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TOXICITY OF FIPRONIL TO THE MIDGE, *Cricotopus lebetis* SUBLETTE

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Fipronil, a relatively new insecticide more recently developed than organophosphates and pyrethroids, has been detected in surface water draining from agricultural and urban-developed areas. This insecticide is primarily lost through subsurface and surface drainage from terrestrial areas where it has been applied. Invasive aquatic plants often need to be managed in these receiving water bodies to prevent loss of recreational and functional values (e.g., drainage), especially in subtropical and tropical areas. One insect of particular interest is the chironomid midge *Cricotopus lebetis* Sublette, which may be a useful augmentative biocontrol agent for the invasive aquatic weed *Hydrilla verticillata* L.f. Royale. Exposure of aquatic organisms, especially insects, to fipronil may significantly impact nontarget populations. These studies investigated the sensitivity of *C. lebetis* to fipronil exposures ranging from 24 to 96 h. The LC_{50} observed for each exposure interval was 7.26 μg/L (24 h), 2.61 μg/L (48 h), 1.78 μg/L (72 h), and 1.06 μg/L (96 h). The LC_{90} values observed were 47.18 μg/L (24 h), 9.55 μg/L (48 h), 6.45 μg/L (72 h), and 4.81 μg/L (96 h). Behavioral changes were seen at all fipronil concentration levels, where larvae exited the plant and exhibited abnormal behavior, such as restricted movement and lack of feeding. Results indicate that acute lethality occurred at environmentally relevant concentrations of fipronil.

Fipronil {5-amino-1-[2,6-dichloro4-4(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile} is a phenylpyrazole insecticide that was approved for use in the United States in 1996 (U.S. EPA, 1996). Fipronil is more toxic to insects than mammals (Hainzl et al. 1998), and is used to control a broad spectrum of terrestrial insects including ants, cockroaches, termites, mosquitoes, locusts, and ticks and fleas in both urban and agricultural areas (Gunasekara et al., 2007). In insects, fipronil binds to the γ-aminobutyric acid (GABA) receptors, disrupting chloride ion control of neuron signaling (Hainzl et al., 1998; Cole et al., 1993), which results in excess neuronal stimulation and ultimately death.

Use of fipronil for terrestrial insect control may expose aquatic ecosystem resources via runoff and drainage water. Fipronil is relatively mobile in soils and susceptible to losses in runoff water due to its water solubility (1.9 mg/L, pH 5; Gunasekara et al., 2007)

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and moderate affinity for soils (K_{oc} 427–1248; Roberts et al., 1999). Fipronil log K_{ow} is 3.5 (Tomlin, 2000).

Fipronil has been detected in surface water bodies in agricultural and urban areas in the United States (Hintzen et al., 2009; Lao et al., 2010; Mize et al., 2008; Sprague and Nowell, 2008; Gilliom et al., 2006; Harman-Fetcho, 2005). Maximal reported concentrations in surface water (6.4 μg/L) were associated with rice field tail waters (Mize et al., 2008). Maximal concentrations reported in surface water associated with urban areas were 0.63 μg/L (Hintzen et al., 2009). A currently unpublished local monitoring study (St. Lucie County, Florida) found that fipronil migrates into surface water retention ponds in residential areas, being detected in more than 50% of samples (with maximum concentrations ranging from 17–207 μg/L) in ponds monitored weekly over a 9-mo period (J. Wu et al., personal communication).

Fipronil is highly toxic to the midge *Chironomus tempperi* Skuse, having an LC_{50} and LC_{90} of 0.43 μg/L and 1.05 μg/L, respectively (Ali et al., 1998). Fipronil is also toxic to other aquatic organisms such as mysid shrimp (LC_{50} = 0.14 μg/L), daphnia (LC_{50} = 190 μg/L), and bluegill sunfish (LC_{50} = 0.083 μg/L) (U.S. EPA 1996).

The chironomid midge *Cricotopus lebetis* Sublette (Diptera: Chironomidae) is a natural enemy of the invasive aquatic weed *Hydrilla verticillata* L.f. Royle (Cuda et al., 2002, 2008, 2011). In preparation for pupation, the larvae mine into the apical stem and feed on meristematic tissues, eventually inducing abscission of the tip (Cuda et al., 2002). *Cricotopus lebetis* may have value as an augmentative biological control agent because it prevents hydrilla from reaching the water surface. When hydrilla reaches the surface it creates dense surface mats, which impede boat traffic and impact recreational and commercial use of water bodies with infestations. Water quality and pesticide contamination may play a key role in determining the distribution of this midge, especially in agricultural and urban drainage basins. Given (1) the observations of fipronil in surface water, (2) its common use pattern, and (3) known toxicity to other invertebrates, the toxicity of fipronil to *C. lebetis* was examined in order to determine the insect potential sensitivity to this insecticide.

