EVALUATING THE EFFECTS OF FUNGICIDES AND OTHER PESTICIDES ON NON-TARGET GUT FUNGI AND THEIR AQUATIC INSECT HOSTS

by

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The following individuals read and discussed the thesis submitted by student Emma R. Wilson, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

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DEDICATION

To my family for their constant support, faith, and encouragement. Also to the fungi that are an endless source of inspiration.

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ABSTRACT

Pesticides are widespread and have been long used to combat the attack and destruction of crops. Fungicides have been used to prevent the establishment of many fungal pathogens, yet little is known about the impacts of fungicides on non-target fungi. With these considerations, it was predicted that trichomycetes, or gut fungi, a group of symbiotic fungi associated with aquatic macroinvertebrates and other arthropods, might be a candidate system to study because of the intimate association with their hosts. Field and laboratory studies were initiated to assess non-target impacts of fungicides on gut fungi. Field surveys were conducted on four streams with varying pesticide inputs in Southwestern Idaho. Larval black flies (Diptera: Simuliidae), hosts to many gut fungi, were analyzed for a suite of currently used pesticides including fungicides. The infestation rate and density of gut fungi in hosts residing in streams within agricultural watersheds was lower than those residing in reference streams. Fungicides were detected in hosts collected from streams within agricultural watersheds, but not in those from reference streams. These findings suggest that there may be an effect of fungicides on non-target fungi. Laboratory investigations were designed to test this hypothesis using both host-fungus microcosms and *in vitro* experiments with axenic fungal cultures. Pure strains of host black fly larvae, *Simulium vittatum* IS-7, and the gut fungus *Smittium simulii*, were exposed to the fungicide azoxystrobin. With direct *in vitro* exposure, a significant decrease in dry weight of the gut fungus was not observed until 0.5 mg/l of azoxystrobin, approximately three orders of magnitude higher than what was detected in

the field. In two of three microcosms, there was no statistically significant effect of fungicides with maximum concentrations as high as 5000 ng/l. Attempts to test the higher concentrations in the microcosm experiments were preempted by 100% mortality of the black fly larvae. It is likely that azoxystrobin alone was not the cause of decreased percent infestation and density observed in the field. Data generated from this study indicate the need for future studies to better understand the effects of fungicides and other currently-used pesticides on non-target fungi.

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LIST OF ABBREVIATIONS

ACE	Acetone
TEG	Triethylene glycol
BHI	Brain Heart Infusion
BHIGTv	Brain Heart Infusion Tryptone Glucose + vitamins

INTRODUCTION

Modern agricultural practices throughout the world make use of pesticides to combat the effects of an array of pathogens on economically important crops. Inevitably, some amounts of pesticides applied to crops are transported to nearby streams via drift, runoff, and infiltration into the groundwater (Rasmussen et al., 2012; Reilly et al., 2012; Relyea and Hoverman, 2006). Pesticide application to agricultural fields and the resulting runoff has been shown to be one of the greatest stressors to aquatic ecosystems (Kolpin et al., 2002; Rasmussen et al., 2012; Relyea and Hoverman, 2006). Many agricultural areas benefit from field-side streams for irrigation purposes. However, these streams and their associated biotic communities may be susceptible to contamination by pesticides at potentially high concentrations (Gilliom, 2007).

Pesticides, at both acute and chronic levels, often impact many non-target organisms, including arthropods. Stream macroinvertebrates can be significantly affected when exposed to non-point source pesticide pollution, and may experience decreases in population density as a result (Hurd et al., 1996). Sorption and accumulation of pesticides has been documented in macroinvertebrates, including in the silk of black fly larvae (Brereton et al., 1999). Other arthropods, including honeybees, are also affected by pesticide application, which may have sublethal physiological effects on development, foraging, feeding behaviors, and learning (Desneux et al., 2007).

Effects of pesticides are varied, depending on whether they are acting singularly or synergistically. For instance, in the presence of a pyrethoid insecticide, the fungicides imidazole and triazole had a synergistic 12-fold increased toxicity in the aquatic crustacean *Daphnia manga* (Norgaard and Cedergreen, 2010). Synergism between pesticides and UV radiation may kill or have sublethal effects on amphibian embryos, larvae, and adults (Blaustein et al., 2003). Similarly, a widely used herbicide, atrazine, may act synergistically with orgahophosphate pesticides, causing increased toxicity to the larval midge *Chironomus tentans* (Pape-Lindstrom and Lydy, 1997).

Fungicides, a group of pesticides that target fungal pathogens, are of particular concern because of the increased tendency for reapplication during the growing season – as much as 10 times per season (Reilly et al., 2012). Recent studies have detected fungicides in surface and ground water, sediments, air and rainfall (Battaglin et al., 2011; Geissen et al., 2010; Schummer et al., 2010; Smalling and Orlando, 2011) at concentrations that have the potential to cause adverse effects on aquatic organisms (Battaglin and Fairchild, 2002; Deb et al., 2010; Gilliom, 2007). The modes of action of fungicides are varied and may be detrimental to non-target organisms, such as macroinvertebrates (Elskus, 2012; Gustafsson et al., 2010). Recently, there has been an increased awareness, interest, and concern about how fungicides may be affecting non-target or non-pathogenic fungi (Dijksterhuis et al., 2011; Maltby et al., 2009). While some studies have focused on leaf decomposing fungi (Bundschuh et al., 2011; Cuppen et al., 2000; Dijksterhuis et al., 2011; Maltby et al., 2009; Rasmussen et al., 2012; Zubrod et al., 2011), a widespread group of fungi that has not yet been studied in this manner are trichomycetes, or gut fungi.

Gut fungi are a cosmopolitan group of symbiotic arthropod associates. They live in the guts of many aquatic macroinvertebrates, including immature stages of aquatic insects such as mayflies (Ephemeroptera), stoneflies (Plecoptera), and black flies (Diptera). The relationship between gut fungus and host is thought to shift depending on environmental conditions (McCreadie et al., 2011). Some studies have shown their mutualistic potential (Horn and Lichtwardt, 1981), while others have demonstrated parasitism (Sweeney, 1981). More generally, gut fungi are regarded as commensalistic (Lichtwardt, 1986). Whether their role is positive, negative, or neutral, the relationship between the gut fungi and their arthropod host reflects the adaptive responses of the symbiotic partners, both on a shorter term and long-term evolutionary time scales (Hibbett et al., 2007; McCreadie et al., 2011; White, 2006).

The gut fungus life cycle is completely entwined with that of its non-predaceous hosts (Lichtwardt, 1986). Upon the ingestion of a spore by an appropriate host, the fungus germinates within the gut lumen and quickly attaches via a holdfast. Gut fungi may attach in the midgut or hindgut, depending on the species and family of fungus. For example, in black fly larvae, *Harpella* spp. reside on the peritrophic matrix (PM) of larval midguts as unbranched thalli, whereas *Smittium* spp. are all branched and attached to the chitinous lining of the hindgut. In contrast to Diptera, where gut fungi can be found in two regions of the digestive tract, gut fungi in mayflies are found only in the hindgut. After each molt, the chintinous lining of the mayfly's hindgut is sloughed off along with any attached fungi.

The PM that lines the black fly midgut is analogous to a conveyer belt that is continuously produced in the anterior region and moved along toward the hindgut, where

it is eventually sloughed off (Valle et al., 2011). This process leads to differences in development or age structure of the individual *Harpella* thalli along the length of the gut. Ingested spores that germinate at the anterior midgut are the most immature, but develop into more mature thalli while being conveyed toward the hindgut. The increase in number and length of attached thalli can be dramatic, and they may seem to fill the whole of the gut lumen. *Harpella* spore maturation is generally restricted to those thalli situated at the posterior region of the midgut lining, just in advance of the hindgut. The deciduous spores of the fungus are released for movement through the digestive tract and with eventual release at the anus to the outside environment (Lichtwardt, 1986).

Black fly larvae and mayfly nymphs were chosen as candidate hosts for the field-based study because they are well-known hosts of gut fungi, and because they play important roles in stream ecosystems. Larval black flies are ecosystem engineers that turn over resources in food webs and serve as a dominant food source for fish and other predators (Wallace and Webster, 1996). Although they are prevalent in many stream ecosystems, black fly larvae are susceptible to many common pesticides (Overmyer et al., 2003; Overmyer et al., 2007). They are also ideal candidates for laboratory toxicity tests as they can be grown in large numbers (Gray and Noblet, 1999; Hyder et al., 2004), have been used in toxicity studies (Hyder et al., 2004; Overmyer et al., 2003), and in studies of the nature of their symbiosis with gut fungi (Beard and Adler, 2002; McCreadie and Beard, 2003; Nelder et al., 2005; Vojvodic and McCreadie, 2008).

Mayflies are a vital part of aquatic ecosystems (Chessman and McEvoy, 1998; Corkum et al., 1995). Their functional roles in ecosystems are as grazers, collectors, and gatherers wherein they consume fine particulate organic matter, biofilm, and periphyton,

thus contributing to nutrient cycling in allochthonous streams (Allan and Castillo, 2007). Mayflies are also a major food-source for predaceous invertebrates (e.g. some species of Plecoptera and Odonata), as well as fish species (Allan and Castillo, 2007). Mayflies are sensitive to oxygen depletion, acidification, and contaminants such as metals, ammonia and pesticides, and have therefore been utilized as bioindicators of stream quality (Savic et al., 2011; Schulz and Dabrowski, 2001). Mayfly nymphs are also hosts to many species of gut fungi (Lichtwardt, 1986). They were only used in the field portion of the study because of the difficulty in obtaining non-inoculated nymphs from the field, and handling difficulty in large-scale rearing conditions; see also (Sweeney et al., 1993).

Among known pesticides, azoxystrobin is an emerging fungicide of interest. It was first sold in 1996 and is used prophylactically, curatively, and eradicatively to inhibit all fungal phyla on a wide range of crops (Adetutu et al., 2008; Bartlett et al., 2002). Battaglin et al. (2011) found that azoxystrobin was the most frequently detected fungicide in 29 surveyed streams across 13 states. It was chosen for use in this investigation because of its widespread use, detectable presence in our field study, and for its potential effect on non-target insects and fungi and other organisms (Adetutu et al., 2008; Bartlett et al., 2002; Battaglin et al., 2011; Dijksterhuis et al., 2011; Rasmussen et al., 2012). Azoxystrobin has a broad-spectrum mode of action in that it inhibits mitochondrial respiration by binding to the Q_o site of cytochrome b, within the cytochrome bc_1 complex located on the electron transport chain of all eukaryotes. Production of ATP is thus halted through the blockage of electron transport from cytochrome b to c_1 (Bartlett et al., 2002).

Several micro- and mesocosm experiments have used azoxystrobin in effects studies. Gustafsson et al. (2010) documented that azoxystrobin significantly altered the

structure and function of model ecosystems and had a direct toxic effect on the invertebrates studied. Warming et al. (2009) found increased stress, metabolic costs, and acute toxicity on *Daphnia*, even at low concentrations (0.026 μg/l). There have been few laboratory tests on the effects of azoxystrobin specifically on fungi. Long-term exposure of soil fungi to azoxystrobin altered the total fungal community structure (Adetutu et al., 2008). In aquatic fungi, species of all fungal phyla tested were more sensitive to azoxystrobin than other fungicides (Dijksterhuis et al., 2011). When fed tebuconazole-treated leaves, the amphipod *Gammarus fosarium* had a significant decrease in leaf consumption, caused by a decreased colonization by aquatic fungi, rendering the leaves less palatable (Bundschuh et al., 2011). Thus, impacts on non-target fungi could have a cascade of effects within aquatic ecosystems. To ascertain the putative and apparent overall effects of the azoxystrobin (and other fungicides) on gut fungi from field observations, a controlled microcosm study was instigated using black fly larvae as hosts.

