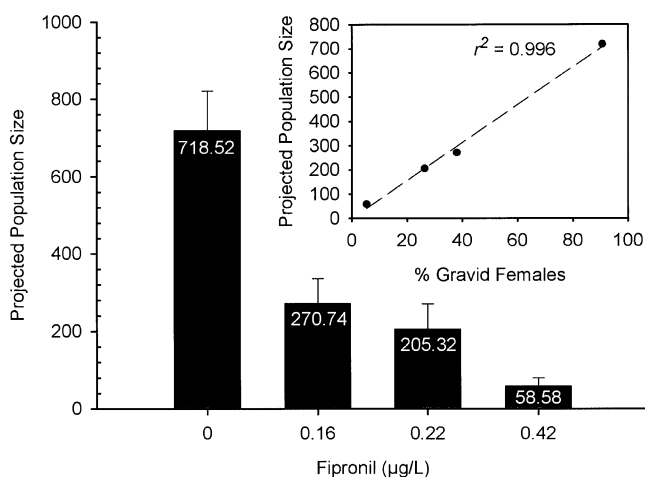


Fig. 6. Leslie-matrix-projected fipronil impacts on population size of *Amphiascus tenuiremis*. Each treatment-specific abundance mean represents 50 replications of the simulated population growth model. The graphical insert shows the correlation between percent of females extruding at least one viable clutch, and the predicted third-generation population size. Error bar =  $\pm 1$  standard deviation.

saltwater and freshwater crustacean fauna, but among saltwater taxonomic groups and even within species. Furthermore, acute toxicity tests with technical- or commercial-grade fipronil may also yield different species-specific acute responses. For example, the estimated adult fipronil 96-h LC50 for red swamp crayfish (*Procambarus clarkii*) is 12.5-fold greater for ICON 6.2 FS-spiked water (180  $\mu\text{g/L}$ ) than for technical-grade fipronil-spiked water (14.3  $\mu\text{g/L}$ ; Table 1; see references therein). Consequently, additional testing with multiple invertebrate species and commercial formulations should be performed to reduce risk uncertainties among the various fipronil formulations.

Within the last few years, Chipco Choice—a fipronil-based commercial product—has been intensively marketed for residential fire ant and turf-grass pest control in the southeastern United States. According to the manufacturer's label, the recommended application rate for Chipco Choice ranges from 0.026 to 0.067 lb a.i./acre. In a recent fipronil risk assessment released by the U.S. Environmental Protection Agency Environmental Fate and Effects Division, a mysid (*A. bahia*)—based acute and chronic toxicity threshold for estuarine invertebrates was defined as 0.14 and 0.005  $\mu\text{g/L}$ , respectively [21]. Based on tier 1 surface-water modeling (Genetic Estimated Exposure Concentration, U.S. Environmental Protection Agency, Office of Pesticide Programs, Environmental Fate and Effects Division, Washington, DC), the estimated peak surface-water fipronil concentrations expected from a single granular minimum (0.0125 lb a.i./acre) or maximum (0.025 lb a.i./acre) application rate were 0.195 and 0.390  $\mu\text{g/L}$ , respectively [21]. Based on tier 2 modeling (Pesticide Root Zone Model/Exposure Analysis Modeling System, USEPA-OPP-EFED, Washington, DC) for South Carolina (USA), the estimated peak surface-water fipronil concentrations expected from a recommended application rate of 0.025 lb a.i./acre every six months is 0.093  $\mu\text{g/L}$  [21]. Throughout the southeastern United States and within South Carolina, these models predict surface-water fipronil concentrations that approach or exceed the acute and chronic toxicity threshold for estuarine invertebrates. Although application of Chipco Choice is prohibited within 18 m of estuarine water-bodies (see manufacturer's label), the Environmental Fate and Effects Division "believes that the efficacy of buffers for fipronil use are uncertain" [21]. Indeed, the risk assessment of the Environmental Fate and Effects Division for broadcast fipronil fire ant control predicted that estuarine surface-water fipronil levels adjacent to treated areas likely would exceed the invertebrate chronic toxicity threshold by more than 50-fold [21].

