EVALUATING HEADLINE AMP® TOXICITY TO *BUFO COGNATUS* TADPOLES IN AN AQUATIC SEDIMENT MICROCOSM

By

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Abstract: Increased use of strobilurin fungicides has expanded the potential for exposure to non-target wetland organisms. An acute toxicity test was performed to compare the toxicity of Headline AMP® to Great Plains toad (*Bufo cognatus*) tadpoles in a water only exposure, and two aquatic sediment microcosms that differ in their texture class and organic matter (OM) content (Nebraska sediment, 6.14% OM, silty clay loam; Texas sediment, 4.15% OM, clay loam). The sediments were predicted to sequester the fungicide at variable rates, thus affecting the exposure and toxicity of the fungicide. Following the acute toxicity test, a fate study was performed to examine partitioning and bioavailability of pyraclostrobin, the active ingredient in Headline AMP®. Ninety-five percent of all tadpole mortalities in the water only exposures occurred within the first 6 hours. Tadpole nominal LC50 values were 8.0 µg/L for water-only exposures, 9.3 µg/L for the Texas sediment system, and 15.3 µg/L for the Nebraska sediment system. Differences in LC50s suggest that Nebraska sediments are more protective than Texas sediments and the water only system. In the fate study, pyraclostrobin dissipated from the water gradually, with mean (±SE) total percent recovery from water at 90±4% at 2 hours to 18±4% at 72 hours for Nebraska sediment. Recovery of pyraclostrobin from water decreased quicker in the Texas sediment system, from 72±2% at 2 hours to 20±3% at 72 hours. Texas sediment treatments averaged 3.8±0.7 times as much suspended sediment as compared to Nebraska sediment treatments. The increased suspended solids in the Texas treatment resulted in more sequestration of analyte by sediments, averaging 30±21% over the course of 72 hr. Tadpoles present during an application of Headline AMP® are at risk of exposure to a lethal dose by spray drift, field runoff, or direct overspray, even at fractions of the label rate, before suspended sediments are able to ameliorate toxicity. Further, different species of amphibians will have different levels of risk based on their species-specific larval periods. Lastly, land use affects deposition, suspension, and texture of sediments in wetlands and thus, can influence partitioning of fungicide to the sediments and exposure scenarios for organisms.

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CHAPTER I

EVALUATING HEADLINE AMP® TOXICITY TO *BUFO COGNATUS* TADPOLES IN AN AQUATIC SEDIMENT MICROCOSM

Cropland covers approximately 20% of the land in the U.S (USDA 2013) and contains wetlands that provide breeding habitat for resident amphibians (Tiner 2003; Marsh et al. 2004). Further, amphibians disperse through the uplands surrounding natal wetlands, which include cultivated land. Consequently, aquatic and terrestrial stage amphibians are at risk of exposure to pesticides in these systems. Currently, there are no regulations that require testing pesticides on amphibians in the United States prior to approving pesticides for commercial use, and exposure risk is only assessed for selected pesticides. The United States Environmental Protection Agency (US EPA) relies on toxicity data from fish (e.g., bluegill sunfish) as a surrogate for aquatic-phase amphibians and birds as a surrogate for terrestrial-phase amphibians (US EPA: Ecological risk assessment; http://www.epa.gov/oppefed1/ecorisk_ders/). Species vary in their sensitivity to toxicants and exposure potential, so using surrogate organisms may not always be appropriate when trying to evaluate toxicity to non-target organisms (Bridges and Semlitsch 2000; Ding et al. 2011). Also, standard toxicity tests are conducted under laboratory conditions when trying to evaluate toxicity to non-target organisms (Bridges and Semlitsch 2000; Ding et al. 2011) which may not be predictive of environmental systems. Thus the need to evaluate pesticides in more

natural systems is fundamental in assessing risk of exposure and effects in the environment.

Headline® (pyraclostrobin; US EPA Reg. No. 7969-186, BASF) is a leading competitor among fungicides and its primary use is to suppress existing fungal diseases, such as Asian Soybean Rust (Shaner et al. 2005). Also, Headline® is labeled for prophylactic use to include increasing yield and tolerance to stressors such as drought, ozone, hail, frost, and heat (BASF Corporation 2012). Strobilurin fungicides bind to the Q_o site on cytochrome b and inhibit mitochondrial respiration (Russell 2005). Application of pyraclostrobin in the U.S. increased from 36,000 kg applied in 2005 to 172,000 kg applied in 2012 on soybean, and corn applications alone reached over 227,000 kg in 2014 (National Agricultural Statistics Service; http://quickstats.nass.usda.gov).

Headline® has increased in use which has led to the exposure of environmental systems and non-target organisms (Smalling et al. 2013a,b). Pyraclostrobin, the main active ingredient in Headline®, was found in 75% of 32 bed sediment (benthic sediments) and suspended sediment samples collected from watersheds in agricultural areas in the US (Smalling et al. 2013b). In addition, pyraclostrobin was the most frequently detected pesticide of 98 pesticides monitored in pacific chorus frog (*Pseudacris regilla*) tissues in northern California (Smalling et al. 2013a). Pyraclostrobin was found in about 30% of 171 tissue samples at concentrations ranging from 2 to 28.7 µg/kg wet weight (Smalling et al. 2013a).

Toxicity of Headline® to aquatic organisms is well documented (Balba 2007; BASF Cooperation 2008; Belden et al. 2010; Hooser et al. 2012; Morrison et al. 2013). Pyraclostrobin has been shown to be acutely toxic to *Daphnia magna* at a median lethal concentration (LC50) of 16.0 µg/L (BASF Cooperation 2012b) and to Rainbow trout (*Oncorhynchus mykiss*) at an LC50 of 6.16 µg/L (BASF Cooperation 2008). Headline® is acutely toxic to tadpoles in aquatic systems at environmentally relevant concentrations (Belden et al. 2010), and studies have shown that

pyraclostrobin is the main contributor to toxicity (Hooser et al. 2012, Morrison et al. 2013), which can occur within hours of exposure (Belden et al. 2010; Hooser et al. 2012; Morrison et al. 2013).

However, only a few studies (Ding et al. 2011; Morrison et al. 2013; Reddy et al. 2013a,b) have explored how sediments can influence bioavailability of pyraclostrobin. The first study to detect pyraclostrobin bound to suspended sediments found that out of 34 measured fungicides, pyraclostrobin was the most frequently detected pesticide (found in 13 bed sediment and 10 suspended sediment samples; n=32) in sites throughout Idaho, Maine, and Wisconsin in areas associated with agricultural production (Smalling et al. 2013b). Another strobilurin fungicide, Azoxystrobin, was found in 20% of bed sediments however it was not detectable in suspended sediments. Pyraclostrobin concentrations (dry weight) were found up to 198 μg/kg in bed sediments and 56.4 μg/kg in suspended sediments (Smalling et al. 2013b). Pyraclostrobin has a log K_{ow} (octanol/water partition coefficient) of 4, among the highest of commercial strobilurins (Bartlett et al. 2002). As indicated by its high K_{ow}, pyraclostrobin is hydrophobic and can be absorbed by organisms and sorb to sediments (Domagalski and Kuivila 1993).

Sediment properties are factors through which contaminants can become sequestered in aquatic systems (Saulnier and Mucci 2000; Cantwell et al. 2002; Eggleton and Thomas 2004). Organic matter (OM) in sediments consists of humic material made from decomposing plant and animal tissue and is the strongest component of sediment driving the sequestration of many contaminants such as penconazole (Sanchez-Martin et al. 2000), metals (Cu, Ni and Pb; Fan et al. 2002) and pyraclostrobin (Reddy et al. 2013a). Soil organic carbon (OC) is the carbon stored in sediment OM and is strongly correlated with OM content, however their ratio is variable depending on other sediment variables such as sediment type, depth, and bulk density (Jain et al. 1997; Westman et al. 2006; Périé and Ouimet 2008). For example, the OC/OM ratio in loamy and sandy loam soils was 0.49 at the topsoil and dropped to 0.27 at 50cm (Périé and Ouimet 2008).

Depending on soil OM and OC content, pesticides such as pyrethroids (insecticides) sorb to OM in the sediment suspensions or to inorganic sediment particles (e.g., clay) when OM content is low (less than 1%) (Yang et al. 2006). In runoff that contains finer particle/aggregate-size sediments there can be higher OC and thus a higher concentration of propiconazole can be sequestered by the sediments than runoff with larger sediment particles (Wu et al. 2003). Pyraclostrobin has a log K_{oc} (OC sorption coefficient) of 3.59 to 5.16 with limited water solubility, indicating it will partition to sediment, organic matter particles suspended in water and thus reduce exposure aquatic organisms (European Commission and the EFSA 2012).

