Toxic Bioassays: LC50 Sediment Testing of the Insecticide Fipronil with the Non-Target Organism, *Hyalella azteca*

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Abstract The use of the insecticide fipronil has dramatically increased in recent years, yet few studies have been performed compared to other popular pesticides. Although fipronil binds readily to organic carbon in soil, past studies have focused on fipronil toxicity in water but not the effects of fipronil toxicity in sediment. This study addresses the need for a broader range in fipronil toxicity research by using LC50 sediment testing to determine the acute toxicity of fipronil and its metabolite degradates, fipronil sulfone and fipronil sulfide, on the non-target organism *Hyalella azteca*. All pesticides were tested on two sediment combinations from different areas within California. Results showed that 9-day LC50 estimates were 227µg/kg for fipronil, 113µg/kg for fipronil sulfone, and 385µg/kg for fipronil sulfide in a San Pablo-Lake Anza sediment mixture. LC50 estimates for fipronil and its degradates tested in Ingram-Pacheco Creek sediment were 385µg/kg, 203µg/kg, and 485µg/kg, respectively. Although fipronil degradates are generally considered to be more toxic than its parent compound to freshwater invertebrates, only the LC50 for fipronil sulfone was found to be more toxic than the parent fipronil (by two-fold) while fipronil sulfide was found to be less toxic. LOEC and NOEC values support the conclusion that fipronil sulfone is toxic at lower concentration levels than the other compounds. This unexpected variability indicates that even within taxonomic groups, fipronil sensitivity may differ, underscoring the importance of exploring different variables such as sediment toxicity testing and use of different test species.

Introduction

Fipronil, an insecticide in the phenylpyrazole class, was first marketed in the U.S. in 1996, replacing several organophosphates in urban pesticide use (University of Minnesota 2005; TDC Environmental 2005a). It acts as a neurotoxin, disrupting the central nervous system by targeting GABA receptor-regulated chloride channels (Hainzl 1998). Although fipronil toxicity is highly selective towards arthropods, it also appears to bioaccumulate in fish, indicating a potential adverse effect to animals in higher trophic levels and to the health of aquatic ecosystems as a whole (US EPA 1996).

Formulated in bait and granular form, and as seed treatment in agriculture, fipronil use has dramatically increased in recent years (TDC Environmental 2005b). However, the number of environmental toxicity tests that have been published to date are few compared to other widely used pesticides (e.g. Roundup and organophosphates such as chlorpyrifos and diazinon). Additionally, past studies have focused on fipronil toxicity in water, although fipronil binds readily to organic carbon in soil. Because many of the non-target organisms it affects are detritivores that eat organic debris, the additional dietary exposure of fipronil may result in higher toxicity levels than previous studies suggest (Schlenk et al. 2001; Chaton et al. 2002).

Results of fipronil toxicity vary in the current body of scientific literature. A recent study done by Stark and Vargas (2005) found that fipronil causes lethal and sublethal effects to the water flea, *Daphnia pulex*, with LC50[1] estimates at 16 µg/L – similar to a Schlenk et al. study (2001) which established LC50 values at 14 and 19 µg/L for two species of crayfish. Chandler et al. (2004) found that even trace concentrations of fipronil significantly affected reproduction and development of the estuarine copepod, *Amphiascus tenuiremis*, with male copepods exhibiting higher acute sensitivity (male=3.5µg/L and female=13.0µg/L). In contrast to the Stark and Vargas (2005) study, Chaton et al. (2002) found that *D.pulex* was insensitive to the range of fipronil concentrations used in laboratory tests. While both papers examined acute toxicity in aqueous medium, they used different grades of fipronil – formulated and unformulated (technical grade) respectively. Specifically, formulated grades represent commercial products such as Regent 4SC (Stark and Vargas 2005) and Icon 6.2 FS™ (Schlenk et al. 2001) in which fipronil is the active ingredient. Technical grade is 98% pure fipronil as identified by gas chromatography (Schlenk et al. 2001; Chandler 2004).

[1] Lethal concentration for 50% mortality in an exposed population

The variability in results – from highly toxic to no effect – can be attributed not only to the different factors (grade of fipronil, test medium, test species) within each study, but also to differences in GABA receptor structure across different species (Schlenk et al. 2001). Table 1 summarizes the different LC50 analyses within current fipronil literature and the factors used to determine those values.

