ENANTIOSELECTIVE TOXICITY, BIOACCUMULATION, AND BIOTRANSFORMATION OF FIPRONIL IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

by

SUZANNE ELIZABETH BAIRD

(Under the direction of Marsha C. Black)

ABSTRACT

Fipronil is a relatively new chiral, phenylpyrazole insecticide used to control both agricultural and household invertebrate pests. Fipronil is applied as a racemate, or equal mixture, of its two enantiomers. Although a number of toxicity studies have demonstrated enantioselective toxicity in aquatic invertebrates, data on enantioselective toxicity in fish is limited. We observed increased toxicity of the (+) enantiomer in larval fathead minnows exposed to the fipronil racemate and each enantiomer seven days. Juvenile fathead minnows were exposed to fipronil-contaminated sediment. We found that fish rapidly bioaccumulated fipronil and transformed it to fipronil sulfone. Fish preferentially transformed the (-) enantiomer, resulting in an increased proportion of the (+) enantiomer in fish tissues. This thesis illustrates the complex behavior of fipronil in fathead minnows and the need for additional research on the fate of fipronil in the aquatic environment.

INDEX WORDS: Acute Toxicity, Subchronic Toxicity, Pesticides, Chiral, Enantioselective

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SUZANNE ELIZABETH BAIRD

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SUZANNE ELIZABETH BAIRD

Major Professor: Marsha C. Black

Committee: A. Wayne Garrison
Robert Bringolf

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Fipronil is a relatively new chiral, phenylpyrazole insecticide used to control both agricultural and household invertebrate pests. As regulations on other pesticides increase, use of fipronil and environmental levels are expected to increase. Fipronil is released into surface waters via runoff from agricultural application and has been detected in both surface waters and sediment. At high concentrations, fipronil is acutely toxic to target and nontarget organisms. At lower, environmentally relevant concentrations, fipronil can depress reproduction and growth in nontarget organisms. Also, fipronil and its metabolites are known to bioaccumulate in aquatic organisms. Single enantiomer or enantiomer enriched formulations could potentially replace the commonly applied racemic mixtures and decrease effects on nontarget organisms. The following is a review of current aquatic toxicity and bioaccumulation data with a discussion of the environmental fate, and chemical structure and mechanism of action. A statement of research objectives and a brief description of experimental approach conclude this chapter.

Literature Review

History of Fipronil Use

Fipronil was developed in 1987 by Rhone Poulenc Ag Company and was approved for use in the USA in 1996 (USEPA, 1996). High selective toxicity, resistance of pests to other

insecticides, and increased regulation of other insecticides have led to increased application of fipronil in the U.S.A. In 1997, 480 tones of fipronil were produced and production nearly doubled to 800 tons in 2000 (Anon. 1997). Production and application of fipronil can be expected to continue increasing as restrictions on other, older pesticides increase.

Toxicity of fipronil to nontarget organisms first received attention after the insecticide was implicated in decreased crayfish harvest in Louisiana's rice producing region (Bedient et al. 2005, Schlenk et al. 2001). In Louisiana, crayfish (*Procambarus sp)* and rice are cultivated in the same fields; rice seeds are planted in flooded fields followed by introduction of crayfish. Fields are then drained and rice is harvested (crayfish burrow in mud). Crayfish are harvested after the fields are reflooded. In 1998, carbofuran insecticides were banned from use in rice agriculture (Bedient et al. 2005) and farmers in Louisiana responded by applying ICON (a pesticide containing fipronil) to rice seeds to control the rice water weevil (Bedient et al. 2005, Schlenk et al. 2001). The following year, the crayfish harvest dropped severely and Louisiana's crayfish industry was hurt (Bedient et al. 2005). Subsequent laboratory and field studies confirmed the acute toxicity of fipronil to crayfish (Schlenk et al. 2001, Overmyer et al. 2007). Farmers in Louisiana have since successfully sued Avantis, the maker of ICON. Although there have been several lawsuits, one recent settlement resulted in a $45 million dollar judgement against Avantis (Associated Press 2004). The economic implications of the decreased crayfish harvest likely helped fuel research on the ecological safety of fipronil. Several other countries are considering a ban, or have banned, fipronil because they are unsure of its potential to negatively affect agriculture. China is considering a ban on fipronil because of its potential to harm agriculture (Anon. 2006) and in 2004 France temporarily banned pesticides containing fipronil because of its acute toxicity to bees (Scott 2004). Although fipronil is no longer used in

Louisiana rice crop, there is still concern regarding toxicity to nontarget organisms. The National Ambient Water-Quality Assessment (NAWQA) detected fipronil in 25% of surface water samples (USGS 2003). In homes, fipronil has been used as a replacement for Dursban, a banned chlorpyrifos formulation, for control of termites, ants, and cockroaches (Anon. 1999). The pesticide is also used for control of mole crickets on golf courses. Irresponsible application by homeowners and use on larger areas (i.e. golf courses) are likely sources for continued inputs of fipronil into surface waters.

Environmental Fate

Fipronil is released into the aquatic environment via runoff from commercial and agricultural application, but its fate in surface waters and sediment is variable. In surface waters and sediment, fipronil undergoes several biotic and abiotic biotic pathways (Figure 1). In aquatic exposures in the laboratory, fipronil photolyzes to desulfinyl fipronil with a half life of 3.6 hours (USEPA 1996). Fipronil is degraded to fipronil sulfone via oxidation and to fipronil sulfide via reduction (Jones et al. 2007). Photolysis of fipronil is abiotic and takes place readily with sunlight (Bobe et al. 1998a), while the oxidation and reduction pathways are biotic and are commonly formed via metabolism by organisms that take up the parent compound (Konwick et al. 2006, Wong et al. 2002) and bacterial communities in soils and sediments (Jones et al. 2007, Bobe et al. 1998b). Fipronil is hydrophobic (log K_{ow}=4.0) and with an average log K_{oc} of 2.9, fipronil can be expected to adsorb to sediment (Roberts et al. 1999, USGS 2003). Some of the degradation products, like fipronil sulfide, have higher log Koc values (Table 1). In addition to being more persistent in the environment, these degradates may be more toxic than the parent compound to some aquatic species (USEPA 1996).

Although fipronil has been detected in the water column (USGS 2003) long term aquatic exposure is likely from sediment. In the Mermentau River Basin, Louisiana, fipronil was applied for rice water weevil control beginning in 1999. By 2000, maximum fipronil concentrations in surface waters ranged from 0.829-5.29 µg/L and the degradation product detected in the highest quantity was desulfinyl fipronil (1.13 µg/L). Fipronil sulfide (0.636-24.8 µg/kg), desulfinyl fipronil (0.55-7.01 µg/kg) and fipronil sulfone (not detected-10.5 µg/kg) were detected in bed sediment at nearly all of the 17 sites sampled, while fipronil was below detection (USGS 2003). The Mermentau survey revealed that over time and distance from source, concentration of degradation products exceeded concentrations of the parent compound. Laboratory studies of the behavior of fipronil in sediment support the trends observed in the Mermentau Basin where degradation products are more persistent than the parent compound. Lin et al. (2008) examined the degradation of fipronil, desulfinyl fipronil, fipronil sulfide, and fipronil sulfone-spiked sediment under facultative and anaerobic conditions and found all degradates more persistent than the parent compound. Under facultative conditions, the half life of fipronil was 18.5 days while the half lives for desulfinyl fipronil, fipronil sulfide, and fipronil sulfone were 388, 588, and 712 days, respectively, and each fit well with a first-order exponential decay model. Degradation under anaerobic conditions was much slower for all forms and degradation products still remained more persistent than fipronil (Lin et al. 2008). First order decay of fipronil was also observed in a study determining fate of fipronil in estuarine mesocosms where fipronil sulfide was determined to be the major degradation product in the system with lesser amounts of fipronil sulfone and desulfinyl fipronil (Walse et al. 2004). Similar findings of anaerobic degradation of fipronil producing primarily fipronil sulfide with lesser amounts of fipronil sulfone were reported in Brennan et al. (2009). Therefore, the degradation products of fipronil,

specifically the sulfide, sulfone, and desulfinyl metabolites, should be taken into consideration when determining appropriate use and application of products containing fipronil.

Mechanism of Action

In insects, fipronil binds at the γ-aminobutyric acid (GABA) receptor and blocks the GABA-gated chloride channel that regulates the passage of chloride across nerve cell membranes (Cole et al. 1993, Ecobichon 1996). Although information on the GABA receptor of aquatic organisms is limited, it is thought to be homologous across arthropods (Konwick et al. 2005, Robinson et al. 1988) and therefore, the mechanism of action of fipronil across arthropod species is likely via the GABA receptor. By binding at the GABA receptor, fipronil interferes with normal nerve cell function resulting in neural excitation and, at sufficient doses, mortality. This mechanism of action is responsible for fipronil's selective toxicity in invertebrates over mammals and is characteristic of phenylpyrazoles (Cole et al. 1993). Fipronil binding affinity at the GABA receptor is much stronger in invertebrates than mammals; Cole et al. (1993) found that a group of phenylpyrazoles (including fipronil) exhibited a 505-1870 fold difference in receptor potency in insects relative to mammals.

Even less is known about fipronil's mechanism of action in fish. In developing zebrafish, fipronil is believed to bind at a glycine receptor (GlyR) subtype, which is structurally related to GABA (Stehr et al. 2006); GABA and GlyR are both chloride-conducting, ligand-gated ion channels (McDearmid et al. 2006). According to McDearmid et al. (2006), GlyRs in the developing spinal cord of larval zebrafish may regulate the cell cycle similar to the role of GABA in the adult zebrafish hippocampus. Stehr et al. (2006) found effects of fipronil in zebrafish embryos and larvae (impaired motility and development) mimicked by a known GlyR

antagonist (strychnine), and not mimicked by a known GABA antagonist. However, zebrafish exposed to gabazine, a potent vertebrate GABA receptor antagonist, exhibited seizure-like activity, which is unlike the effects observed in fipronil-treated fish larvae and juveniles. Bloomquist et al. (1993, 2003) examined the mechanism of action of avermectin and cyclodiene insecticides and found that GABA-gated chloride channel to be an important site of action. However, Bloomquist suggests other ligand- and voltage-gated chloride channels may also play a part in the toxicity of these insecticides. Perhaps both GABA and GlyR receptors are responsible for the toxicity of fipronil in fish.

Chirality of Fipronil

Fipronil is chiral, and unlike most chiral chemicals centered around a carbon atom (Smith 2009), the chiral center of fipronil is located at the sulfur atom (Figure 2). Approximately 25% of pesticides in current use are chiral, and that amount is expected to grow as more complex chemicals are developed (Liu et al. 2005), so issues related to the chirality of fipronil are not unique. Although degradates of some chiral chemicals (i.e. the fungicide triadimefon, Kurihara et al. 1997) maintain their chirality, the major metabolites of fipronil (fipronil sulfide, fipronil sulfone, and desulfinyl fipronil) are not chiral. Since metabolism acts upon atoms or groups at or near the sulfur chiral center, these degradates lose their chirality.

Fipronil occurs as two enantiomers, designated (R,-) and (S,+) based on the molecule's rotation on a plane and three dimensional structure (Solomons 1990, Teicher et al. 2003). Enantiomers rotate the plane of polarized light by equal amounts in opposite directions; this differentiation is described by the " + " and " – " designations. The "R" (clockwise) and "S" (counterclockwise) designations describe the orientation of atoms or groups around the chiral

center (Smith 2009). Enantiomers have identical physical and chemical properties and are degraded equally by abiotic mechanisms (i.e. photolysis, hydrolysis), but may behave differently in biological systems (Jones et al. 2007, Liu et al. 2005, Smith 2009). Different behavior between enantiomers is referred to as enantioselectivity. For example, enantioselective acute toxicity of fipronil has been documented in aquatic invertebrates (Konwick et al. 2005, Overmyer et al. 2007) and enantioselectivity in transformation and elimination has been observed in some species of fish (Konwick et al. 2006, Wong et al. 2002). Although fipronil is applied as a racemate, biological processes may degrade one enantiomer at a faster rate than the other, resulting in mixtures enriched in either enantiomer following environmental exposure.

