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Acute toxicity of three strobilurin fungicide formulations and their active ingredients to tadpoles

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Abstract Fungicide applications in the United States have increased tenfold in the last 5 years. Formulations and active ingredients (AIs) have been demonstrated to cause acute mortality to amphibian life stages. However, there has been little to no discrimination between the toxicity of fungicide formulations and their AIs. Therefore, we compared the acute toxicity of the active ingredients and formulations of the fungicides Headline®, Stratego®, and Quilt® using *Bufo cognatus* tadpoles exposed to four concentrations and a control. All fungicides, including AIs and formulations, demonstrated toxicity to tadpoles, with Headline® and Stratego® causing 100 % mortality at the highest concentrations. Exposure to Quilt® formulation and its AIs resulted in 50–60 % tadpole mortality. Overall, toxicity was comparable between AIs and formulations for all fungicides and concentrations, with the exception of Headline® at 5 μg/L, where formulation exposure resulted in 79 % mortality versus no mortality from exposure to the AI. Results suggest the AIs are responsible for most mortality for Quilt® and Stratego®. Results for Headline® however suggest that although the AI is toxic to tadpoles at environmentally relevant concentrations, adjuvant(s) in the Headline® formulation also contribute to mortality, making it the most toxic of the fungicides studied.

Keywords Fungicide · Strobilurin · Amphibian · Toxicity · *Bufo*

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Introduction

Fungicide applications on corn, soybean, and wheat acreage in the United States have increased from less than 2 % treated in 2004 and 2005 (National Agricultural Statistics Service; http://www.pestmanagement.info/nass/app_usage.cfm) to 25–30 % treated in 2009 (letter from the Universities Regarding the strobilurin, pyraclostrobin, supplemental label, 2009, http://www.epa.gov/pesticides/regulating/headline-letter.pdf). Similarly, Great Britain has experienced an increase in the use of strobilurin fungicides within the last decade, with the use of pyraclostrobin nearly doubling between 2007 and 2008, from 72,836 to 123,330 kg applied (http://pusstats.csl.gov.uk/). This is in part due to recent disease outbreaks, such as soybean rust (*Phakopsora pachyrhizi*) (Deb et al. 2010), where recently developed strobilurin fungicides have been deemed the only effective means to combating the disease (Shaner et al. 2005). Strobilurins have a non-specific mode of action, binding to the Q_0 site in the mitochondria and thus blocking electron transfer between cytochrome b and c complexes and inhibiting cellular respiration of any organism with mitochondria (Bartlett et al. 2002; Balba 2007). Application also is not a selective process as most fungicides are sprayed aerially and sometimes directly with a ground sprayer (BASF 2006; Bayer Crop Science 2008; Syngenta Crop Protection 2009). Strobilurin fungicides are considered preventative by nature (Bartlett et al. 2002; Balba 2007) and labels claim they promote increased nutrient uptake, thus increasing yield (BASF Corporation 2008a, 2008b) and overall plant health (Bayer Crop Science 2008). Recent labelling changes allowing for prophylactic use of these products is also at least partially responsible for their increased usage (BASF Corporation 2008b; Bayer Crop Science 2008; Syngenta Crop Protection 2009).