Table 1 – Past studies examining fipronil toxicity

Study	Test species	Grade	Medium	Type of toxicity test	Results
Chandler et al. 2004a	*Amphiascus tenuiremis*	Technical	Aqueous	Acute and life-cycle	96hr LC50 = 6.8µg/L (male=3.5µg/L; female=13.0µg/L)
Chandler et al. 2004b	*Amphiascus tenuiremis*	Technical	Sediment	Chronic	Reproductive effects at 0.25-0.5µg/L for parent compound
Chaton et al. 2002	*Daphnia pulex*	Technical	Aqueous	Acute	No significant effects to *D. pulex*
Schlenk et al. 2001	*2 species Procambarus*	Formulated (Icon 6.2 FS™)	Aqueous		96hr LC50 = 14-19 µg/L
Stark and Vargas 2005	*Daphnia pulex*	Formulated (Regent 4SC)	Aqueous	Acute	48hr LC50 = 16µg/L

This variability in toxicity estimates for fipronil also exists in studies of fipronil degradates – sulfone, sulfide, and desulfinyl – compounds generally considered to be more toxic than the parent fipronil (U.S. EPA 1996; Connelly 2001; Schlenk et al. 2001). The level at which these degradates are classified as more toxic vary, with the U.S. EPA (1996) establishing the metabolite sulfone as 6.6 times more toxic and the metabolite sulfide and photodegradate desulfinyl as 2 times more toxic to freshwater invertebrates than the parent fipronil compound.

The dramatic increase of fipronil use in recent years coupled with its varying effects across different species justifies establishing a wide range of toxicity data for a variety of species, both in the laboratory and in situ. To date there has been no research testing fipronil with *Hyalella azteca*, a standard specimen widely used in pesticide toxicity tests because of their sensitivity to pollutants and important ecological role in many aquatic food chains (US EPA 2000; Watts 2002). Similarly, few studies (Chandler et al. 2004*b*) have addressed the acute toxic effects of fipronil in sediment despite its high affinity for soil particles; particularly the sulfone and sulfide metabolites have K_{OC} values that indicate a very high persistence in soil environments[2] (Connelly 2001).

The purpose of this study is to determine the acute toxicity of fipronil and its metabolite degradates, sulfone and sulfide, to *H. azteca* by finding the lethal concentration necessary to kill 50% of an exposed population (LC50) and to find the lowest observable effect concentration (LOEC)[3]. I hypothesize that the sulfone and sulfide degradates will be at least two times more toxic, exhibiting lower LC50 and LOEC values, than the parent fipronil compound. The results of this project can be directed towards the development of more appropriate pesticide regulation, especially in areas of new urban development in which fipronil is frequently applied.

Methods

This experiment was designed to establish the LC50 and LOEC levels for *Hyalella azteca*, a freshwater amphipod, exposed to technical-grade fipronil obtained from ChemService (West Chester, PA, USA). 9-day LC50 tests were conducted according to the standard protocols for testing sediment toxicity with freshwater invertebrates as outlined in the EPA manual (U.S. EPA 2000). *H. azteca* is considered to be an ideal specimen in pesticide toxicity research because it is more sensitive to pollutants than other species; therefore if a pesticide is non-toxic towards *H. azteca*, it will also be non-toxic to other organisms (Ibid). In addition to values for fipronil, this experiment will determine the LC50 and LOEC for the sulfone and sulfide metabolites of fipronil.

Fipronil, fipronil sulfone, and fipronil sulfide toxicities were tested using two different sediment combinations, San Pablo-Lake Anza and Ingram Creek-Pacheco Creek. Both sediments have high total organic carbon (TOC)[4] levels that are reflective of the soil environments in which fipronil would normally be applied. The combination of Lake Anza and San Pablo Dam sediment yielded a TOC of 2.09%, and the combination of Ingram Creek and Pacheco Creek sediments had a TOC level of 5.00% (higher pesticide concentrations were used in the latter to account for the higher TOC). Data was collected from two 9-day tests (one for

[2] K_{OC} : organic carbon-water partitioning coefficient. K_{OC} for fipronil is 803, while K_{OC} values for sulfone and sulfide are 4209 and 2719 respectively.
[3] LC50 and LOEC estimates are common parameters used to develop regulations on pesticide toxicities.
[4] Total organic carbon (TOC): pesticides bind to the organic carbon content in soil; therefore sediment with a higher TOC will have greater affinity for the pesticide.

each sediment combination). Six different concentrations were tested for each pesticide based on results of a range finding test[5], and three replicate beakers were set up for the different sediment-pesticide groups with ten juvenile *H. azteca* in each sample beaker.