There are several ways to describe the proportions of enantiomers in a mixture. Consider a mixture of fipronil with 80% of the (S,+) enantiomer (E_A) and 20% of the (R,-) enantiomer (E_B) (Smith 2009):

Enantiomer ratio (ER):

$$ER = E_A/E_B = 0.80/0.20 = 4.0$$

Enaniomer excess (EE):

$$EE = \left| E_A + E_B \right|, \text{ where } E_A + E_B = 1 \text{ (or 100\%)}, = \left| 0.80 - 0.20 \right| = 0.6$$

Enantiomeric fraction (EF):

$$EF = E_A/(E_A+E_B) = 0.80/(0.80+0.20) = 0.8$$

Gues et al. (2000) recommends the use of enantiomeric fractions because unlike enantiomer ratio and enantioer excess, EF values clearly indicate relative amounts of each enantiomer. For example, in mixtures of fipronil, the EF of the racemate is 0.50, a mixture with a greater

proportion of the (S,+) enantiomer has an EF>0.50 and a mixture with a greater proportion of the (R,-) enantiomer has an EF<0.50. Another advantage of EF is that they allow for correct calculations of means and standard deviations. For these reasons, EFs will be used to describe fipronil mixtures for the remainder of this literature review and in the following chapters.

Potential Mechanisms of Enantioselective Toxicity

Several theories have been proposed to explain enantioselectivity among different species and within organisms. Stereospecific uptake is unlikely. In fish, uptake of hydrophobic compounds from the gastrointestinal tract (Konwick et al. 2006) and across the gills (McKim et al. 1984) is a passive diffusion process and not likely to be enantioselective. In arthropods, in which fipronil is known to bind at the GABA receptor (Cole et al. 1993, Ecobichon 1996), enantioselective toxicity may result from differential receptor binding by enantiomers at the GABA receptor (Konwick et al. 2006). In mammals, receptors with chiral dependence are well documented and include increased activity of S(-)-propranol vs R(+)-propranol at an andrenoceptor (Popp et al. 2004, Ranade et al. 2005), and inhibition of GABA by the (S,+) enantiomer of an antiepileptic (vigabatrine) the (R,-) enantiomer is inactive (Gidal et al. 1999). Alternatively, one enantiomer may be metabolically deactivated and eliminated allowing the other enantiomer to accumulate and elicit toxic effects. Konwick et al. (2006) found that trout exposed via diet preferentially transformed the (+) enantiomer; this ability of trout to enantioselectively eliminate chiral compounds has been observed with other chemicals, including *trans*-chlordane and chlorbiphenyl 136 (Wong et al. 2002).

Aquatic Toxicity

No clear pattern emerges for enantioselective toxicity with fipronil with lethality (LC50) as the endpoint. Enantioselectivity varies between species and life stages, and toxicity of the racemate can be dependent on sex of organism. Acute enantioselective toxicity (LC50) of fipronil has been found in several aquatic species, including the daphnid, *Ceriodaphnia dubia*, (Konwick et al. 2005), which like the crayfish are more sensitive to the (+) enantiomer, and larval grass shrimp (*Palaemonetes pugio*) which is more sensitive to the (-) enantiomer (Overmyer et al. 2007). However, acute enantioselectivity (LC50) has not been observed in the clam (*Mercenaria mercenaria*), adult grass shrimp, larval black flies, (*Simulium vittatum*, or the Japanses medaka (*Oryzias latipes)* Overmyer et al. 2007, Nillos et al. 2009) (Table 2). In tadpoles of African clawed frog (*Xenopus laevis*) the (S,+) enantiomer caused significant toxicity earlier than the (R,-) enantiomer and racemate, but the (R,-) enantiomer caused slightly higher (though not statistically significant) mortality overall (Overmyer et al. 2007). In copepods (*Amphiascus tenuiremis)*, the racemate is more toxic to males (96-hr LC50=3.5 µg/L) than females (96-hr LC50=13.0 µg/L) (Chandler et al. 2004). Nillos et al. (2009) found the racemate to be significantly more toxic than the enantiomers to primary rainbow trout hepatocytes after 24 hours.

Sensitivity to degradation products is also highly variable. For example, in the daphnid, the desulfynil photoproduct (48-hr LC50=355 µg/L) was found to be significantly less lethal than the fipronil racemate (48-hr LC50=17.7 µg/L) (Konwick et al. 2005). The oxidation degradation product fipronil sulfone is 6.3 times more lethal to rainbow trout and 3.3 times more lethal to bluegill (*Lepomis macrochirus*) than the parent compound (USEPA 1996). In red swamp crayfish, desulfinyl fipronil (96-hr LC50=68.6 µg/L) was four to five fold less toxic than

fipronil (96-hr LC50=14.3 µg/L), fipronil sulfone (96-hr LC50=11.2 µg/L), and fipronil sulfide (96-hr LC=15.5 µg/L; Schlenk et al. 2001). Differential toxicity of degradation products may be partially explained by differential receptor site binding strengths. Hainzl et al. (1998) indicated that the more toxic sulfone metabolite has a stronger affinity for the GABA receptor in invertebrates than the parent compound. Similarly, the desulfinyl photodegradation product has a stronger affinity for the GABA receptor in mice (male albino Swiss-Webster mice) and houseflies (*Drosophilia sp.*) than the less toxic parent compound (Hainzl and Casida 1996).

Although acute toxicity data are important in identifying sensitive species and comparing between species, these data lack environmental realism because studies are conducted at concentrations well above those detected in the environment. Studies of nonlethal endpoints are conducted at lower doses over longer, more realistic time periods and are less prevalent than acute toxicity studies. However, chronic studies are more likely to capture effects at environmentally relevant levels. In the daphnid, the (S,+) enantiomer (LOEC = 2 µg/L) reduced reproduction at lower exposure concentrations than either the racemate (LOEC = 15 µg/L) or the (R,-) enantiomer (LOEC = 30 µg/L) (Wilson et al. 2007). The (+) enantiomer also impaired motility at lower concentrations and was more toxic to neonates than both the racemate and the (-) enantiomer (Wilson et al. 2007). In a reproductive study with only the racemate, fipronil did not significantly decrease egg production in adult female grass shrimp (Volz et al. 2003), but it has been shown to change the sex ratio of grass shrimp at concentrations below 15 µg/L (Wirth et al. 2004). The racemate decreased development in male and female copepods (*A. tenuiremis*) and decreased female egg extrusion at 0.22 µg/L with almost complete elimination of reproduction at 0.42 µg/L (Chandler et al. 2004). The racemate also inhibited growth of larval rainbow trout (LOEC = 0.015 µg/L; USEPA 1996). Chronic effects, such as those described

above, are more likely to occur at lower, environmental levels, but their effects on populations are more insidious and difficult to quantify than lethality.

Bioaccumulation and Biotransformation

Bioaccumulation is also a concern with many aquatic contaminants and fipronil is no exception. Fipronil has been shown to bioaccumulate in a range of aquatic species under a variety of exposure conditions. Juvenile rainbow trout rapidly accumulated fipronil when fed food spiked with 7.68 μg/g racemic fipronil (Konwick et al. 2006). The trout also transformed fipronil as evidenced by changing EF; although fed racemic fipronil, EF of bioaccumulated fipronil decreased and the (-) enantiomer was more prominent than the (+) enantiomer. In a study of bioaccumulation of fipronil in nontarget arthropods, bioaccumulation was highly variable; after 48-hrs aquatic exposure to racemic fipronil, accumulation factors ranged from <1 in *Chaoborus crytallinus* to >60 in *Chironomus annularius* (accumulation factor is, approximately, the ratio of the concentration of radiolabeled fipronil in the organism to that in the medium; Chaton et al. 2002). In the sediment dwelling amphipod *Hyalella azteca* fipronil, fipronil sulfide, and fipronil sulfone bioaccumulated when the organisms was exposed via sediment contaminated with the three chemicals (Kroger et al. 2009). Bioaccumulation in *H. azteca* varied according to site conditions (i.e. drought, vegetation). As evidenced by the studies cited above, fipronil can bioaccumulate in organisms regardless of source (food, water, sediment). Higher level organisms (i.e., fish) could potentially be at greater risk of fipronil bioaccumulation as they are exposed not only to contaminated water and sediment, but may also consume contaminated prey (i.e. benthic macroinvertebrates – a common prey item in aquatic food webs) and introduce the potential for trophic transfer.

Conclusions

At least 25% of current-use insecticides are chiral and uncertainty surrounding enantioselective toxicity is not unique to fipronil (Garrison 2006). Single enantiomer or enantiomer-enriched formulations have been introduced in the US and Europe and both will allow for decreased application of chemical and a potential decrease in nontarget organism toxicity. For example, the acetanilide herbicide S-metalochlor enriched to 86% of the active enantiomer was applied at 60% of the typical racemate application and yielded equal pest control (Garrison 2006). Enantiomer specific formulations of fipronil could potentially be equally effective against pests with decreased effects on nontarget organisms. Laboratory experiments with selected target organisms show the racemate and both enantiomers to be equally acutely toxic (Chaton et al. 2001, Teicher et al. 2003). Therefore, application of one specific enantiomer could be equally effective as application of the racemate while minimizing nontarget organism enantioselective toxicity. Decreased application of enantiomer specific formulations would also partly alleviate nontarget organism sensitivity to degradation products; less fipronil applied translates to less fipronil degradation products in runoff. More research is required to determine the overall ecological safety of fipronil and feasibility and efficacy of enantiospecific application.

Objectives and Outline of Thesis Research

The purpose of this research was to determine enantioselective toxicity of fipronil in larval fathead minnow (*Pimephales promelas*). Although there has been a great deal of research into the behavior of fipronil in invertebrates, there are few data regarding the effect of fipronil on fish. I hypothesized that fipronil would be toxic to fish in water-borne exposures; however, I did

not hypothesize any enantioselective trends (enantioselectivity is variable among aquatic organisms and is difficult to predict). I tested my hypothesis by exposing larval fathead minnows to racemic fipronil, (+) enantiomer and (-) enantiomer in three 7-day aquatic toxicity experiments (Chapter 2). A second objective of this research was to determine the behavior (degradation, transformation, bioaccumulation) of fipronil in a sediment-water-fish system. I hypothesized that fipronil would be degraded into fipronil sulfide in the sediment and fish may bioaccumulate fipronil. To test these hypotheses, I exposed juvenile fathead minnows to sediment-bound fipronil in a 42-day bioaccumulation experiment (Chapter 3). In Chapter Four, I discuss how the results of the bioaccumulation experiment aid in understanding the trends in toxicity observed in the aquatic toxicity experiment. I also discuss the significance of these findings, and future work that would aid in understanding the toxicity of fipronil in fathead minnow.

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Table 1.1. Average log Koc (USGS 2003) and log Kow (Roberts et al. 1999, Walse et al. 2004) values for fipronil and degradation products (USGS 2003).

Chemical	Log K_{oc}	Log K_{ow}*
Fipronil	2.9	4.0
Fipronil sulfide	3.4	3.7
Fipronil sulfone	3.6	NA
Desulfinyl fipronil	3.1	NA

* NA = Data not available

Table 1.2. Summary of acute and enantioselective toxicity of fipronil to a range of aquatic organisms. Different letters indicate significantly different LC50 values.