Inorganic portions of sediment, such as clay, also effect sorption and transport of a contaminant (Sanchez-Camazano and Sanchez-Martin 1994; Li et al. 2003; Wu et al. 2003). Clay particles have a high surface area to volume ratio and negative charge, and compared to larger soil particles they have more available area for adsorption of chemicals. Because clay has a hydrophilic characteristic which is driven by its surface inorganic cations, it makes hydrophobic organic contaminants compete for sequestration by clay particles (Sanchez-Camazano and Sanchez-Martin 1994). In addition, pyraclostrobin readily partitions to clay-rich sediments (e.g., sandy and silty clay loam, >20% clay; Deb et al. 2010; Reddy et al. 2013b). When clay particles are removed from the soil, 34-46% of pyraclostrobin desorbed from the sediments compared to control clay sediments with 22% of desorption, demonstrating influence of clay on contaminant sequestration (Reddy et al. 2013a).

In laboratory systems with only water, contaminants can only partition to organisms or experimental tank walls. Pyraclostrobin rapidly partitions into deposited bed sediment in aquatic microcosms, as demonstrated by Morrison et al. (2013), who examined Headline® toxicity to *Hyalella azteca* in the presence of bed sediment in an aquatic microcosm. *Hyalella azteca* LC50 increased five-fold in the sediment microcosm compared to a water only exposure. Measured

water concentrations of pyraclostrobin declined to 69% by 168 hours while sediment concentrations increased to 56% from the initial pyraclostrobin measurements. Partitioning of fungicide to the bed sediments is most likely responsible for the decrease in toxicity to the invertebrates. Although protection was found for *H. azteca* in an unmixed system, it is unclear how protection is different for more sensitive non-target organisms, like tadpoles, in a more naturally turbid system.

Adding suspended sediment to the system can sequester organic contaminants and thus further reduce the toxic effects compared to bed sediments alone (Eggleton and Thomas 2004; Yang et al. 2006; Fojut and Young 2011). Natural systems such as depressional wetlands are often turbid and contain suspended particles (Smith 2003). In the presence of suspended sediment (200 mg/L), pyrethroid (organic insecticide) toxicity (LC50) to *Daphnia magna* decreased 2.5 to 13 times the toxicity in a system with no sediment (Yang et al. 2006). Sediments that tend to suspend in aquatic systems have different physiochemical properties, such as the higher specific surface area and OC content than bed sediments (Wu et al. 2003; Fojut and Young 2011). These suspended sediment properties sequester pyrethroid insecticides more readily than bed sediments, as shown by a greater than two-fold increase in K_d (solid-water distribution coefficient) for suspended sediments (Fojut and Young 2011).

Sediment composition and soil OM content varies among wetlands due to differences in hydrology, geomorphic setting, and sediment compositions (Allen et al. 1972; Luo et al. 1997, 1999; Scanlon et al. 2010). For example, the soil OM content of playas in the Rainwater Basin (RWB) in south-central Nebraska can reach 10% while playas in the Southern High Plains (SHP) of Texas may range between 0.5% and 5.9% (United States Department of Agriculture. Web Soil Survey; http://websoilsurvey.nrcs.usda .gov/). In comparison, the OC content throughout playas within the SHP is often less <2% (Luo 1994). In general, playas may not contain as much OC as other wetlands because their frequent drying results in aerobic soil conditions (Anderson and

Smith 2002). Clay content in RWB wetlands range from 0-25% clay (silt loam) within surface soils and >40% clay (silty clay or clay) in subsurface soils (Stutheit et al. 2004). Playas in the Texas SHP consists of clay loam soils 89% of the time (>35% clay; Scanlon et al. 2010). Clay content within SHP playas typically exceeds 50% (Smith 2003). Sediments in playas can also differ depending on locations within the depressions as well as the geographic location of the playa. Clay content in SHP playas increased to approximately 60% at the center of cropland playas compared to ~20% clay in the sediments along the edge (Luo et al. 1999). Soil OC was greater in native grassland playas compared to cropland playas in the SHP and RWB (O'Connell 2011). Because OC is a primary driver of contaminant sequestration, maintaining grassland playas may be beneficial to reduce aquatic organism's exposure to organic contaminants. This variability in composition among wetland sediments results in the possibility for different contaminant exposure scenarios, with some systems potentially being more effective than others in ameliorating exposure and toxic effects.

Evaluating the protective qualities that sediments have on the toxicity of Headline AMP®, a recent reformulation of Headline®, will provide a more realistic scenario for addressing pesticide exposure risk to non-target organisms. Thus, this study was designed to compare the acute toxicity of Headline AMP® to Great Plains toad (*Bufo cognatus*) tadpoles in a water only exposure, and 2 aquatic sediment microcosms that differ in their texture class and OM content. Headline AMP® (US EPA Reg. No. 7969-291, BASF) contains the same primary active ingredient as Headline®. However, the toxicity of Headline AMP® might differ from Headline® formulation due to the removal of naphthalene, a reduced pyraclostrobin concentration (13.64 vs. 23.6% by mass), and the addition of a secondary fungicide, metconazole (5.4% by mass; Table 1.1; BASF 2008; BASF 2012). Further, a secondary fate study was performed, similar to Morrison et al (2013), to explain the rate of pyraclostrobin partitioning to sediments and it's bioavailability of pyraclostrobin to tadpoles.

Methods:

Fungicide formulation:

Headline AMP® fungicide was acquired from a local distributor. Concentrations were based on predicted environmental concentrations of pyraclostrobin from direct overspray of 16cm of water using the maximum Headline® label rate for corn (150 µg/L pyraclostrobin; Belden et al. 2010). The predicted environmental concentration of Headline AMP®, based on maximum application rate in corn in 16cm of water, falls within the range of dosing concentrations (95 µg/L of pyraclostrobin, see below).

Test sediments:

Sediments were collected from a native grassland playa in the Southern High Plains of Texas (TX) and from a waterfowl production area (WPA) playa in the Rainwater Basin of Nebraska (NE). Sediment was collected from 3 random locations within each wetland and combined to account for potentially variable soil compositions throughout regions within wetland (i.e., ~60% clay in center vs ~20% at edge; Luo et al. 1999). Sediments were dried and mixed prior to being sifted to <2mm to break up large aggregates and remove large plant material (sticks, leaves, roots). Soil composition and texture class was determined at the Oklahoma State University's Soil, Water and Forage Analytical Laboratory, Stillwater, OK, USA. Texture class of NE sediment was silty clay loam (13.8% sand, 56.3% silt, and 30.0% clay) and had an OM content of 6.14%. Texas sediment consisted of clay loam (27.5% sand, 40.0% silt, and 32.5% clay) with an organic matter (OM) content of 4.15%.

Test organisms:

Adult *B. cognatus* were captured from roads in central Oklahoma in May and June 2014 (Kingfisher and Caddo Counties, Oklahoma) and housed on-site at Oklahoma State University for 4 weeks prior to breeding. Males (n=3) were hormonally primed by injecting 1 µg/g body weight of the synthetic analogue of luteinizing hormone releasing hormone (LHRHa) into the dorsal lymph sac using a 29ga insulin syringe. Seventy-two hours after the initial priming, males were injected with 2 µg/g body weight of LHRHa intraperitoneally. Six hours later, 3 females were injected with LHRHa (2 µg/g body weight) and paired with males in glass aquaria with 38 L dechlorinated water for egg deposition. Adults were removed after oviposition and water tanks continuously aerated. Tadpoles were fed TetraMin® mix and kept on a 13:11 hour light dark cycle at 21º C for the duration of the experiment. All animal procedures were conducted under an animal use protocol approved by the Oklahoma State University's Institutional Animal Care and Use Committee (protocol number AS-14-10).

Microcosm toxicity test:

There were 3 microcosm treatments (water only, TX sediment, NE sediment) with varying concentrations of Headline AMP®. In the water-only exposure, 3 replicates of 8 concentrations of Headline AMP® were used (150, 62.5, 26.0, 10.9, 5.4, 2.7, 1.4, and 0.7 µg pyraclostrobin/L) and a vehicle control (deionized water). Fungicide concentrations were mixed with deionized water to replicate dosing concentrations based on expected aquatic exposure of maximum Headline® label application rates for corn (Belden et al. 2010). For the sediment treatments (NE and TX), 3 replicates of the 6 highest concentrations of Headline AMP® and the vehicle control were used, anticipating a shift in toxicity due to the presence of sediment. Sediment-water microcosms were constructed by adding 280 mg of dry soil to 3.5 L of dechlorinated water and mixing for 30

seconds to suspend the sediment. Dechlorinated water was obtained by carbon filtration of Oklahoma State University tap water (pH: 7.5–7.7; hardness: 80–100 mg calcium carbonate/L; dissolved oxygen: 6.7–9.0 mg/L). Sediment suspensions were maintained via continuous aeration with air stones and a linear piston air compressor (JEHM Co. model LPH120). Fifteen tadpoles were placed in each aquarium 30 minutes post-suspension. One mL of dosing solvent or deionized water control was added to each tank to achieve the desired concentration. Tanks were arranged 2 rows deep on 3 shelves. Replicates from all treatments were randomized on their respective shelves. Tadpole mortality in the water-only exposure was checked every 2 hours for the first 6 hours, at 12 hours, then every 24 hours through 72 hours. Due to high suspended sediment content in the sediment treatments and the inability to see tadpoles throughout the toxicity test without disturbing the sediment, mortality was only checked for the sediment treatments at the end of the 72 hours. Tadpoles that were missing or unresponsive to probing with a glass rod were considered dead.

