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Direct and indirect toxicity of the fungicide pyraclostrobin to *Hyalella azteca* and effects on leaf processing under realistic daily temperature regimes☆

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A B S T R A C T

Fungicides in aquatic environments can impact non-target bacterial and fungal communities and the invertebrate detritivores responsible for the decomposition of allochthonous organic matter. Additionally, in some aquatic systems daily water temperature fluctuations may influence these processes and alter contaminant toxicity, but such temperature fluctuations are rarely examined in conjunction with contaminants. In this study, the shredding amphipod *Hyalella azteca* was exposed to the fungicide pyraclostrobin in three experiments. Endpoints included mortality, organism growth, and leaf processing. One experiment was conducted at a constant temperature (23 °C), a fluctuating temperature regime (18–25 °C) based on field-collected data from the S. Llano River, Texas, or an adjusted fluctuating temperature regime (20–26 °C) based on possible climate change predictions. Pyraclostrobin significantly reduced leaf shredding and increased *H. azteca* mortality at concentrations of 40 μg/L or greater at a constant 23 °C and decreased leaf shredding at concentrations of 15 μg/L or greater in the fluctuating temperatures. There was a significant interaction between temperature treatment and pyraclostrobin concentration on *H. azteca* mortality, body length, and dry mass under direct aqueous exposure conditions. In an indirect exposure scenario in which only leaf material was exposed to pyraclostrobin, *H. azteca* did not preferentially feed on or avoid treated leaf disks compared to controls. This study describes the influence of realistic temperature variation on fungicide toxicity to shredding invertebrates, which is important for understanding how future alterations in daily temperature regimes due to climate change may influence the assessment of ecological risk of contaminants in aquatic ecosystems.

1. Introduction

Decomposition of organic matter is an essential ecosystem process in many aquatic environments (Vannote et al., 1980). Microbes (i.e., bacteria and fungi) and macroinvertebrate shredders are responsible for much of the breakdown of this material, releasing nutrients and carbon into aquatic food webs (Cummins and Klug, 1979; Hieber and Gessner, 2002). Shredding invertebrates, such as the amphipod *Hyalella azteca*, often preferentially feed on microbially conditioned leaf material, consuming the fungi and bacteria that has colonized this organic matter as a source of nutrition and energy (Arsuffi and Suberkropp, 1989). However, fungicides entering aquatic ecosystems via agricultural applications may impact these beneficial fungi, potentially reducing microbially-mediated decomposition as well as limiting food resources for shredding detritivores (Flores et al., 2014; Forrow and Maltby, 2000). These compounds may enter aquatic environments via spray drift, overspray, or run-off, and few studies have investigated the ecotoxicity of fungicides on non-target aquatic organisms. Previous literature has demonstrated fungicide toxicity to shredding invertebrates and impacts on leaf processing via alterations in leaf-associated fungal and microbial biomass (e.g., Flores et al., 2014; Zubrod et al., 2014, 2010).

Despite potential ecological impacts, the number of fungicides applied and the frequency of detections in the environment has rapidly increased. In the United States, agricultural usage of the strobilurin fungicide, pyraclostrobin, has increased from

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approximately 1.13 × 10^5 kg of active ingredient (a.i.) applied in 2002 to over 9.07 × 10^5 kg a.i. applied in 2010 (USGS, 2011). Several recent surveys have detected fungicides including pyraclostrobin in surface waters (Reilly et al., 2012) and sediments (Smalling et al., 2013). Maximum environmental concentrations of pyraclostrobin detected in surface waters range from 0.054 μg/L (Battaglin et al., 2011) to 0.239 μg/L (Reilly et al., 2012), but concentrations of 150 μg/L have been predicted after overspray of surface waters based on label application rates (Belden et al., 2010). Because this fungicide inhibits cellular respiration in mitochondria by blocking electron transfer and reducing energy production, it may also impact non-target organisms (Bartlett et al., 2002). Toxicity of pyraclostrobin and its formulations have been reported in amphibians and aquatic invertebrates. Morrison et al. (2013) estimated a 96-h median lethal concentration (LC50) for an aqueous exposure to *Hyalella azteca* to be 25.1 μg/L, and Hooser et al. (2012) determined a 72-h LC50 of 10.0 μg/L for *Bufo cognatus* tadpoles. Presently, no studies have investigated pyraclostrobin effects on endpoints associated with functional ecosystem processes, such as organic matter breakdown.

To our current knowledge no studies have investigated the effects of temperature on toxicity of pyraclostrobin and few data exist for temperature effects on toxicity of fungicides in general (Seeland et al., 2012). Temperature is an important environmental variable responsible for altering metabolic rates as well as contaminant toxicokinetics and toxicodynamics. Numerous studies have identified other pesticide classes as having temperature-dependent toxicity (Cairns et al., 1975; Harwood et al., 2009; Lydy et al., 1999). Willming et al. (2013) found that a daily temperature fluctuation (21–31 °C) over a 24 h period altered the toxicity of the fungicide chlorothalonil to *H. azteca* compared to a constant 24 °C. Additionally, climate change is predicted to increase global temperatures approximately 2–4 °C (IPCC, 2013), and may shift the pattern of daily temperature fluctuation by increasing daily minimums at a higher rate than daily maximum temperatures (Easterling et al., 1997). Therefore, in the present study we also investigated the effects of realistic daily temperature patterns on the toxicity of pyraclostrobin. Specifically, we compared two fluctuating temperature regimes based on field collected data from a river in Texas. One temperature treatment represented a daily temperature fluctuation observed in the field (18–25 °C), and the other was based on temperature shifts predicted under a climate change scenario (20–26 °C). Ectotherms experiencing realistic temperature fluctuation may become more sensitive to effects of climate change or other stressors compared to a constant temperature (Paaijmans et al., 2013).