	Species	LC50 (µg/L)			Exposure duration (hr)	Freshwater (F) or Saltwater (S)	Source
		Racemic fipronil	(S,+) enantiomer	(R,-) enantiomer			
Fish	*Lepomis macrochirus* (bluegill sunfish)	83	-	-	96	F	USEPA 1996
	Oncorhynchus mykiss (rainbow trout)	246	-	-	96	F	USEPA 1996
	Oryzias latipes (Japanese medaka)	94.2[a]	95.4[a]	98.3[a]	96	F	Nillos et al. 2009
	Cyprinodon variegatus (sheepshead minnow)	130	-	-	96	S	USEPA 1996
Invertebrates	*Ceriodaphnia dubia* (daphnid)	17.7[a]	10.3[b]	31.9[a]	48	F	Konwick et al. 2005
	Daphnia magna (daphnid)	190	-	-	48	F	USEPA 1996
	Procambarus clarkii (red swamp crayfish)	14.3	-	-	96	F	Schlenk et al. 2001
	Procambarus clarkii (red swamp crayfish)	124.89[a,b]	81.7[a]	163.5[b]	96	F	Overmyer et al. 2007
	Procambarus zonangulus (white river crayfish)	19.5	-	-	96	F	Schlenk et al. 2001
	Simulium vittatum (black fly larvae)	0.65[a]	0.72[a]	0.74[a]	48	F	Overmyer et al. 2007
	Amphiascus tenuiremis (copepod)	3.5–13.0	-	-	96	S	Chandler et al. 2004
	Mercenaria mercenaria (clam)	177[a]	208[a]	187[a]	96	S	Overmyer et al. 2007
	Mysidopsis bahia (mysid)	0.14	-	-	96	S	USEPA 1996
	Palaemonetes pugio (adult grass shrimp)	0.32[a]	0.37[a]	0.32[a]	96	S	Overmyer et al. 2007
	Palaemonetes pugio (larval grass shrimp)	0.68[a]	0.54[a]	0.35[b]	96	S	Overmyer et al. 2007

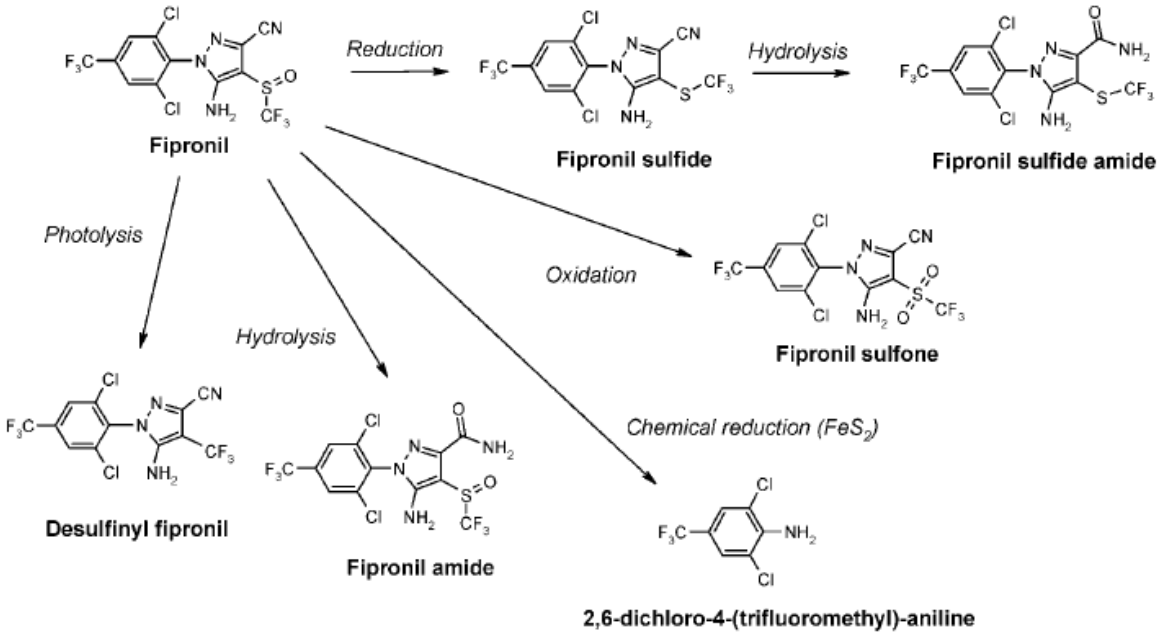

Figure 1.1. Biotic (noted with *) and abiotic degradation pathways and products of fipronil (from Jones et. al 2007, reproduced with permission).

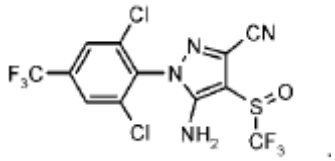

Figure 1.2. Molecular structure of fipronil (chiral center located at sulfure atom) (from Jones et. al 2007, reproduced with permission).

CHAPTER 2

ACUTE AND SUBCHRONIC ENANTIOSELECTIVE TOXICITY OF FIPRONIL TO THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)[1]

[1]Baird. S, A.W. Garrison, J. Avants, M.C. Black. To be submitted to *Archives of Environmental Contamination and Toxicology.*

Abstract

Fipronil is a relatively new chiral, phenylpyrazole insecticide used to control both agricultural and household invertebrate pests. Fipronil is applied as a racemate, or equal mixture, of its two enantiomers. As regulations on older pesticides increase, production and application of fipronil is expected to increase, leading to increased inputs into aquatic environments. Although a number of toxicity studies have demonstrated acute and chronic enantioselective toxicity in aquatic invertebrates, data on enantioselective toxicity in fish is limited. We conducted three 7-day aquatic toxicity experiments to determine the acute and subchronic toxicity of the fipronil racemate and each enantiomer to larval fathead minnows (*Pimephales promelas).* Acute (96-hr) LC50s for the fipronil racemate and each enantiomer were not significantly different. However, enantioselective toxicity was observed in fathead minnow exposures with longer exposure durations (7-d), with increased toxicity of the racemate (7-day LC50 = 191 µg/L) and (+) enantiomer (7-day LC50 = 207 µg/L) observed compared to the (-) enantiomer (7-day LC50 = 312 µg/L). Reduced fish growth was also observed in fish exposed to the (+) enantiomer and racemate, compared to the (-) enantiomer. Linear regression of concentration vs. fish weights at 7 days revealed significantly increased slopes for the (+) enantiomer and racemate versus the (-) enantiomer ($p<0.05$). Curiously, for both chronic endpoints toxicity of the racemate and (+) enantiomer was not significantly different, even though the racemate contains 50% of the (+) enantiomer and 50% of the less toxic (-) enantiomer. Metabolic processes could potentially change the enantiomeric fraction present in the organism, so that fathead minnows selectively transform and eliminate the (-) enantiomer, increasing the proportion of the more toxic (+) enantiomer in the racemic exposure.

Introduction

Fipronil is a chiral phenylpyrazole pesticide used to control household and agricultural pests and is applied as a racemate, or equal mixture of its two enantiomers. The two enantiomers are designated (R,-) and (S,+) based on their optical rotation and three dimensional structure (Solomons 1990, Teicher et al. 2003). Fipronil was introduced for use in the US in 1996 and is applied increasingly as a replacement for the banned carbofuran pesticides and other restricted use pesticides (USEPA 1996). In invertebrates, fipronil causes toxicity by interfering with the GABA-gated chloride channels of nerve cells and has a strong affinity for invertebrate GABA receptors compared to mammals (Cole et al. 1993, Ecobichon 1996). Cole et al. (1993) found that a group of phenylpyrazoles (including fipronil) exhibited a 505 to 1870 fold difference in receptor potency in insects relative to mammals. This selectivity contributes to fipronil's popularity because it minimizes toxic effects in humans and domesticated animals at doses that are effective on insects. Little is known about the mechanism of action of fipronil in fish. In developing zebrafish, fipronil is believed to bind at a glycine receptor (GlyR) subtype, which is structurally related to GABA (Stehr et al. 2006). Stehr et al. (2006) found effects of fipronil in zebrafish embryos and larvae (impaired motility and development) mimicked by a known GlyR antagonist, and not mimicked by a known GABA antagonist. Effects observed in fipronil-treated zebrafish were almost identical to those induced by strychnine, a GlyR antagonist. However, zebrafish exposed to gabazine, a potent vertebrate GABA receptor antagonist, exhibited seizure-like activity, which is unlike the effects observed in fipronil-treated fish larvae and juveniles.

Fipronil is released into the aquatic environment via runoff from commercial and agricultural application and the pesticide is becoming more common in surface waters. The

National Ambient Water-Quality Assessment (NAWQA) detected fipronil in 25% of surface water samples (US Geological Survey [USGS] 2003). In the USGS study, maximum surface water concentrations of fipronil ranged from 0.8 to 5.3 μg/L and the degradation product detected in the highest quantity was desulfinyl fipronil (1.1 μg/L) (USGS 2003). In surface waters and sediment, fipronil undergoes several degradation pathways: fipronil photolyzes to desulfinyl fipronil, oxidizes to form fipronil sulfone, and fipronil sulfide is formed via reduction (Jones et al. 2007, Ramesh et al. 1999). In the Mermentau River Basin, Louisiana, where fipronil was applied for rice water weevil (*Lissorhoptrus oryzophilus*) control in 1999, the parent compound and several degradates were detected in water and sediment. Some degradates are more toxic to some aquatic species than the parent compound, which can cause difficulties in assessment of nontarget toxicity. Fipronil's chirality further complicates evaluation of its potential to harm nontarget organisms in the aquatic environment. Although fipronil is applied as a racemate, biological processes within organisms or in the environment can alter fipronil's enantiomeric fraction (EF), resulting in enrichment in one enantiomer as the other is degraded (EF>>0.5 or EF<<0.5) (Garrison 2006). Therefore environmental exposures may consist of mixtures enriched in either enantiomer, even though fipronil is applied as a racemate,.

Fipronil received attention for its toxicity to nontarget organisms after it was found responsible for low crayfish harvest in Louisiana after the pesticide was used in rice agriculture. After carbofuran pesticides were banned in 1998, farmers in Louisiana applied fipronil to control the rice water weevil in fields where crayfish (*Procambarus clarkii*) and rice were co-cultivated (Bedient et al. 2005, Schlenk et al. 2001). Crayfish harvests were significantly lower after fipronil was introduced compared to previous years (Bedient et al. 2005). Subsequent laboratory studies confirmed the acute toxicity of fipronil to crayfish (Schlenk et al. 2001, Overmyer et al.

2007) and further investigation revealed crayfish are significantly more sensitive to the (+) enantiomer in acute exposures (Overmyer 2007). The economic implications of the decreased crayfish harvest helped fuel research on the ecological safety of fipronil. Acute enantioselective toxicity (LC50) of fipronil has been found in several other aquatic species, including the daphnid, *Ceriodaphnia dubia*, (Konwick et al. 2005), which like the crayfish are most sensitive to the (+) enantiomer, and larval grass shrimp (*Palaemonetes pugio*) which is more sensitive to the (-) enantiomer (Overmyer et al. 2007). Nillos et al. (2009) found the racemate to be significantly more toxic to primary rainbow trout hepatocytes after 24 hours. However, fipronil toxicity has not been found to be enantioselective in the marine clam (*Mercenaria mercenaria*), adult grass shrimp, larval black flies (*Simulium vittatum*) (Overmyer et al. 2007), or the Japanese medaka (*Oryzias latipes*) (Nillos et al. 2009). In all cases, concentrations of fipronil that cause acute lethality are not commonly found in surface waters.

Chronic studies of fipronil toxicity are more representative of possible exposure scenarios in the environment. However, there are fewer studies investigating chronic toxicity and rarely is enantioselective toxicity of fipronil evaluated in chronic studies. In a study of the effect of fipronil on copepod (*Amphiascus tenuiremis*) growth and reproduction, the racemate decreased development in both males and females and decreased female egg extrusion at 0.22 µg/L with almost complete elimination of reproduction at 0.42 µg/L (Chandler 2004). Fipronil racemate decreased larval trout growth at 15 µg/L (USEPA 1996) and skewed the sex ratio (increased number of male organisms) in grass shrimp at 0.15 µg/L (Wirth et al. 2004). In one of the few chronic studies on enantioselective toxicity of fipronil, the (+) enantiomer was significantly more toxic (LOEC = 2 µg/L) to *C. dubia* reproduction than either the racemate (LOEC = 15 µg/L) or the (-) enantiomer (LOEC = 30 µg/L) (Wilson et al. 2008).

The objective of this study was to evaluate toxicity of fipronil and its two enantiomers to fish using the larval fathead minnow (*Pimephales promelas)*, an EPA small fish model. Specifically, our objectives were to (1) measure acute toxicity of fipronil racemate and enantiomers to the fathead minnow, with mortality as the endpoint, (2) measure growth as a subchronic endpoint of exposure to fipronil racemate and enantiomers, and (3) identify enantioselectivity, if present. A previous study of enantioselective toxicity of fipronil in larval Japanese medaka found no acute enantioselective toxicity after 96-hrs (Nillos et al. 2009). Other studies on the effect of fipronil on fish are limited to short term (<96 hrs) exposures often using only the fipronil racemate. In our study, we will evaluate lethality of fipronil enantiomers at 96 h and subchronic toxicity via survival and growth after a 7-d exposure. Determining enantioselective toxicity of fipronil could potentially lead to single enantiomer or enantiomer-enriched formulations, which may decrease effects on nontarget organisms.