MATERIALS AND METHODS

Source and Culturing of Insects and Plants

Hydrilla was collected from Lake Tohopekaliga, Osceola Co., FL (28.2° N, 81.4° W), and *C. lebetis* was collected from Lake Rowell, Bradford Co., FL (29.9° N, 82.1° W). Both cultures were maintained at the University of Florida/IFAS Biological Control Research and Containment Laboratory (UF/IFAS-BCRCL), Fort Pierce, FL. Hydrilla was propagated by placing stems in 10-cm-diameter pots containing a layer of potting soil (Fafard 3B Mix, Conrad Fafard, Inc., Anderson, SC) covered by sand (Sakrete Multipurpose Sand; Sakrete Inc., Cincinnati, OH). The pots were placed into 378-L tanks in a greenhouse and covered with 60% shade cloth. *Cricotopus lebetis* was reared by placing hydrilla tips in a large aerated container within a cage constructed from polyvinyl chloride (PVC) tubing covered with a fine-mesh cloth. Containers were filled with well water from the UF/IFAS-BCRCL. Well water had the following characteristics: pH 7.9, alkalinity 290 mg/L as $CaCO_3$, hardness 146 mg/L as $CaCO_3$, and electrical conductivity 0.885 mS/cm. Egg masses were placed in the containers and adults that emerged were collected using an aspirator. Adults were transferred to a 250-ml separatory funnel containing approximately 15 ml well water as described by Cuda et al. (2002). Females oviposited on the water surface and egg masses were collected by opening the stopcock on the separatory funnel.

Evaluation of Toxicity

Toxicity of fipronil was evaluated using static, nonrenewal assays. A preliminary range-finding susceptibility study was conducted with *C. lebetis* to identify a narrowed range of toxic

concentrations. Test solutions were made by dissolving fipronil (99% pure, ChemService, West Chester, PA) in well water to achieve concentrations of 0.0, 0.02, 0.2, 2, 20, 200, and 2000 μg/L. In total, 20 ml of each solution was placed into a 35-ml test tube using a transfer pipette. A single, undamaged, healthy hydrilla tip (3–5 cm in length) was placed in each tube, along with one 8-day-old larva. Five replicate tubes were used for each test concentration. Test tubes were placed in a rack in an environmental growth chamber at 25°C and 14 h:10 h light/dark photoperiod (model E36L, Percival Scientific, Inc., Perry, IA). Larval survival was visually assessed every 24 h for 96 h. Larvae were considered dead if they exited the plant, were stiff, and showed no signs of movement. Two separate definitive assays were conducted using a narrower range of fipronil concentrations (0.0, 0.5, 2, 5, 10, 15, and 20 μg/L). Concentrations during the definitive test were confirmed only at the beginning of the assays. Concentration measurements were not taken at the end of the study due to limited sample volumes. For confirmation of concentrations, fipronil was extracted from duplicate water samples from each treatment using solid phase extraction techniques. The surrogate (4,4-dibromooctafluorobiphenyl, 4,4-D) was spiked into each sample duplicate to achieve a concentration of 10 μg/L before extraction. Hypersep C18 cartridges (500 mg/3 ml; ThermoScientific, Waltham, MA) were first cleaned by passing 6 ml methylene chloride through each. Each cartridge was activated by passing 6 ml acetone followed by 6 ml nanopure water. Each sample was then drawn through each cartridge, taking care to never allow drying once activated. Following extraction, cartridges were vacuum dried for 1.5 h. Fipronil was eluted from the cartridges with 8 ml methylene chloride and acetone (1:1 ratio). The elution solvent was evaporated to dryness using a RapidVap system (speed: 55%, temperature: 60°C, pressure: 330 mBar; Labconco, Kansas City, MO), and the extract was redissolved in 1 ml methyl *tert*-butyl ether (MTBE). Extracts were then analyzed by gas chromatography with electron capture detection (GC-ECD) using the equipment and conditions described by Wu et al. (2010). Quality control elements included instrument and method blanks, calibration check standards, matrix spikes, and matrix spike duplicates. Calibration curve R^2 values were >.99 and recoveries ranged from 92 to 110% (fipronil) and from 89 to 97% (4,4-D). Percent mortality at each concentration was compared to controls using analysis of variance (ANOVA) with Dunnett's means separation test ($p = .05$) for each time period (SAS 2008). Results also were analyzed using PROC PROBIT to predict the median lethal concentrations (LC_{50}) and concentrations lethal to 90% of the organisms (LC_{90}) during each time interval (SAS 2008).