Sediment and test organism preparation Test sediments were collected from San Pablo Dam (El Sobrante, CA), a drinking water reservoir devoid of agricultural runoff and other non point source pollutants (Amweg and Weston, pers. comm), and from Lake Anza (Berkeley, CA), a site similarly free of pesticide residues. Sediments were sieved through a 1mm sieve to ensure a roughly homogenous texture in sediment grain sizes, and then mixed to achieve a 20/80 combination of Lake Anza to San Pablo Dam sediment. Sediments from Ingram Creek (Stanislaus County, CA) and Pacheco Creek (Santa Clara County, CA) were also sieved and then combined in a 50/50 blend.

Using data from a range-finding test, a control group, solvent-control group, and spiking concentrations ranging from 17-600µg/kg for San Pablo-Lake Anza and 61-2667µg/kg for Ingram-Pacheco Creek sediments were chosen to predict a theoretical survival range between 0 – 100%. The San Pablo-Lake Anza (SL) sediment and Ingram-Pacheco Creek (IP) sediment was separated into groups, each spiked with a different concentration of technical-grade fipronil dissolved in an acetone carrier for a stock dilution of 0.25µg/ml. The amount of solvent in each concentration group was normalized with =260µl of acetone in SL and =1316µl acetone in IP. The spiked sediment was then homogenized with a drill fitted with a steel auger and aged for 12 days at 4°C[6]. The aging process allows the pesticide to combine with the organic carbon in the soil, therefore decreasing the bioavailability of the pesticide to the test organisms (reflecting conditions that are more realistic).

Three days prior to commencement of the test, juvenile *H. azteca* were removed from mature cultures using a 500 µm sieve and retained on a 350µm sieve. Retained juveniles were incubated at 23°C and fed a mixture of trout chow, yeast, and cyanobacteria until initiation of the tests.

Experimental procedure One day prior to commencement of the tests, three replicate 400mL beakers were set up for each concentration of fipronil-spiked sediment and for the control. 50ml sediment samples were added to each beaker along with 300ml of moderately hard

[5] Range finding test: small-scale experiment testing wide range of pesticide concentrations from 1-1000µg/kg to yield preliminary data for further tests.

[6] The half-life of fipronil under photolysis is slow, about 34 days (Connelly 2001). Sediment groups are kept at a constant temperature to ensure that fipronil does not degrade during the testing process.

water (Milli-Q deionized water reconstituted with salts). The beakers were allowed to equilibrate overnight in a 24°C water bath. Samples of the sediment were also removed for chemical analyses to verify the actual concentration of fipronil within one of the groups.

This same procedure was used for sediments spiked with fipronil degradates, sulfone and sulfide. At day zero, ten *H. azteca* juveniles were counted under a microscope and added to each beaker. The beakers were placed in the water bath for the duration of 10 days on a 16:8hr light:dark cycle. Beakers received automatic water changes twice a day and were fed daily with 1.0mL of trout chow-yeast-cyanobacteria slurry. Before water renewal, water samples were removed on the 2nd and 10th day and water quality parameters were analyzed for dissolved oxygen, temperature, alkalinity, hardness, ammonia, pH, and conductivity. On the tenth day, the contents of each beaker were sieved through a 425µm screen, and surviving *H. azteca* counted.

Data analysis Toxicity data was analyzed using ToxCalc 5.0 Software (Tidepool Scientific Software). LC50 values were determined using the trimmed Spearman-Karber method with Abbott's correction and the significance of LOEC and no observable effect concentration (NOEC) values were determined using Dunnett's one-tailed t-test. The trimmed Spearman-Karber method uses the input of pesticide concentration levels and mortality data to calculate LC50s with a 95% confidence interval (Hamilton 1977). Specifically, the alpha value represents the percent of extreme values to be trimmed from each end/tail of distribution (or maximum and minimum likelihood).