Accordingly, because fipronil is moderately persistent and associates with sediments [7], estuarine water-bodies both adjacent to and miles from the point of application are likely receiving bodies because of rainfall runoff. In this study, fipronil induced strong developmental and reproductive effects on *A. tenuiremis* at concentrations likely to occur in estuaries. Development of female and male *A. tenuiremis* was not delayed in the lowest fipronil treatment (0.16  $\mu\text{g/L}$ ; Fig. 3), but female egg production was reduced (but not significantly) by 58% (Fig. 4), and significantly delayed by 2 d (Fig. 5). At a slightly higher concentration (0.22  $\mu\text{g/L}$ ), fipronil significantly delayed development of copepodites into adults by more than 2 d (Fig. 3), and inhibited and delayed female egg production by more than 70% and 2 d, respectively (Figs. 4 and 5). Although life-cycle survival was  $>91\%$  in all treatments (Table 2), copepod egg production was almost completely eliminated

at the highest fipronil concentration tested (0.42  $\mu\text{g/L}$ ; Fig. 4), and projected population growth was reduced >60% at only 0.16  $\mu\text{g/L}$  (Fig. 6). The reproductive median effects concentration was 0.14  $\mu\text{g/L}$  (95% confidence interval estimate = 0.08, 0.19); thus, at least for *A. tenuiremis*, a fipronil concentration of 0.05 to 0.10  $\mu\text{g/L}$  represents a reasonable aqueous reproductive lowest-observed-effect concentration.

Acute fipronil neurotoxicity (e.g., abnormal mating behavior) seems unlikely because male and female copepods appeared healthy and were reproductively active at all fipronil concentrations tested. In fact, multiple spermatophore sacs on microwell bottoms—an observation indicative of active copulation—were noted in all treatments throughout the monitored mating period (9 d). Thus, because crustacean reproduction is tightly linked to neurotransmitter-mediated endocrine signaling [22], sublethal fipronil-induced reproductive malfunctions such as alterations in female vitellogenesis or male spermatogenesis likely accounted for the low reproductive success at >0.2  $\mu\text{g/L}$ .

Male copepods exhibited fourfold greater acute sensitivity to fipronil than did nongravid females (Table 1), and similar sex-specific sublethal effects may occur differentially in males at low fipronil concentrations. Sex-specific differences in toxicant sensitivity have been found with other marine copepods. For example, males of the marine planktonic calanoid *Acartia tonsa* are twice as sensitive to water-borne cypermethrin (log  $K_{\text{OW}} = 6.32$ ) [23], and males of the estuarine meiobenthic harpacticoid *Microarthridion littorale* are twice as sensitive to sediment-associated Aroclor 1254 (Monsanto, St. Louis, MO, USA) (log  $K_{\text{OW}} = 6.50$ ) [24] and 10 times as sensitive to sediment-associated azinphosmethyl (log  $K_{\text{OW}} = 2.75$ ), an organophosphorous insecticide, than are females [25]. Because fipronil is lipophilic, female *A. tenuiremis* may resist fipronil endocrine or reproductive toxicity simply by sequestering fipronil in their higher body lipid content [25]. Nongravid female copepods have approximately 45% higher total lipid levels than do male copepods [25]. Because fipronil has a relatively high log  $K_{\text{OW}}$  (= 4.01), females likely have a greater capacity to reduce toxicant bioavailability in vivo. However, although fipronil does not accumulate in the tail muscle of red swamp crayfish exposed in experimental rice paddies [26], to date no published data are available on long-term (>94-h) bioaccumulation in lipid-rich crustacean tissues (e.g., the hepatopancreas). Unfortunately, we did not measure male or female copepod lipid or fipronil body burdens in these experiments.

In this study, trace fipronil concentrations significantly affected development and reproduction of *A. tenuiremis*, and even lower nominal concentrations (0.005  $\mu\text{g/L}$ ) have affected mysid reproduction [7]. Currently, the best analytical detection limits for fipronil quantitation in our laboratory and abroad span 0.05 to 0.10  $\mu\text{g/L}$  [10]. Many estuarine surface-water samples collected miles from Louisiana rice fields have shown fipronil at or near detection limits (U.S. Geological Survey, Louisiana Division, Baton Rouge, LA, USA, unpublished data), and similar low-level concentrations may exist in other estuarine systems receiving runoff from lawns and golf courses. Fipronil is rapidly gaining use in coastal areas and beyond, thus estuarine crustacean populations exposed to near-detectable or undetectable levels of fipronil may potentially suffer severe reproductive impacts with little notice by the management and regulatory communities. Because this copepod is normally a sediment-ingesting, infaunal species, and fipronil

has high fugacity for sediments, future studies should test whether fipronil exhibits similar reproductive toxicity in the sediment-associated state.

**Acknowledgement**—This research was funded by the U.S. Environmental Protection Agency through grant R-82-7397 to T. Chandler, G. Scott, J. Quattro, E. Wirth, J. Ferry, and M. Fulton. This research has not been subject to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the U.S. Environmental Protection Agency and no endorsement should be inferred.