Headline® (BASF), Stratego® (Bayer Crop Science), and Quilt® (Syngenta Crop Protection) are three strobilurins commonly sold in the United States. Although their application is prohibited in aquatic systems (BASF Corporation 2008a; Bayer Crop Science 2008; Syngenta Crop Protection 2009), contamination can occur through spray drift, overspray, and runoff. This is particularly true for depressional wetlands, the dominant aquatic feature of the intensively cultivated Great Plains (Smith et al. 2008), where cropping practices often extend to the edge or through the wetland center (O'Connell et al. 2011). This increased usage, potential exposure, and the demonstrated acute toxicity toward vertebrate (USEPA 2007) and invertebrate (Ochoa-Acuna et al. 2009) aquatic organisms (e.g., pyraclostrobin, the active ingredient in Headline®) lead Belden et al. (2010) to assess the toxicity of Headline®, Stratego®, and Quilt® on larval and recently metamorphed Great Plains toads, *Bufa cognatus*. Headline®, and to a lesser extent, Stratego®, were shown acutely toxic to larvae and metamorphs at environmentally relevant concentrations (Belden et al. 2010). However, only fungicide formulations were tested, with no discrimination among active ingredients (AIs), adjuvants, and inert ingredients. Studies with glyphosate formulations and adjuvant chemicals showed that adjuvant components (surfactants) cause anuran mortality at concentrations below that of AIs (Mann and Bidwell 1999; 2001). Headline® formulation contains polycyclic aromatic hydrocarbons, such as naphthalene (BASF Corporation 2008a, 2008b), which causes toxicity to various organisms (Barron et al. 1999; Stohs et al. 2002). Additionally, the 96 h-LC_{50} of pyraclostrobin, the AI in Headline®, is 11.4 µg/L (USEPA 2007) and 14 µg/L (Ochoa-Acuna et al. 2009) for bluegill (*Lepomis macrochirus*) and water flea (*Daphnia magna*), respectively, concentrations well within environmentally relevant levels. Stratego® is also reported to have petroleum distillates and xylene or xylene range aromatic solvents as part of its formulation (Bayer Crop Science 2008); therefore, adjuvants, in addition to the AIs, may contribute to the toxicity of these fungicide formulations.

Evaluating the contribution of both AIs and formulation additives toward toxicity provides a more complete analysis of the risks associated with exposure to pesticides. Thus, in this study we compare the acute toxicity of the AIs vs. complete formulations of Headline®, Stratego®, and Quilt® to larval *B. cognatus*. *Bufo cognatus* were used because of existing toxicity data (Belden et al. 2010), their risk of exposure as tadpoles in wetlands adjacent to croplands, and their distribution throughout much of the Great Plains. The results reported herein provide further understanding of potential risks of fungicides on non-target organisms and the formulation components responsible for the toxicity of these fungicides.

Materials and methods

Fungicide formulations

Headline® Fungicide (EPA Reg. No. 7969-186, BASF), Stratego® Fungicide (EPA Reg. No. 264-779, Bayer Crop Science), and Quilt® Fungicide (EPA Reg. No. 100-1178, Syngenta Crop Protection) formulations that would be commonly applied aerially were purchased through a local distributor. Formulation solutions were created by diluting formulations with deionized water (Table 1). These four concentration solutions were constructed around those of Belden et al. (2010) in an attempt to capture a measurable dose response (e.g., the range of Headline concentrations were reduced, as Belden et al. (2010) had 100 % mortality at all concentrations). Analytical standard grade (Pestanal®) AIs (pyraclostrobin, propiconazole, trifloxystrobin, and azoxystrobin) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved using HPLC grade acetone to produce the same concentrations as the formulations (Table 1). Fungicides were applied to experimental aquaria by adding 0.5 ml of the appropriate solution. Acetone (0.5 ml) was also added to formulation treatments and the control.

Test organisms

Adult *B. cognatus* were captured in central Oklahoma in May and June 2011 during and after rain events and housed in our in-house animal facility. Tadpoles were obtained from three adult toad pairings induced to breed by injecting luteinizing hormone-releasing hormone analog (LHRHa) at 20 µg LHRHa/10 g body mass into the dorsal lymph sacs. Pairs were placed in 38 L of dechlorinated water with a piece of nylon mesh to aid in oviposition. Adults were removed after oviposition and aeration added to the aquaria. Tadpoles were fed a mix of commercial rabbit food and TetraMin® (Tetra). Tadpoles were 6 days old (ca. Gosner stage 25; Gosner 1960) at the start of the toxicity test.