Fate test:

To determine the rate of partitioning of fungicide and the total suspended sediment in the previously described microcosm system, a secondary test was designed as described for the toxicity test but with a single pyraclostrobin concentration of 5.4 µg/L. Contents of tanks were collected at 5 time points (2, 7, 12, 24, and 72 hr) and included whole water (water + suspended sediment), water fraction (water without suspended sediment), suspended sediment fraction (isolated suspended sediment from the water), and bed sediment (deposited benthic sediments) samples. One liter aliquots of whole water were removed from the top 6cm of water from each replicate at each time point, and separated into 3 aliquots. These included 100 mL for whole water, 300 mL for separation into water fraction and suspended sediment fraction, and 100 mL

for determination of suspended sediment concentrations. Due to more suspended sediment buildup on SPE cartridges compared to the acute toxicity test, extractions required >2 cartridges/sample and prolonged each extraction to >6 hr/cartridge. Thus, not enough extraction space and time was available to accommodate the whole water extractions in conjunction with fractionated water samples by methods illustrated above. Whole water samples were extracted from only 1 of the 3 replicates at 2 hr, 24 hr, and 72 hr. Total suspended sediment was assessed by running 100 mL of whole water samples through dry, pre weighed fiberglass filters (Whatman™) and drying at 100° C overnight to a constant weight. Final dry weights were subtracted from the original filter weights and sediment quantities were extrapolated back to the original volume (3.5 L) of each tank. Percent solids for each sediment were determined by weighing 5.0 g of wet treatment sediment from each tank and drying them overnight at 60º C to a constant weight, followed by ashing samples in the muffle furnace overnight, where final weights were then taken. Dry weights were divided by the weight of the ashed samples to calculate the percent solids in each tank.

Analytical determination of fungicide in water and sediment

A 100 mL whole water sample (water and suspended sediment) was collected from all of the 10.8 µg/L and 150 µg/L NE and TX sediment tanks at 2, 24, and 72 hours post-treatment (n=36; percent recoveries were pooled from both concentrations for results). Samples were passed through 1000 mg C8 AccuBond® SPE cartridge (Agilent Technologies, Santa Clara, CA, USA) following conditioning with 5-mL methanol and 10-mL distilled water. Samples were extracted at a rate of approximately 3.5 mL/min. Fungicide was removed from cartridges with 10 ml of ethyl acetate, which was evaporated under a stream of nitrogen to a final volume of 1 ml. Samples with high suspended sediment loads were run through 2 cartridges. Both cartridges were extracted

as described above, extracts pooled, and evaporated to a final volume of 1 ml. Treatment concentrations for the water-only tanks were assessed at 72 hr by extracting a 1 L composite water sample from the 3 replicates of each treatment concentration, as described above.

Whole water samples from the fate test were extracted by the SPE procedure described above and evaporated under a stream of nitrogen to a final volume of 1 ml. Suspended sediment fraction and the water fraction were collected by splitting the 300 mL sample into 6 50 mL aquilots, which were centrifuged for 10 minutes at 1000 rpm. Water was pipetted off the soil pellet and recombined to make up the water fraction. Water fractions were extracted by the SPE procedure described above. Fungicide was extracted from the sediment pellet by adding 4 mL of ethyl acetate and enough sodium sulfate to absorb the excess water in each centrifuge tube. Tubes were vortexed for a minimum of 1 minute to coat the sediment and unbind it from the centrifuge tube. Ethyl acetate from the 6 tubes (from 1 replicate) was pipetted off the top of the sediment and recombined into a single vile. Ethyl acetate (200 µL) was used to rinse around the edges of each of the 6 centrifuge tubes to ensure full extraction, recombined into the single vile, and stored at -20º C. Prior to analysis, samples were evaporated to dryness under nitrogen and brought to a final volume of 1 mL in ethyl acetate.

Bed sediment samples were collected by decanting the overlying water, homogenizing the sediment and removing a 20 g subsample. Bed sediment was centrifuged (Fisher Scientific accuSpin 3R) at 1000 rpm for 10 minutes, excess water decanted, and sediment stored at -20º C for <30 days prior to analysis. Samples were extracted by homogenizing 1.5 g of collected bed sediment with 750 mg diatomaceous earth, 900 mg Florisil, and 50 mg primary secondary amine-bounded silica in a mortar. Then 14 mL solvent solution of hexane:ethyl ether (1:2, v/v) was passed through an extraction column that contained, in order, 0.5 g activated silica gel, 1 g sodium sulfate, and the homogenized sediment sample. Collected effluent was evaporated to

dryness under a stream of nitrogen and brought to a final volume of 0.5 mL in ethyl acetate. Method precision and accuracy was evaluated by analyzing sediment blanks spiked with fungicide formulation (n=8, 4 for each sediment). Mean (±SE) percentages of sediment extraction recoveries for pyraclostrobin and metconazole were 121±2.5% and 55±6.4% respectively. Sediment blanks were analyzed for pyraclostrobin and metconazole and no detectable quantities were found.

Gas chromatography / mass spectrometry

Extracts were analyzed by gas chromatography/mass spectrometry (GC/MS Agilent 5975c, Santa Clara, CA, USA) using electron ionization 70eV. GC inlet temperature was set at 260° C and oven program started at 130° C and increased over 16 min to 295° C. Separation was achieved using a 15 m x 250 µm x 0.25 µm HP-5 capillary column. Select ion monitoring was utilized with the following qualifier ions: pyraclostrobin (132:164, 325) and metconazole (125:250, 319). Chrysene D12 (240:241) and perylene D12 (264:265) were used as internal standards for calibration. All extracted samples were diluted to within the calibration range prior to analysis and calibration verified by continuous calibration verification throughout all analyses. Continuous calibration verification was performed during each analytical run by randomly re-analyzing calibration standards.

Statistical analysis

A binomial Probit regression was used to determine median lethal concentrations (LC50), and 95% confidence intervals of tadpole mortalities based on nominal concentrations (IBM® SPSS® Statistics 20, Armonk, NY: IBM Corp). Water only LC50s were calculated for all 8

concentrations (see Appendix 1.f), and with the 6 concentrations used for the sediment treatments for treatment comparisons. Percent mortality per tank was calculated at 72 hours for each treatment. A two-way analysis of variance with interaction (ANOVA) was used on logit transformed percent mortality to test for differences among treatments (NE, TX, water-only) and concentrations and the control (R version 3.2.0). The 2 highest concentrations (150 µg/L and 62.5 µg/L) in which mortality was 100% were taken out to remove the one-inflated class of concentrations. A protected post-hoc Tukey's Honestly Significant Difference (HSD) test was used for main effects (α=0.05). A secondary two-way ANOVA was performed across all concentrations with a protected post-hob Tukey's HSD. A Dunetts test was used to determine no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) for each treatment.

Water concentrations from the fate study were used to calculate average measured concentrations over time following methods from Morrison et al. (2013). An average time-weighted water concentration was calculated as pyraclostrobin concentrations in the fate study declined by first-order decay throughout the exposure. A water concentration decay curve was generated to fit pyraclostrobin dissipation from the water over time and then the equation was used to fit a time-weighted average using the equation

$$\text{Weighted water conc.} = \frac{\int_c^b f(x)dx}{b - a}$$

where a=0 hr, b=6 hr, and f(x) is a first-order exponential decay function to calculate water concentrations within that given time frame. A time weighted average concentration over the first 6 hours was used because this is where the majority (95%) of mortality occurred in the water only

exposure from the acute toxicity test. LC50 values from the corrected water concentrations were calculated using Probit regression (IBM® SPSS® Statistics 20, Armonk, NY: IBM Corp).

Results:

Toxicity Test

Mean (±SE) percent recovery of pyraclostrobin in whole water samples for NE and TX treatments were 84±6% and 83±6% of nominal in the first 2 hours, which dropped to 27±14% and 33±7% at 24 hours, and further to 16±5% and 18±5% at 72 hours (recoveries pooled from treatment 10.8µg/L and 150µg/L; Fig. 1.1), respectively. For water only treatments mean recovery of pyraclostrobin for all concentrations (minus the 2 highest concentrations due to broken sample vials) was 16±2% after 72 hours. Mean recovery of pyraclostrobin and metconazole in spiking solutions were 99±8% and 85±16%, respectively.

Tadpole mortality in the water only control treatment was 4±2%, compared to 9±9% and 11± 4% for NE sediment control and TX sediment control, respectively. Mortality was low (6±3%) in all fungicide concentrations through 5.4 µg/L, whereafter it increased to 100% at 26.0 µg/L through 150 µg/L in all sediment treatments, except for 2 NE sediment replicates at 26 µg/L (Fig. 1.2). However, mortality differed among sediment treatments at 10.8 µg/L with water-only and TX sediment treatments averaging 89±8% and 69±12%, respectively, which were twice that of the NE sediment treatment at 36±6%. The LOEC and NOEC for all treatments were 10.9 µg/L and 5.4 µg/L (Fig. 1.2). Ninety-five percent of mortality in the water only treatments occurred within the first 6 hours of exposure. Based on nominal concentrations, the 72 hour LC50 (95% confidence intervals) for NE sediment treatment was 15 µg/L (12.3-19.5), which was almost

twice that of water only and TX at 8 µg/L (6.7 – 9.7) and 9 µg/L (7.6-12.1) respectively (Table 1.2). The LC50 based on the average water concentrations from 0-6 hours was 11.5 µg/L (9.2-15.3) for NE sediments and 7.6 µg/L (5.9-10.8) for TX sediments.