Several laboratory studies provide insights into the symbiosis between gut fungi and black fly larvae. McCreadie and Beard (2003) found that *Smittium culisetae* has an uneven distribution along the hindgut section of the digestive tract, with a higher prevalence in the posterior colon and rectum of the host than the anterior colon. Additionally, certain species of *Smittium* differentially colonize different species of black flies (Nelder et al., 2005), and when multiple species of *Smittium* are present, there may be competition between the symbionts within the gut (Vojvodic and McCreadie, 2008). However, none of these studies were conducted in the presence of toxic stressors such as pesticides. In other studies, black fly larvae (without gut fungi) were tested for the potential effects of pesticides. When exposed to the insecticide chlorpyrifos, Hyder et al.

(2004) described a higher sensitivity to toxicity in mid-instar larvae. Also, in the presence of common lawn-care insecticides, there was a greater than additive toxicity to the larvae when exposed to mixtures of pesticides (Overmyer et al., 2003).

This is the first field-based study to investigate the impact of pesticides on gut fungi and their immature black fly and mayfly hosts. Samples from two agricultural streams were compared to two non-agricultural (reference) streams. Non-target gut fungi residing in hosts from agriculturally dominated streams containing fungicides were predicted to have a lowered percent infestation and density compared to non-agricultural streams. Specific field-based objectives were to: 1) survey surface waters known to contain pesticides and determine percent infestation and density of gut fungi, while assessing dissolved concentrations of fungicides and other pesticides in the water column, 2) measure pesticide concentrations in black fly host tissue, and 3) compare these metrics with those collected from reference streams.

This study is also the first laboratory-based attempt to investigate the impact of fungicides on gut fungi. Microcosm experiments exposed black fly larvae, *Simulium vittatum* IS-7, and the hindgut dwelling gut fungus, *Smittium simulii* (JAP-51-5), to a range of azoxystrobin concentrations. *In vitro* experiments directly exposed the same fungal species in axenic culture. It was predicted that the gut fungi in the microcosms would have decreased density within the host and a decreased dry weight *in vitro*. Also, it was suspected that black fly larvae grown in the same fungicide treatment might have greater fitness (estimated by head capsule width measurements) when inoculated with gut fungi than without. Specific objectives were to: 1) enumerate the gut fungi in inoculated black fly larvae and compare across fungicide doses, 2) assess black fly fitness when

grown with or without gut fungi while being exposed to fungicides, and 3) obtain and compare gut fungus dry weight when grown in different fungicide doses.

MATERIALS AND METHODS

Field Study

Site Descriptions and Sample Collection

Two of the four streams selected for this study drain agricultural land are known to contain fungicides, and are referred to as agricultural sites (Reilly et al., 2011): Sand Run Gulch at Highway 95 (USGS Station Number 13210360) and a ditch near Wanstad Road (herein referred to as Wanstad Ditch) (USGS Station Number 13213008). Both sites are near Parma, Idaho. Over 37% of the Sand Run Gulch watershed contains land used for agricultural purposes, primarily alfalfa (25%), corn (15.9%), and winter wheat (14.3%). At Wanstad Ditch, approximately 90% of the watershed is agricultural with dominant crops of winter wheat (27.5%), hay (18.1%), and corn (17.7%) (Fig. 1) (Reilly et al., 2011). Both streams had substrates containing sand, silt, and small pebbles. The riparian vegetation adjacent to the stream was dominated by grass that was often extended to the water column.

The two other streams in this study are referred to as reference sites. Both Cottonwood Creek (USGS Station Number 433711116110700) and Dry Creek at Bogus Basin Road near Boise, Idaho (USGS Station Number 434006116112100) drain non-agricultural land. Both streams had 0.1% or less of their watershed containing agricultural land (Fig. 1) (Reilly et al., 2011). Both streambeds contained sand, pebbles, and cobbles. The riparian vegetation adjacent to both streams contained grasses, trees, and shrubs.

Reference stream locations were selected based on proximity, accessibility, and similarity of streams as much as possible. The two streams were also selected because they are known to contain insect larvae with robust and known populations of gut fungi (Bench and White, 2012; Kandel and White, 2012).

Host and surface water sampling was conducted using the same procedures at all sites. Samples were collected approximately every three weeks starting in April 2010 (except starting in June for Dry Creek), through early December 2010. This timeframe encompasses the growing season in Idaho and several weeks after the last pesticide application (Mike Thornton, University of Idaho, personal communication). Sample collections did not specifically target runoff events or other hydrological conditions.

Water samples were obtained by submerging pre-cleaned amber glass bottles [1 L for pesticide analysis and 125 mL for dissolved organic carbon (DOC)] and a 1 L polyurethane bottle for water quality parameters (specific conductance, pH, and turbidity) once at each site at a depth of not less than 0.1 m below the water surface. The same location within the stream was visited for each sampling event. Water temperature was recorded at the time of sample collection using an alcohol-filled thermometer. Water samples were shipped on ice overnight to the United States Geological Survey (USGS) Organic Chemistry Laboratory in Sacramento, California for analysis. Methods and quality assurance for surface water analysis conducted by the USGS followed that of Reilly et al. (2012) and can be found in Appendix A.

Host Sampling and Dissection

At each sampling event, a 10–20 m stream section was sampled for immature stages of black flies and mayflies using either kick-nets or methanol-cleaned forceps to

pick specimens from dangling vegetation and rocks from riffle zones. A minimum 20 each of black fly larvae and mayfly nymphs were collected for transport on ice to Boise State University, where they were stored in stream water at 4°C for up to 48 hours until dissected. If 20 hosts were not found within 90 minutes of searching, all collected insects were dissected and used exclusively for fungal metrics. For black fly larvae, when greater than 20 individuals were recovered, subsamples were also partitioned and held for later tissue analysis. Those held for later analysis were placed in glass vials, using methanol-cleaned forceps, for storage at -20°C prior to extraction and analysis of tissue. Based on the detection frequency and maximum concentrations observed in surface water samples, field-collected black fly larvae were also analyzed directly for 4 fungicides and 8 herbicides. Eleven of these pesticides were found in their tissue. In total, 17 larval tissue samples were analyzed (5 from Cottonwood Creek, 4 from Dry Creek, 7 from Sand Run Gulch and one from Wanstad Ditch). The methods for tissue analysis, as conducted by collaborators at the USGS, are described in Appendix A and B. No tissue analysis was performed for mayfly nymphs due to low numbers recovered from the field.

Within 48 hours of collection, hosts were dissected per Bench and White (2012) in a drop of distilled water on a glass slide with the aid of a stereomicroscope (Olympus SZ60, equipped with indirect ring lighting from the base) and using fine-tipped jeweler's forceps and insect mounting needles secured in pin vices (Grobet, USA). Larval black fly midguts were placed on fresh slides for fungal identification and enumeration. If hindgut fungi were present, they were similarly prepared on a fresh slide and in either case, fixed to identify the gut fungi to genus, also following the methods of Bench and White (2012). Density metrics were not calculated for mayfly midguts because these insects only have

hindgut fungi (Lichtwardt, 1986). However, percent infestation (number of hosts containing gut fungi divided by the number in the sample (Beard and Adler, 2002; Nelder et al., 2005)) was calculated for both black fly larvae and mayflies for all sampling events.

Fungal Density and Sporulation in Larval Black Fly Midguts

Images of dissected, slide-mounted larval black fly peritrophic matrices (PMs) were used to assess the density of thalli (individual fungal hyphae) and trichospores (asexual sporangiospores) of *Harpella* spp. (Fig. D.1). Before fixation, the PM, freshly mounted in distilled water, was viewed using a Nikon Eclipse 80i microscope (equipped with both phase and interference optics). Digital images were captured using a 2 MP Spot Color Mosaic camera (Diagnostic Instruments, Sterling Heights, Michigan USA) and accompanying Spot Advanced software (version 4.6). Thalli and trichospores were counted directly. Previous studies (Beard and Adler, 2002; McCreadie and Beard, 2003) used grids and ratios to estimate the amount of fungi within the gut. In the present study, an exact count was made by focusing on and enumerating holdfasts, even when the unbranched, overlapping thalli were present as dense masses. The density of thalli and spores in the PM was normalized for gut size by counting the number of thalli and spores within the gut and dividing it by the PM area to give the number of thalli or spores per μm^2 of PM.

Laboratory Study

Fungicide Doses and Preparation

Across microcosm experiments, azoxystrobin doses ranged from 5 ng/L to 5 mg/L. For the microcosm experiments, azoxystrobin was dissolved in acetone (ACE) as a vehicle to obtain stock solutions of 100 mg/L and 500 mg/L. An additional 500 mg/L stock solution was made using triethylene glycol (TEG) as a vehicle. ACE was originally chosen as a carrier for azoxystrobin, since it has been used in other studies to dissolve pesticides (Avenot and Michailides, 2007; Gustafsson et al., 2010; Overmyer et al., 2003; Warming et al., 2009). TEG was chosen as another carrier solvent because it is virtually non-toxic for invertebrates (Ballantyne and Snellings, 2007). Since ACE was used in the initial microcosm it was used for subsequent experiments to ensure consistency.

The stock solutions were dissolved in moderately hard water that was prepared following the recipe of Iburg et al. (2011) to achieve the desired concentrations. The following compounds were dissolved in a 25 L carboy: Sodium bicarbonate (2.4 g), Calcium sulfate dehydrate (1.5 g), Magnesium sulfate (1.5 g), and Potassium chloride (0.1 g).

Azoxystrobin concentrations for microcosm experiment MC-1 were 5, 250, and 750 ng/L, whereas MC-2 and -3 had an additional treatment of 5000 ng/L. MC-3 also included additional treatments at 0.5 and 5 mg/L using the TEG-vehicle stock solution. The ACE vehicle control was 8.3 µl/L and the TEG vehicle control was 10 ml/L. Doses for the rearing and experimental containers were made in batches to avoid within-treatment variability.

For the *in vitro* experiments, azoxystrobin was dissolved in TEG in a stock solution of 5000 mg/L. The maximum concentration, 250 mg/L, was chosen based on previous fungal assessments of azoxystrobin (Dijksterhuis et al., 2011). Treatments in Experiment 1 were 0.005, 0.5, 25, and 250 mg/L azoxystrobin, 50 ml/L TEG (vehicle control), and control (CON). Experiment 2 treatments were 0.005, 0.05, 0.01, and 5 mg/L azoxystrobin, 10 ml/L TEG, and CON. To test for differences in fungal growth between ACE and TEG, an experiment was conducted where the fungus was grown in 20 µl/L ACE, which is the maximum concentration with no effect on invertebrates (Hutchinson et al., 2006), as well as 10 ml/L and 50 ml/L TEG.