Materials and Methods

Test organisms

Larval *Pimephales promelas* were obtained from the U.S. Environmental Protection Agency, Region 5 laboratory in Cincinnati, OH. Fertilized *P. promelas* eggs were shipped and fish hatched both *en route* and overnight in the laboratory. Upon arrival, eggs and larval fish were gradually brought to test temperature (25± 1º C) and transferred to a small aquarium containing 50% transport water and 50% synthetically prepared moderately hard water (MHW). Larval fish were fed newly hatched *Artemia sp.* nauplii *ad libitum* in the evening prior to test initiation and twice daily during the exposure. Fish were not fed during the final 12 hours of the exposure.

Chemical

Fipronil (± 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4[(trifluoromethyl)-sulfinyl]-1H-pyrazole-3-carbonitrile; 97.8% pure) was purchased from ChemService (West Chester, PA, USA). Enantiomer separation was performed by Chiral Technologies (Exton, PA, USA). The process of enantiomer separation is described in Konwick et al. (2005).

Solutions

Stock solutions of 0.01 mg/L fipronil were prepared in 99.6% HPLC-grade acetone (Fisher Chemicals, Fair Lawn, NJ, USA) for each chemical form [racemic fipronil (±), (+) enantiomer (+), and (-) enantiomer (-)] at the beginning of each toxicity test. Based on a range finder test (data not shown), stock solutions of fipronil racemate and enantiomers were diluted in MHW to 50, 100, 200, 400, and 800 µg/L. Acetone concentrations were held constant at 0.1% of final solution volume and a vehicle control (0.1% v/v acetone in MHW) was tested to evaluate potential effects of acetone toxicity. Fresh test solutions were prepared daily with MHW. Subsamples of test solutions were collected daily for analytical confirmation of test concentrations. During the first toxicity test, daily samples were collected and analyzed separately for concentration confirmation. During the second and third toxicity tests, composite samples were collected. In the second toxicity test, two sets of composite samples were collected per concentration after renewal, compositing samples from days 0-3 and days 3-6. Based on accuracy of measured concentrations in the first and second toxicity tests, a single sample was composited for all 7 days for each chemical form and concentration during the third toxicity test.

Test solution analysis

Subsamples of test solutions were analyzed to confirm nominal fipronil concentrations. Fipronil concentrations were determined with solid phase extraction followed by gas chromatography-mass spectrometry (HP 6890/5973) using a chiral column (BGB-172 Analytik, AG, Anwil, Switzerland). This process is described in detail in Konwick et al. (2005). Average recovery of fipronil spiked into water at 100 µg/L was 99 ± 0% (n = 3).

Acute and subchronic toxicity tests

Toxicity tests were conducted in triplicate and according to EPA protocol (Method 1000.0, USEPA 2002). Larval fathead minnows were exposed to the fipronil racemate, (+) enantiomer, or (-) enantiomer for 7 days with daily, 80% renewal of test solutions. Larval fish were exposed to fipronil in 600-mL glass beakers with 300 mL test solution and ten fish in each beaker. There were four replicates (beakers) per concentration and twelve vehicle control replicates. Test vessels were randomly arranged in an incubator and maintained at 25 ± 1º C with a photoperiod of 16 h light:8 h dark. Water quality (DO [YSI Model 55, Yellow Springs, OH], pH and temperature [Orion model 290a, Beverly, MA]) was monitored twice daily in one replicate per concentration to minimize disturbance to test organisms. Survival was monitored daily and dead organisms were removed from test vessels. After 7 days of exposure, surviving larval fathead minnows were euthanized with buffered MS-222 (Argent Chemical Laboratories, Inc., Redmond, WA), rinsed with deionized water and placed on pre-weighed aluminum pans (FisherBrand, Thermo Fisher Scientific, Waltham, MA) by replicate. Fish were dried for 24 hrs at 60ºC (Thelco Laboratory Oven, Thermo Fisher Scientific, Waltham, MA) and then weighed to the nearest 0.01 mg on an analytical balance.

Statistical analysis

Median lethal values (LC50) and associated 95% confidence intervals for fathead minnows exposed to racemic fipronil, (+) enantiomer, and (-) enantiomer were calculated using the trimmed Spearman-Karber method (ToxStat V 3.3). LC50s were first calculated for each test repetition within each form of fipronil; if LC50s were not significantly different (overlapping confidence intervals), data were combined and analyzed as a single data set to compare toxicities of the fipronil racemate, (+) enantiomer and (-) enantiomer. LC50s were considered significantly different if confidence intervals did not overlap (APHA 1995).

Average dry weights of surviving fish were checked for normality with Shapiro-Wilks test and homogeneity of variance with Bartlett's test. Data meeting assumptions of normality and homogeneity were analyzed with analysis of variance (ANOVA) followed by a post hoc t-test with Bonferroni adjustment to identify NOEC and LOEC (Tox Stat V 3.3). Growth of surviving fish was also regressed against exposure concentrations (SAS V 9.1) to evaluate toxicity trends. In order to combine tests within each chemical form, weights were expressed as percentage of control weight within each test. Despite a minor residual pattern for the racemate, a linear model of the weight as percentage of control vs. concentration was deemed both appropriate (according to residual plots) and useful ($r^2>0.5$). Transformations of the concentration data did not correct the minor residual pattern observed for the racemate, nor did they produce different results versus untransformed data.

Results

Test solution analysis

Average measured fipronil solutions ranged from 82 to 101% of nominal concentrations (Table1). The greatest departure (82%) from nominal concentration occurred in the 100 µg/L racemic exposure with an average measured concentration of 81.8 µg/L fipronil. Nominal concentrations were used in data analysis.

Test Conditions

Daily water quality in test vessels remained within acceptable values (USEPA 2002) during the three tests. In all test vessels monitored, dissolved oxygen was > 4 mg/L, pH ranged from 7.30-8.00 and temperature was maintained at 25±1 °C.

Acute and Subchronic Lethality of Fipronil

Fathead minnow mortality increased with increasing concentration for racemic fipronil and each enantiomer. After 96 hrs, there were no significant differences in acute toxicity for fipronil racemate or the two enantiomers; LC50 values ranged from 446 to 448 µg/L. After 7-days, the LC50 of the (-) enantiomer was significantly higher than both the racemate and the (+) enantiomer (Table 2).

Effects of Fipronil on Fish Growth

Weights of fish exposed to racemic fipronil and both enantiomers decreased with increasing chemical concentration. NOECs and LOECs calculated for fish weights for the enantiomers and racemate did not show clear trends of effects of fipronil on fish weights (Table 3). However, linear regression of weights (expressed as a percentage of control weight) and

concentration revealed a significantly lower slope (non-overlapping 95% confidence interval of slope estimate) for the (-) enantiomer compared with similar slopes (overlapping 95% confidence interval of slope estimate) for the racemate and (+) enantiomer (Figure 1; Table 4).

Discussion

Acute toxicity of fathead minnows (LC50) to racemic fipronil is similar to values measured for other fish species. In our study of fipronil toxicity, fathead minnows are slightly more sensitive to racemic fipronil (96-hr LC50 = 448.49 µg/L) than channel catfish (96-hr LC50 = 560 µg/L) and more tolerant than bluegill (96-hr LC50 = 83 µg/L), sheepshead minnow (96-hr LC50 = 130 µg/L), rainbow trout (96-hr LC50 = 246 µg/L), and Japanese medaka (96-hr LC50 = 94.2 µg/L) (USEPA 1996, Nillos et al. 2009). Based on acute test comparisons, fathead minnows are not extremely sensitive to racemic fipronil. Furthermore, even the highest known concentrations of racemic fipronil detected in surface waters (5.3 µg/L) are well below the 96-hour and 7-day LC50s for fathead minnows determined in the present study.

In the present study, enantioselective toxicity was observed only after seven days exposure; acute toxicity (96-hr LC50) was not enantioselective. Nillos et al. (2009) also did not find enantioselectivity in 96-hr LC50s for fipronil and its two enantiomers in Japanese medaka; however, this exposure was terminated after 96 hours. Perhaps with longer exposure times (7 d) enantioselectivity may have occurred with time as was observed in the present study. Several theories have been proposed to explain enantioselectivity among different species and within organisms. Stereospecific uptake is unlikely. In fish, uptake of hydrophobic compounds from the gastrointestinal tract (Konwick et al. 2006) and across the gills (McKim et al. 1984) is a passive diffusion process and not likely to be enantioselective. In arthropods, in which fipronil is

known to bind at the GABA receptor (Cole et al. 1993, Ecobichon 1996), enantioselective toxicity may result from differential receptor binding by enantiomers and racemate (Konwick et al. 2006). Alternatively, one enantiomer may be metabolically deactivated and eliminated allowing the other enantiomer to accumulate and elicit toxic effects (Wong et al. 2002, Konwick et al. 2006). We observed an interesting trend of the (-) enantiomer being less toxic than both the racemate and the (S, +) enantiomer. Selective biotransformation could account for this trend in enantioselective toxicity. For example, fathead minnows may selectively biotransform and eliminate the (-) enantiomer, increasing the proportion of the more toxic (+) enantiomer. Similarly, in racemic exposures, the (+) enantiomer may reach the binding site before the (-) enantiomer or at higher concentrations and flood the binding sites.

Although NOEC and LOEC data for enantiomers revealed no enantiomer-specific toxicity trends among the three chronic experiments (Table 3), the slopes of the dose-response curves of fish growth plotted vs. concentration may give some insight into enantioselective toxicity (Landis et al. 2004). The higher (steeper) slopes of dose – response curves for the racemate and (+) enantiomer compared to the significantly lower (flatter) slope of the dose – response curve for the (-) enantiomer may indicate a similar relationship between toxicity and increasing concentration for the racemate and (+) enantiomer compared to the (-) enantiomer (Table 3). Higher slopes generated by the (+) enantiomers and racemate indicate that relatively small increases in their concentrations can result in large increases in toxicity. The lower slope for the (-) enantiomer indicates larger increases in chemical concentration are required to produce the same increases in toxicity. For both acute and subchronic exposures, the (-) enantiomer is consistently the least toxic. Although not tested in this experiment, perhaps the (-) enantiomer is more rapidly metabolized and eliminated, thus relative to the (+) or racemic

fipronil, much larger exposure concentrations are required to ensure enough chemical is available within the organisms to elicit toxic effects. More research is required to determine the underlying cause of enantioselective toxicity of fipronil in the fathead minnow observed in the present study.

One goal of this and similar research into the enantioselective toxicity of fipronil is to determine the feasibility of single enantiomer or enantiomer-enriched formulations for application. Single enantiomer or enantiomer-enriched formulations of other pesticides have been introduced in the US and Europe and allow for decreased application of chemical and a potential decrease in nontarget organism toxicity. For example, the acetanilide herbicide S-metalochlor was enriched to 86% of the active S enantiomer and was applied at 60% of the typical racemate application and yielded equal pest control (Garrison 2006). Enantiomer specific formulations of fipronil could potentially be equally effective against pests with decreased effects on nontarget organisms. Laboratory experiments with selected target organisms show the racemate and both enantiomers have equal acute toxicity to certain insect pests (Chaton et al. 2001, Teicher et al. 2003). Therefore, application of one specific enantiomer could be equally effective as application of the racemate while minimizing nontarget organism enantioselective toxicity for fipronil, as the target species toxicity is equivalent for both enantiomers. According to the limited studies of the enantioselective toxicity of fipronil, the (-) enantiomer appears to be the least toxic form in freshwater organisms exhibiting enantioselective toxicity. However, although many freshwater species are more sensitive to the (+) enantiomer compared to the (-) enantiomer (Konwick et al. 2005, Overmyer et al. 2007, Wilson et al. 2008), the magnitude of toxicity varies. To reliably recommend application of one enantiomer over the other, more

research into chronic enantioselective toxicity, including full-life cycle assessments and behavioral studies, is required.

Conclusions

In our study of toxicity of fipronil to the fathead minnow, we found significant enantioselective toxicity via larval fish mortality and growth in subchronic (7-d) exposures, indicating increased toxicity of the racemate and (+) enantiomer relative to the (-) enantiomer. Although enantioselective toxicity of fipronil has been documented in aquatic invertebrates (Konwick 2005, Overmyer 2007, Wilson 2008), our study is the first to show subchronic enantioselective effects on survival and growth in fish. In the future, enantiospecific application of fipronil could decrease application rates and minimize nontarget organism toxicity. However, additional experiments are needed to investigate mechanisms for enantioselective toxicity and determine if enantiospecific application is feasible in the environment.

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Table 2.1. Average (n=3) measured concentrations of fipronil racemate, (+), and (-) enantiomer.