Results

LC50, LOEC, and NOEC[7] estimates were calculated using the mortality data at each of the different sediment-spiked concentration groups. Mortality results were generally consistent across the three replicate beakers of each sediment-pesticide group. Each data point in the population-level dose response graphs of Figures 1, 2, and 3 represent the average mortality data of the three replicates in San Pablo-Lake Anza sediment (Figures also indicative of Ingram-Pacheco Creek response). A regression line was generated using these points, from which the LC50 estimate was then interpolated.

LC50 analysis yielded zero survival at the highest concentrations and 80-100% survival at the lowest concentrations with some variability within the control. Due to the inconsistencies in

[7] NOEC – No Observable Effect Concentration

the control group but the high survival rates among the lower concentrations, these data points were treated as controls, and the trimmed Spearman-Karber method was chosen to analyze the rest of the data.

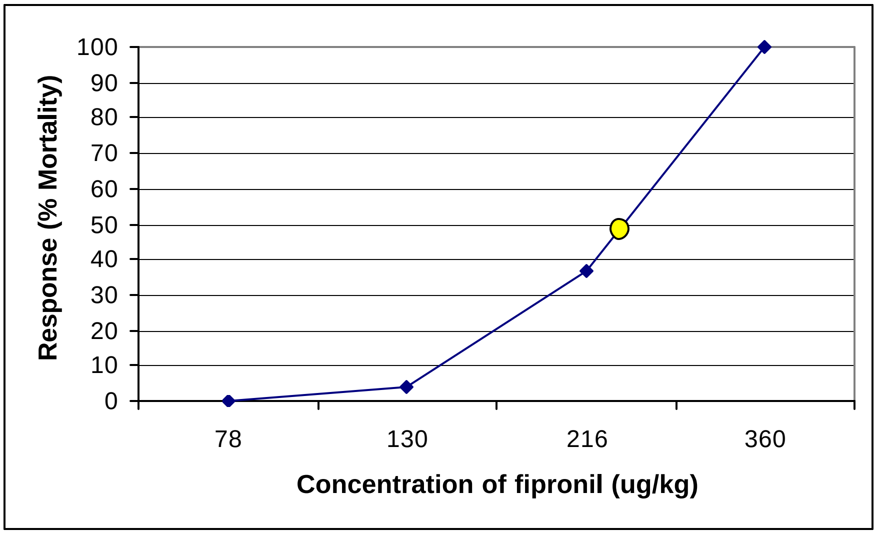

Figure 1. Mortality of *Hyalella azteca* exposed to varying concentrations of fipronil in San Pablo-Lake Anza sediment. Circular point on graph represents fipronil LC50 at 227µg/kg.

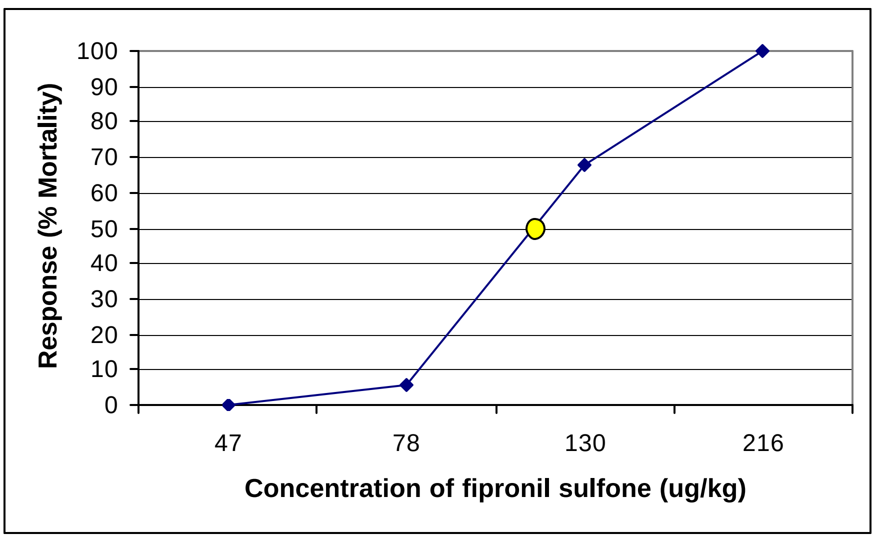

Figure 2. Mortality of *H. azteca* exposed to varying concentrations of fipronil sulfone in San Pablo-Lake Anza sediment. Circular point on graph represents fipronil sulfone LC50 at 113µg/kg.