The interaction between sediment treatment was not significant ($F_{12,42}$=1.55, p=0.145), and removing the 2 highest concentrations (150 µg/L and 62.5 µg/L) did not change the result ($F_{20,42}$=1.30, p=0.28). There were differences in mortality among concentrations ($F_{8,30}$=78.24, p<0.001) and sediment treatment ($F_{2,42}$=5.02, p=0.011). Again, removing the 2 highest concentrations did not change the results for either concentration ($F_{4,30}$= 39.90, p<0.001) or sediment treatment ($F_{2,30}$=5.02, p=0.013). Tadpole mortality in NE sediment treatment (48±9.5%) was significantly lower than TX (57±9.1; p=0.047) and water only exposures (58±10%; p=0.02), which did not differ from one another (p=0.96). Tadpole mortality from concentration 0 and 5.4 µg/L did not differ from each other, but they differed from all other concentrations (p<0.01). Mortality from concentrations 26 µg/L, 62 µg/L and 150 µg/L did not differ from each other but they differed from all other concentrations (p>0.01). Mortality in the 10.9 µg/L concentration was intermediate (64±9%) of mortality in concentrations 0-5.4 (5.7±1.4%) and 26-150 µg/L (99±0.1%) and significantly differed from these groups (p<0.05).

Fate test:

Mean total (water fraction + suspended sediment fraction + bed sediment) percent recovery of nominal pyraclostrobin concentrations for NE and TX sediment treatments were 114± 3% and 112±4% in the first 2 hours, respectively (Fig. 1.3). Total percent recovery dropped through 24 hours to 61±7% for NE and 70±6% for TX. Finally, total recoveries at 72 hours were 34±4% and 76±1% for NE and TX, respectively. A 2 hr TX bed sediment replicate (21.4 µg/L; 7X the 2 hr

mean) and 12 hr TX suspended sediment replicate (10.3 µg/L; 10X the 12 hr mean) were excluded as outliers (Grubbs outlier test; $p<0.05$).

Pyraclostrobin recovery differed among the 3 different sample types (i.e., water fraction, suspended sediment, and bed sediment; Fig. 1.4). Pyraclostrobin recovery was initially highest in the water fraction for both sediments, which decreased over time from about 22 µg to 6 µg in NE treatments and 21 µg to 14 µg in TX treatments. Pyraclostrobin recovery in suspended sediment and bed sediment fractions remained relatively low (<5 µg), with little variation, except for the TX suspended sediment recovery at 72 hours (7.6±1.1µg; Fig. 1.4). At 2 hours, the amount of pyraclostrobin in the water fraction was 90±4% and 72±2% of nominal, in NE and TX treatments, respectively. Pyraclostrobin recovery in the water fraction fell to 18±4% and 20±3% at 72 hours in NE and TX sediment treatments, respectively. Pyraclostrobin dissipation in the water fraction is consistent with the decrease in whole water concentrations from the acute toxicity test. However, the total recovery in the TX exposure remained four-fold higher at the 72 hour time point in the fate study (fate: 76 ±0.0% vs. toxicity test: 18±11%; Fig 1.4). Variation in total recovery between the toxicity and fate test was expected because the fate total includes a bed sediment component. Nonetheless, the TX suspended sediment at 72 hours in the fate test was driving the main difference in recovery. Pyraclostrobin concentrations in whole water samples were 20.4% and 18.9% at 2 hours, 21.0% and 18.9% at 24 hours, and 3.2% and 6.5% at 72 hours for NE and TX sediment treatments, respectively (n=1).

Total pyraclostrobin in the suspended sediment fraction was 1.4±0.4 µg and 3.7±1 µg at 2 hours, which reached 1.7±0.5 µg and 7.6±1.1 µg at 72 hours, for NE and TX sediments, respectively (Fig. 1.4). Total pyraclostrobin recovery in the bed sediment averaged 1.8±0.3 µg for NE treatments and 2.9±0.3 µg for TX treatments and varied little across time points (Fig. 1.4).

Total suspended sediment was 3.8±0.7 times higher in the TX sediment treatments compared to NE treatments over the course of the experiment (Fig. 1.5). Concentrations of TX suspended sediment were 1.3±0.04 g/L at 2 hours, which fell to 0.13±0.08 g/L at 72 hours. Concentrations of NE suspended sediment started well below TX concentrations at 0.5±0.08g/L and fell to 0.06±<0.01 g/L at 72 hours.

Discussion

Significant mortality occurred in all 3 sediment treatments when tadpoles were exposed to Headline AMP® at environmental relevant concentrations. Water-only exposures resulted in a nominal LC50 of 8.0 µg/L (6.7 – 9.7) which was twice that reported by Hooser et al. (2012) for the previous Headline® formulation (LC50 3.7 µg/L). Although pyraclostrobin is the main contributor to toxicity in Headline® and Headline AMP, as indicated by an LC50 of 10 µg/L for the active ingredient, the original Headline® formulation contained additional compounds (Hooser et al. 2012). For example, naphthalene is a toxic polycyclic aromatic hydrocarbon (Barron et al. 1999), and was in the original formulation but was removed from Headline AMP®. An added AI, metconazole, a triazole fungicide, constitutes 5.14% of total AMP® formulation (BASF Cooperation 2012b). Metconazole, has an LC50 of 2.2 mg/L (US EPA 2007a) to *O. mykiss* which is well above the exposure levels in this study. Nonetheless, despite the two-fold increase in Headline AMP® LC50 compared to Headline®, both LC50 values are essentially equal to estimated environmentally relevant maximum concentrations of pyraclostrobin for wetland field runoff, which is predicted to be 13.7 µg/L in soybean fields (Deb et al. 2010). This estimated concentration is a worst-case scenario if pyraclostrobin was applied three times during the

growing season at its maximum label rate. Headline AMP® may be less toxic than its former formulation, however it remains acutely toxic in aquatic environments at environmentally relevant concentrations.

Organism exposure to contaminants is dependent on conditions such as timing of application, bioavailability, and sorption, among others (Eggleton and Thomas 2004; Lenhardt et al. 2013; Reddy et al. 2013a). Within aquatic sediment systems, toxicity to tadpoles occurs faster than the sequestration of pyraclostrobin by sediment. Pyraclostrobin concentrations in the water were maintained much longer after initial application compared to a previous study (Morrison et al. 2013). In their aquatic microcosm with only deposited bed sediments, water concentrations of pyraclostrobin declined to 28± 2% within the first 4 hours, indicating a more immediate reduction in bioavailability (Morrison et al. 2013). Increased concentrations of suspended sediments facilitates more analyte sorption (Yang et al. 2006), however, more available sediment may create a higher potential for desorption, possibly prolonging bioavailable water concentrations. In contrast, pyraclostrobin partitions strongly to sediment and has little desorption (Reddy et al. 2013a) and the longer the contaminant is sequestered by a solid, the slower the rate of desorption over time (Kan et al. 1994). Pyraclostrobin concentrations in the water fraction did not fall below 50% of the initial dose until sometime between 24 and 72 hours for both sediment treatments. So if tadpoles are exposed to a high enough dose in the water column, the sediments may not able to sequester the majority of pyraclostrobin (>50%) rapidly enough to reduce toxic effects. In the first 24 hours, where greater than 99% of the mortality took place, water fraction concentrations were 10.1 μg/L ± 1 for NE and 9.9 μg/L ± 0.5 for TX. These water concentrations represent approximately 10% of expected environmental concentrations from direct overspray of maximum label rate of Headline AMP® (95 μg/L). Within the first 24 hours of exposure these bioavailable water concentrations are above lethal doses and will cause mortality to tadpoles.

To estimate risk, it is important to consider the degree of temporal overlap between application of a pesticide and the presence of susceptible organisms. Amphibian species travel to ephemeral pools for breeding and egg deposition at various times of the year and some reports indicate significant overlap between the estimated application times of pesticide and the presence of amphibians moving to and from breeding ponds (Lenhardt et al. 2013). The maximum number of Headline AMP® applications is 4 times per season for corn (BASF 2012). Headline AMP® is advertised for use in corn in conjunction with an in-furrow application of Xanthion® fungicide (23.6% pyraclostrobin; BASF Cooperation 2014) followed by pre-tassel application of Priaxor® fungicide (28.28% pyraclostrobin; BASF Cooperation 2012c) as part of a multiple spray program. Therefore, although Headline AMP® has a reduced pyraclostrobin content, a single use of pyraclostrobin may not always be the case in a given field. Breeding seasons for many amphibian species in temperate environments spans between March and August, and often occurs after precipitation events (Krupa 1994; Todd and Winne 2006; Semlitsch and Hall 2013). *B. cognatus* typically have larval periods that last anywhere between 18 to 49 days (Krupa 1994). Other temperate species like spadefoot toads, *Scaphiopus couchi* and *Scaphiopus multiplicatus*, have larval periods ranging from 8 to 40 days and 14 to 44 days, respectively (Wright and Wright 1949; Mayhew 1965; Newman 1989; Pfennig et al. 1991). Some species have much longer larval periods such as the green frog (70 days to > 1 year; Martof 1965) and wood frog (40 to 90 days; Martof 1970). The duration of larval periods is influenced by factors such as hydroperiod, resource availability, predators, and pesticide presence (Pfennig et al. 1991; Relyea 2000; Buck et al. 2012). Breeding seasons and larval periods vary between species, which leads to different pesticide exposure potentials based on their aquatic life stage. Although pyraclostrobin is toxic to tadpoles (Belden et al. 2010; Hooser et al. 2012), tadpole embryos can be more tolerant, however not resistant, to organic pesticide because of their protective casing (Bridges and Semlitsch 2000;

Howe et al. 2004). However, the temporal overlap of breeding migrations and larval periods with fungicide application make exposure of pyraclostrobin to sensitive life stages a possibility.