RESULTS

The actual threshold concentration where 50% mortality occurred (based on analysis of variance [ANOVA] with Dunnett's means separation, $p = .05$) varied by one treatment concentration between the two separate studies for some of the exposure periods (data not shown). For example, results from the two separate assays indicated that 50% mortality occurred at 5 and 10 μg/L following 24-h exposure, between 2 and 5 μg/L following 48-h exposure, 2 μg/L after 72-h exposure (both assays), and at 0.5 and 2 μg/L after 96-h exposure (data for each separate assay not shown). In all cases no mortality occurred in controls. After 24-h exposure, 100% mortality did not occur at any concentration, though it was 90% in one study. However, 100% mortality did occur during the exposure periods longer than 24 h, although onset was variable between the two assays (i.e., 100% mortality occurred at 5 and 15 μg/L after 48-h exposure and at 5 and 10 μg/L after both 72- and 96-h exposures (data for each separate assay not shown).

Since the probit-transformed dose-response curves with their associated 95% confidence intervals (CI) for the two definitive assays overlapped, all toxicity data were combined and the LC_{50} and LC_{90} were estimated using the combined data. The LC_{50} (95% CI)

values for each exposure interval were 24 h: 7.26 μg/L (4.92–10.89 μg/L), 48 h: 2.61 μg/L (1.78–3.55 μg/L), 72 h: 1.78 μg/L (1.18–2.47 μg/L), and 96 h: 1.06 μg/L (0.6–1.57 μg/L) (Figure 1). The LC_{90} values (95% CI) were 24 h: 47.18 μg/L (25.75–155.81 μg/L), 48 h: 9.55 μg/L (6.72–16.09 μg/L), 72 h: 6.45 μg/L (4.47–11.16 μg/L), and 96 h: 4.81 μg/L (3.16–9.3 μg/L) (Figure 1).

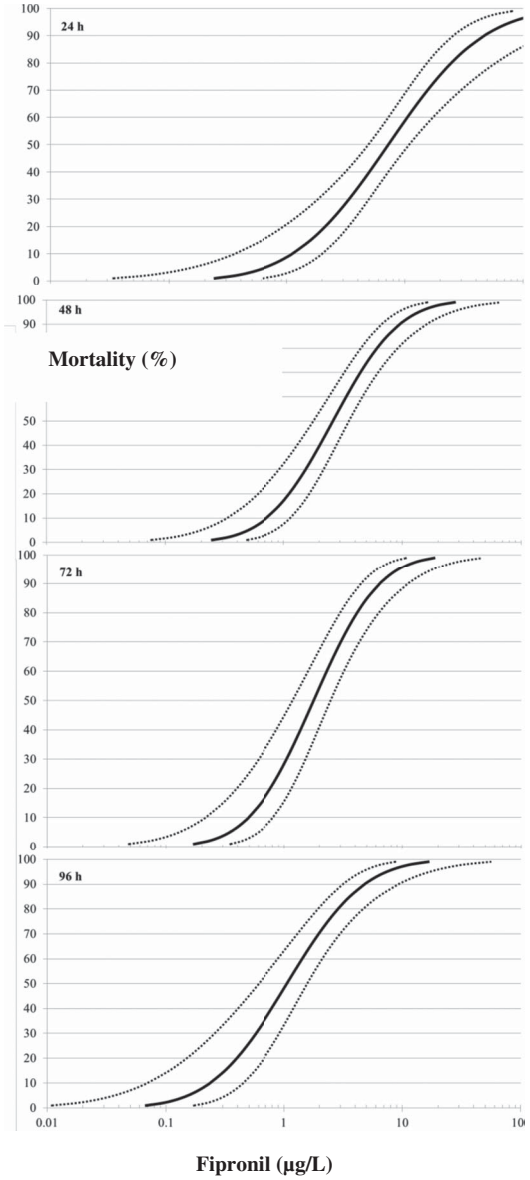

FIGURE 1. *Cricotopus lebetis* larvae mortality following exposure to fipronil for 24, 48, 72, and 96 h. Solid line depicts the predicted lethal concentrations and dotted lines indicate 95% confidence intervals.