Toxicity test

Seven haphazardly selected tadpoles from each pair were placed into each aquarium (21 tadpoles per tank) and allowed to acclimate for 24 h prior to the beginning of the toxicity test. Tanks consisted of standard 9.5 L glass aquaria and contained 6 L dechlorinated water. All tanks were washed 2× with acetone and rinsed 3× with water prior to the start of the experiment. After acclimation,

Table 1 Environmental concentration of AI based on direct overspray of 16 cm of water using the maximum label rate for corn predicted by Belden et al. (2010), and corresponding nominal concentrations of four dosing solutions applied to test chambers

Fungicide formulation	Active ingredients	Environmental concentration (μg/L)	Concentration (μg/L)			
			1	2	3	4
Headline®	Pyraclostrobin	150	15	5	1.7	0.5
Stratego®	Trifloxystrobin	74	500	160	50	15
	Propiconazole	74	500	160	50	15
Quilt®	Azoxystrobin	44	1,200	400	130	40
	Propiconazole	74	2,000	650	215	70

fungicide formulations or AIs (0.5 ml) were mixed into each tank. Each treatment consisted of a chemical type (formulation or AI) and one of the four concentrations used for the fungicide or the control (Table 1). Aquaria were assigned a chemical type and concentration using a random sampling technique where numbers were drawn to assign tanks and treatments were stratified by shelf. Each treatment was replicated 3× ($n = 3$; 75 experimental units). Tests were conducted at 25 ± 2 °C. Dissolved oxygen remained above 5.4 mg/L, and the photoperiod held constant at a 13:11 h light dark cycle. Mortality was defined as the failure to move after gentle probing with a glass rod and was checked every 2 h for the first 12 h and then every 12 h through 96 h. Dead tadpoles were removed during each check period. Tadpoles surviving to the end of the study were euthanized using 0.5 % tricaine methanesulfonate (MS-222). All procedures were conducted under protocols approved by Oklahoma State University Institutional Animal Care and Use Committee.

Quality Control

Fungicide concentrations in tank water were confirmed for the lowest and highest concentrations, and the controls. A 100 ml sample was taken from two of the three replicates ($n = 2$) for the concentrations analyzed and passed through a 1,000 mg C8 AccuBond® SPE cartridge (Agilent Technologies, Santa Clara, CA, USA). Cartridges were conditioned with methanol and distilled water and samples extracted at a rate of ~3.5 ml/minute. Samples were stored frozen on the cartridge for <60 days. Fungicides were eluted from the columns with 8 ml of ethyl acetate and the extracts evaporated under a stream of nitrogen to a 1 ml final volume. Analysis was performed using gas chromatography/mass spectrometry (GC/MS) (Agilent 5975c, Santa Clara, CA, USA). The GC inlet temperature was set at 240 °C and the oven program started at 70 °C and ramped over 30 min to 290 °C. Select ion monitoring was utilized with the following quantitation ions: pyraclostrobin 132, azoxystrobin 344, trifloxystrobin 116, propiconazole 173. Internal calibration was used with Chrysene D12 as the internal standard (quantitation ion: 240). Method precision and accuracy was monitored by spiking laboratory water with the AIs at 2 μg/L ($n = 4$). Percent extraction efficiencies (and standard deviations) for the AIs were 104 (14), 75 (15), 83 (12), and 87 % (12) for pyraclostrobin, trifloxystrobin, azoxystrobin, and propiconazole, respectively.

Additionally, the concentration of naphthalene was measured in the Headline® formulation. The formulation was dissolved into acetone and then further diluted into methylene chloride. The sample was analyzed using the chromatography conditions above. The quantitation ion for naphthalene was 128 and naphthalene D8 was used as an internal standard.

Statistical analysis

Percent mortality at 72 h was determined for each replicate and presented to parallel the investigation done by Belden et al. (2010). No additional mortality was observed beyond 72 h. A one-way analysis of variance (ANOVA; SAS 9.2; SAS Institute Inc., Cary, NC, USA) was used to compare chemical treatments (e.g., Headline® AI and Headline® formulation at all concentrations and the control) to each other. Each fungicide was tested separately to the controls. A protected Tukey's multiple range test was used to determine individual treatment effects. Significance was determined at alpha = 0.05. 72 h-LC_{50} values were also determined using a graphical method to account for the lack of partial mortalities in most tests (Lewis et al. 1994). However, confidence intervals were not reported for data that lacked any partial mortality and thus should be interpreted cautiously.