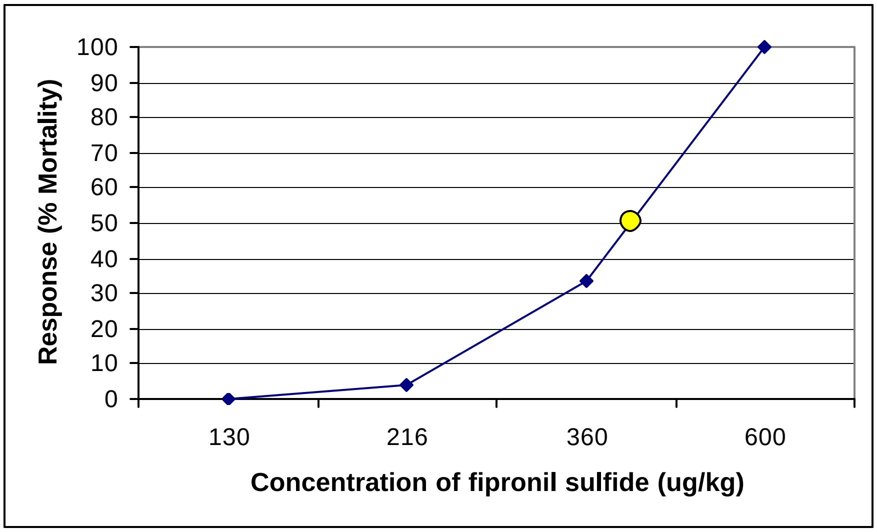

Figure 3. Mortality of *H. azteca* exposed to varying concentrations of fipronil sulfide in San Pablo-Lake Anza sediment. Circular point on graph represents fipronil LC50 at 385µg/kg.

The LC50 values for fipronil and its metabolite degradates, sulfone and sulfide, are listed in Table 2. Analysis of the survival data showed that the LC50 values are 227µg/kg (ppb) for fipronil, 113µg/kg for fipronil sulfone, and 385µg/kg for fipronil sulfide in San Pablo-Lake Anza sediment. LC50 values are 385µg/kg for fipronil, 203µg/kg for fipronil sulfone, and 485µg/kg for fipronil sulfide in Ingram-Pacheco Creek sediment combination. The values are higher in the Ingram-Pacheco Creek sediment (because of TOC) but fipronil sulfone's higher toxicity is consistent in both sediment combinations, with the LC50 for fipronil sulfone almost two times lower (becoming toxic at lower levels) than fipronil itself. The LC50 of fipronil sulfide is less toxic in both sediment combinations. Figure 4 shows the side by side comparison of these values.

Table 2. LC50, median lethal concentration values, with 95% CI for each different pesticide two different sediment combinations, San Pablo-Lake Anza and Ingram Creek-Pacheco Creek.

Pesticide	San Pablo-Lake Anza LC50 (µg/kg dry sediment)	Ingram-Pacheco LC50 (µg/kg dry sediment)
Fipronil	227 (207-248)	385 (359-412)
Fipronil Sulfone	113 (104-124)	203 (178-230)
Fipronil Sulfide	385 (352-421)	485 (426-553)