Although dissipation of pyraclostrobin in water was slow compared to toxicity in this study, a reduction in lethal concentrations over 72 hours is rapid. Only organisms residing in an exposed wetland during an application of Headline AMP® are at risk of a lethal dose, assuming an appropriate concentration. After 72 hours, tadpoles will have a greater chance of survival based on the rapid removal of pyraclostrobin from aquatic systems. At 72 hours, pyraclostrobin in the water reduced to ~20% of the initial dose in both sediment systems. In the TX system around 56% of the total recovered was found sequestered to sediments at 72 hours and the NE system lost a total of 66% of the initial dose by the end of the test. The aqueous photolysis half-life of pyraclostrobin in water is 0.05 and 0.08 days at pH 5 and the anaerobic soil metabolism half-life is 3.0-3.14 days (US EPA 2007b). Therefore, a majority of the total pyraclostrobin loss can be explained by microbial degradation, photolysis (US EPA 2007b), and uptake of non-lethal doses into the tadpoles.

Nebraska sediment systems had consistently higher LC50s for Headline AMP® than TX sediment systems. LC50s and their CIs were calculated based on nominal concentrations and calculated average water concentrations to accurately compare what is being added to the system with what concentrations are available to aquatic organisms. The CI for NE LC50 based on nominal concentrations did not overlap with TX and water only LC50s. However, for LC50s based on calculated water concentrations, CIs overlapped, indicating minimal toxicity differences among sediment treatments. Calculated LC50s are a more accurate representation of actual toxicity as they are based on measured exposure concentrations and not nominal values. Mortality in the NE sediment treatment was different than the TX sediment and water only exposures. The OM content of NE sediment was higher than TX (6.14% vs. 4.15%) and likely played a role in the differences in toxicity. Indeed, organic contaminants have a higher affinity for sorption to

organic matter compared to clay availability (Sanchez-Camazano and Sanchez-Martin 1994; Sheng and Boyd 2000). Nonetheless, despite the shift in NE LC50, NE sediments are not able ameliorates toxic effects in time (within 6 hours) if tadpoles are exposed to a lethal dose.

Over the 72 hours, TX sediment treatments had 3.8 times the amount of suspended sediment in the water compared to NE treatments, which is consistent with the higher analyte concentrations bound to the TX suspended solids. These results are supported by the linear sorption equilibrium model (the percentage of sediment bound pesticide increases with increasing sediment concentration) (Wu et al. 2003). Four times the total amount of pyraclostrobin was recovered in suspended sediment at 72 hours for the TX treatments compared to NE treatments. Sediment treatments had similar clay content (TX 32.5% vs. NE 30%), however TX sediments consisted of finer particle sizes indicated by the increase in sediment suspension. Finer particle sizes sequester more contaminants (Wu et al. 2003), explaining the increase in analyte bound to TX suspended sediments at 72 hours. More sediment suspended in the water column results in more surface area for sorption by hydrophobic organic compounds (Yang et al. 2006). In contrast, Morrison et al. (2013) observed a 68% reduction in pyraclostrobin water concentrations in an aquatic deposited sediment system, with little suspended sediment, where analyte quickly moved into bed sediments by 48 hours. There was a much more rapid partitioning of pyraclostrobin to the bed sediment compared to the bed sediments in the fate study. Although finer particle sizes and suspended sediment can facilitate more analyte sequestration (Yang et al. 2006), suspended sediments may prolong water concentrations over time compared to a clear aquatic system. A grassland playa with higher water clarity and a higher OC in its sediments than cropland playas (O'Connell 2011) may be more effective at sequestering pesticides. Further, wetlands within highly cultivated regions, such as the SHP (Bolen et al. 1989), contain more sediments than

grassland playas, which may prolong contaminants in the water and increase exposure to non-target organisms.

However, based solely on the higher TX suspended sediment content and increased fungicide sequestration in the fate test, TX sediments are expected to be more protective than the NE sediments. Conversely, NE sediments had a higher LC50 compared to the TX sediment system in the acute toxicity test. At concentrations near the threshold of toxicity, TX sediments may be able to quickly sequester pyraclostrobin from the water and reduce initial fungicide concentrations faster than NE sediments, as seen in the fate test, before lethal exposures can take effect. For concentrations above the threshold, NE sediments may protect tadpoles enough over time to decrease toxicity, as seen in the acute toxicity test, however the mechanism of protection is unknown. It is not clear whether the higher OM content of NE sediments or the finer particle size and decrease in bioavailable fungicide of TX systems would make for a more protective scenario in the environment. The variability between test systems makes it difficult to discern the characteristics responsible for decreases in toxicity.

Different larval species of anurans use different strata within the water column (Wells 2007). This variation in behavior can influence exposure potential. For example, certain benthic species of tadpoles may be exposed to higher concentrations in the sediment/water interface (pore water) before partitioning takes place (Liu et al. 2014; Guo et al. 2015). Surface sediment layers may have higher partitioning compared to deeper bed sediments that do not have the same opportunity for sequestering available analyte in the water column. This leads to a higher contaminant exposure in pore water (Booij et al. 2003) to tadpoles that may use the sediment/water interface for habitat and feeding. Concentrations of pyraclostrobin in bed sediments made up on average 9-20% of the total analyte recovery in both systems while concentrations in suspended sediment made up 3-57% of total recovery. However, because the total mass of suspended sediment was orders of magnitude less than bed sediments, suspended

sediments sequestered more pyraclostrobin per gram compared to bed sediments. In conclusion, bed sediments play a smaller role in protectiveness compared to suspended sediment. Further, desorption of pyraclostrobin sequestered by sediment is minimal (Morrison et al. 2013; Reddy et al. 2013a). Morrison et al. (2013) showed less than 2% of pyraclostrobin applied directly to bed sediments were recovered in the overlaying water column over time, supporting that benthic species of tadpoles are not at risk of exposure from desorbed analyte.

Direct application of Headline AMP®, even at 10% of expected exposure (95 µg/L pyraclostrobin), will result in acute toxicity to tadpoles. Fungicide water concentrations do not dissipate from the water rapidly within the first 24 hours, where 99% of mortality took place. However, concentrations decrease significantly over the course of 72 hours, and thus exposure after this time will not likely result in toxic effects. Nebraska playa sediments, with their higher OM content, had less mortality compared to Texas playa sediments indicating some systems may decrease toxicity more than others. In contrast, increased suspended solid content, as seen in the Texas system, enables more analyte to partition to the available sediments, reducing bioavailable concentrations to aquatic organisms over time. Because of the rapid toxic effects of pyraclostrobin, mortality may still occur before sediment sorption if a high enough concentration enters the aquatic system. A lethal dose of Headline AMP® is possible for tadpoles that experience a direct overspray, however if an organism enters the wetland after 72 hours they will more likely be protected by sequestration and degradation of pyraclostrobin. Further, as discussed above, different amphibian species will have different exposure potentials depending on their species-specific larval periods and other life-history traits. Also, because land use affects deposition, suspension, and composition of sediments in wetlands, it can influence partitioning of fungicide to sediments and effect the exposure scenario for wetland organisms. Grassland buffers around wetlands will decrease sediment input and aid in filtering pesticides, thus reducing

contaminants entering wetland habitats. Field investigations are needed to properly evaluate the risk of non-target organisms and develop risk assessments for wetland systems.

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Figure Captions

Figure 1. Mean (± SE) whole water (water + suspended sediment) percent recovery of pyraclostrobin based on the total amount added to the system of 2 concentrations (150 µg/L and 10.9 µg/L) in 2 microcosms (Nebraska and Texas) over 72 hours from the acute toxicity test (n=6). Sediments were collected from a Nebraska wetland and a Texas wetland.

Figure 2. Mean (± SE) percent mortality of *B. cognatus* tadpoles exposed to 4 Headline AMP® concentrations and a control for 3 aquatic microcosm treatment groups: Water only, Nebraska sediment (NE) Texas sediment (TX). Sediments were collected from a Nebraska wetland and a Texas wetland. Mortality was calculated by averaging replicates (n=3) for each concentration at 72 hours. Fifteen tadpoles were used in each replicate. Letters above bars indicate significant differences between the fungicide concentrations ($p < 0.05$). NE sediment treatment is significantly different than both TX and water-only treatments (*$p<0.05$).