Results and discussion

Control mortality was <2 % for the entire experiment. For all treatments with 100 % mortality, death occurred within

the first 12 h, with over 95 % of the remaining mortalities occurring within the first 24 h. Fungicide concentrations for the lowest and highest treatments were all near the targeted concentrations after 3 h in the aquaria water (Table 2). However, some AIs were found at lower concentrations at 96 h. For example, trifloxystrobin, in the lowest concentration treatment, declined the most with 96 h concentrations at 6–10 % of the initial concentration. Additionally, pyraclostrobin, also at the lowest concentration treatment, was determined at 96 h to be at lower concentrations in the AI treatment than the formulation treatment. Belden et al. (2010) quantified these same compounds over 72 h in similar conditions and found the concentrations to be relatively constant, suggesting that concentrations declined primarily late in the test. Further, toxic effects on amphibians were only seen at higher concentration levels and thus, changes in lower concentration treatments likely did not affect our results. Finally, because most mortality occurred within 12–24 h, actual concentrations likely deviated little from initial target concentrations. Nominal concentrations are used for comparisons among treatments.

Stratego® formulation and AIs and Quilt® formulation treatments resulted in significant mortality ($P \leq 0.0012$), although there were no differences between formulation and AI for either Stratego® or Quilt® at any concentration (Table 3; Fig. 1b, c). Stratego® resulted in 100 % mortality at 160 and 500 µg/L but minimal mortality at all other concentrations. Quilt® showed a lesser response, with mortality averaging less than 60 % for both formulation and AIs at the highest concentration, and essentially no mortality at lower concentrations. Headline® exposure resulted in significant ($P < 0.0001$; Table 3; Fig. 1a) mortality at highest tested concentrations, with differences in toxicity between the AI and formulation at 5 µg/L only.

Previously, Belden et al. (2010) evaluated the toxicity of these fungicide formulations to *B. cognatus* tadpoles at concentrations of 0.1, 1, and 10X the maximum label rate for corn (BASF Corporation 2008a; Bayer Crop Science 2008; Syngenta Crop Protection 2009) applied directly to water body 16 cm deep. These values are dependent on the depth of the water, and therefore may fluctuate higher or lower depending on water volume. They demonstrated toxicity for Headline® and Stratego® but not Quilt® (Belden et al. 2010). In their study, it was unclear whether the AI or adjuvant was responsible for the observed toxicity (Belden et al. 2010). Our goal was to assess the role of

Table 3 One-way ANOVA comparing fungicide chemicals (fungicide formulation vs. active ingredient) and the control on mortality of exposed *B. cognatus* tadpoles

	DF	F ratio	*P* value
Headline®	8	124.93	<0.001
Stratego®	8	590.06	<0.001
Quilt®	8	5.60	0.0012

Each fungicide was tested independently to the control ($P < 0.05$)

Table 2 Estimates of median lethal concentrations (72 h-LC_{50}) of fungicide formulations and AI following exposure to *B. cognatus* tadpoles

Fungicide chemical	72 h-LC_{50} (µg/L)	Average % recovery (standard deviation)			
		High (3 h)	High (96 h)	Low (3 h)	Low (96 h)
Headline® AI					
Pyraclostrobin	10.0		–[a]	84(48)	22(12)
Headline® formulation					
Pyraclostrobin	3.7	98(6)	–[a]	126(11)	91(25)
Stratego® AIs	100.3				
Trifloxystrobin		75(4)	–[a]	71(4)	6(1)
Propiconazole		73(2)	–[a]	107(8)	99(5)
Stratego® formulation	104.1				
Trifloxystrobin		68(4)	–[a]	68(1)	10(4)
Propiconazole		66(16)	–[a]	118(4)	85(13)
Quilt® AIs	1241.5				
Azoxystrobin		112(23)	73(5)	117(10)	77(9)
Propiconazole		75(20)	53(4.2)	75(1)	48(5)
Quilt® formulation	1029.5				
Azoxystrobin		129(29)	63(4)	139(13)	66(6)
Propiconazole		68(14)	38(8)	86(14)	46(2)