Maintenance and Growth of Black Fly Larvae

The experimental design and setup for the microcosm experiment was adapted from McCreadie and Beard (2003). An image of the rearing and experimental units is shown in Fig D.2. Eggs of the black fly *Simulium vittatum* IS-7 were obtained from the University of Georgia colony, which is free of trichomycetes, nematodes, microsporidia, and other symbionts (Adler et al., 2004). The eggs were placed in 1 L glass rearing units, each filled with 600 mL of fungicide-dosed water, and allowed to grow for 21 days in order to be large enough to handle and dissect. The fungicide-dosed water was changed every two days to maintain optimal conditions for the larvae and to ensure that the concentration of azoxystrobin was not decreasing due to photodegradation (Adetutu et al., 2008). Black fly larvae were fed daily a mixture of ground TetraFin goldfish flakes (Tetra Holding, Virginia USA) and certified organic rabbit food (Oxbow Enterprises, Nebraska USA) similar to the protocol of Overmeyer et al. (2003). The goldfish flakes tested negative for pesticide presence and the rabbit food was assumed to not have any

fungicides because of its certified organic label. The food was blended at a ratio of 0.5 g each of rabbit and fish food into 500 mL distilled water, and strained through a methanol-cleaned 60 μm metal sieve. Each rearing unit received 6 mL of food slurry, whereas the experimental units received 3 mL of food daily.

An incubator (Fisher Scientific model 3724, Ohio USA) fitted with fluorescent light fixtures (programmed for a 16/8-hour light/dark cycle) was used to hold the microcosm rearing and experimental units. For each fungicide dose, there was one rearing unit and at least 3 experimental units, unless otherwise noted. Each unit was fitted with an aquarium air-stone attached via silicone tubing, and held in place by stiff polyethylene plastic tubing inserted through the top of the unit's lid (Fig D.2). The silicone tubing was attached to a manifold unit, which was supplied with compressed air regulated to 3–5 psi, and pumped through an air filter (Campbell Hausfeld, Ohio USA).

Maintenance of Fungal Culture for Microcosm

The gut fungus *Smittium simulii* (isolate JAP-51-5) was selected for the microcosm and *in vitro* studies based on similarity to the morphospecies observed in black fly larvae in our field study and prior knowledge of the widespread occurrence of this fungus in black fly larvae (Lichtwardt, 1986). The isolation technique was adapted from Horn (1989) and McCreadie and Beard (2003), and from personal communication with Beard and Horn. To isolate spores for the microcosm, cultures of *S. simulii* were grown on 100 mm petri dishes of 1/10 Brain Heart Infusion (BHI), a medium known to produce prolific sporulation (Lichtwardt, 1986), for 10 days. On days 4 and 9, 3 mL of sterile distilled water was added as an overlay. At the end of day 10, the water overlay was filtered through glass roving (Pyrex, Corning, New York USA) into a media bottle.

The filtrate slurry was distributed into as many 50 mL conical tubes as there were treatments (5 tubes for MC-1, 6 for MC-2, and 7 for MC-3) with a maximum volume of 35 mL. The slurry was centrifuged (Sorvall Centrifuge, Thermo Fisher Scientific, North Carolina USA) at 950 G for two minutes, decanted, filled with distilled water to 20 mL to resuspend the spores, centrifuged again, decanted, and filled to 10 mL with distilled water. The spores were then enumerated using a C-Chip Neubauer Improved disposable hemocytometer (INCYTO, Korea) wherein the number of refractive, non-extruded (both of which signified they were viable, Fig C.1) spores in each grid were counted three times and averaged. This was repeated twice for each treatment to estimate the number of spores per milliliter. A volume needed to inoculate each experimental unit with the same concentration of spores was then calculated (see Appendix C for spore enumeration and dosage calculations, Table C.1.). Once the spore dosage was calculated, each conical tube received an azoxystrobin or vehicle concentration that corresponded to each treatment in the microcosm.

Microcosm Experimental Protocol

The same protocol was used for all microcosms: three replicates of each fungicide treatment including a control and vehicle control, plus an additional set of replicates (2 to 3 units) to compare the metrics of black fly larvae with and without spore inoculum added. In all, MC-1 had 15 experimental units with spore inoculum (+spore) and 15 without spore inoculum (−spore), MC-2 had 18 +spore and 12 –spore, and MC-3 had 20 +spore and 12 –spore treatments. Due to spatial constraints in the incubator, only two replicates per treatment of the −spore were completed in MC-2 and -3, and the TEG treatment in MC-3 did not have any –spore treatments.

On day 21, black fly larvae from the rearing units were randomly separated into the experimental units. Twenty larvae in MC-1 and 15 larvae in MC-2 and -3 were added to each experimental unit, which consisted of 500 ml glass jars filled with 300 mL of fungicide-dosed water. The larvae were allowed to acclimate for 24 hours prior to spore inoculation. The spores were added at each water change at the same concentration for all experimental units: 1200 spores/mL in MC-1, 500 spores/ml in MC-2 and -3. After exposure to spores for a 6-day period, all larvae from the experimental units were removed and placed in 60 mm petri dishes for 24 hours to allow for evacuation of the food bolus from the hindgut prior to dissection.

At the end of each experiment, all black fly larvae were dissected within 24 hours to ensure consistency. Only the black fly hindguts were extracted, following the methods of McCreadie and Beard (2003) because *S. simulii* does not naturally grow in the PM (Lichtwardt, 1986). The wet-mounted hindgut was placed on a glass slide and imaged at 100x before fixing and staining with infiltration of lactophenol cotton blue under the coverslip (Fig. D.3). Upon dissection, head capsules were preserved in 100% ethanol. After preservation in 100% ethanol, the head capsules were placed in 10% KOH for at least 12 hours for clearing. They were then placed in a drop of glycerin on a depression slide. The distance between the antennal buttresses (Fig. D.4) was measured to estimate larval fitness (McCreadie et al., 2005; McCreadie and Colbo, 1990). Some head capsules were damaged during dissection, resulting in an unequal sample size.

Prior to enumeration of the hindgut fungi, a composite image of the hindgut was made in Adobe Photoshop (CS5 version 12.0) and a 1 mm^2 digital grid overlay was added to the image. Using the counting tool in Photoshop, the number of grids containing

the gut area was recorded. Only sections taking up more than half of the grid were included to avoid confusion when counting. Relative abundance of hyphae was calculated according to McCreadie and Beard (2003) by determining the ratio of grids containing thalli to the total number of grids occupied by the gut (Fig. D.3).

Microcosm Quality Control for Fungicide Concentrations

For each microcosm, two samples each of "day 0", freshly prepared solutions, and "day 2" water, in which the larvae had been residing for two days, were analyzed for azoxystrobin concentrations (Table A.5). Based on the consistency of previous quality control measures, the concentrations for MC-3 were expected to be within range of those demonstrated during the previous experiments, therefore no water samples were taken for the final microcosm experiment. Quality control was the same for day 0 and 2, except day 0 was not filtered since a fresh solution was made that day. These methods were the same as the field analyses conducted by the USGS, but only azoxystrobin was analyzed (see Appendix A, Table A.3).

In vitro Experimental Protocol

Smittium simulii (JAP-51-5) was grown on 100 mm petri dishes of Brain Heart Infusion + Tryptone Glucose and vitamin (BHIGTv) agar media with a sterile water overlay for 5 days prior to the experiment. The culture was then transferred using a sterile loop to six 250 mL Erlenmeyer flasks, each filled with 50 mL BHIGTv liquid media. Next, the culture was grown in an incubated shaker (New Brunswick Excella E24, Edison, New Jersey USA) for 4 days at 200 rpm and 24°C (Williams and Lichtwardt, 1972) (Fig. D.5). The culture was homogenized using a commercial Waring blender set

on high for 20 seconds. One mL of the homogenized inoculum was transferred to new flasks using a sterile transfer pipette. Both trials used the stock solution of 5000 mg/L azoxystrobin with TEG as a carrier. Experiment 1 had a vehicle control of 50 ml/L TEG, and Experiment 2 had a vehicle control of 10 ml/L TEG. Three replicates of each fungicide dose were randomly placed and grown in the same incubated shaker for 4 days at 200 rpm in the dark (to avoid photodegradation). The fungus was then filtered on pre-dried and weighed filter paper (Whatman #1 90 mm) (Fig. D.5) and then dried in an 80°C oven (Lipshaw, Detroit, Michigan USA) for 24 hours, weighed, then dried another 24 hours and re-weighed to confirm actual dry weight. Prior to filtering, slides of the cultures were prepared as vouchers (Lichtwardt, 1986).

For the vehicle comparison, the culture was grown on solid media as above, and homogenized by scraping the culture (moistened with sterile water overlay) into a sterile 50 mL eppendorf tube and vortexing for 1 minute. One mL of this mixture was then transferred to BHIGTv shake cultures, as described above. Dry weights were calculated for this test, but no slide vouchers were prepared.

Statistical Analysis

With minimal replication (only four streams) statistical analyses were not possible for the field study. Therefore, only descriptive statistics are presented. Results of the microcosm experiments were analyzed with a one-way analysis of variance with fungicide dose as the main effect, followed by a Tukey test for means comparison. A Kruskal-Wallis test, the non-parametric equivalent of an ANOVA, was used to analyze the results of the *in vitro* experiments and *in vitro* vehicle comparions. Post-hoc analysis of treatment differences for the *in vitro* experiments was done using a Wilcoxon test.

Because there were only three replicates for each treatment in the *in vitro* experiments, differences could not be detected at an alpha of 0.05. Therefore, to explore the potential for differences between treatments, the alpha value was adjusted to 0.10. Head capsules were compared using a two-way ANOVA with fungicide treatment and inoculation as the predictor variables. Microcosm and *in vitro* analyses were conducted in JMP Pro 10 (SAS Institute Inc., Cary, NC, 1989-2012), vehicle comparisons and head capsule analyses were conducted in R version 2.15.2 (http://cran.r-project.org/).

RESULTS

Field Study

Percent Infestation of Gut Fungi

Black fly larvae were present in sufficient numbers ($n \geq 20$) at both reference sites and for 11 of the 12 sampling events at Sand Run Gulch. By contrast, black fly larvae were absent for 6 of the 12 sampling events at Wanstad Ditch, even though the habitat appeared suitable for black flies (Adler et al., 2004). During 3 of the 6 sampling events in which black fly larvae were present, only 1 to 8 individuals were collected. Percent infestation of black fly larvae with gut fungi was almost always higher in the reference sites than in the agricultural sites (Fig. 2). At the reference sites, Cottonwood Creek had 100% infestation rates across all sampling events (Fig. 2a), and Dry Creek had an average 93% infestation rate (Fig. 2b). At the agricultural sites, Sand Run Gulch had an average infestation rate of 54% (Fig. 2c) and Wanstad Ditch had an average infestation rate of 33% (Fig. 2d).

Mayfly nymphs were present in sufficient numbers ($n \geq 20$) for all 11 sampling events at the reference sites, and 10 out of 12, and 5 of the 12 sampling events at Sand Run Gulch and Wanstad Ditch, respectively. Although the percent infestation rate was lower overall than in black fly larvae, clear differences were still observed between reference and agricultural sites (Fig. 3). In the reference sites, mayfly nymphs had an average percent infestation of 60% at Cottonwood Creek (Fig. 3a) and 18% at Dry Creek

(Fig. 3b). At the agricultural sites, Sand Run Gulch had an average percent infestation of only 0.8% with gut fungi detected on only two sampling events (Fig. 3c). Gut fungi were not detected in any mayflies collected at Wanstad Ditch (Fig. 3d).