Figure 3. Mean (± SE) total percent recovery of pyraclostrobin based on total amount added to the system in 2 aquatic sediment microcosms (Nebraska and Texas) over 72 hours from the fate study (n=3, except for TX 2 hr and TX 72 hr n=2). Sediments were collected from a Nebraska wetland and a Texas wetland. Total percent recovery is a combination of the recovered concentrations from the 3 measured components from the sediment systems: water fraction + suspended sediment fraction + and bed sediment concentrations. SE for 72 hour TX mean is 1%. Pyraclostrobin was added to the system at 5.4 µg/L.

Figure 4. Mean (± SE) measured water fraction, suspended sediment, and bed sediment amount of pyraclostrobin in 2 aquatic sediment microcosms over 72 hours (n=3, except for TX 2 hr bed sediment and 72 hr suspended sediment n=2). Sediments were collected from a (A) Nebraska wetland and a (B) Texas wetland. Pyraclostrobin was added to the system at 5.4 µg/L of for a total of 19.005µg/L (100%) in the whole system (3.5L).

Figure 5. Mean (± SE) suspended sediment concentrations in 2 aquatic sediment microcosms (Nebraska and Texas) over time. Sediments were collected from a Nebraska wetland and a Texas wetland. A total of 280 g of sediment was added to the system. Error bars on some data points are not visible because of the low variance between replicates (n=3).

Table 1.1. Percent of active ingredients and maximum corn application rates in Headline® and Headline AMP® fungicide formulations.

Active Ingredients:	Headline® (%)	Headline AMP® (%)
Pyraclostrobin	23.6	13.64
Naphthalene	30	0
Metconazole	0	5.14
Maximum application rate for corn:	12 fl ozs/acre (877 mL/ha)	14.4 fl ozs/acre (1052 mL/ha)

Table 1.2. Estimated median lethal concentrations (72 h-LC50) of Headline AMP® exposure to *Bufo cognatus* tadpoles for 3 aquatic microcosm treatment groups: Water only, Nebraska sediment (NE) Texas sediment (TX). Sediments were collected from a Nebraska wetland and a Texas wetland. Estimates are based on µg of pyraclostrobin (the primary active ingredient) per unit volume. LC50 values for each treatment were calculated based on nominal concentrations and calculated average water concentrations over the first 6 hours.

	Concentrations based on added to the system (nominal)		0-6 hr average water concentration (calculated)	
Treatment	LC50 (µg/L)	CI (95%)	LC50 (µg/L)	CI (95%)
Water only	8.0	6.7 - 9.7		
Nebraska	15.3	12.3-19.5	11.6	9.2-15.3
Texas	9.3	7.6-12.1	7.6	5.9-10.8

Fig. 1.1

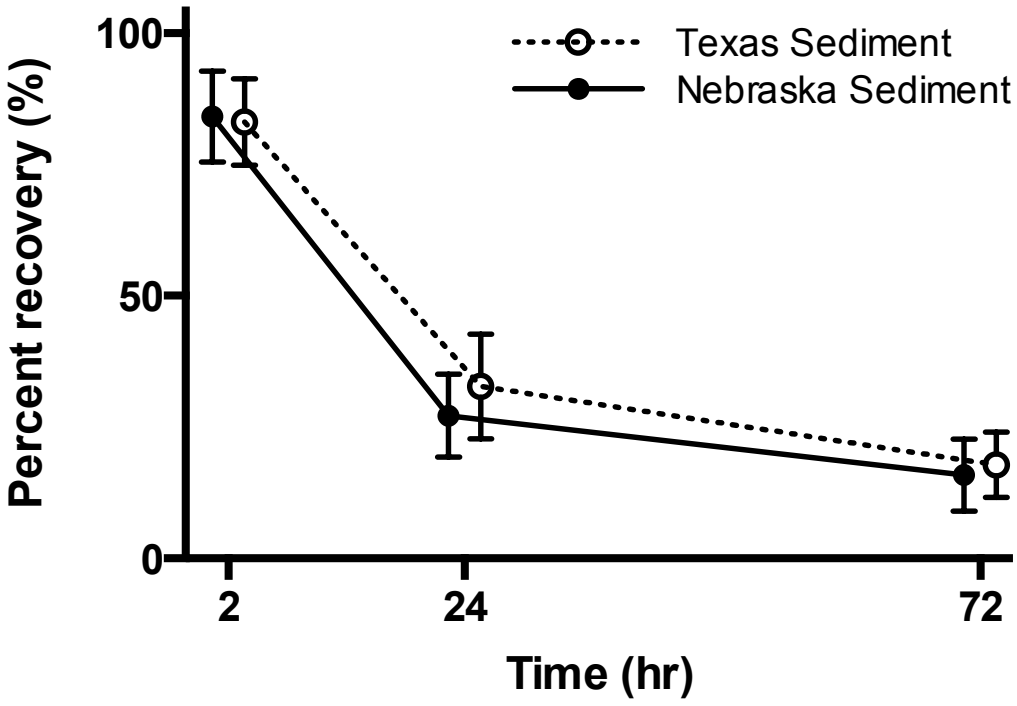

Texas Sediment
Nebraska Sediment
Percent recovery (%)
100
50
0
2
24
72
Time (hr)

Fig. 1.2

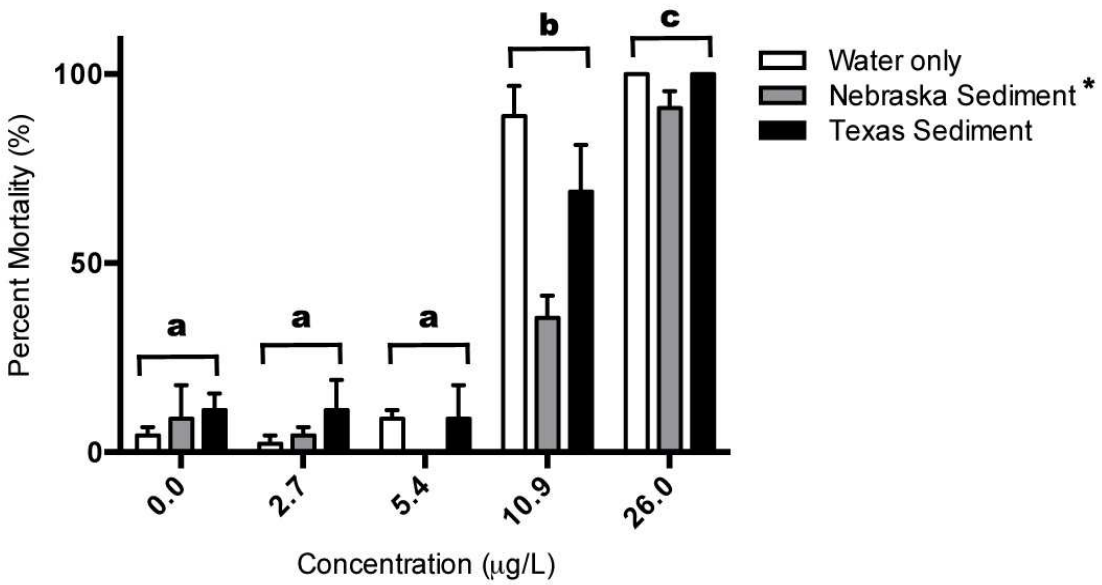

Percent Mortality (%)
100
50
0
a
a
a
b
c
0.0
2.7
5.4
10.9
26.0
Concentration (μg/L)
Water only
Nebraska Sediment *
Texas Sediment

Fig. 1.3

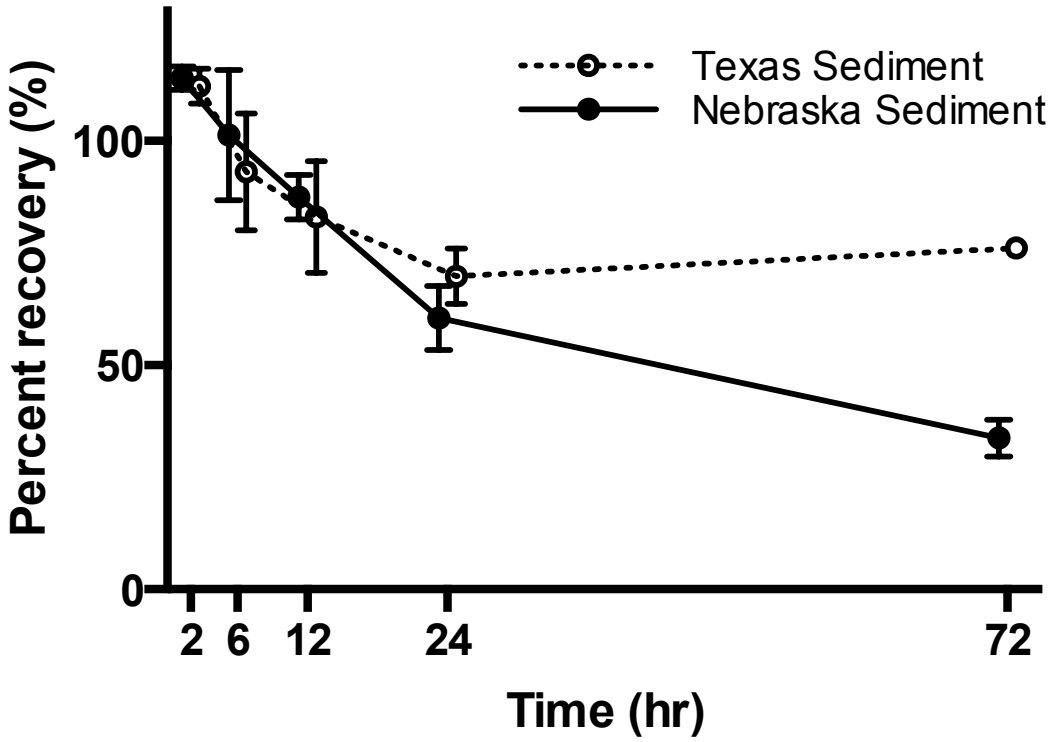

Texas Sediment
Nebraska Sediment
Percent recovery (%)
100
50
0
2
6
12
24
72
Time (hr)