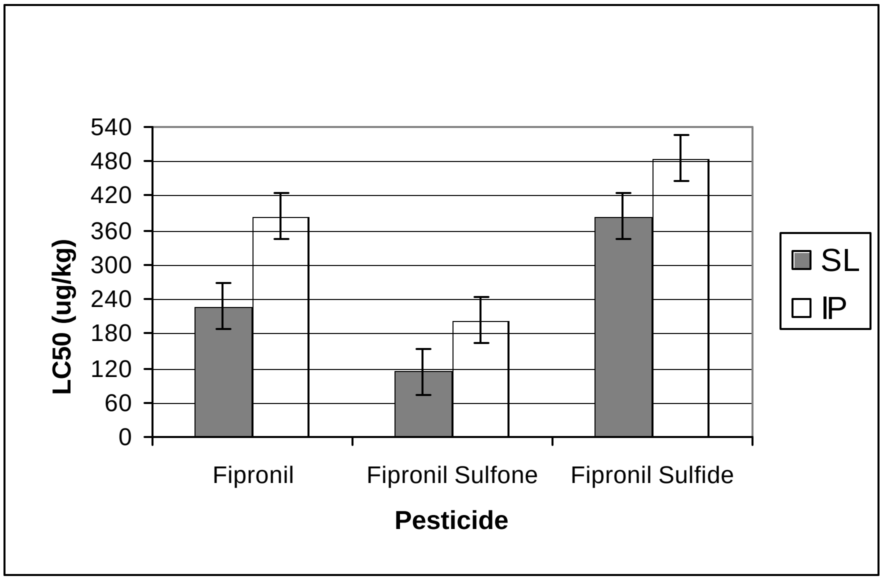

Figure 4. Comparative LC50 for fipronil and its two derivatives, sulfone and sulfone, in two different sediment combinations, San Pablo-Lake Anza (SL) and Ingram Creek-Pacheco Creek (IP). Error bars represent a 95%CI.

Survival data was arcsin square root-transformed and Dunnett's one-tailed t-test was applied to compare the treatment data to the control in the LOEC analysis. The Shapiro-Wilks test indicates a normal distribution ($p>0.01$) for fipronil, fipronil sulfone, and fipronil sulfide. Control means were not significantly different ($p=0.95$) for all three compounds. Dunnett's test uses the same values as those assigned to the sediment groups and does not use the interpolation method of the Spearman-Karber for LC50 values. LOEC and NOEC results are shown in Table 3 and 4. Similar to the results of the LC50 analysis, LOEC and NOEC values are lower for fipronil sulfone than the parent compound, while fipronil sulfide values are higher (indicating observed toxicity at higher concentration levels).

Table 3. LOEC and NOEC data for fipronil and its metabolite degradates in a combination of San Pablo-Lake Anza sediment

Pesticide	San Pablo-Lake Anza LOEC (µg/kg dry sediment)	San Pablo-Lake Anza NOEC (µg/kg dry sediment)
Fipronil	360	216
Fipronil Sulfone	130	78
Fipronil Sulfide	600	360

Table 4. LOEC and NOEC data for fipronil and its metabolite degradates in a combination of Ingram Creek-Pacheco Creek sediment

Pesticide	Ingram-Pacheco LOEC (µg/kg dry sediment)	Ingram-Pacheco NOEC (µg/kg dry sediment)
Fipronil	340	204
Fipronil Sulfone	170	102
Fipronil Sulfide	345	207

Discussion

The analysis of the results indicates that the LC50 value for fipronil is moderately toxic to *Hyalella azteca* with median toxicity levels established at 226µg/kg (ppb) in San Pablo-Lake Anza sediment. Fipronil sulfone was two-times more toxic than the parent fipronil compound with LC50 estimate at 113µg/kg. The results in Table 2 also suggest, unexpectedly, that fipronil sulfide is less toxic. These results for fipronil sulfide contrast with previously established acute toxicity for freshwater invertebrates in which the sulfide degradate was found to be more toxic than fipronil (U.S. EPA 1996). Although the test medium and species are different, the LC50 analysis conducted by the EPA is comparable to the results obtained using *H. azteca*[8]. This difference can be attributed to GABA receptor sensitivity in different species and to differences in fipronil toxicity tested in sediment and in the water column.

LC50, LOEC, and NOEC results of fipronil and its metabolite degradates in Ingram-Pacheco Creek sediment further confirm the conclusion that fipronil sulfone is two times more toxic than fipronil itself, and that the degradate sulfide is less toxic. The LC50 values are higher by a factor of about 1.5 in Ingram-Pacheco Creek sediment than in San Pablo-Lake Anza sediment because of the difference in TOC levels. However, the LC50 results were not directly proportional, suggesting that fipronil is not as hydrophobic as other pesticides such as pyrethroids (Weston, pers. comm).