Density and Spore Production of Gut Fungi

Fungal density and spore production in larval black fly PMs varied over time in all of the sampled sites. There was a greater thallial density and number of trichospores of *Harpella* spp. per μm^2 of PM in black fly larvae at reference sites compared to the agricultural sites (Fig. 4 and 5). For reference sites, the maximum number of thalli/μm^2 was $2.9x10^{-4}$ and $2.6x10^{-4}$ at Cottonwood Creek and Dry Creek, respectively (Fig. 4a, b). Cottonwood Creek had a maximum number of spores/μm^2 of $3.6x10^{-4}$ whereas Dry Creek had $3.2x10^{-4}$ spores/μm^2 (Fig. 5a, b). At agricultural sites, the maximum number of thalli/μm^2 was $0.8x10^{-4}$ and $0.04x10^{-4}$ at Sand Run Gulch and Wanstad Ditch, respectively (Fig. 4c, d). The maximum number of spores/μm^2 at Sand Run Gulch was only $0.4x10^{-4}$. Wanstad Ditch had even fewer with $0.08x10^{-4}$ spores/μm^2 (Fig. 5c, d).

Pesticides in Surface Water and Black Fly Tissue

At the agricultural sites, 22 pesticides were detected: 10 herbicides, 8 fungicides, 2 insecticides, and 2 degradates. Two of the top three most detected pesticides were fungicides (Table 1). The fungicides azoxystrobin and boscalid were detected in 11 of the 12 (92%) water samples collected from Wanstad Ditch and in 8 of the 12 (67%) sampling events at Sand Run Gulch. At the reference sites, three pesticides were detected: two herbicides and one insecticide, each detected on one sampling occasion (Table 2). No fungicides were detected in any surface water samples collected from the reference sites.

A total of 11 pesticides (4 fungicides and 7 herbicides) were detected in larvae from agricultural sites with detections of 50–88% (Table 3). Composite and estimated individual concentrations of pesticides from black fly larvae from each sampling event are in Appendix Tables B.2 and B.3. At the agricultural sites, the maximum estimated concentration in individual black fly larvae was 270 μg/g-wet weight. All of the pesticides detected in the tissue were also detected in the surface water, with the exception of the fungicide pyraclostrobin (Tables 1 and 3). Imazalil and simazine were only detected once in water (Table 1), but in 88% and 50% of tissue samples, respectively (Table 3). The fungicides azoxystrobin, boscalid and imazalil and the herbicide pendimethalin were the most frequently detected pesticides in tissue (each at 88%) (Table 3). Although not detected in the surface water, pyraclostrobin had the second highest maximum composite concentration (0.84 μg/g) and the highest estimated individual tissue concentration (270 μg/g wet weight) while boscalid had the highest composite concentration of 0.93 μg/g (Table 3). Azoxystrobin was detected in 88% of the tissue samples, with a maximum estimated individual concentration of 222 μg/g (Table 3).

No fungicides were detected in larval tissue from the reference sites, but three herbicides, atrazine, simazine, and trifluralin (Table 3) were detected in the immature aquatic hosts' tissues at concentrations of 22%, 67%, and 67%, respectively. The maximum composite concentration was 0.234 μg/g wet weight of simazine (Table 3).

Laboratory Study

Microcosm Experiment

The relative abundance of *Smittium simulii* hyphae in black fly hindguts varied between microcosms. In MC-1, the concentrations tested were 750, 250, and 5 ng/L of azoxystrobin. There was no significant difference in the main effect in MC-1 ($F_{(4,138)} = 1.58$, $p = 0.1811$). The lowest hyphal abundance was in the 5 ng/L treatment with 37% of grids containing hyphae, whereas the highest abundance was in the 750 ng/L treatment with 53% of grids having hyphae (Fig. 6a). In MC-2, the fungicide concentrations tested were 5000, 750, 250, and 5 ng/L azoxystrobin, and a significant affect was observed ($F_{(5, 98)} = 6.82, p < 0.0001$). The 250 ng/L-exposed larvae had the lowest abundance of hyphae with a mean of 20%, and was significantly different from the 750 ng/L, ACE, and CON (Fig. 6b). The 250 ng/L treatment in MC-2 may have been a anomalous result, as the effect was not observed in any other microcosm. An overall effect of azoxystrobin was still observed when the 250 ng/L treatment was removed from analysis ($F_{4,85} = 4.01$, $p = 0.005$) (Fig. 7). In this alternative analysis, 5000 ng/L (mean = 32%) and ACE (mean = 51%) were significantly different, but no other treatments differed significantly from each other. MC-3 tested the same fungicide concentrations as MC-2, but no significant differences were observed for any of the treatments ($F_{(6, 161)} = 1.27, p = 0.2699$). The 250 ng/L treatment and CON both had the lowest abundance of hyphae (40%), while the highest abundance of hyphae was in the TEG treatment (57%) (Fig. 6c).

Survivorship (i.e., the percent of black fly larvae alive at the end of the experimental week) was relatively stable throughout the course of the experiment. MC-1 had 6 replicates per treatment and 60–93% of black fly larvae survived (Fig. 8a). In MC-

2, survivorship was 55–100% (Fig. 8b), and in MC-3 it was from 0–100% (Fig. 8c). In MC-3, the treatments that had no survivors included the highest concentration of 5000 ng/L and the TEG (vehicle) treatment. At the beginning of MC-3, concentrations of 0.5 and 5 mg/L azoxystrobin also were applied to the rearing containers, but within 24 hours post-hatching, there was 100% mortality of the black fly larvae.

In vitro Experiment

The dry weight of *Smittium simulii* was significantly affected by azoxystrobin in both *in vitro* experiments (Experiment 1: $p = 0.0131$, $H = 14.4$, $df = 5$; Experiment 2: $p = 0.0408$, $H = 11.6$, $df = 5$). In Experiment 1, a significant decrease in dry weight was observed when the concentration of azoxystrobin was increased from 0.005 to 0.05 mg/L ($p = 0.0809$), and again between 0.05 to 25 mg/L ($p = 0.0809$) (Fig. 9). Fungi exposed to 25 and 250 mg/L had the lowest dry weight (Fig. 9). In Experiment 2, concentrations of azoxystrobin varied between 0.005 and 5 mg/L. The only significant difference in dry weight among treatments occurred at a concentration of 5 mg/L ($p = 0.0809$) (Fig. 9). With $\alpha = 0.10$, a significant difference was found between ACE and TEG ($p = 0.0992$, $H = 4.62$, $df = 2$). Exposure to the 2.5 ml/L TEG vehicle yielded significantly lower biomass than the ACE ($p = 0.081$) (Table 5).

Black Fly Head Capsules

Head capsule widths of black fly larvae varied across treatments and microcosms (Fig. 10 and Table 4). When comparing +spore and -spore treatments within microcosms, significant differences were observed in MC-1 with the ACE treatments and in MC-3 with the CON treatments. In MC-1 ACE, the head capsules were an average of 88 μm

wider when larvae were inoculated. However, in MC-3 CON, the head capsules were an average of 99.4 μm narrower when inoculated (Fig. 10 and Table 4). For all other microcosms, no statistically significant differences were observed between inoculations treatments within fungicide doses.

There was no clear trend between fungicide concentration and head capsule size (Fig. 10). On average, in MC-1 the smallest head capsules were observed in the ACE –spores (369.2 ± 14.7 μm) treatment. This value was significantly smaller than those in the 5ng/L and CON (both with and without spore inoculation) and the ACE +spores (Table 4). The widest head capsules in MC-1 were observed in CON +spores (509.5 ± 14.7 μm). In MC-2, significant differences were observed between the narrowest head capsules, CON –spores (432.9 ± 17.2 μm) treatment, and the widest head capsules, 250 ng/L +spores (506.8 ± 16.7 μm) treatment; however, these two treatments were not significantly different from any other treatment in MC-2 (Fig. 10, Table 4). In MC-3, the smallest head capsule was observed in the 250 ng/L –spores (282.6 ± 17.5 μm; n = 8) treatment, which was significantly different from 5000 ng/L, ACE, CON, and TEG (both with and without spore inoculation) (Table 4). The widest head capsule in MC-3 was observed in the CON –spores (488.9 ± 48.2 μm; n = 7) treatment, which was significantly different from CON +spores, TEG, ACE +spores, 5 ng/L, 250 ng/L, and 750 ng/L (both with and without spore inoculation), and 5000 ng/L +spores (Table 4).

DISCUSSION

Pesticides are frequently detected in surface water and especially in streams near agricultural land (Gilliom, 2007; Kolpin et al., 2002; Reilly et al., 2012; Smalling and Orlando, 2011). With the emergence of new pathogens, the amount and number of pesticides used (and detected) is expected to increase in coming years (Battaglin et al., 2011). Numerous studies have shown effects of pesticides on non-target organisms (Blaustein et al., 2003; Brereton et al., 1999; Desneux et al., 2007; Gustafsson et al., 2010; Hurd et al., 1996; Norgaard and Cedergreen, 2010; Pape-Lindstrom and Lydy, 1997). However, comparatively few studies have examined the effects of fungicides on non-target aquatic fungi. When they have, these studies have mainly focused on free-living hyphomycetes, as a dominant player in stream ecosystems, which were negatively effected by a range of fungicides, including azoxystrobin (Bundschuh et al., 2011; Cuppen et al., 2000; Dijksterhuis et al., 2011; Gustafsson et al., 2010; Maltby et al., 2009; Rasmussen et al., 2012; Zubrod et al., 2011).

The novelty of the present study is that it is the first assessment of pesticide effects on insect-associated gut fungi using field and laboratory techniques, with the aim of providing a better understanding of pesticide impacts across trophic levels in aquatic systems. Although no statistical inferences could be made in the field study, clear patterns were observed. Lower fungal infestation, density, spore production, and hosts were detected in streams with fungicides present than without.

Field Study

Pesticides have been detected in up to 97% of surface water near agricultural land, and in most cases at least two pesticides are present per sample (Gilliom, 2007). Over the eight months of the present field study, 22 pesticides were detected in agricultural streams, and on two occasions 10 or more pesticides were detected (Table 1). At these sites, fungicides were detected in nearly 80% of the samples, undoubtedly corresponding to repeated field application throughout the growing season (Reilly et al., 2012). Given this high frequency of detection, organisms living in these waterways are potentially receiving a chronic exposure to pesticides.

The metrics used for assessing effects on insect hosts in the field were abundance of both black fly larvae and mayfly nymphs, and an estimation of body burden of select pesticides in black fly larvae. Potential habitats for black flies are varied, and include impacted areas such as irrigation channels and drainage ditches (Crosskey, 1990) similar to those sampled at the agricultural sites. However, agricultural streams are often associated with decreased black fly abundance and diversity (Pramual and Kuvangkadilok, 2009). Black fly larvae were absent from approximately half of the sampling events at Wanstad Ditch the agricultural site. The reduced number of black fly hosts may have been due to the presence of contaminants, life history, or environmental conditions (Beard et al., 2003). Less than half of the samples from Wanstad Ditch had a sufficient number ($n \geq 20$) of mayfly nymphs, suggesting that the system may have been stressed (Allan and Castillo, 2007; Chessman and McEvoy, 1998; Savic et al., 2011; Schulz and Dabrowski, 2001).