Also, recoveries of AIs for high and low concentration treatments at 3 and 96 h are reported. –[a] = These treatments were not tested because all individuals were dead within 24 h

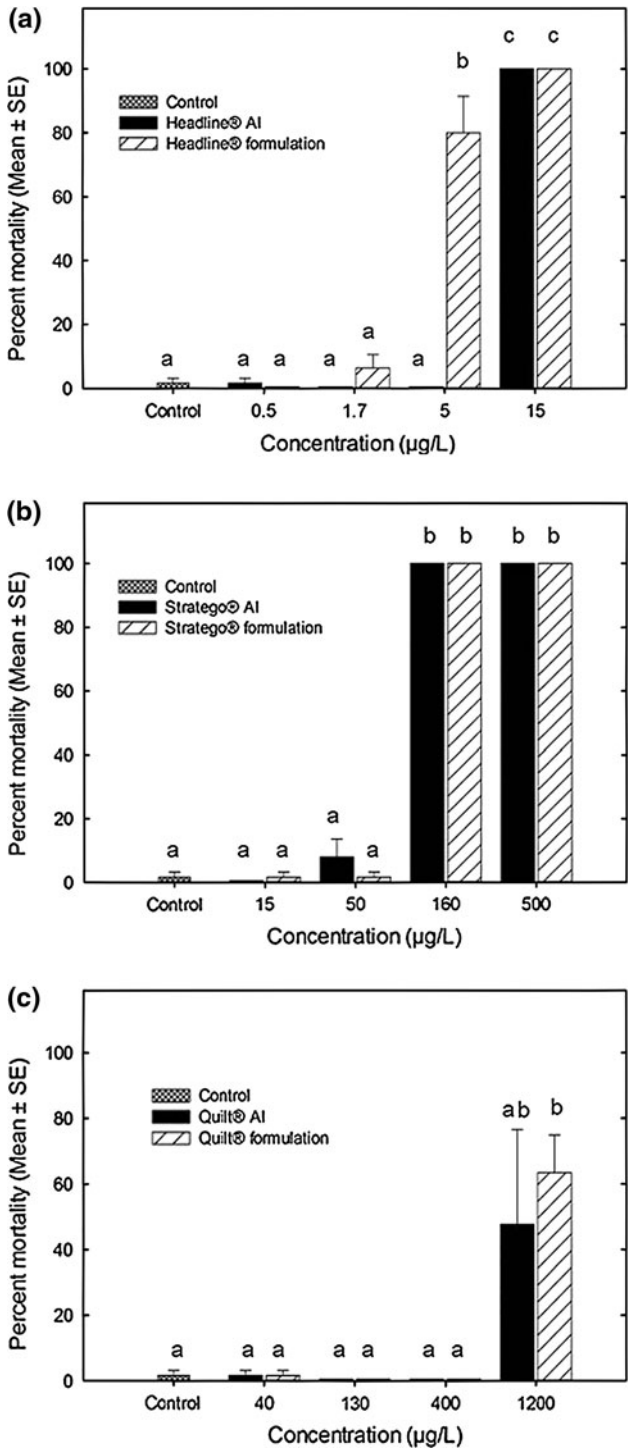

Fig. 1 Mean (±SE) percent mortality of *B. cognatus* tadpoles exposed to formulations and the AIs of Headline® (**a**), Stratego® (**b**), and Quilt® (**c**) at four concentrations plus a control. Stratego® and Quilt® contain two AIs. Refer to Table 1 for nominal concentrations of each AI. Control animals were exposed to solvent carrier used for AI treatments (acetone). Each treatment consisted of three replicates ($n = 3$). Lower case letters above bars indicate significant differences between the concentrations and/or chemical treatment ($P < 0.05$)