The objectives of the present study were to assess the direct toxicity of pyraclostrobin in a water-borne exposure and indirect toxicity via a food selection experiment to *Hyalella azteca* and effects on leaf processing. *Hyalella azteca* was chosen as a model species because it is a common toxicity testing organism (USEPA, 2000) and is also a representative shredding detritivore. Waterborne toxicity and leaf decomposition were also assessed under varying daily temperature patterns to determine possible interactions of temperature regime and fungicide concentration under a more environmentally realistic scenario.

2. Materials and methods

2.1. Experiment 1: preliminary exposure

A preliminary experiment was performed as a range-finding test to determine a concentration range for possible effects. Leaf disks (2.3 cm diameter) were cut from rewetted *Acer saccharum* (sugar maple) leaves collected shortly after abscission near Benton Harbor, Michigan, USA. Disks were placed in a drying oven at 60 °C for 24 h, and then initial dry mass was measured using a microbalance. After weighing, individual leaf disks were placed in numbered, plastic tissue cassettes. These cassettes allowed each leaf disk to be individually identified while permitting water flow to the disk and facilitating establishment of a microbial community on all disks during conditioning. All tissue cassettes were conditioned in a 9.5 L tank in aerated, reconstituted moderately hard water (RMHW) (Smith et al., 1997) with whole leaf material pre-conditioned for 14–21 d in stream water used as an inoculum for the microbial community. Stream water was collected from the South Llano River, near Junction, Texas at the Texas Tech University Llano River Field Station. Stream water was considered to be free of contaminants with no major sources of upstream contaminant input at this site. Leaf disk conditioning occurred for 16 d at 23 °C.

Hyalella azteca obtained from laboratory cultures were sieved to collect animals passing through a 710 μm sieve and retained on a 500 μm sieve, which corresponded to approximately 7–14 d old animals (USEPA, 2000). Cultures were maintained on *A. saccharum* leaves and Tetramin flake food. Sieved organisms were held for 24 h in culture water at a constant 23 °C to prevent use of any animals injured during handling.

Test solutions were made from a stock solution of pyraclostrobin (99.9% purity, Sigma–Aldrich) dissolved in analytical grade acetone. All test solutions were made using RMHW with the control consisting of RMHW only. Nominal test concentrations of pyraclostrobin were 0, 20, 40, and 80 μg/L pyraclostrobin with six replicates at each concentration. Maximum acetone concentrations were less than 0.1% which was below toxicity thresholds for the endpoints measured. Exposure concentrations were selected based on previous pyraclostrobin toxicity studies for aquatic species (Hooser et al., 2012; Morrison et al., 2013). Static exposures were conducted in 400 mL beakers containing 200 mL of test solution. Five *H. azteca* were pipetted into each experimental chamber and a single conditioned leaf disk was added to each replicate. Experimental chambers were placed in a temperature controlled water bath at a constant 23 °C for five days with a 16:8 h light:dark cycle. Endpoints included *H. azteca* mortality and leaf mass loss. At the end of the exposure period, leaf disks were removed from beakers, dried at 60 °C for 24 h, and weighed to determine final dry mass. Leaf ash-free dry mass (AFDM) was determined by ashing dried leaf disks in a muffle furnace for 1 h at 500 °C and measuring remaining ash mass. Total number of surviving *H. azteca* in each replicate was recorded. Survival was assessed by gently probing motionless organisms with a plastic pipette.

2.2. Experiment 2: leaf disk choice

In order to assess whether *H. azteca* would preferentially shred a leaf disk conditioned in a control medium versus a leaf disk exposed to pyraclostrobin, animals were given a choice of two leaf disks to shred. Pyraclostrobin may impact *H. azteca* food resources by targeting leaf-associated fungi and reducing fungal biomass. Therefore, pyraclostrobin treated disks may represent a substrate containing a lower quality food resource for *H. azteca*, especially if preferred fungal species are impacted. Animals may preferentially shred the untreated leaf disk, which could contain more abundant food resources and represent a more labile form of organic matter (Bundschuh et al., 2011). This experiment assessed effects on shredding without direct aqueous toxicity to *H. azteca*. Additionally, because pyraclostrobin has a reported log K_{oc} of 4.0 (Bartlett et al., 2002), it is likely that a portion will bind to organic matter. Given the choice between two leaf disks, *H. azteca* may exhibit a chemical avoidance behavior by choosing to feed on the untreated disk (Zubrod et al., 2015).