The percent infestation of host insects also showed that there may be an impact in the system. Nearly all black fly larvae from reference sites were 100% infested with fungus compared to only 33-50% at agricultural sites. These lower rates are not unlike those found by others when surveying black fly larvae for gut fungi (Beard et al., 2003; Nelder et al., 2006). However, the actual density of fungi in the gut is only rarely taken into account in other studies. For example, a population of insects may be 100% colonized with gut fungi (see August 2010 for black fly larvae at Sand Run Gulch, Fig. 2c), but each larva may only have a few thalli per gut, compared to dozens or up to hundreds per gut in reference streams. The colonization rates of certain gut fungi can differ amongst species of black fly larvae, although PM-dwelling *Harpella* spp. typically have the highest colonization rates regardless of host species (Beard and Adler, 2002; Lichtwardt and Williams, 1988). In the present study, larval *Simullium* spp. were collected (see Table D.1 for species identifications), but it was assumed that all larvae had an equal probability of being colonized by *Harpella* spp.

The density and spore production in black fly PMs was much higher in the reference sites compared to those in agricultural sites. However, there was a great deal of variation in both number of thalli and spores over time. Seasonality has been documented in several other studies on gut fungi where fungal infestation and species composition can change over time and across environmental gradients (Beard and Adler, 2002; Beard et al., 2003; Bench, 2009; Nelder et al., 2006). Until the present study, the influence of chronic non-point source pesticide exposure had not been explored. Future investigations that model season, environment, host species, and other factors (including pesticide

levels) would offer a window onto the forces driving fungal colonization rates and densities observed in this symbiotic system.

The pathway of exposure of the fungicides and/or other pesticides for the PM-dwelling fungi inside the black fly larvae is unknown. One exposure pathway could be the food bolus, which contains potentially pesticide-accumulated periphyton and biofilm (Kreutzweiser et al., 1995; Montuelle et al., 2010; Sweeney et al., 1993; Tlili et al., 2011). Alternatively, the gut fungus could be exposed directly from the host as it accumulates pesticides from the water column.

The peritrophic matrix (PM) that lines the midgut also could play a role in the exposure of gut fungi to pesticides. Hegedus et al. (2009) detailed the structure and function of the PM and presents it as a system comprised of the ectoperitrophic space, located between the PM and the epithelial cells of the midgut, the PM itself, and the endoperitrophic space where the gut lumen is presented and food bolus moves through the digestive tract. The PM functions as a selectively permeable molecular sieve, moving nutrients from digested food out to the ectoperitrophic space (Hegedus et al., 2009). It was speculated from a study on mosquito larvae (also lower Diptera) that the PM may sequester and possibly detoxify contaminants, including pesticides (Hegedus et al. 2009). Although this detoxification may take place, and assuming the endoperitrophic space is devoid of the pesticides, *Harpella* spp. and other PM-dwelling gut fungi attach to the PM via a holdfast. *Harpella* holdfasts do not penetrate the PM, as other midgut-dwelling gut fungi can (e.g., *Stachylina penetralis* (Lichtwardt, 1986)). Instead, the holdfasts of *Harpella* form a series of secretory pores or “digits” that are surrounded by adhesive glue (Reichle and Lichtwardt, 1972). The function of the holdfast as a point of attachment

versus an active zone (and its sensitivity to any chemicals) is an avenue for future research. The same can be said of the filamentous gut fungi exposed to the passage of material through the gut itself while resident in their hosts.

While most studies involving mayfly-associated gut fungi focus on their discovery and taxonomy (Bench and White, 2012; Kandel and White, 2012; Lichtwardt and Williams, 1992; Strongman, 2007; Strongman and White, 2008, 2011; Valle et al., 2011; White and Lichtwardt, 2004; White et al., 2006; Williams and Lichtwardt, 1999), only four studies report the percent infestation in mayflies (Lichtwardt and Williams, 1988; Valle and Santamaria, 2002a, b; White, 2003). In Baetidae nymphs, the gut fungi *Baetimyces ancorae* and *Legeriomyces ramosus* was detected in 70% and 20% of these mayflies, respectively (Valle and Santamaria, 2002a). In Leptophlebiidae nymphs, *Tectimyces* spp. ranged from 5-80% infestation (Valle and Santamaria, 2002b). In Caenidae nymphs, gut fungi were found in eight of eleven insects sampled (White, 2003). Lichtwardt and Williams (1988) surveyed over 400 mayfly nymphs that were between 1.4–49% infested. The percent infestation observed in mayflies from reference sites falls within the wide ranges that are possible, but the near absence of gut fungi in the agricultural sites in this study is in striking contrast to what has been previously documented. As mayflies are ecological indicators (Allan and Castillo, 2007; Chessman and McEvoy, 1998; Corkum et al., 1995; Savic et al., 2011), the percent infestation of gut fungi observed in field collections should be more frequently included.

No fungicides were detected in the surface water or black fly tissue from reference sites, although three herbicides were detected in black fly tissue. Herbicides are used in many non-agricultural settings and, like other pesticides, can be non-point source

pollutants (Kegley et al., 2011; Relyea and Hoverman, 2006). Atrazine is one of the most commonly applied and detected pesticides in the country (Gilliom, 2007; Thurman and Cromwell, 2000). It has even been found in pristine areas through aerial deposition (Thurman and Cromwell, 2000). Atrazine was detected once in surface water and twice in larval tissue from Dry Creek. Simazine had the highest estimated individual tissue concentration in reference sites (131 ng/g wet weight, Table 3). It was one of the most frequently detected urban-herbicides in a national assessment (Gilliom, 2007), and was detected once in surface water from the reference site Dry Creek in the present study. Trifluralin is another widespread herbicide and was in the top 10 most detected agricultural-herbicides nationally (Gilliom, 2007), but not found in surface water at either reference site. Trifluralin was detected in 67% of the black fly tissue samples but had relatively low composite concentrations within tissues.

Eleven pesticides were detected in the tissue analysis from agricultural sites. All of these, except for pyraclastrobin, were also detected in the surface water. Pyraclostrobin is a strobilurin fungicide (the same class as azoxystrobin), and regularly binds to sediment (Bartlett et al., 2002; Battaglin et al., 2011). Although pyraclostrobin was not detected in surface water samples taken in 2010, it was detected frequently in 2009 at the same sites (Table A.5). Therefore, there may have been some persistence from 2009 or an undocumented flush of the fungicide in 2010 that was missed during sampling episodes.

The magnitude of pesticide accumulation in individual larval tissue is striking, even when using approximated values (see Appendix B). The composite concentrations in larval black fly tissue are similar to measurements from other organisms (Dugan et al., 2005; Sapozhnikova et al., 2004; Smalling et al., 2010). However, the estimated

individual concentration in each black fly larva was substantially higher, at least one order of magnitude higher in the tissues than in the surface water samples. The maximum estimated concentration of azoxystrobin in individual black fly larva tissue from the agricultural sites was 222 μg/g, which is approximately three orders of magnitude higher than the maximum concentration of azoxystrobin in the surface water (40 ng/l) (Table 1).

At agricultural sites, black fly larvae were often collected at the stream surface or edge on dangling vegetation and rarely from the benthic region of the stream. These larvae are generally filter feeders that ingest sloughed biofilm and periphyton, but occasionally can be grazers (Adler et al., 2004; Allan and Castillo, 2007). Since periphyton and biofilm accumulate pesticides (Kreutzweiser et al., 1995; Sweeney et al., 1993; Tlili et al., 2011), indirect exposure from the ingestion of pesticide-contaminated food may be a pathway of exposure.

The occurrence and numbers of mayfly nymphs were too low to pursue tissue analysis. However, mayflies may also be exposed to pesticides as many of them reside in the benthos where particle bound-pesticides can accumulate (Battaglin et al., 2011; De Haas et al., 2005). Sweeney et al. (1993) documented accumulation of the insecticide chlordane in the mayfly *Cloeon triangulifer*, and suggested that it could be used as a test organism for toxicity screening. It would be of value to determine if field-sampled mayflies also have high concentrations of pesticides in their tissue. Since mayflies are critical for stream functioning (Allan and Castillo, 2007), the accumulation of pesticides in their tissue could impact other aquatic biota, possibly through accumulation up trophic levels.

It is difficult to place these data into context as, to our knowledge, there are no field studies documenting fungicide detection in aquatic insect tissue. Several laboratory studies have documented the effects of organophosphate insecticides (chlorpyrifos and diazinon) on aquatic insects (Buchwalter et al., 2004; Stuijfzand et al., 2000). Stuijfzand et al. (2000) suggested that pesticide impacts on larval insects are related to the timing of pesticide occurrence in the stream and the life stage of the organism. Other field studies have documented current-use pesticides in other aquatic tissues including crab embryos (Smalling et al., 2010) and sand crabs (Anderson et al., 2010; Dugan et al., 2005) as well as fish (Anderson et al., 2010; Sapozhnikova et al., 2004).

Laboratory Study

In the microcosm experiments, no significant difference in fungal abundance was observed in MC-1 or -3. There was a less than 20% difference between maximum and minimum fungal abundances in MC-1 and -3, and the lowest abundance was never observed in the highest fungicide treatments. In MC-2, there was a significant difference in treatments and more than a 30% range of abundances (Fig. 6).

In the *in vitro* experiments, a significant decrease in fungal dry weight was observed at 0.5 mg/L azoxystrobin. This is two orders of magnitude greater than the highest concentration tested in the microcosm experiment (5000 ng/L), and three orders of magnitude higher than what was detected in the field. Dijksterhuis et al. (2011) exposed *Mucor hiemalis*, which belongs within the same traditional phylum of the gut fungi (Zygomycota), to azoxystrobin and the NOEC (lowest test concentration where no or slight effects were observed (Maltby et al., 2009)) and EC_{100} (concentration with the maximal effect (Maltby et al., 2009)) values were recorded. The NOEC when grown on

minimal media (MM) was 0.23 mg/L, and 0.014 mg/L when grown in malt extract broth (MEB). The EC_{100} values were 15 mg/L and 235 mg/L when grown on MM and MEB, respectively. The medium for the gut fungus *Smittium simulii* was BHIGTv, which is very nutrient rich (Lichtwardt, 1986). If a maximal effect was observed in *Mucor* at a lower concentrations of azoxystrobin on MM, perhaps *Smittium simulii* would be more sensitive when grown on a different medium. Given this possibility, future experiments could test the responses of gut fungi using varying media and fungicides.

The two concentrations that showed an impact on the fungus *in vitro* (0.5 and 5 mg/L) were also included in MC-3, but all of the black fly larvae for these treatments died within 24 hours of exposure. This was likely due to acute azoxystrobin toxicity (EC_{100}), since the vehicle control (10 ml/L TEG) was not as affected, although the larvae did not survive as well as other treatments. Warming et al. (2009) found similar acute toxicity levels within 48 hours for *Daphnia* exposed to azoxystrobin using an acetone vehicle (1ml/L). Although the acute LC_{50}-value for *Daphnia manga* is 190 µg/L, even lower concentrations of azoxystrobin can also be acutely toxic to macroinvertebrates (Gustafsson et al., 2010).