The different grade of fipronil used, the medium to which it is applied, and the types of toxicity test performed are factors that have attributed to the variability in past research. In Chandler et. al (2004), LC50 analysis using the sediment-dwelling *Amphiascus tenuiremis* showed median toxicity levels at 6.8μg/L – an estimate 28-times lower (or more highly toxic) using *Daphnia magna*, a water column dweller, in the same test. Furthermore, 96-hour LC50 tests in Schlenk et. al's (2001) study using *Procambarus clarkii* exposed to technical grade fipronil were more toxic (LC50=14.3μg/L) than *P. clarkii* exposed to formulated fipronil, from the product ICON 6.2 FS™ (LC50=180μg/L), in a different study by Biever et al. (2003). Furthermore, the results of Schlenk et al.'s (2001) crayfish study classifies desulfinyl as less toxic than fipronil, and the sulfone and sulfide derivatives as having the same lethality as the parent compound – results very different from the U.S. EPA (1996) findings.

Based on these past findings, I hypothesized that fipronil sulfone and fipronil sulfide would be at least two times more toxic than the parent compound. The results of this study support the higher toxicity of the sulfone degradate, but sulfide degradate was less toxic than the parent fipronil in both sediment combinations tested.

LOEC and NOEC analyses support this conclusion. LOEC estimates show that fipronil sulfone is toxic to *H. azteca* at much lower levels than that of fipronil (360μg/kg in San Pablo-Lake Anza; 340μg/kg in Ingram-Pacheco Creek) or fipronil sulfide (600μg/kg in San Pablo-Lake Anza; 354μg/kg in Ingram-Pacheco Creek). Similarly, NOEC values in Table 3 and 4 prove that test organisms can sustain higher levels of fipronil and fipronil sulfide at no effect than fipronil sulfone (i.e. a three-fold difference).

Since LOEC and NOEC analyses use only the values assigned to the sediment-spiked concentration groups, the numbers for San Pablo-Lake Anza sediment are actually higher than the LC50 estimate. This error is due to the small dataset and steep regression line generated between data points with 0% and 100% mortality (Fig. 1, 2, and 3). In addition, survivals at the lowest concentrations were interpreted as controls because of the inconsistencies in the control

[8] The EPA study used freshwater daphnia in a 48-hour exposure that yielded an LC50 of 190ppb.

group itself, resulting in a smaller dataset. Although these results do not correspond accurately to the LC50 value (the expected lowest observable effect concentration should be lower than LC50), the numbers are representative of the relative proportion at which one compound is more or less toxic than the other. LOEC and NOEC values for Ingram-Pacheco Creek in Table 4 accurately correspond to the LC50 (this dataset had a consistent 100% survival in the control group). Because LOEC and NOEC analyses use values of the actual concentration groups, a more detailed test with a narrower series of concentration levels could yield a more precise LOEC.

The LC50 values correlate with the estimates previously established in a range-finding test. However in the San Pablo-Lake Anza test, the lower-than-expected survival rates in the control can be attributed to factors such as miscounting juvenile *H. azteca* at the start of the tests. There were a number of dead juveniles in the batch that was used so there was a possibility that a weak organism was placed in the beaker. Therefore it is hard to verify the health of an organism, an important aspect in a study that depends on the survival/mortality of an individual test organism.

Additionally, due to time constraints the content of the beakers were sieved on the 9^{th} day instead of the 10^{th}. Although statistically this makes no difference in the LC50 analysis, the actual LC50 could be higher than if test organisms were removed on the 10^{th} day as originally planned.

Future research may address the sediment toxicity of the fipronil photodegradate, desulfinyl, which was not included in this study due to financial constraints. Furthermore, it is important to develop literature that will supplement the current fipronil toxicity literature on water column organisms by using other sediment dwelling test species such as *Chironomous tetanus*, in addition to *H. azteca*. LC50 results from this study can also be applied to future studies of field sites in new residential development areas treated with fipronil; concentrations used in the lab study could be much lower or higher than the amount that would be found in situ.

The results measured here expose a different species and different medium to fipronil toxicity research, results that have broader implications in terms of pesticide regulation in that policy may differ depending on what grade of fipronil is used and what non-target species are expected to be affected. The LC50 values here were the first established using *Hyalella azteca* and can be applied towards future sediment toxicity tests of fipronil, not only to broaden the range in scientific literature but also to address the ecological impacts of increased pesticide use.

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