AI(s) vs. adjuvants as toxic agents to tadpoles. With the exception of Headline® at the 5 µg/L concentration, exposure to AI(s) and formulations for each fungicide produced parallel results, demonstrating that AIs are toxic and primarily responsible for the observed mortality. However, formulation ingredients other than the AI(s) may still play a role in toxicity for Stratego® and Quilt®. For example, Stratego® contains petroleum distillates (Bayer Crop Science 2008) which may contribute to toxicity and investigating effects between 50 and 160 µg/L would elucidate the toxicity of adjuvants. A similar case may exist for Quilt®, although toxicity of this fungicide was not observed until the highest concentration, which was 27X greater than the predicted concentrations from Belden et al. (2010).

Although propiconazole, one of the AIs of Quilt®, is likely to persist in the environment due to its high water solubility (110 mg/L) and long half-life in soil (110 days) (Deb et al. 2010), levels of only 0.01–1 µg/L have been found in streams in the United States (Battaglin et al. 2011), well below that shown to be toxic to amphibians in this study. Thus, neither the AIs nor formulation of Quilt® appear to pose a risk toward amphibian larvae under normal field use conditions. Similarly, toxicity of Stratego® in this study is not apparent until 160 µg/L; however Belden et al. (2010) showed 40 % mortality of tadpoles exposed to 74 µg/L Stratego® formulation, the concentration predicted from a label rate application over 16 cm of water. Our results of no effect at 50 µg/L and 100 % mortality at 160 µg/L, coupled with the results of Belden et al. (2010), suggest a relatively steep response curve for Stratego® and a smaller margin of error during application than observed for Quilt®. Our results stem from a single application, thus toxicity of Stratego® to non-target organisms may be an issue under conditions (e.g., disease presence) where it may be sprayed every 7 days (Bayer Crop Science 2008). Trifloxystrobin and propiconazole have soil half-lives of 5 and 110 days (Deb et al. 2010), respectively, allowing toxic levels to potentially accumulate.

Headline formulation resulted in significant mortality at 5 µg/L, which is approximately 3 % of the concentration predicted from a direct overspray of a 16 cm deep aquatic body. Further, both formulation and AI resulted in complete kill of tadpoles at the next highest concentration (10 % of predicted concentration) and overall, these results demonstrate a response curve even steeper than observed for Stratego®. These concentrations are environmentally relevant for wetlands as shown by Deb et al. (2010) who modeled the maximum annual average input levels in field runoff of pyraclostrobin to be 13.7 µg/L, which exceeds the 72 h-LC_{50} values (Table 2) for *B. cognatus* determined in this study for both AI and formulation (10.0 and 3.7 µg/L, respectively). In contrast to the other formulations, we observed differences between AI and formulation toxicity

with Headline®, indicating that formulation ingredients other than the AI likely play a role in the observed toxicity. Naphthalene and 2-methylnaphthlane constituted 6.2 and 13.7 %, respectively, of the total formulation. Assuming these percentages, concentration ranges of naphthalene and 2-methylnaphthalene are 1.3–3.9 and 2.7–8.07 µg/L, respectively, for the two highest Headline® concentrations. Naphthalene as reported on the label may constitute up to 9 % of the formulation (BASF Corporation 2008a), so these values may fluctuate in other scenarios. Nonetheless, these concentrations fall within the range reported to be toxic in combination with other petroleum distillates, such as hydrocarbons and aromatics (Barron et al. 1999). Similarly, naphthalene alone was 100 % lethal to larval marine invertebrates at concentrations of 8 and 12 µg/L (Sanborn and Malins 1977). Other than having the potential for additive toxicity, naphthalene could influence the toxicodynamics of pyraclostrobin and increase toxicity. However, naphthalene is volatile and unlikely to stay in the testing chambers or wetlands for very long, but increased toxicity could occur during the initial exposure and may explain why most of the formulation mortality was within 24 h. Yet, a majority of mortality induced by the AIs also occurred within 24 h, making it difficult to discern interactions between chemical types and the timing of the response.