Head capsule size of larval black flies can be an indirect measurement of their fitness (McCreadie et al., 2005). Differences in the width between antennal butresses were observed between inoculated and non-inoculated larvae within fungicide treatments in microcosms. This contradicts a study by McCreadie et al. (2005) where no difference in head capsule width was observed in black fly larvae inoculated with various spore doses. In the present study, MC-1 head capsules were wider in ACE treatments that were inoculated with fungi, but were narrower in the +spore CON treatment. It should be noted

that the delicate larvae were susceptible to damage when dissecting, therefore sample sizes differ between many of the treatments in the head capsule analysis because they could not be measured reliably (see Table 4 for sample sizes). This especially could be a factor in MC-3 where more than half of the samples had less than 15 measurable head capsules. These considerations aside, there was no clear relationship between head capsule size and azoxystrobin concentrations.

The level of spore inoculation did not seem to present any differences in the overall result between the microcosms. Spore dose was reduced for the final two microcosms, down from 1200 spores/mL in MC-1 to 500 spores/mL in MC-2 and -3. In other studies where black fly larvae were inoculated with gut fungi, 4000 spores/mL or more have been used (McCreadie and Beard, 2003; McCreadie et al., 2005; Nelder et al., 2005; Vojvodic and McCreadie, 2008, 2009). From our own preliminary laboratory experiments prior to conducting these microcosms, it was determined that a spore dosage that high always resulted in nearly 100% abundance (i.e., all grids contained thalli) for all treatments. Therefore, a lower spore load was chosen to avoid occluding the gut and potentially confounding interpretations of the effects of the fungicide. The difference between this study and others also could stem from the variability among *Smittium* species used. Others (McCreadie and Beard, 2003; McCreadie et al., 2005; Nelder et al., 2005; Vojvodic and McCreadie, 2008, 2009) have used *S. culisetae*, which is more frequently found in mosquito and midge larvae than in black fly larvae. The present study used *S. simulii*, which is often found in black fly larvae, and possibly reflects a higher success rate in establishing in the gut. Future studies should consider the fungal taxa used and the host insect that is naturally occurring in the field.

The role and responsiveness of symbiotic gut fungi within their arthropod hosts is not completely understood. They have been considered commensalistic, but can also be mutualistic under times of stress (Lichtwardt, 1986). Studies in many mammals are showing the positive impact that gut microflora can have at the whole organism level (Eckburg et al., 2005; Hooper and Gordon, 2001). This could also be the case of gut fungi, but on a smaller scale. If fungicides in streams are impacting potentially mutualistic fungi they may not be available to respond to the needs of their host in times of stress (i.e., as with exposure to other pesticides and/or toxicants). As mayflies are generally considered ecological indicators of stream health, their decreased abundance observed in this study may be a result of the impact on gut fungi. Few studies have investigated the prevalence of gut fungi in mayflies (Lichtwardt and Williams, 1988; Valle and Santamaria, 2002a, b; White, 2003), yet these hosts are established stream health indicators (Allan and Castillo, 2007; Chessman and McEvoy, 1998; Corkum et al., 1995; Savic et al., 2011). Indeed, gut fungi occur in other aquatic bioindicators, including both Plecoptera and Trichoptera (Lichtwardt, 1986), but to date there have not been any determination as to how their sensitivity may be related to their gut symbionts and how that might be translated through trophic cascades.

Pesticides may be more toxic when acting in mixtures than when applied singularly (Blaustein et al., 2003; DeLorenzo et al., 2001; Norgaard and Cedergreen, 2010; Pape-Lindstrom and Lydy, 1997). In the present laboratory study, a single-fungicide assessment was done with azoxystrobin. When these data are compared to the *in vitro* experiment, it is perhaps not surprising that little effect was seen. No consistent effect was observed when applied to the symbiotic system of black fly larvae and fungus.

However, a significant decrease in biomass in the *in vitro* exposure occurred at 0.5 mg/L. This fungicide concentration is approximately three orders of magnitude higher than what was observed in the field. Future studies should test fungicide mixtures, and combinations of other pesticides to see if an effect can be seen. More research will be needed to distinguish between the significance of natural environmental conditions and the presence of dissolved pesticides as possible stressors affecting the percent infestation of gut fungi in impacted habitats.

SUMMARY

This study was the first assessment of how pesticides may be affecting a novel symbiotic system between gut fungi and their aquatic insect hosts. In agricultural streams, 22 pesticides were detected and often occurred in mixtures. Aquatic insects in those habitats had substantially fewer gut fungi than those in reference sites, and had high concentrations of pesticides in their tissue. When attempts were made to replicate these observations in the lab, no clear effect was observed in a single fungicide test until concentrations were above those considered to be field relevant. Therefore, the impacts observed in the field were likely due to synergistic effects of pesticides, possibly also interacting with the environmental conditions. Future studies should continue to decipher the mechanisms driving the decreased prevalence of gut fungi in agriculturally impacted streams, with a particular focus on hosts that are beneficial to stream health.

Table 1. Summary of pesticides detected, pesticide type, detection frequency, median and maximum concentrations from agricultural surface water samples (F, fungicide, H, herbicide, I, insecticide, D, degradate).

		Total (n = 24) Frequency (%)	Sand Run Gulch (n = 12)		
Pesticide	**Type**		**Frequency (%)**	**Median (ng/l)**	**Maximum (ng/l)**
Metolachlor	H	95	92	21.6	170.5
Azoxystrobin	F	79	67	4.4	20.2
Boscalid	F	79	67	10.5	37.8
Atrazine	H	50	50	6.6	28.1
Pendimethalin	H	33	33	36.6	45.9
Trifluralin	H	33	17	1.6	2.0
Ethalfluralin	H	29	25	5.9	9.0
Hexazinone	H	25	33	116.9	770.6
s-ethyl dipropylthiocarbamate (EPTC)	H	21	25	22.9	23.3
Simazine	H	13	8	na	10.6
Imazalil	F	8	8	na	205.2
Triflumizole	F	8	8	na	149.6
Alachlor	H	4	nd	nd	nd
3,5-dichloroaniline (DCA)	D	4	nd	nd	nd
Chlorothalonil	F	4	nd	nd	nd
Chlorpyrifos	I	4	nd	nd	nd
Clomazone	H	4	nd	nd	nd
Diazinon	I	4	nd	nd	nd
p,*p*'-DDD	D	4	nd	nd	nd
Propiconazole	F	4	8	na	4.8
Pyrimethanil	F	4	8	na	5.2
Tetraconazole	F	4	8	na	4.8
Pyraclostrobin	F	nd	nd	nd	nd

na: median not calculated for compounds detected only once

nd: not detected

Table 1 (cont.). Summary of pesticides detected, pesticide type, detection frequency, median and maximum concentrations from agricultural surface water samples (F, fungicide, H, herbicide, I, insecticide, D, degradate).

		Wanstad Ditch (n = 12)		
Pesticide	**Type**	**Frequency (%)**	**Median (ng/l)**	**Maximum (ng/l)**
Metolachlor	H	100	77.7	565.7
Azoxystrobin	F	92	3.6	40.4
Boscalid	F	92	11.7	36.6
Atrazine	H	42	4.6	15.2
Pendimethalin	H	33	49.3	154.0
Trifluralin	H	50	4.5	40.9
Ethalfluralin	H	33	9.6	14.4
Hexazinone	H	17	56.5	66.9
s-ethyl dipropylthiocarbamate (EPTC)	H	17	23.1	25.2
Simazine	H	17	14.8	15.6
Imazalil	F	8	na	176.0
Triflumizole	F	8	na	68.8
Alachlor	H	8	na	14.1
3,5-dichloroaniline (DCA)	D	8	na	8.8
Chlorothalonil	F	8	na	3.6
Chlorpyrifos	I	8	na	5.0
Clomazone	H	8	na	35.8
Diazinon	I	8	na	10.6
p,*p*'-DDD	D	8	na	17.0
Propiconazole	F	nd	nd	nd
Pyrimethanil	F	nd	nd	nd
Tetraconazole	F	nd	nd	nd
Pyraclostrobin	F	nd	nd	nd

na: median not calculated for compounds detected only once

nd: not detected

Table 2. Summary of pesticides detected, pesticide type, detection frequency, median and maximum concentrations from reference site surface water samples (H, herbicide, I, insecticide).

		Total (n = 21)	Cottonwood Creek (n = 11)			Dry Creek (n = 10)		
Pesticide	**Type**	**Frequency (%)**	**Frequency (%)**	**Median (ng/l)**	**Maximum (ng/l)**	**Frequency (%)**	**Median (ng/l)**	**Maximum (ng/l)**
Atrazine	H	5	nd	nd	nd	10	na	21.4
Simazine	H	5	nd	nd	nd	10	na	142.2
Fipronil	I	5	9	na	14.4	nd	nd	nd

na: median not calculated for compounds detected only once

nd: not detected

Table 3. Detection frequency (%) and maximum concentrations (µg/g wet weight) of pesticides detected in black fly samples composited from the agricultural and reference sites. For a description of the estimated pesticide concentration calculations see Appendix B.

	Reference Sites			Agricultural Sites		
	Detection frequency	Maximum composite concentration	Maximum estimated individual concentration	Detection frequency	Maximum composite concentration	Maximum estimated individual concentration
Fungicides						
Azoxystrobin	nd	nd	nd	88	0.42	222
Boscalid	nd	nd	nd	88	0.93	100
Imazalil	nd	nd	nd	88	0.37	188
Pyraclostrobin	nd	nd	nd	63	0.84	270
Herbicides						
Atrazine	22	0.18	99.4	50	0.25	90.9
Ethalfluralin	nd	nd	nd	50	0.07	17.5
Hexazinone	nd	nd	nd	50	0.10	62.0
Metolachlor	nd	nd	nd	63	0.44	181
Pendimethalin	nd	nd	nd	88	0.25	131
Simazine	67	0.23	131	50	0.23	51.3
Trifluralin	67	0.03	36.5	63	0.04	21.7

nd: not detected

Table 4. Means comparisons for black fly head capsule width between treatments and spore inoculations in microcosms. N represents the number of larvae measured. See Figure 10.

Treatment	Inoculated (+/-)	N	Mean	Standard Error (±)	Means Comparison Letters
			MC-1		
CON	+	25	509.5	± 14.7	a
CON	-	29	504.2	± 14.7	a
ACE	+	23	457.7	± 20.5	ab
ACE	-	20	369.2	± 14.7	c
5 ng/l	+	26	476.9	± 17.4	ab
5 ng/l	-	25	459.5	± 13.4	ab
250 ng/l	+	15	411.9	± 20.3	bc
250 ng/l	-	25	438.1	± 20.7	abc
750 ng/l	+	18	433.0	± 17.5	abc
750 ng/l	-	15	437.8	± 20.3	abc
			MC-2		
CON	+	24	465.2	± 14.7	ab
CON	-	23	432.9	± 17.2	b
ACE	+	24	505.2	± 10.5	a
ACE	-	17	477.0	± 20.0	ab
5 ng/l	+	21	478.0	± 14.5	ab
5 ng/l	-	22	467.5	± 17.2	ab
250 ng/l	+	27	506.8	± 16.7	a
250 ng/l	-	17	497.1	± 19.5	ab
750 ng/l	+	22	475.7	± 13.4	ab
750 ng/l	-	23	474.3	± 14.3	ab
5000 ng/l	+	31	491.0	± 8.5	ab
5000 ng/l	-	17	495.7	± 12.7	ab

Table 4 (cont.). Means comparisons for black fly head capsule width between treatments and spore inoculations in microcosms. N represents the number of larvae measured. See Figure 10.