## REFERENCES

1. Cole LM, Nicholson RA, Casida JE. 1993. Action of phenylpyrazole insecticides at the GABA-gated chloride channel. *Pestic Biochem Physiol* 46:47–54.
2. Ratra GS, Kamita SG, Casida JE. 2001. Role of human GABA<sub>A</sub> receptor  $\beta 3$  subunit in insecticide toxicity. *Toxicol Appl Pharmacol* 172:233–240.
3. Gant DB, Chalmers AE, Wolff MA, Hoffman HB, Bushey DF. 1998. Fipronil: Action at the GABA receptor. *Rev Toxicol* 2:147–156.
4. Ratra GS, Casida JE. 2001. GABA receptor subunit composition relative to insecticide potency and selectivity. *Toxicol Lett* 122: 215–222.
5. Hainzl D, Cole LM, Casida JE. 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. *Chem Res Toxicol* 11:1529–1535.
6. Robinson TN, Olsen RW. 1988. GABA. In Lunt GG, Olsen RW, eds, *Comparative Invertebrate Neurochemistry*. Cornell University Press, Ithaca, NY, USA, pp 90–123.
7. U.S. Environmental Protection Agency. 1996. New pesticide fact sheet. EPA 737-F-96-005. Office of Pesticide Programs, Washington, DC.
8. Schlenk D, Huggett DB, Allgood J, Bennett E, Rimoldi J, Beeler AB, Block D, Holder AW, Hovinga R, Bedient P. 2001. Toxicity of fipronil and its degradation products to *Procambarus* sp.: Field and laboratory studies. *Arch Environ Contam Toxicol* 41:325–332.
9. Key PB, Chung KW, Wirth EF, Fulton MH. 2003. Toxicity of the insecticides fipronil and endosulfan to selected life stages of the grass shrimp (*Palaemonetes pugio*). *Bull Environ Contam Toxicol* 70:533–540.
10. Vilchez JL, Prieto A, Araujo L, Navalón A. 2001. Determination of fipronil by solid-phase microextraction and gas chromatography–mass spectrometry. *J Chromatogr A* 919:215–221.
11. Lang K. 1948. *Monographie der Harpacticiden*. Hakan Ohlsson, Lund, Sweden.
12. Chandler GT. 1986. High density culture of meiobenthic harpacticoid copepods within a muddy sediment substrate. *Can J Fish Aquat Sci* 43:53–59.
13. Chandler GT, Green AS. 1996. A 14-day harpacticoid copepod reproduction bioassay for laboratory and field contaminated muddy sediments. In Ostrander GK, ed, *Techniques in Aquatic Toxicology*. Lewis, Boca Raton, FL, USA, pp 23–39.
14. Hurlburt SH. 1984. Pseudoreplication and the design of ecological field experiments. *Ecol Monogr* 54:187–211.
15. Leslie PH. 1945. On the use of matrices in certain population mathematics. *Biometrika* 33:183–212.
16. Ferson S, Ginzburg L, Silvers A. 1989. Extreme event risk analysis for age-structured populations. *Ecol Model* 47:175–187.
17. Power M, Power G. 1995. A modelling framework for analyzing anthropogenic stresses on brook trout (*Salvelinus fontinalis*) populations. *Ecol Model* 80:171–185.
18. Akçakaya HR, Burgman MA, Ginzburg LR. 1999. *Applied Population Ecology: Principles and Computer Exercises using RAMAS EcoLab 2.0*, 2nd ed. Applied Biomathematics, Setauket, NY, USA.
19. Hamilton MA, Russo R, Thurston RV. 1977. Trimmed Spearman–Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol* 11:714–718.
20. American Society for Testing and Materials. 2002. Standard guide for conducting acute toxicity tests on test materials with fishes,

- macroinvertebrates, and amphibians. E 729-96. Philadelphia, PA, USA, p 22.
21. U.S. Environmental Protection Agency. 2001. Fipronil environmental fate and ecological effects assessment and characterization for a Section 3 for broadcast treatment with granular product to control turf insects and fire ants (addendum). Report 08-MAY-2001. Internal Memorandum. Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC.
  22. Fingerman M. 1997. Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. *Invertebr Reprod Dev* 31:47-54.
  23. Medina M, Barata C, Telfer T, Baird DJ. 2002. Age- and sex-related variation in sensitivity to the pyrethroid cypermethrin in the marine copepod *Acartia tonsa* Dana. *Arch Environ Contam Toxicol* 42:17-22.
  24. DiPinto LM, Coull BC, Chandler GT. 1993. Lethal and sublethal effects of the sediment-associated PCB Aroclor 1254 on a meiobenthic copepod. *Environ Toxicol Chem* 12:1909-1918.
  25. Klosterhaus SL, DiPinto LM, Chandler GT. 2003. A comparative assessment of azinphosmethyl bioaccumulation and toxicity in two estuarine meiobenthic harpacticoid copepods. *Environ Toxicol Chem* 22:2960-2968.
  26. Biever RC, Hoberg JR, Jacobson B, Dionne E, Sulaiman M, McCahon P. 2003. ICON® rice seed treatment toxicity to crayfish (*Procambarus clarkii*) in experimental rice paddies. *Environ Toxicol Chem* 22:167-174.