Fig. 1.4

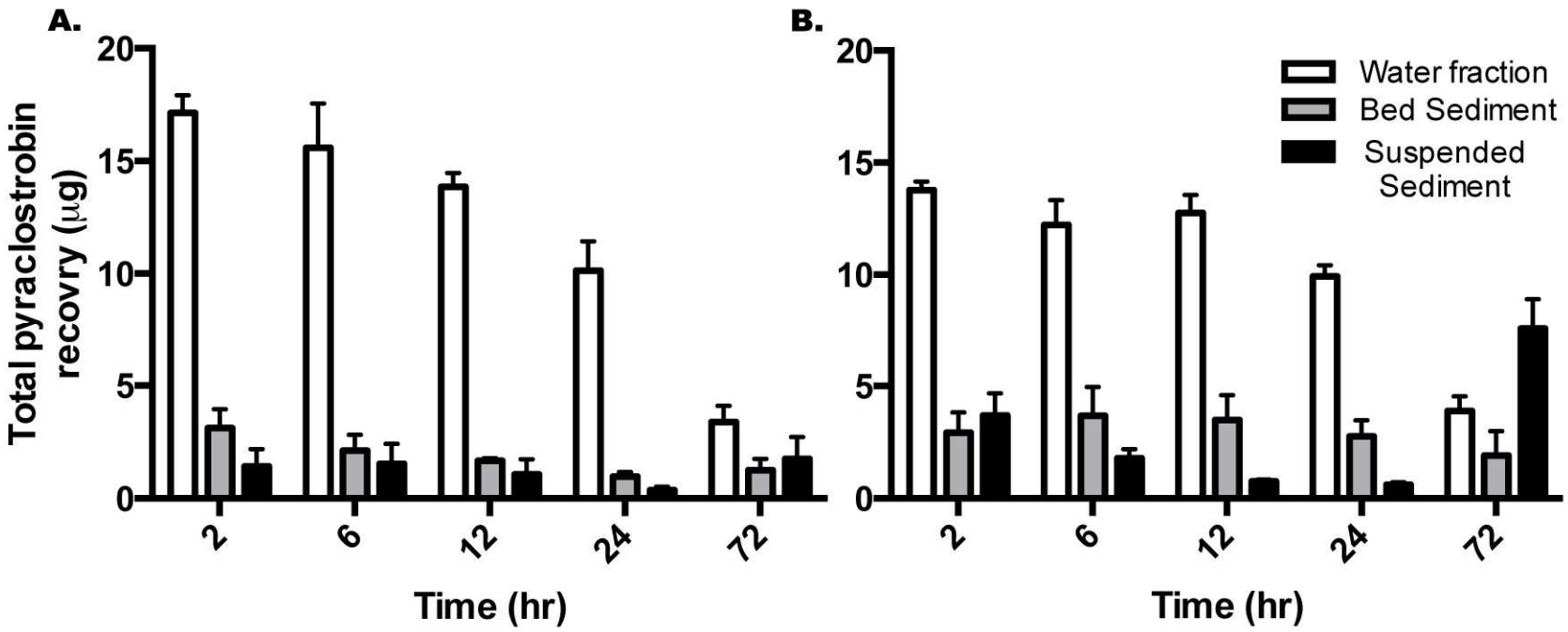
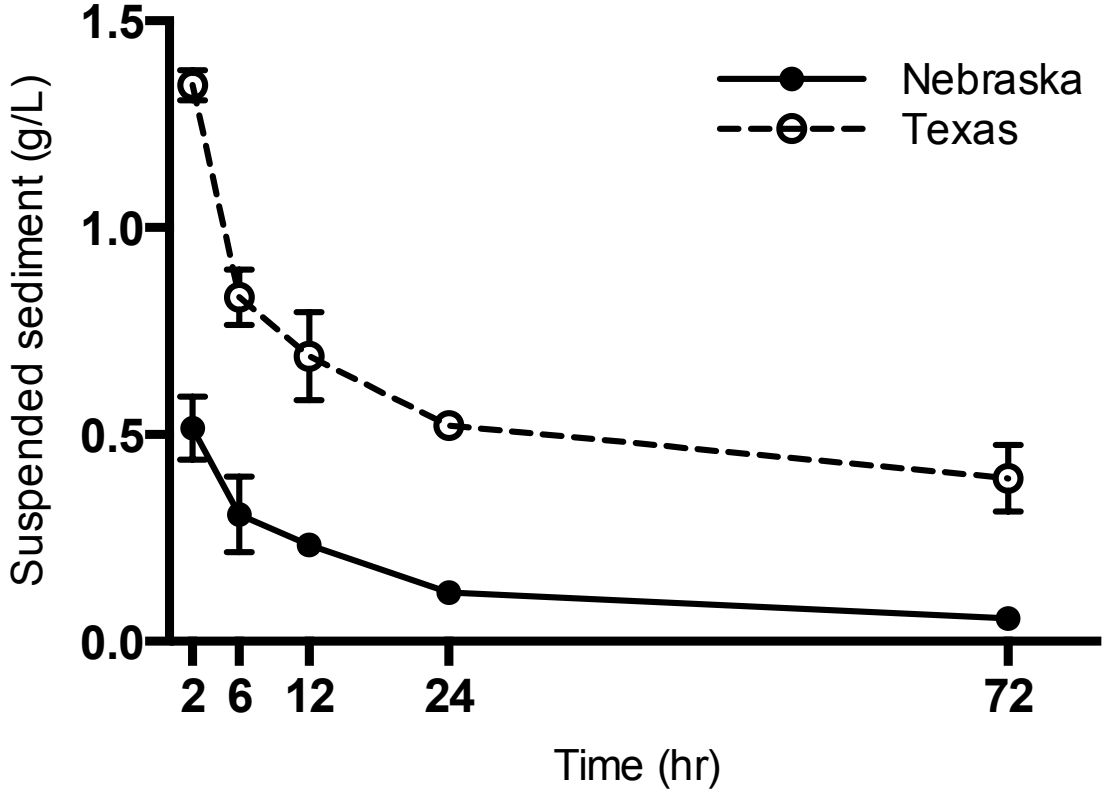

A.
B.
Total pyraclostrobin recovry (µg)
20
15
10
5
0
2
6
12
24
72
Time (hr)
Water fraction
Bed Sediment
Suspended Sediment

Fig. 1.5


1.5
1.0
0.5
0.0
Suspended sediment (g/L)
Nebraska
Texas
2
6
12
24
72
Time (hr)

APPENDICES

Appendix 1.a. Mean (± SE) percent mortality of *B. cognatus* tadpoles exposed to 8 Headline AMP® concentrations and a control for 3 aquatic microcosm treatment groups: Water only, Nebraska sediment (NE) Texas sediment (TX). The highest 6 concentrations and the control were used for the sediment treatment exposure. Sediments were collected from a Nebraska wetland and a Texas wetland. Mortality was calculated by averaging replicates (n=3) for each concentration at 72 hours. Fifteen tadpoles were used in each replicate.

Concentration (µg/L)	Water only		Nebraska Sediment		Texas Sediment	
	Mean mortality (%)	SE	Mean mortality (%)	SE	Mean mortality (%)	SE
0	4.4	2.2	8.9	8.9	11.1	4.5
0.7	2.2	2.2	-	-	-	-
1.4	0	0	-	-	-	-
2.7	2.2	2.2	4.4	2.2	11.1	8.0
5.4	8.9	2.2	0	0	8.9	8.9
10.9	88.9	8.0	35.5	5.9	68.9	12.4
26	100.0	0	91.0	4.5	100.0	0
62.5	100.0	0	100.0	0	100.0	0
150	100.0	0	100.0	0	100.0	0

Appendix 1.b. Mean (± SE) whole water (water + suspended sediment) percent recovery of pyraclostrobin based on the total amount added to the system of 2 concentrations (150 µg/L and 10.9 µg/L) in 2 aquatic sediment microcosms (Nebraska and Texas) over 72 hours from the acute toxicity test (n=6). Sediments were collected from a Nebraska wetland and a Texas wetland.