Treatment	Inoculated (+/-)	N	Mean	Standard Error (±)	Means Comparison Letters
			MC-3		
CON	+	23	389.5	± 11.0	bcde
CON	-	7	488.9	± 48.2	a
ACE	+	21	369.5	± 16.2	cde
ACE	-	14	403.5	± 11.6	abc
5 ng/l	+	20	339.2	± 12.4	cdef
5 ng/l	-	11	306.2	± 16.8	ef
250 ng/l	+	19	362.5	± 13.0	cdef
250 ng/l	-	8	282.6	± 17.5	f
750 ng/l	+	17	352.2	± 18.8	cdef
750 ng/l	-	10	315.4	± 16.7	def
5000 ng/l	+	17	388.6	± 16.1	bcde
5000 ng/l	-	7	460.6	± 18.2	ab
TEG	+	13	393.4	± 16.7	bcd

Table 5. Dry weights of *Smittium simulii* grown in acetone (ACE) and triethylene glycol (TEG). N = 3 for all treatments.

Treatment	Vehicle Concentration	Mean Dry Weight (g)	Standard Error (±)
ACE	20 µl/l	0.142	0.002
TEG-1	0.5 ml/l	0.136	0.002
TEG-2	2.5 ml/l	0.134	0.001

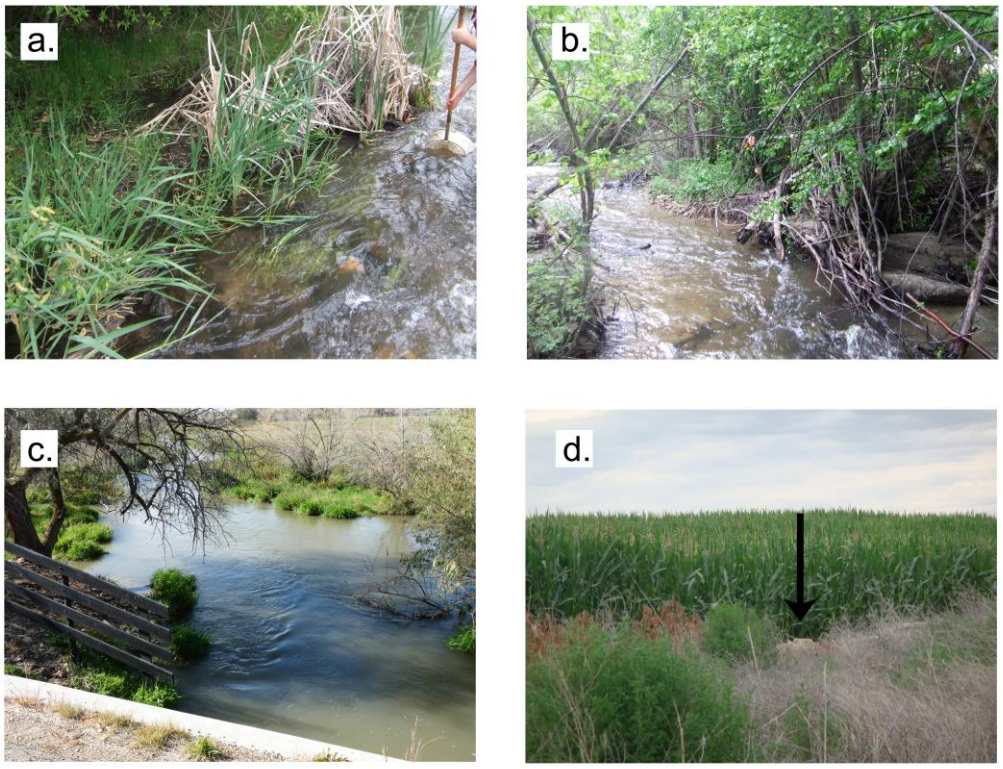

Figure 1. Images of Reference Sites: (a) Cottonwood Creek, (b) Dry Creek, and Agricultural Sites: (c) Sand Run Gulch, (d) Wanstad Ditch (indicated by arrow).

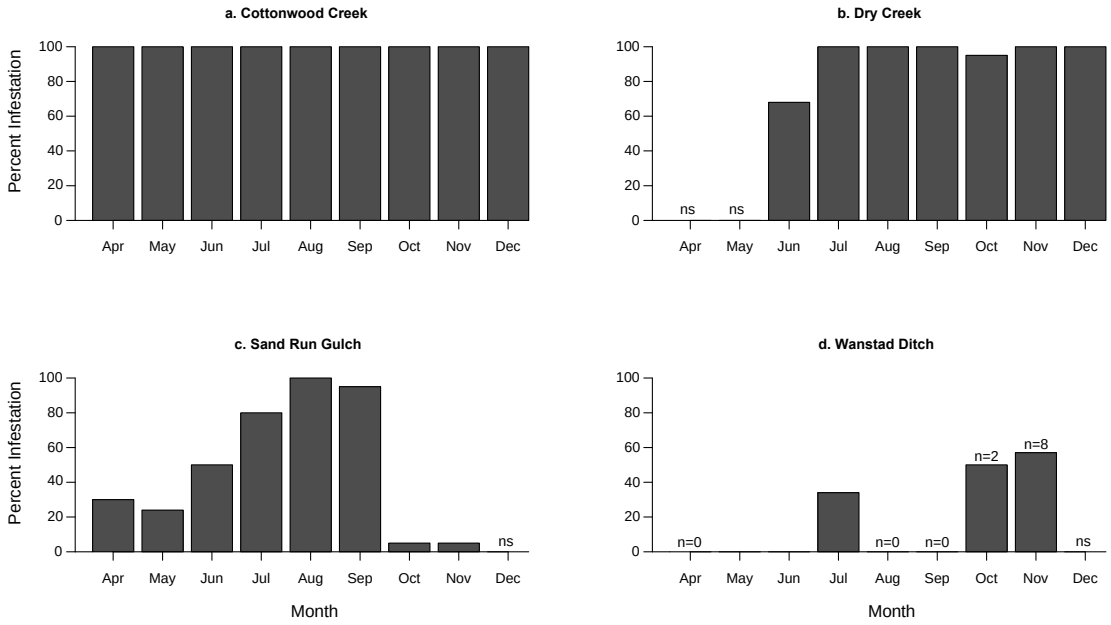

Figure 2. Percent infestation of gut fungi in black fly larvae from reference sites (a) Cottonwood Creek, (b) Dry Creek, and agricultural sites (c) Sand Run Gulch, (d) Wanstad Ditch in Idaho. All samples have n=20 unless noted otherwise; ns: not sampled.

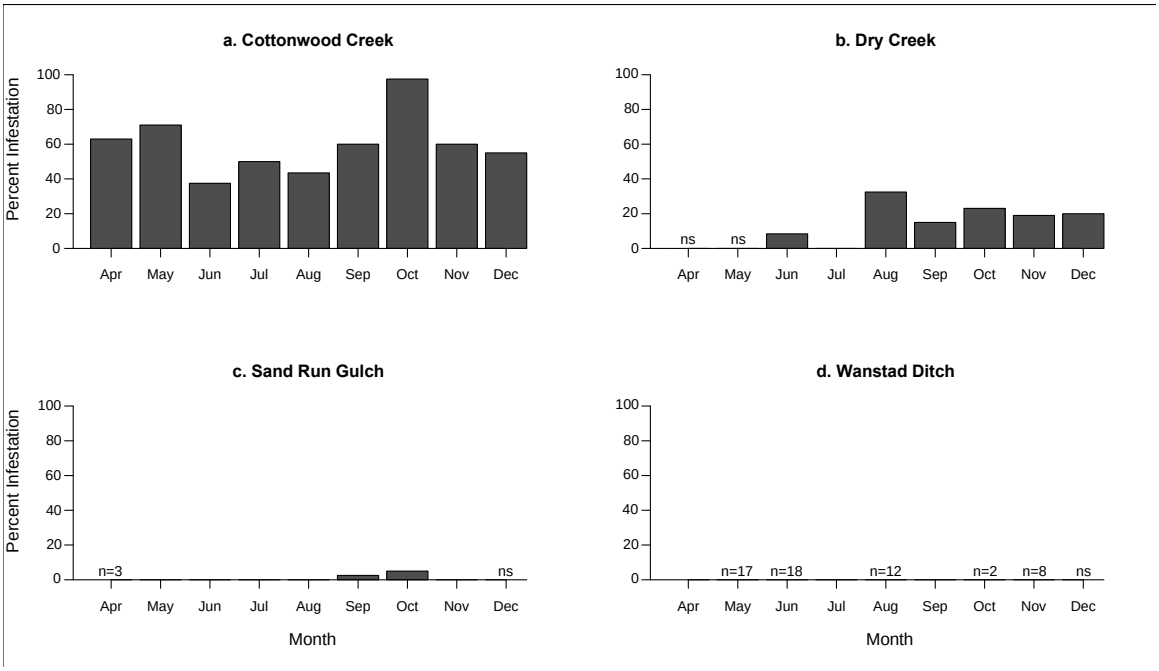

Figure 3. Percent infestation of gut fungi in mayfly nymphs from reference sites (a) Cottonwood Creek, (b) Dry Creek, and agricultural sites (c) Sand Run Gulch, (d) Wanstad Ditch in Idaho. All samples have n=20 unless noted otherwise; ns: not sampled.