Nominal Concentration (μg/L)	Chemical	Measured Concentration (μg/L) (average ± standard error)
50	Racemate	50.4 (± 0.9)
	(+) Enantiomer	48.5 (± 0.7)
	(-) Enantiomer	50.1 (± 0.2)
100	Racemate	81.8 (± 18.1)
	(+) Enantiomer	85.1 (± 11.8)
	(-) Enantiomer	90.7 (± 9.9)
200	Racemate	171.4 (± 27.7)
	(+) Enantiomer	173.7 (± 27.3)
	(-) Enantiomer	174.6 (± 23.3)
400	Racemate	372.4 (± 26.3)
	(+) Enantiomer	338.6 (± 64.1)
	(-) Enantiomer	350.7 (± 45.1)

Table 2.2. Subchronic toxicity [median lethal concentration (LC50)] of racemic fipronil, (+) enantiomer, and (-) enantiomer to larval *Pimephales promelas* after seven days exposure. Median lethal concentrations were calculated with trimmed-Spearman Karber (ToxStat V 3.3). Different letters indicate significantly different LC50s ($p < 0.05$).

Chemical	µg/L	
	7-day LC50*	95% Confidence Interval
Racemate	208[a]	191-224
(+) Enantiomer	227[a]	201-243
(-) Enantiomer	365[b]	333-397

Table 2.3. NOEC and LOEC of larval *P. promelas* exposed to racemic fipronil, (+) enantiomer, and (-) enantiomer for seven days. NOECs and LOECs calculated with ToxStat V 3.3.

Test	Chemical	NOEC	LOEC
	Racemate	50	100
1	(+) Enantiomer	0	50
	(-) Enantiomer	0	50
	Racemate	50	100
2	(+) Enantiomer	0	50
	(-) Enantiomer	0	50
	Racemate	50	100
3	(+) Enantiomer	50	100
	(-) Enantiomer	50	100

Table 2.4. Results of linear regression (slope and associated 95% confidence interval) (SAS V9.1) of concentration versus percentage control weight for larval *P. promelas* exposed to racemic fipronil, (+) enantiomer, and (-) enantiomer for seven days. Different letters indicate significantly different slopes ($p < 0.05$).

Chemical	Slope	Lower 95% Confidence Limit	Upper 95% Confidence Limit
Racemate	-0.00276[a]	-0.00322	-0.00223
(+) Enantiomer	-0.00287[a]	-0.00328	-0.00247
(-) Enantiomer	-0.00196[b]	-0.00216	-0.00176

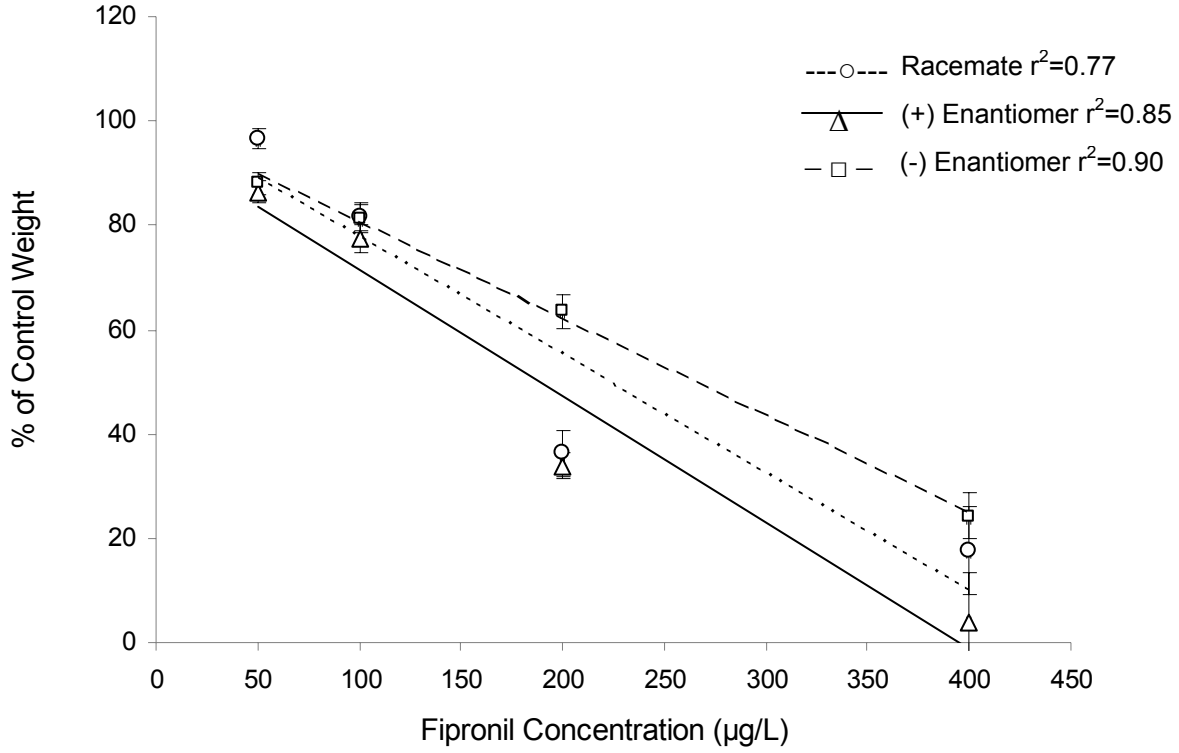

Figure 2.1. Reductions of growth of fipronil racemate, (+) enantiomer, and (-) enantiomer exposed *P. promelas*. Each point is mean % of control weight (± one standard error) of three tests based on nominal test solution concentration.

CHAPTER 3

BEHAVIOR OF FIPRONIL IN A SEDIMENT-WATER-FISH SYSTEM[1]

[1]Baird. S, A.W. Garrison, J. Avants, J. Jones, R. Bringolf, M.C. Black. To be submitted to *Environmental Science and Technology*.

Abstract

Fipronil is a widely used pesticide and as regulations on older pesticides increase, production and application of fipronil is expected to increase, leading to increased inputs into aquatic environments. Fipronil is hydrophobic and can be expected to sorb to sediments when introduced to aquatic environments. We exposed juvenile fathead minnows to fipronil spiked sediment to determine behavior of fipronil in a sediment-water-fish system. In sediment, fipronil was transformed primarily to the fipronil sulfide. Fish rapidly accumulated fipronil and immediately transformed it to fipronil sulfone. This study suggests that there are several potential exposures scenarios of fipronil in aquatic environments. First, in anaerobic sediments, sediment dwelling organisms may be exposed primarily to fipronil sulfide. Second, by excreting fipronil sulfone, fish may contribute to exposure of fipronil sulfone in sediment dwelling organisms. Also, fathead minnows had body burdens of fipronil, fipronil sulfone and fipronil sulfide, allowing for possible trophic transfer and/or bioaccumulation of all three chemicals if fish were consumed by larger organisms. Dominant degradation products and enantioselectivity in degradation are dependent on site specific conditions, such as redox potential. Therefore, site-specific knowledge is necessary to determine potential exposure scenarios of fipronil in the environment. Also, more research on nontarget organism toxicity of the sulfide and sulfone degradates will aid in understanding potential deleterious effects in different exposure scenarios.

Introduction

Fipronil is a chiral, phenylpyrazole pesticide used to control household and agricultural pests and is applied as a racemate, or equal mixture of its two enantiomers. The two enantiomers are designated (R-) and (S+) based on the molecule's three dimensional structure and rotation on

a plane (Solomons 1990, Teicher et al. 2003). Fipronil was introduced for use in the US in 1996 and is applied increasingly as a replacement for the banned carbofuran and other restricted use pesticides (USEPA 1996). Fipronil is released into the aquatic environment via runoff from commercial and agricultural application, but its fate in surface waters and sediment is variable. In surface waters and sediment, fipronil undergoes several degradation pathways: fipronil photolyzes to desulfinyl fipronil, oxidizes to form fipronil sulfone, and fipronil sulfide is formed via reduction (Figure 1) (Jones et al. 2007, Ramesh et al. 1999). However, the predominant metabolite is dependent on site specific conditions; for example, fipronil sulfide would likely be the predominant metabolite in anaerobic soils and sediments and fipronil sulfone would likely be the predominant metabolite in aerobic soils and sediments (Rhône-Poulenc 1996). With the increasing use of fipronil, it is important to understand its fate in the aquatic environment because this will determine potential harmful effects on nontarget aquatic organisms.

Fipronil is hydrophobic, and with an average log K_{oc} of 2.9, can be expected to sorb to sediment (USGS 2003). Although fipronil has been detected in the water column [the National Ambient Water-Quality Assessment (NAWQA) detected fipronil in 25% of surface water samples (USGS 2003)], long term exposure in the environment is likely from sediment. In the Mermentau River Basin, Louisiana, fipronil was applied for rice water weevil (*Lissorhoptrus oryzophilus*) control beginning in 1999. By 2000, maximum fipronil concentrations in surface water ranged from 0.829 to 5.29 µg/L and the degradation product detected in the highest quantity was desulfinyl fipronil (1.13 µg/L). Fipronil sulfide (0.636-24.8 µg/kg), desulfinyl fipronil (0.55-7.01 µg/kg) and fipronil sulfone (not detected-10.5 µg/kg) were detected in bed sediment at nearly all of the 17 sites sampled, while fipronil was below detection (USGS 2003).

The Mermentau survey revealed that over time and distance from the source, concentrations of degradation products exceeded the concentration of the parent compound.

Laboratory studies of the behavior of fipronil in sediment support the trends observed in the Mermentau Basin, where degradation products are more persistent than the parent compound. Lin et al. (2008) examined the degradation of fipronil to desulfinyl fipronil, fipronil sulfide, and fipronil sulfone in fipronil-spiked sediment under facultative and anaerobic conditions and found all degradates to be more persistent than the parent compound. Under facultative conditions, the half- life of fipronil was 18.5 days while the half-lives for desulfinyl fipronil, fipronil sulfide, and fipronil sulfone were 388, 588, and 712 days, respectively, with degradation following a first-order decay model. Degradation under anaerobic conditions was much slower for all forms and degradation products remained more persistent than fipronil (Lin et al. 2008). First order decay of fipronil was also observed in estuarine mesocosms (Walse et al. 2004). Walse et al. (2004) found fipronil sulfide to be the major degradation product in the system with lesser amounts of fipronil sulfone and desulfinyl fipronil. Similar findings under anaerobic conditions were reported in Brennan et al. (2009). In addition to being more persistent in the environment, some degradates may be more toxic than the parent compound to some aquatic species; the oxidation degradation product fipronil sulfone is significantly more toxic than the parent compound to both rainbow trout (*Oncorhynchus mykiss)* and bluegill (*Lepomis macrochirus*) in waterborne exposures (USEPA 1996).

Fipronil's chirality further complicates evaluation of its fate in aquatic systems. Although fipronil is applied as a racemate, biological degradation can alter the enantiomeric fraction (EF = [concentration of (+) enantiomer]/[concentration of (+) enantiomer + concentration (-) enantiomer]). The enantiomers can be degraded at different rates thereby

increasing or decreasing the EF (Garrison 2006). In one study, enantioselective degradation in sediment was dependent on the microbial community; sulfidogenic sediments preferentially degraded the (+) enantiomer and methanogenic sediments preferentially degraded the (-) enantiomer (Jones et al. 2007). Nillos et al. (2009) found preferential degradation of the (+) enantiomer in anaerobic sediments and non-enantioselective degradation in aerobic or slightly reducing conditions. Fipronil also exhibits enantioselectivity in its toxicity to aquatic organsisms. Acute enantioselective toxicity (LC50) of fipronil has been found in several aquatic species, including the daphnid (*Ceriodaphnia dubia*) (Konwick et al. 2005), which like the crayfish and larval grass shrimp (Overmyer et al. 2007) are more sensitive to the (+) enantiomer. In one of the few studies measuring chronic enantioselective toxicity, the (+) enantiomer (LOEC = 2 µg/L) was significantly more toxic to daphnid reproduction than either the racemate (LOEC = 15 µg/L) or the (-) enantiomer (LOEC = 30 µg/L) (Wilson et al. 2008). Baird et al. (in prep.) found subchronic enantioselective toxicity in larval fathead minnows; the racemate and (+) enantiomer were more toxic than the (-) enantiomer to fish survival and growth.