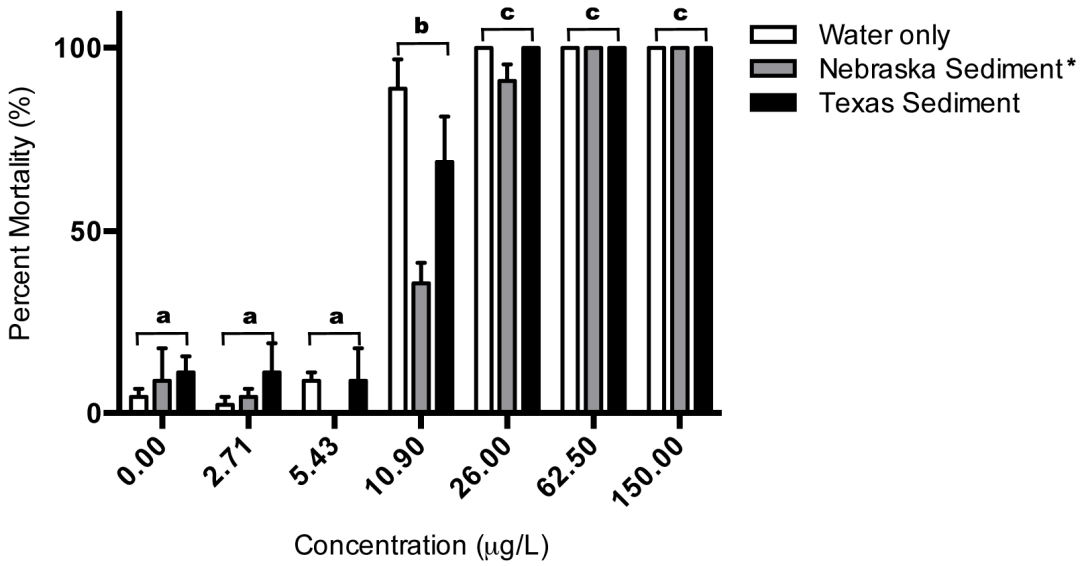
Acute Toxicity	Nebraska sediment		Texas sediment		Water only	
Hour	Percent recovery (%)	SE	Percent recovery (%)	SE	Percent recovery (%)	SE
2	84.1	8.7	83.1	8.3		
24	27.1	7.9	32.8	10.0		
72	15.9	6.9	17.8	6.2	15.8	2.4

Appendix 1.c. Appendix 1.c. Mean (± SE) total percent recovery of pyraclostrobin based on total amount added to the system in 2 aquatic sediment microcosms (Nebraska and Texas) over 72 hours from the mass balance fate study (n=3, except for TX 2 hr and TX 72 hr n=2). Sediments were collected from a Nebraska wetland and a Texas wetland. Total percent recovery is a combination of the recovered concentrations from the 3 measured components from the sediment systems: water fraction, suspended sediment fraction, and bed sediment concentrations. Pyraclostrobin was added to the system at 5.4 µg/L.

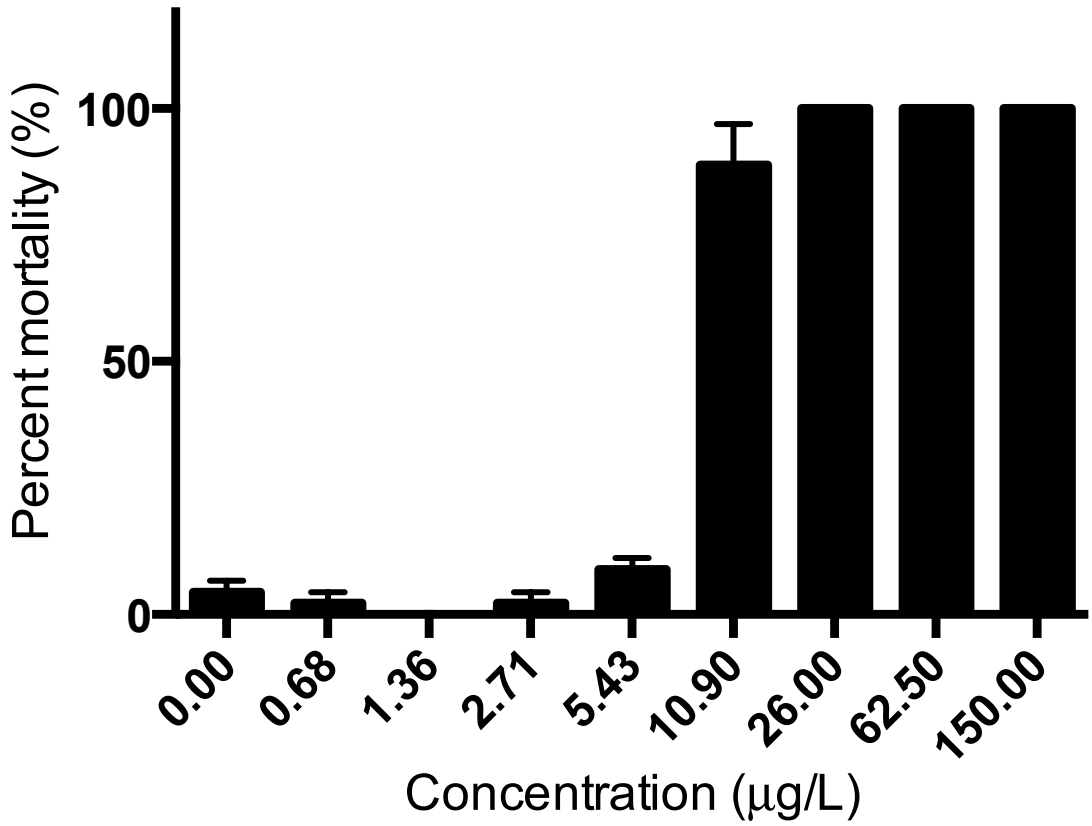
		Nebraska sediment		Texas sediment	
Fate test	Hour	Percent recovery (%)	SE	Percent recovery (%)	SE
Total					
	2	114.1	2.6	112.2	3.9
	6	101.3	14.6	93.1	13.0
	12	87.4	5.1	83.1	12.5
	24	60.5	7.1	69.8	6.2
	72	33.8	4.1	76.1	0.8
Water fraction	2	17.1	0.8	13.8	0.4
	6	15.6	2.0	12.2	1.1
	12	13.9	0.6	12.8	0.8
	24	10.1	1.3	9.9	0.5
	72	3.4	0.7	3.9	0.7
Bed sediment	2	3.1	0.5	2.9	0.9
	6	2.1	0.4	3.7	1.3
	12	1.7	0.1	3.5	1.1
	24	1.0	0.1	2.8	0.7
	72	1.3	0.3	1.9	1.1
Suspended sediment	2	1.4	0.4	3.7	0.7
	6	1.6	0.5	1.8	0.7
	12	1.1	0.4	0.8	0.7
	24	0.4	0.1	0.6	0.7
	72	1.8	0.6	7.6	1.4

Appendix 1.d. Mean (± SE) suspended sediment concentrations in 2 aquatic sediment microcosms (Nebraska and Texas) over time. Sediments were collected from a Nebraska wetland and a Texas wetland. A total of 280 g of sediment was added to the system.

	Nebraska sediment		Texas sediment	
Hour	Suspended sediment (g/L)	SE	Suspended sediment (g/L)	SE
2	0.5153	0.0767	1.3447	0.0360
6	0.3073	0.0916	0.8320	0.0677
12	0.2327	0.0100	0.6893	0.1068
24	0.1177	0.0092	0.5220	0.0250
72	0.0557	0.0068	0.3943	0.0800



Appendix 1.e. Mean (± SE) percent mortality of *B. cognatus* tadpoles exposed to 6 Headline AMP® concentrations and a control for 3 aquatic microcosm treatment groups: Water only, Nebraska sediment (NE) Texas sediment (TX). Sediments were collected from a Nebraska wetland and a Texas wetland. Mortality was calculated by averaging replicates (n=3) for each concentration at 72 hours. Fifteen tadpoles were used in each replicate. Letters above bars indicate significant differences between the fungicide concentrations ($p < 0.05$). NE sediment treatment is significantly different than both TX and water-only treatments (*$p<0.05$).



Appendix 1.f. Mean (± SE) percent mortality of *B. cognatus* tadpoles exposed to 8 Headline AMP® concentrations and a control in a water only exposure. Mortality was calculated by averaging replicates (n=3) for each concentration at 72 hours. Fifteen tadpoles were used in each replicate. LC50 in a water only exposure is 8.7 µg/L (6.5-12.5).

Appendix 1.g. GPS coordinates and additional information for collected sediments from Nebraska and Texas used in the acute toxicity and fate study.

Sediments	GPS coordinates	Type	Additional details
Nebraska	N 40.466, W -97.900	Waterfowl Production Area (WPA)	175 acres, Clay county, Eckhardt
Texas	N 35.265, W -100.92	Grassland playa	Gray county

VITA

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2014 - present - The Herpetologists' League