Behavioral changes were seen at all fipronil concentration levels, but not in the controls. Affected organisms typically swam erratically after exiting the host plant, before becoming sessile, followed by mortality. In the sessile state, organisms were characterized as twitching, moving slowly, and with mouths slowly opening and closing. Every organism that died exited the host plant. At the treatment concentrations of 0.5, 2, and 5 μg/L, larvae exhibited these symptoms for longer periods of time until mortality occurred, indicating more tolerance to lower concentrations. In certain instances these symptoms occurred for over 24 h, but at higher concentrations symptoms were observed for 24 h or less due to mortality. No marked effects on the hydrilla were observed.

DISCUSSION

These results indicate that *C. lebetis* is sensitive to fipronil, although not as sensitive as some other species reported (Table 1). Ali et al. (1998) reported 48-h LC_{50} values of 0.43 μg/L for the mosquito larvae *Aedes taeniorhynchus* and *Anopheles quadrimaculatus,* and 23 μg/L for *Aedes albopictus*. Ali et al. (1998) also noted a 48-h LC_{50} of 0.42 μg/L for *Chironomus crassicaudatus* and *Glyptotendipes paripes*. Overmyer et al. (2005) showed a 48-h LC_{50} of 0.18–0.31 μg/L for the aquatic insect black fly larvae (*Simulium vittatum*). Thus, it appears that *C. lebetis* may not be the most ideal species to use in ecological risk assessments since it was not the most sensitive at 48-h exposures to fipronil. However, the 96-h LC_{50} for *C. lebetis* (1.06 μg/L) was markedly lower than that reported for the crayfish *Procambarus clarkia* (14.3 μg/L) and *Procambarus zonangulus* (19.5 μg/L) (Shlenck et al., 2001). The higher 48-h LC_{50} observed in the present study may have resulted from the fact that these tests included hydrilla cuttings in the assay. Some of the fipronil may have been sorbed to the plant surfaces, rendering it unavailable. However, this approach more closely resembles exposures that might occur in the natural environment.

TABLE 1. Median Lethal Concention (LC_{50}) Values for Organisms Exposed to Fipronil

Species	LC_{50} (μg/L)	Exposure (h)	Source
Mysidopsis bahia (mysid)	0.14	Not reported	U.S. EPA, 1996
Simulium vittatum IS-7 (black fly)	0.18–0.31	48	Overmyer et al., 2005
Palaemonetes pugio (adult grass shrimp)	0.32	96	Key et al., 2003
Chironomis crassicaudatus (midge)	0.42	48	Ali et al., 1998
Glyptotendipes paripes (midge)	0.42	48	Ali et al., 1998
Aedes taeniorhymchus (mosquito)	0.43	48	Ali et al., 1998
Anopheles quadrimaculatus (mosquito)	0.43	48	Ali et al., 1998
Culex migropalpus (mosquito)	0.87	48	Ali et al., 1998
Macrobrachium rosenbergii (shrimp)	0.98	96	Shan et al., 2003
Cricotopus lebetis (midge)	1.06	96	Current study
Amphiascus tenuiremis (copepod)	3.5–13.0	96	Chandler et al., 2004
Macrobrachium nipponensis (shrimp)	4.3	96	Shan et al., 2003
Eriocheir sinensis (crab)	8.5	96	Shan et al., 2003
Procambarus clarkia (crayfish)	14.3	96	Shlenck et al., 2001
Ceriodaphnia dubia (daphnid)	17.7	48	Konwick et al., 2005
Procambarus zonangulus(crayfish)	19.5	96	Shlenck et al., 2001
Aedes albopictus (mosquito)	23	48	Ali et al., 1998
Procambarus clakii (crayfish)	62.9–179.2	96	Overmyer et al., 2007
Lepomis macrochirus (bluegill sunfish)	83	96	U.S. EPA, 1996
Oncorhynchus mykiss (rainbow trout)	246	96	U.S. EPA, 1996