Fipronil has been shown to bioaccumulate in a range of aquatic species under a variety of exposure conditions (Chaton et al. 2001, Chaton et al. 2002). Juvenile rainbow trout rapidly accumulated fipronil when fed food spiked with 7.68 µg/g racemic fipronil (Konwick et al. 2006). Trout also biotransformed fipronil as evidenced by changing EF. Although trout were fed racemic fipronil, the EF of bioaccumulated fipronil decreased and the (-) enantiomer was more prominent than the (+) enantiomer (Konwick et al. 2006). In a study of bioaccumulation of fipronil in nontarget arthropods, bioaccumulation was highly variable; after 48-hrs aquatic exposure to racemic fipronil, accumulation factors (ratio of the concentration of radiolabeled fipronil in the organism to that in the medium) ranged from < 1 in the phantom midge

(*Chaoborus crystallinus*) to > 60 in the bloodworm (*Chironomus annularis*) (Chaton et al. 2002). The sediment dwelling amphipod *Hyalella azteca* bioaccumulated fipronil, fipronil sulfide, and fipronil sulfone when exposed to contaminated sediment (Kroger et al. 2009). Otherwise, there is a paucity of data on bioaccumulation of fipronil from sediment and no data were found for fish exposed in a sediment:water system.

The objective of this study was to measure bioaccumulation of fipronil and fipronil degradation products by the fathead minnow (*Pimephales promelas)* via sediment exposure. Fathead minnows are known to ingest sediment (McCarthy et al. 2003) and are expected to be exposed primarily via sediment sorbed fipronil and, to a lesser extent, waterborne fipronil. Specifically, our objectives were to determine (1) if fathead minnows bioaccumulate fipronil from sediment exposure, (2) quantify major metabolites formed in fish, sediment, and water over the course of the exposure, and (3) identify any enantioselectivity in fipronil biotransformation and degradation. Although several studies document bioaccumulation of fipronil from food, water and sediment (though only in invertebrates), to our knowledge, there are no studies on the bioaccumulation of fipronil in fish via sediment exposure. Sediment is the primary source of chronic fipronil exposures in the environment and it is important to determine risks to fish of this exposure route. It is also important to identify enantioselectivity in degradation because of the well documented enantioselective toxicity and differential toxicity of degradation products.

Materials and Methods

Sediment collection

Sediment was collected from a freshwater pond at the USDA field research station near Watkinsville, GA. Large debris was removed. Sediment from this site was previously

characterized by Jones et al. (2007) as actively methanogenic with a pH of 6.7, weight percent total organic carbon of 4.0, and 0.30 weight percent total nitrogen. Fipronil was not detected in sediment from this site. Sediment was stored in 5-gal sealed, plastic buckets with a blanket of N_2 in the headspace and held in the dark at 4° C prior to sediment spiking.

Chemicals

Fipronil (± 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4[(trifluoromethyl)-sulfinyl]-1H-pyrazole-3-carbonitrile; 97.8% pure) was purchased from ChemService (West Chester, PA, USA).

Sediment Preparation

Prior to spiking and aging, sediment from the three 5-gal buckets were combined and thoroughly mixed to ensure homogeneity. Mixing was accomplished with an electric drill fitted with a paint mixer attachment. Sediment was then divided equally and placed into two new plastic 5-gal buckets for preparation of control and spiked sediments. Fipronil in acetone was slowly added to the sediment while mixing. Nitrogen gas was continuously added to the mixing container to maintain anoxic conditions and samples were mixed for 24 hours at 4 ° C. One batch of fipronil spiked sediment was prepared with a target spike of 5 ppm. One batch of control sediment was prepared with acetone only as a solvent control. After mixing, sediment containers were purged with N_2, sealed, and sediments were aged for 6 months to allow for complete sorption of fipronil into the sediment. Prior to test initiation, samples of control and fipronil spiked sediment were analyzed for concentration of fipronil and metabolites and calculation of fipronil EF.

Test organisms

Juvenile fathead minnows ranging in age from 101-120 days old were used in the bioaccumulation experiment. Fish were obtained from U.S. Environmental Protection Agency, Region 5 laboratory in Cincinnati, OH and acclimated in flow-through tanks in the laboratory for two months prior to testing. During the holding period, fish were fed twice daily and water quality was monitored once daily. Laboratory conditions (pH = 6.9-7.5, temperature=18.9-24.0, DO>4.0 mg/L, photoperiod= 16 h light:8 hr dark) during the holding period were similar to test conditions.

Bioaccumulation Test

Control and fipronil spiked sediments were each divided into three 10-gal aquaria with 2.5 L of sediment added to each tank. Eighteen L of test water (dechlorinated, filtered (5µm, UV) tap water) were added slowly to minimize sediment disturbance. Tanks were allowed to further settle overnight. The following morning 58 fish were randomly added to each tank. A subsample of 15 fish sacrificed on day zero had no detectable body burdens of fipronil, fipronil sulfide, or fipronil sulfone. Ammonia, pH, DO, and temperature were monitored three times daily for the first week, after which water quality was monitored twice daily. To maintain water quality, 2 L of water were exchanged with clean test water renewed in each tank twice/week for the first two weeks and once/week thereafter. Fish were fed flake food (Tetrafin, Aquatic Eco-Systems Inc, Apopka, FL) *ad libitum* twice daily. On sampling days, fish were not fed within 8 hours of sampling.

Fish, sediment, and water were sampled on days seven, 14, 21, 28, 35, and 42 from each tank within each treatment (control and fipronil spiked sediment). Water and sediment samples

were collected in glass vials; 200 mL of water and 15 g sediment were removed and stored overnight at 4 ° C prior to analysis. Typically, 10 fish were sampled from each tank, but because of sporadic mortality, some samples contained less than 10 fish. On day 35, nine fish were removed from one control tank, and eight to nine fish were removed from the three fipronil exposure tanks. On the final day of the experiment, all remaining fish were removed from control tanks (one from the first tank, zero from the second tank, and six from the third tank) and fipronil exposure tanks (seven from the first tank and five each from the second and third tanks). Fish were maintained overnight in test water removed at sampling to allow them to empty their gut contents, so that fipronil body burdens would not include fipronil sorbed onto sediment in their GI tract. Fish were euthanized with an overdose of buffered MS-222, rinsed with deionized water, placed in plastic storage bags and transported on ice to the EPA facility in Athens, GA. At the end of the experiment, to determine if fipronil and metabolites were evenly distributed within tanks, sediments were analyzed for fipronil from seven locations within the representative tank.

Sample analysis

Samples of overlying water, sediments, and fish were analyzed for concentrations of fipronil, fipronil sulfide, fipronil sulfone, and fipronil EF. In water, concentrations were determined with solid phase extraction followed by gas chromatography-mass spectrometry (HP 6890/5973) using a chiral column (BGB-172 Analytik, AG, Anwil, Switzerland). This process is described in detail in Konwick et al. (2005). Average recovery of fipronil spiked into water at 100 µg/L was 99 ± 0% (n = 3). The minimum detection limit (MDL) of fipronil in water was 1 ppb. Fish tissue and sediment samples were analyzed similar to Konwick et al. (2007). A recovery standard, PCB 65, was added to the samples prior to extraction so as to give a concentration of 0.5 mg/L in the final

extract that was used for GC-MS analysis. Samples (sediment or freeze dried fish carcasses) were homogenized twice in methanol (10 mL for sediment and 5 mL for fish). The methanol was separated, combined and evaporated to 5 mL (fish) or 2 mL (sediment). This methanol extract was then combined with distilled water (25 mL for fish and 10 mL for sediment) and extracted with methyl-tert-butyl ether (MTBE) (5 mL for fish and 3 mL for sediment). This final extract was then centrifuged to separate as much MTBE as possible from the methanol-water layer and the MTBE was analyzed by GC-MS as described above for the water samples. Fish and sediment extract concentrations were corrected to PCB 65 recovery, which averaged 46 ± 2% (mean ± SE) over all fish samples and 54 ± 2% (mean ± SE) over all sediment samples. The MDLs of fipronil, fipronil sulfide, and fipronil sulfone in both fish and sediment were 20, 50, and 50 ppb, respectively.

The enantiomeric fraction of fipronil was calculated as:

$$EF = [E_A]/([E_A]+[E_B]) \quad (1)$$

where $[E_A]$ and $[E_B]$ are the concentrations of the first and second eluting enantiomers (Jones et al. 2007). The (+) enantiomer was the first eluting enantiomer and EFs > 0.5 have an increased proportion of the (+) enantiomer. The (-) was the second eluting enantiomer and EF < 0.5 indicated an increased proportion of the (-) enantiomer.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's mean separation test (α=0.05) was used to determine significant differences among chemical types (fipronil, fipronil sulfide, and fipronil sulfone) in fish (n = 3) and sediment (n = 3) within each timepoint and significant differences within chemical types (in fish and sediment) across timepoints. Proportional EF data

was arcsine transformed and significant differences of EF between fish and sediment were determined with a t-test at each timepoint. Significant differences of EF within fish and sediment across timepoints were analyzed with ANOVA followed by Tukey's mean separation test. All ANOVA and t-test procedures were conducted with SAS (V 9.1). Z-statistic ($z = (EF_{mean} - 0.50)/(\sigma_{EF})$) was calculated in Microsoft Excel to determine if measured EF's were significantly different from 0.50. A z-statistic > 1.96 indicated EFs significantly higher than 0.5.

Bioaccumulation factors (BAF) were calculated using average chemical concentrations:

$$BAF = C_{fish}/C_{(sed+water)} \quad (2)$$

where C_{fish} is the concentration of fipronil and all metabolites detected in fish and $C_{(sed+water)}$ is the concentration of fipronil and all degradates detected in the sediment and water.

Results

Fish Health

Fish in both fipronil and control tanks experienced sporadic mortality, which never exceeded 12%. Mortality in the three fipronil tanks was 3, 9, and 9% and mortality in the control tanks was 3, 7 and 12%. Mortality in all tanks never exceeded the chronic limit of 20% (USEPA 2002).

Water Quality

Water quality was similar among all tanks. Temperatures ranged from 19.6 to 23.1 ° C, with 24 hour temperature changes not exceeding ± 1 ° C. Dissolved oxygen was greater than 4.0 mg/L and pH ranged from 6.6-7.2. The highest un-ionized ammonia concentration observed in the control tanks was 0.06 mg/L in one tank on day seven. In the fipronil tanks, the highest un-

ionized ammonia (0.09 mg/L) was observed in one tank on day 12. By day 23, un-ionized ammonia in fipronil and control tanks dropped below 0.002 mg/L and 0.007 mg/L, respectively, and remained low for the remainder of the exposure. In all tanks, un-ionized ammonia levels were below toxic levels for chronic exposure. Thurston et al. (1986) found no effects on survival or growth of fathead minnows at un-ionized ammonia concentrations of 0.44 mg/L and no histopathologic (brain lesions) effects at levels at or below 0.11 mg/L.

Water

Following equilibration with contaminated sediments, fipronil was detected in the water, although at considerably lower concentration than in both fish and sediment. The highest concentration of fipronil detected was 20 ppb on day seven. Fipronil was not detectable in the water by the final day of the test (Figure 2). Enantiomeric fraction of fipronil in water was greater than 0.5 at all sampling timepoints with detectable fipronil concentrations (an EF>0.5 indicates that the proportion of (+) enantiomer is higher than (-) enantiomer) (Figure 3). The degradation products fipronil sulfone and fipronil sulfide were not detected in water.

Sediment

Fipronil sulfide was the primary chemical form in sediment (Figure 4) and was detected at significantly ($p<0.0001$) higher concentrations than fipronil and fipronil sulfone for the majority of the experiment. Sediment fipronil sulfide concentrations ranged from 1.2 to 2 ppm and did not vary significantly over the course of the study. Fipronil concentrations in sediment were highest on day zero and decreased significantly ($p<0.0001$) until day 14, after which they remained below 0.5 ppm and did not vary significantly through day 35 and fipronil was not

detected in the sediment on the final day of the exposure (day 42). Fipronil sulfone was not detected until days 28-35 and concentrations remained below 0.06 ppm (Figure 4). Fipronil EF in sediments was significantly higher than 0.5 (z-statistic ranged from 3.9 to 13.6) for the duration of the experiment (Figure 3). Except for a significant ($p=0.01$) increase from day seven to day 14, EF did not vary significantly over the course of the study.