Overall, this study demonstrates that Headline®, and possibly Stratego®, has the capacity to be toxic at environmentally relevant concentrations, and that adjuvant ingredients in Headline® can contribute to the toxicity. Pyraclostrobin's toxic effect at low concentrations raises concern as its application has become popular in the United States and Great Britain. The use of pyraclostrobin in Great Britain has increased from 20 to 40 % from 2005 to 2008 in cereal crop fields, whereas the other strobilurin/triazole AIs discussed in this paper have decreased and stabilized at or below 20 % (http://pusstats.csl.gov.uk/). Headline® has been ranked as the top fungicide for no-till farmers four consecutive years in the United States since 2006 (http://www.agproducts.basf.com/news-room/press-releases/current-press-releases/headline-fungicide-from-basf-named-a-no-till-product-of-the-year.html). Further, it is now registered in Canada and used on 90 different crops controlling 50 different diseases (http://agproducts.basf.us/products/headline-fungicide.html). The continued proclamations of increase in crop yield (BASF Corporation 2008a, 2008b; http://agproducts.basf.us/products/headline-fungicide.html) and incentives, discounts, and finance plans for purchasing Headline® will make it an attractive product, which may increase use and thus the risk of exposure to non-target organisms.

Although, mode of action studies are lacking for non-target animals, this study along with others suggest that the non-specific mode of action of the strobilurin AIs may be responsible for the toxicity toward non-target organisms (Belden et al. 2010; Ochoa-Acuna et al. 2009). Maltby et al. (2009) addressed the issue that the mode of action of fungicides is non-specific, and concluded that the sensitivity of non-target organisms (fish, invertebrates, and primary producers) to fungicides that affect energy production (e.g., the strobilurins) is approximately the same among taxonomic groups (Maltby et al. 2009). Additional studies on the fate of fungicides in the environment, the potential for exposure, and cause of mortality will shed light to how these fungicides may affect non-target species in field settings.

Data for fungicide acute toxicity in an amphibian model is limited (reviewed by Sparling et al. 2010). Considering the fungicide concentrations found in streams in the United States where all fungicides quantified were under 1 µg/L (Battaglin et al. 2011) and the non-strobilurin fungicide LC_{50} values for tetraconazole and chlorathalnil are 3.85 mg/L (USEPA 2004a) and 76 µg/L (Ernst et al. 1991), respectively for fish, these non-strobilurin fungicides are not predicted to have an acute mortality effect at environmentally relevant concentrations. However, fungicide exposure to non-target organisms at sublethal concentrations is a greater concern (reviewed by Sparling et al. 2010). Although few studies have used an amphibian model, studies investigating non-strobilurin fungicide exposure to fish have demonstrated endocrine disruption (e.g., alteration in 17β-estradiol and testosterone; Villeneuve et al. 2007), change in gene regulation (e.g., up regulations of CYP1A or CYP3A; Hegelund et al. 2004), and growth retardation (e.g., larval growth; USEPA 2004b). These effects seen in fish are predicted to be similar in an amphibian model. Because the fungicides under investigation inhibit cellular respiration, they may have the ability to affect non-target organisms at a sublethal level, which may translate into population effects. Exposure to azoxystrobin has been shown to cause up-regulation of growth factor-binding proteins which when bound to important growth factors can potentially retard individual growth (Olsvik et al. 2010). Further, the non-strobilurin fungicide chlorothalonil has been shown to affect survival of *Osteopilus septentrionalis* and alter sublethal endpoints such as corticosterone levels and tissue morphology (McMahon et al. 2011), demonstrating the importance of future studies on fungicides using multiple endpoints to understand the potential environmental effects.

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