A simple method for screening for ecological risks is to consider the ratio of the expected environmental concentration (EEC) to the LC_{50} (EEC/LC_{50}/). A ratio greater than 0.5 indicates possible adverse acute risks, while a ratio less than 0.5 indicates a margin of safety. Using the maximum concentrations of fipronil reported in surface water from rice production areas (6.4 μg/L; Mize et al., 2008), the risk ratios are 0.89 (24-h exposure), 2.49 (48-h), 3.65 (72-h), and 6.13 (96-h), indicating significant risks after 24-h exposure. The risk ratios for the maximum noted urban surface water concentrations (0.63 μg/L; Hintzen et al., 2009) are 0.08 (24 h), 0.24 (48 h), 0.35 (72h), and 0.59 (96 h), indicating low risks of toxicity up to 72 hr exposure. Fipronil concentrations in surface water are likely to be highest close to the land areas where it is applied for insect control, especially in areas where it is broadly applied (as opposed to spot applications), and at surface water discharge outfalls from canals and ditches serving land areas where fipronil is used. Thus, augmentative biocontrol of hydrilla using *C. lebetis* may be most influenced by fipronil in these areas where concentrations are expected to be the highest.

In this study, there was variation between the two definitive studies during the 48- and 72-h time periods, which suggests that this time period is critical for larval survival. In the first assay, mortality was higher between 48 and 72 h at 5 μg/L. Some larvae were able to tolerate the conditions for a longer period of time before experiencing mortality, whereas other individual larvae could not tolerate these conditions for as long. This time period may be the threshold for which some larvae can survive if conditions are reversed within this time period. This variation is important when considering management approaches for water bodies containing fipronil. More research focused on potential recovery associated with different exposure intervals is needed.

Some sources of uncertainty in this study are the actual concentration and disposition of fipronil throughout the exposure periods. Concentrations and chemical identity were not determined during and at the end of the assays due to limited sample volumes (extra treatment solutions made at beginning to allow for initial extraction and analysis). These results are more representative of toxicity associated with a single pulsed exposure event, integrating the

effects of the parent compound along with possible degradation products. Given its high log K_{ow} (3.5) and K_{oc} (427–1248), some of the fipronil is expected to sorb to hydrophobic materials. Kroger and Moore (2008) investigated the fate of fipronil in mesocosm wetland systems planted with 4 different plant species and reported relative losses of 28–45% for fipronil during the 12-h flow-through studies. Sorption to plant materials was not significant due to observations of no differences in fipronil concentrations between nonplanted controls and any of the planted treatments (Kroger and Moore 2008). Sorption to sediments, tank surfaces, and fipronil delivery tubing may have contributed more to the losses, as well as degradation of fipronil.

Fipronil is susceptible to degradation under certain conditions. Under oxidative conditions, fipronil may be converted to fipronil sulfone and fipronil-desulfinyl. Both of these degradation products retain equal or greater toxicity to fish and aquatic invertebrates and are more persistent than parent fipronil (Gunasekara et al., 2007). Oxidative conditions are assumed to occur in the current study because of the lack of *C. lebetis* mortality in the controls. Fipronil is also susceptible to degradation by photolysis, with fipronil-desulfinyl being the major degradation product in field studies (Gunasekara et al., 2007). In the Kroger and Moore (2008) study, reductions in fipronil concentrations were accompanied by concomitant gains of the oxidative degradate, fipronil sulfone. These findings were attributed to accumulation in the strongly oxidative environment within the mesocosms and also to photolysis (Kroger and Moore, 2008). Photodegradation is possible, but not likely as significant as in the Kroger and Moore (2008) study due to the lower light intensity in incubators (fluorescent lights) relative to field conditions. Degradation by hydrolysis was likely not significant given the pH of 7.9. Fipronil has been shown to be stable in aqueous solution at acidic and circum-neutral pH (Gunasekara et al., 2007).

Overall, the results from this study indicate that *C. lebetis* is one of the more sensitive known aquatic organisms (96-h LC_{50} = 1.06) (Table 1). Efficacy of this organism as an augmentative biological control agent for hydrilla management may be limited in areas where fipronil concentrations routinely exceed toxicity thresholds for extended periods of time. These results also may be useful in ecological risk assessments where species sensitivity distributions are used for characterizing the potential effects of fipronil on nontarget aquatic organisms.

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