Fish

Fish accumulated fipronil rapidly and biotransformed it primarily to the sulfone metabolite. Fish BAF ranged from 2.08 to 5.12 (Table 1). Fish body burdens of fipronil sulfone were significantly ($p<0.0001$) higher than fish and sediment concentrations of fipronil and fipronil sulfide on days seven through 42. Fish body burdens of fipronil and fipronil sulfide remained below 2 ppm over the course of the study, and concentrations of these forms in fish tissue were similar until day 35, after which fipronil sulfide concentrations were higher ($p<0.0001$) than fipronil for the remainder of the study. Fish and sediment concentrations of fipronil and fipronil sulfide were similar for the majority of time points. Fipronil EF in fish was significantly higher than 0.5 (z-statistic ranged from 3.7 to 26.2) throughout the experiment. Overall, EF in fish increased significantly ($p<0.0001$) over the course of the study (Figure 3) and EF was not significantly different in fish and sediment on days seven through 35.

Discussion

Fipronil and its degradation products have high K_{oc} values (all greater than 2.09); therefore, it is essential to consider the behavior of fipronil and degradates in sediments to determine potential effects of fipronil contamination in aquatic environments. Fipronil sulfide,

produced when fipronil is reduced in anaerobic conditions, is a common degradation product in many laboratory studies and field surveys (Gunasekera et al. 2007, Tingle et al. 2003, Lin et al. 2008, USGS 2003, Aajoud et al. 2003), and was the primary form observed in the present study (Figure 3). Similar results, degradation of fipronil to fipronil sulfide, were found by Jones et al. (2007) with methanogenic sediments collected from the same location as the present study. Furthermore, Jones et al. (2007) found preferential transformation and removal of the (-) enantiomer resulting in an EF>0.5 throughout their study. We observed a similar trend: the sediment EF remained enriched in the (+) enantiomer over the course of the experiment (Figure 4). However, enantioselectivity of fipronil degradation varies according to sediment redox conditions. In sulfidogenic saltwater sediments, Jones et al. (2007) found preferential transformation of the (+) fipronil enantiomer to fipronil sulfide by microbial communities (resulting in an EF<0.5) while Nillos et al. (2009) found increased transformation of the (+) enantiomer in three different sediments under anaerobic conditions. However, significant enantioselectivity in degradation has not been observed in sediment under aerobic conditions (Nillos et al. 2009, Tan et al. 2008). Enantioselective toxicity of fipronil and variable toxicity of degradation products is well-documented (Baird et al. in prep, Overmyer et al. 2007, Schlenk et al. 2001, USEPA 1996, Wilson et al. 2007, Konwick et al. 2005). Therefore, site specific conditions (i.e. redox potential) that dictate dominant enantiomers and metabolites will also dictate potential deleterious effects on nontarget organisms.

Fathead minnows bioaccumulated fipronil but rapidly metabolized it to fipronil sulfone; fipronil sulfone concentrations in fish exceeded concentrations of both fipronil and fipronil sulfide in the sediment. Fish likely convert fipronil to fipronil sulfone via Phase 1 oxidation, a common detoxification process used by fish to detoxify xenobiotics (Newman et al. 2003,

Kleinow et al. 1987). Furthermore, fipronil sulfone is a known oxidation product in both mice and invertebrates (Brookhart 1994, Hainzl 1996) and was also observed in trout exposed to fipronil via diet (Konwick et al. 2007). The log K_{ow} of fipronil sulfone (3.7) (Walse et al. 2004), and greater biomagnification factor (BMF) of 4.78 for fipronil sulfone compared to fipronil (BMF=0.04) (Konwick et al. 2007) support the preferential accumulation of fipronil sulfone observed in the present study. Bioaccumulation factors of fipronil sulfone in fathead minnows in the current study were very high (data not shown). However, because fish may be a source of fipronil sulfone in sediment (via elimination), it would be incorrect to calculate BAF based on the concentration of fipronil sulfone in fish and concentration of fipronil sulfone in sediment. Because all forms of fipronil present in the sediment are a potential source of all forms of fipronil in the fish, the BAF of summed concentrations may be more appropriate.

Fish may play an interesting role in the fate of fipronil and the potential toxicity of fipronil to other nontarget species. Fish are a potential source of fipronil sulfone detected in sediments in the present study; fipronil sulfone was not detected in the sediment until day 28. However, the presence of fipronil sulfone in the sediment may also be a result of oxidation of sediment; fipronil sulfone has been observed in aerobic and flooded soils (Tan et al. 2008). Although the sediment was aged under anoxic conditions, the water column was aerated during the exposure, potentially allowing for oxidation of the top layer of sediment. Conversion of fipronil to fipronil sulfone is not likely beneficial to fathead minnows. Although toxicity of fipronil sulfone to fathead minnows is unknown, the sulfone metabolite is 6.3 times more toxic to rainbow trout and 3.3 times more toxic to bluegill than the parent compound (USEPA 1996).

Konwick et al. (2007) observed preferential transformation of the (+) enantiomer in trout exposed via diet. This is opposite to the trend observed in the present study. Based on overall

increase of EF in fish (Figure 4), fathead minnows appeared to selectively transform the (-) enantiomer leading to an increased proportion of the (+) enantiomer. Like conversion to fipronil sulfone, fathead minnow's preferential transformation of the (-) enantiomer may be detrimental to the organism. In an acute test with larval fathead minnows, the (+) enantiomer and racemate were significantly more toxic than the (-) enantiomer (Baird et al. in prep). If fathead minnows selectively transform the less toxic enantiomer, as observed in the present study, and retain the more toxic metabolite (fipronil sulfone), this could explain the enantioselective toxicity observed in acute exposures (Baird et al. in prep). In racemic exposures fish may be retaining the more toxic enantiomer while also converting the less toxic enantiomer to a more toxic metabolite.

Small amounts of fipronil (<20 ppb) were detected in the overlying water in the present study, and likely resulted from desorption from sediments resuspended by fish activity (McCarthy et al. 2003). However, fipronil concentrations and EF's in water were not analyzed for significance over time because (1) amounts were magnitudes lower than amounts detected in fish and sediment and (2) the inability to determine whether amounts in water were sourced from fish excretion or equilibration with sediment. Nevertheless, we can contrast the concentrations of fipronil in the water and sediment to known toxic levels in fathead minnows. Waterborne fipronil is acutely toxic to larval fathead minnows (7-day LC50 of racemic fipronil = 208 ppb) (Baird et al. in prep). Although the concentration of fipronil in the water column (<20 ppb) was well below this level, the concentration of fipronil in the sediment (>2 ppm) was well above the LC50 for larval fathead minnows. Even though fathead minnows ingest sediment (McCarthy et al. 2003), fipronil did not appear to cause mortality in excess of control mortality in the present study. There are several possible explanations for the lack of mortality despite the high concentrations of chemical in the sediment. First, the juvenile fish used in the present study are

likely much less sensitive than larval fish (<7 days of age). This trend is well documented in the aquatic toxicity of other chemicals to fathead minnows (Hoang et al. 2004, Devlin et al. 1982, Mayes et al. 1983). Also, fipronil toxicity may be decreased when fish are exposed via ingested sediment because of the potential reduced bioavailability of sediment-bound fipronil. Although sediment-sorbed contaminants may be less bioavailable, the presence of fipronil and fipronil sulfone in fish tissue samples show that fish are indeed taking up the chemical and there may only be minimal protection afforded by sorption of fipronil to sediment.

This study suggests that there are several potential scenarios for fipronil exposure in aquatic environments. First, in anaerobic sediments, sediment dwelling organisms may be exposed primarily to fipronil sulfide. Second, by excreting fipronil sulfone, fish may contribute to exposure of fipronil sulfone in sediment dwelling organisms. Also, fathead minnows accumulated body burdens of fipronil, fipronil sulfone and fipronil sulfide, allowing for possible trophic transfer and/or bioaccumulation of all three chemicals if fish were consumed by larger organisms.

Conclusions

While the present study demonstrated the fate of fipronil in a simplified sediment-water-fish system, it also introduced several important factors to be considered when evaluating potential deleterious effects of fipronil in the aquatic environment. First, fish may be a source of fipronil sulfone (fipronil sulfone was not detected in sediment until well after it was being produced in fish) presenting an increased risk to sediment dwelling organisms with greater sensitivity to fipronil sulfone. For example, fipronil sulfone is slightly (though not significantly) more toxic than fipronil to crayfish (*Procambarus clarkii)* when exposed via water in acute

exposures (Schlenk et al. 2001) and in both acute and chronic endpoints of larval *Chironomus tentans* when exposed via sediment (Maul et al. 2008). And, based on data for bluegill and rainbow trout (USEPA 1996), conversion of fipronil to fipronil sulfone may also be detrimental to the fish itself. This study also added to the body of knowledge demonstrating the variable fate of fipronil in the aquatic environment. Dominant degradation products and enantioselectivity in degradation are dependent on site specific conditions, such as sediment redox potential. Therefore, site-specific knowledge is necessary to determine potential exposure scenarios for fipronil in the environment. Also, more research on nontarget organism toxicity of the sulfide and sulfone metabolies will aid in understanding potential deleterious effects of fipronil in different exposure scenarios.

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Table 3.1. Bioaccumulation factors of fipronil, fipronil sulfide, and fipronil sulfone in fish exposed to fipronil spiked sediment by sampling timepoint.

Day	BAF
7	3.90
14	5.12
21	3.02
28	2.14
35	2.08
42	3.83

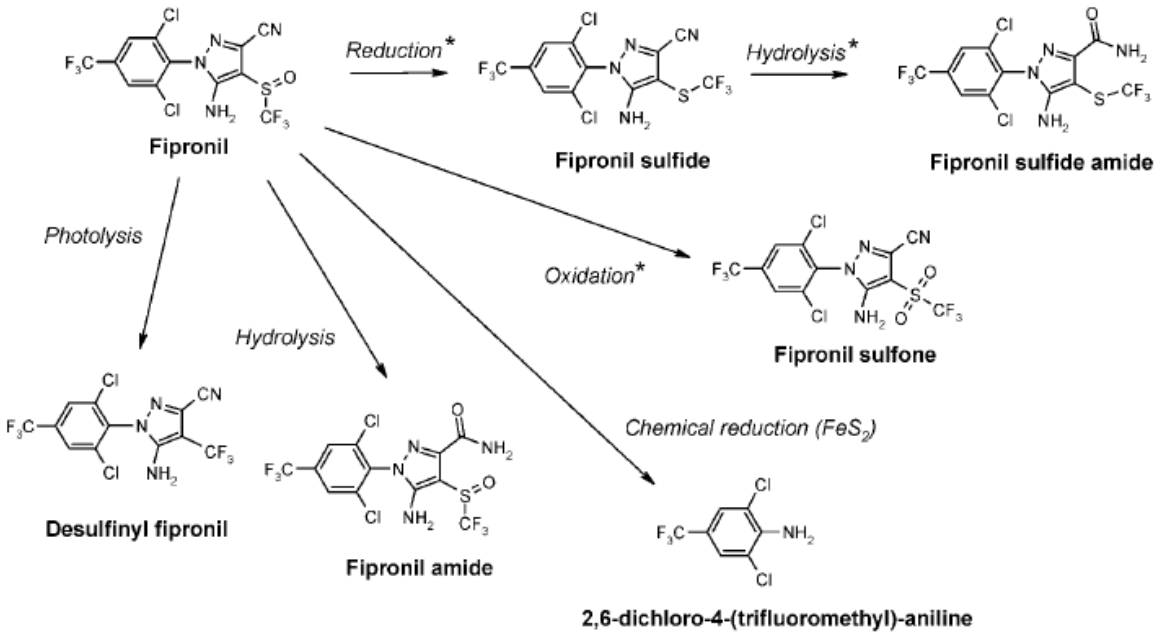

Figure 3.1. Biotic (noted with *) and abiotic degradation pathways and products of fipronil (From Jones et. al 2007, reproduced with permission)

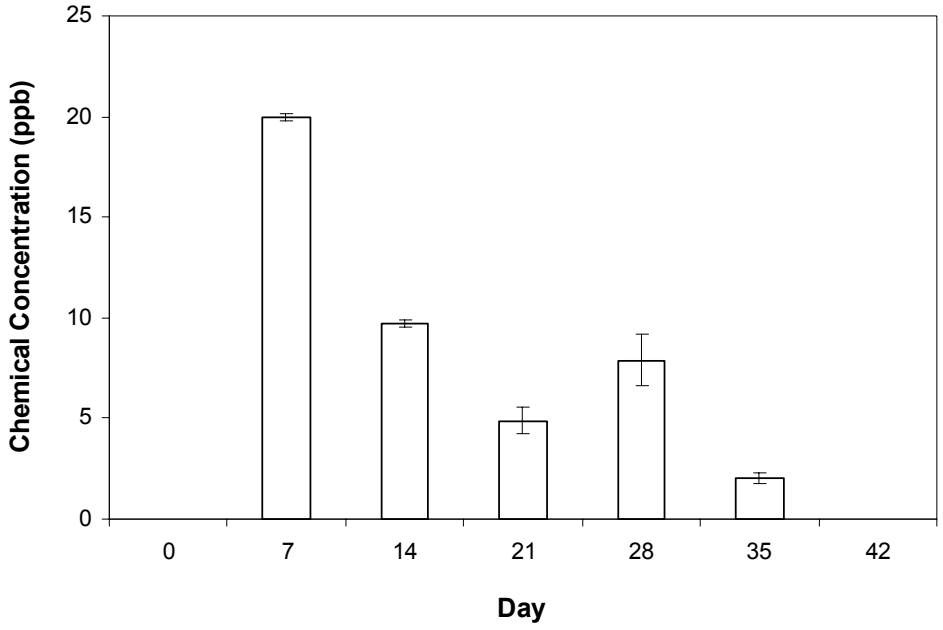

Figure 3.2. Average concentration (mean ± standard error) of fipronil in overlying water by sampling day. The degradation products fipronil sulfone and fipronil sulfide were not detected in water samples.

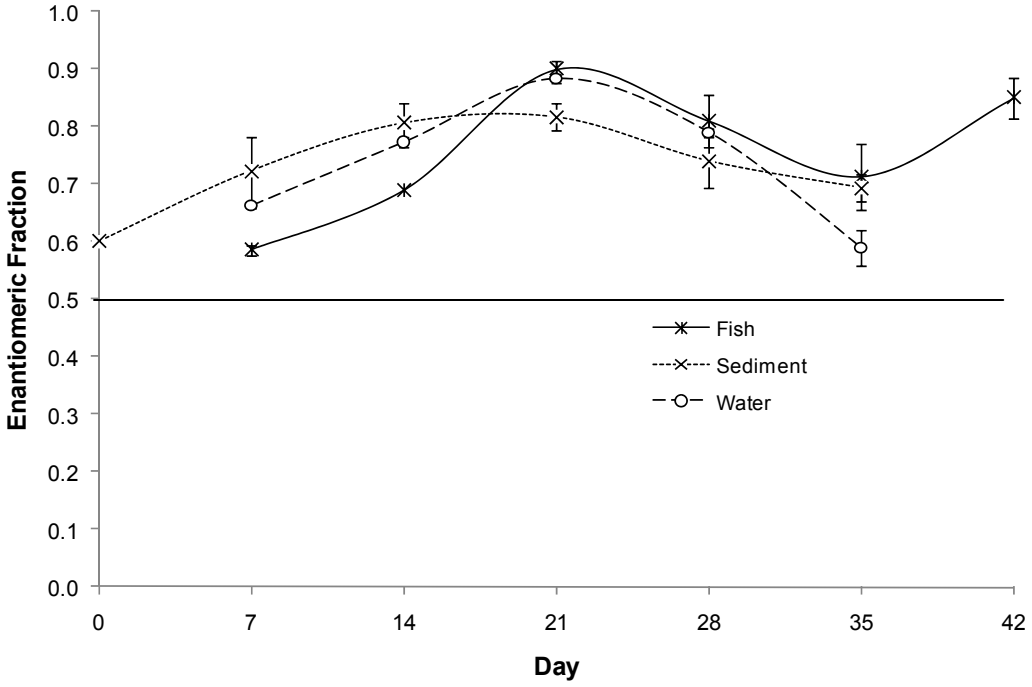

Figure 3.3. Average enantiomeric fraction (mean ± standard error) of fipronil in fish, sediment, and water by sampling day.

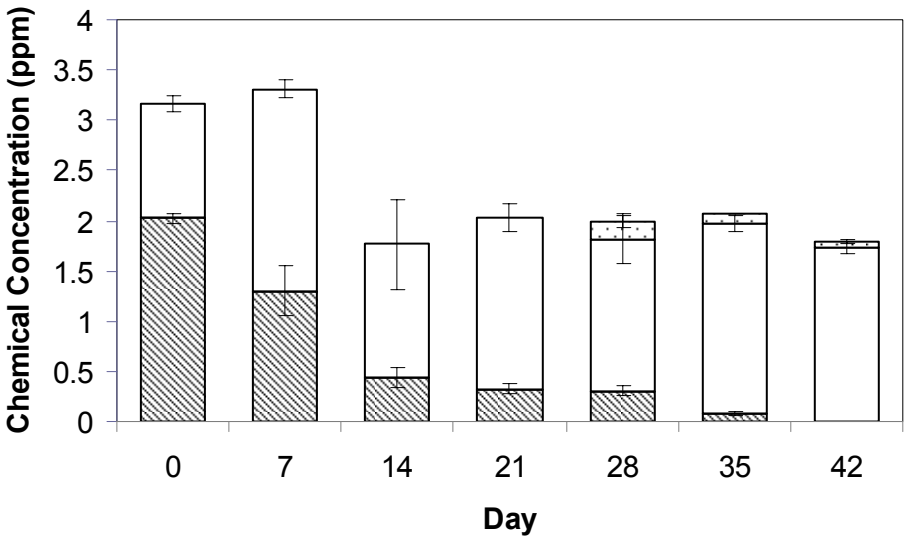

Figure 3.4. Average concentrations (mean ± standard error) of fipronil sulfone ⁘, fipronil □ , and fipronil sulfide ▨ in sediment by sampling day. Fipronil sulfone only detected in samples from days 28, 35, and 42.

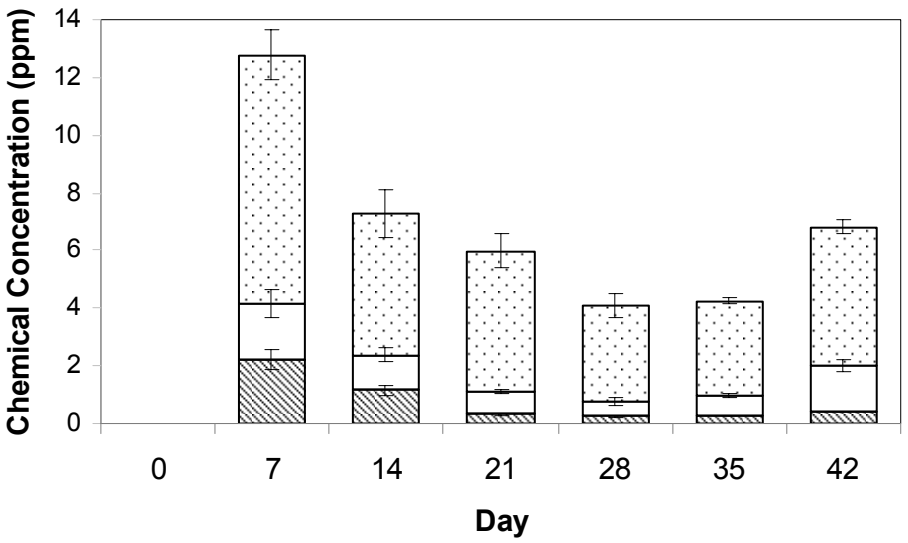

Figure 3.5. Average concentrations (mean ± standard error) of fipronil sulfone ⬚, fipronil ☐, and fipronil sulfide ▨ in fish by sampling day.

CHAPTER 4

CONCLUDING REMARKS

Pesticide contamination in surface waters and biota is a widespread problem in the USA. In a 10-year study, Gillom et al. (2006) sampled 178 streams for pesticides in surface water, shallow groundwater, bed sediment, and fish tissues. In agricultural areas, pesticides were detected in over 90% of surface water and fish tissue samples and in over 50% of groundwater and sediment samples. In urban areas, pesticides were detected in over 90% of surface water and fish samples, 55 % of groundwater samples, and 80% of sediment samples. Although undeveloped areas were the least effected, there was still a significant amount of detections; in surface water and fish tissue, pesticides were detected in around 60% of samples and in less than 30% of groundwater and sediment samples. It is likely that some of these detections were fipronil, based on field studies of fipronil pollution (Bedient et al. 2005, USGS 2003) and the increasing production and application of fipronil. Therefore, it is important to understand fipronil's potential negative effects on nontargets in the environment.

There have been a number of studies regarding toxicity to nontarget organisms such as daphnids, bluegill, crayfish, and rainbow trout. To contribute to this data, in Chapter 2, we determined the enantioselective toxicity of fipronil in larval fathead minnows (*Pimephales promelas*). We observed increased toxicity of the (+) enantiomer in larval fathead minnows exposed to the fipronil racemate and each enantiomer seven days. Curiously, the toxicity of the

racemate and (+) enantiomer was not significantly different, even though the racemate contains 50% of the (+) enantiomer and 50% of the less toxic (-) enantiomer. Metabolic processes could potentially change the enantiomeric fraction present in the organism, so that fathead minnows selectively transform and eliminate the (-) enantiomer, increasing the proportion of the more toxic (+) enantiomer in the racemic exposure. To better understand the interesting trend we discovered in Chapter 2, in Chapter 3, we exposed juvenile fathead minnows to fipronil contaminated sediment. We found that fish rapidly bioaccumulated fipronil and transformed it to fipronil sulfone. Fish preferentially transformed the (-) enantiomer, resulting in an increased proportion of the (+) enantiomer in fish tissues. If fathead minnows selectively transform the less toxic enantiomer, as observed in Chapter 3, and retain the most toxic metabolite (fipronil sulfone), this could explain the enantioselective toxicity observed in Chapter 2. Fish may be retaining the more toxic enantiomer while possibly converting the less toxic enantiomer to a more toxic metabolite.

Another aim of this research was to contribute to data on enantioselective toxicity in support of development of single-enantiomer or enantiomer-enriched formulations of fipronil. At least 25% of current-use insecticides are chiral and uncertainty surrounding enantioselective toxicity is not unique to fipronil (Garrison 2006). Single enantiomer or enantiomer-enriched formulations of other pesticides have been introduced in the US and Europe and both will allow for decreased application of chemical and a potential decrease in nontarget organism toxicity. Enantiomer specific formulations of fipronil could potentially be equally effective against pests with decreased effects on nontarget organisms. Laboratory experiments with selected target organisms show the racemate and both enantiomers to be equally acutely toxic (Chaton et al. 2001, Teicher et al. 2003). Therefore, application of one specific enantiomer could be equally

effective as application of the racemate while minimizing nontarget organism enantioselective toxicity. Decreased application of enantiomer specific formulations would also partly alleviate nontarget organism sensitivity to degradation products; less fipronil applied translates to less fipronil degradation products in runoff. Unfortunately, no clear pattern emerges for enantioselective toxicity with fipronil with lethality (LC50) as the endpoint. Enantioselectivity varies between species and life stages, and toxicity of the racemate can be dependent on sex of organism. Acute enantioselective toxicity (LC50) of fipronil has been found in several aquatic species, including the daphnid, *Ceriodaphnia dubia*, (Konwick *et al.* 2005), which like the crayfish are more sensitive to the (+) enantiomer, and larval grass shrimp (*Palaemonetes pugio*) which is more sensitive to the (-) enantiomer (Overmyer *et al.* 2007). However, acute enantioselectivity (LC50) has not been observed in the clam (*Mercenaria mercenaria*), adult grass shrimp, larval black flies, (*Simulium vittatum*, or the Japanses medaka (*Oryzias latipes)* vermyer *et al.* 2007, Nillos et al. 2009). Furthermore, enantioselectivity in degradation is dependent on site specific conditions, such as redox potential. So, even if an enantiomer-enriched formulation were applied, this would not guarantee that organisms would be exposed to that formulation. Therefore, widespread use of enantiomer-enriched formulations is unlikely. However, enantiomer-enriched formulations may be useful in minimizing negative effects to specific economically important or endangered species. Otherwise, minimizing application of fipronil while continuing to research the toxic mechanism of action in nontarget organisms are the best ways to respond to and understand effects of fipronil on nontarget organisms.

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