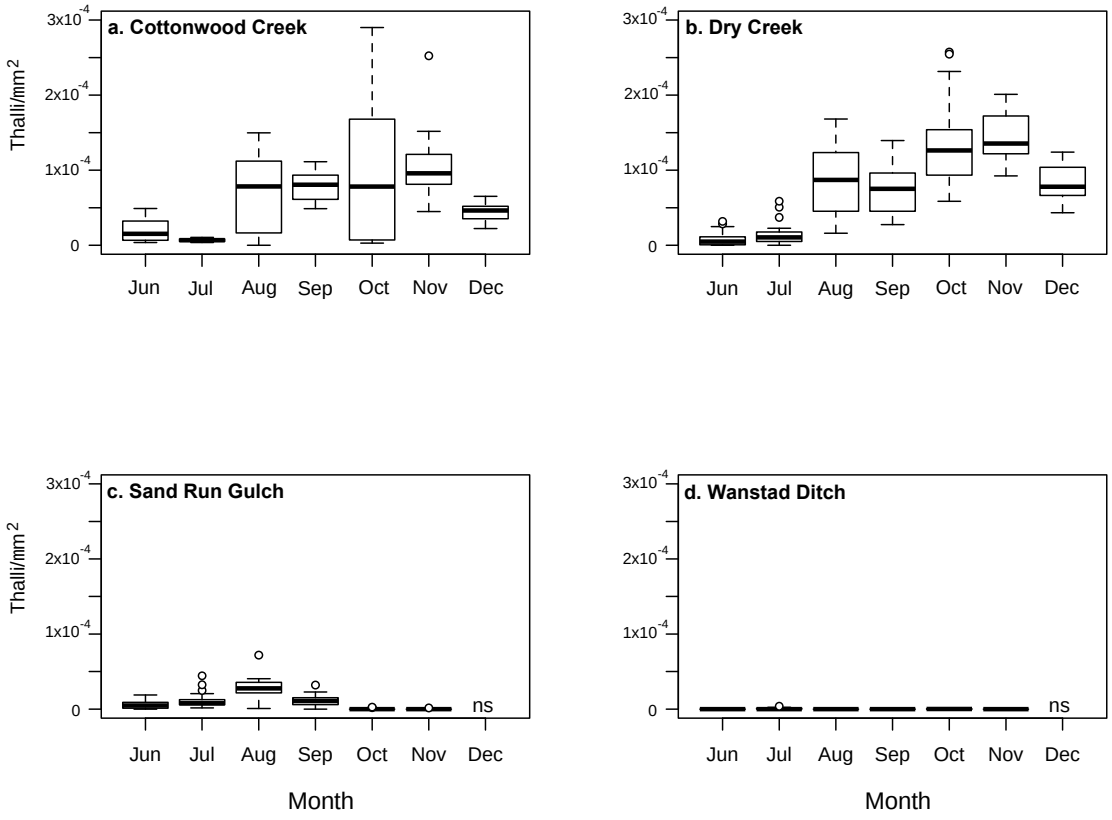

Figure 4. Number of thalli per µm^2 of peritrophic matrix in black fly larvae from reference sites (a) Cottonwood Creek, (b) Dry Creek, and agricultural sites (c) Sand Run Gulch, (d) Wanstad Ditch.

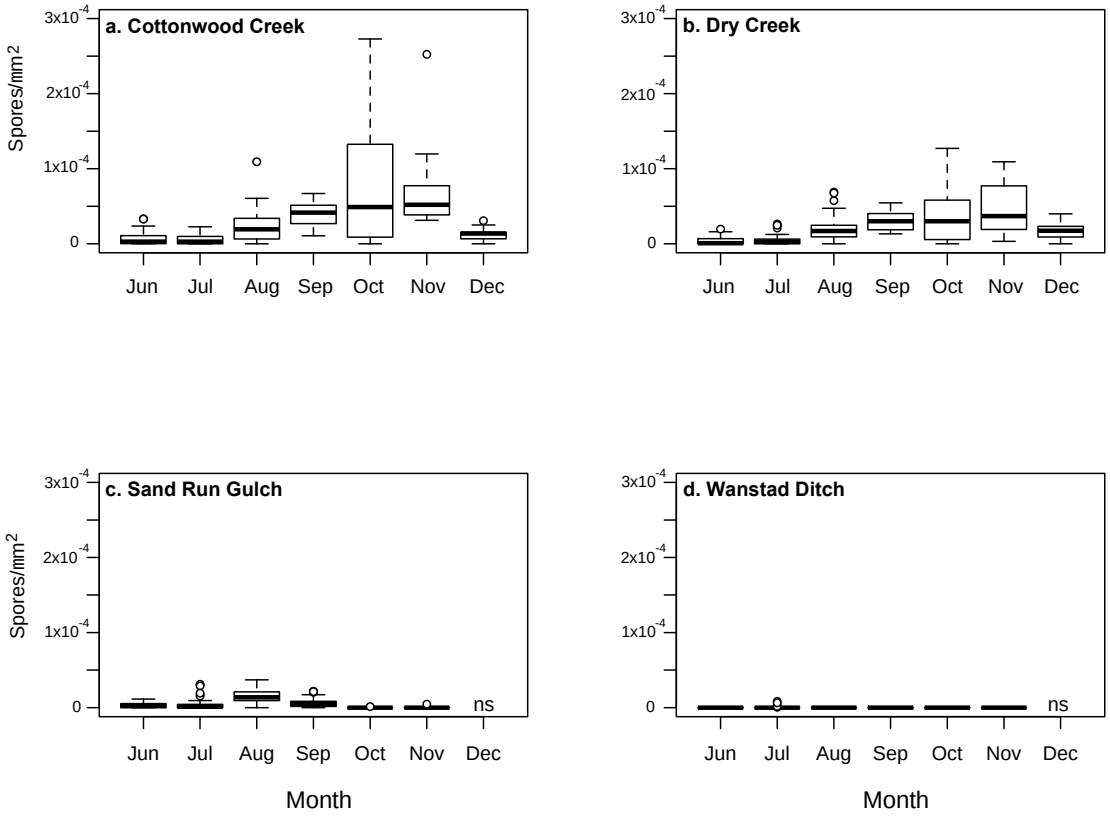

Figure 5. Number of spores per µm^2 of peritrophic matrix in black fly larvae from reference sites (a) Cottonwood Creek, (b) Dry Creek, and agricultural sites (c) Sand Run Gulch, (d) Wanstad Ditch.

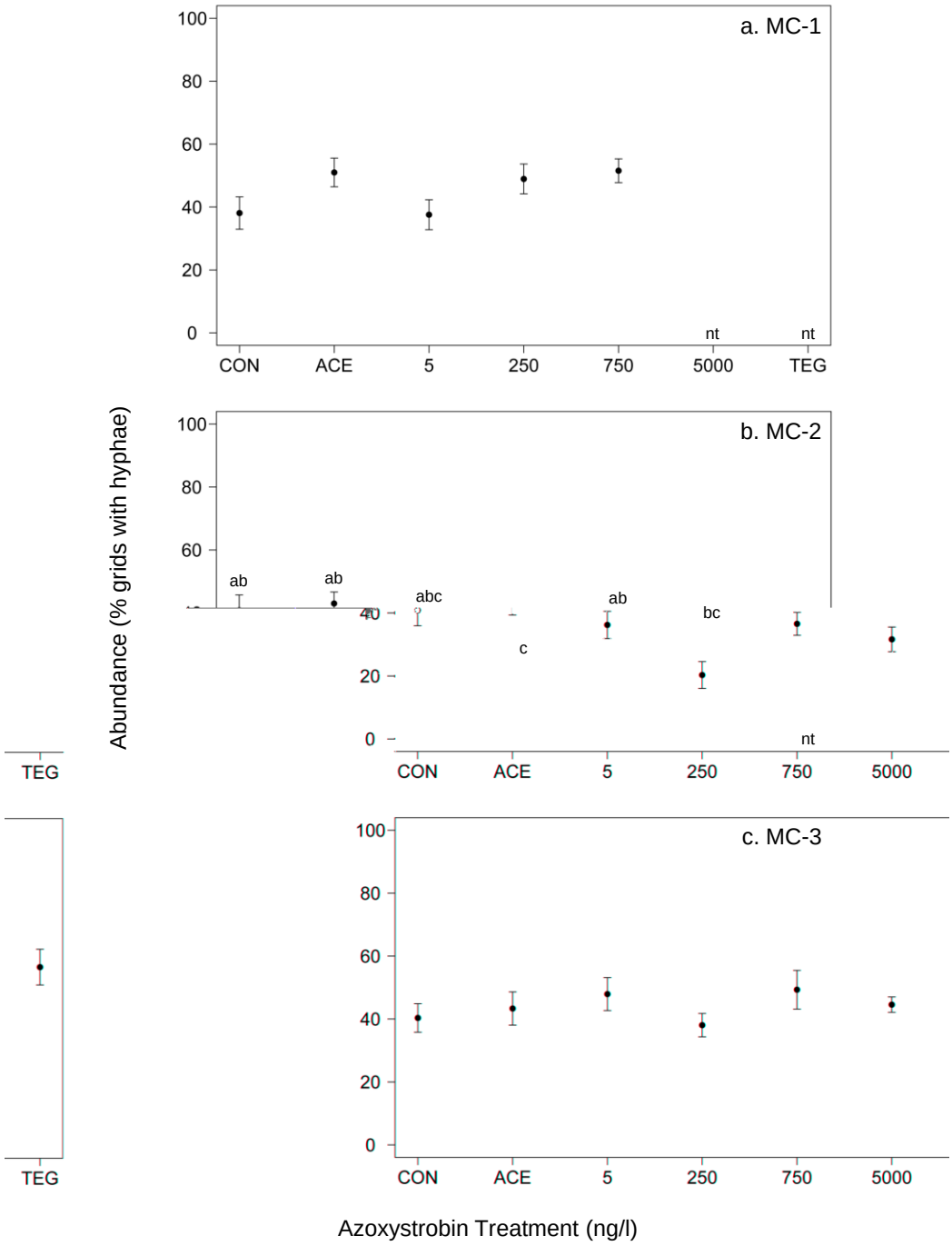

Figure 6. Mean (± standard error) of the relative hyphal abundance in microcosm experiments (a) MC-1, (b) MC-2, and (c) MC-3. Letters indicate significant differences. CON: control treatment; ACE: acetone vehicle treatment (8.3 µl/L); TEG: triethylene glycol vehicle treatment (10 ml/L).

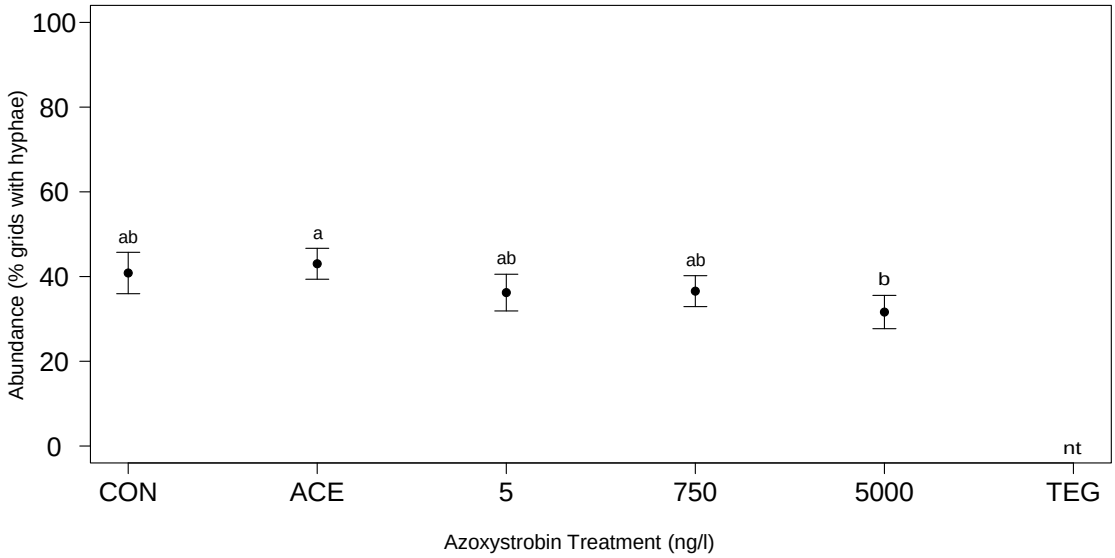

Figure 7. Mean (± standard error) of the relative hyphal abundance in MC-2 without the addition of the 250 ng/L treatment. Letters indicate significant differences.

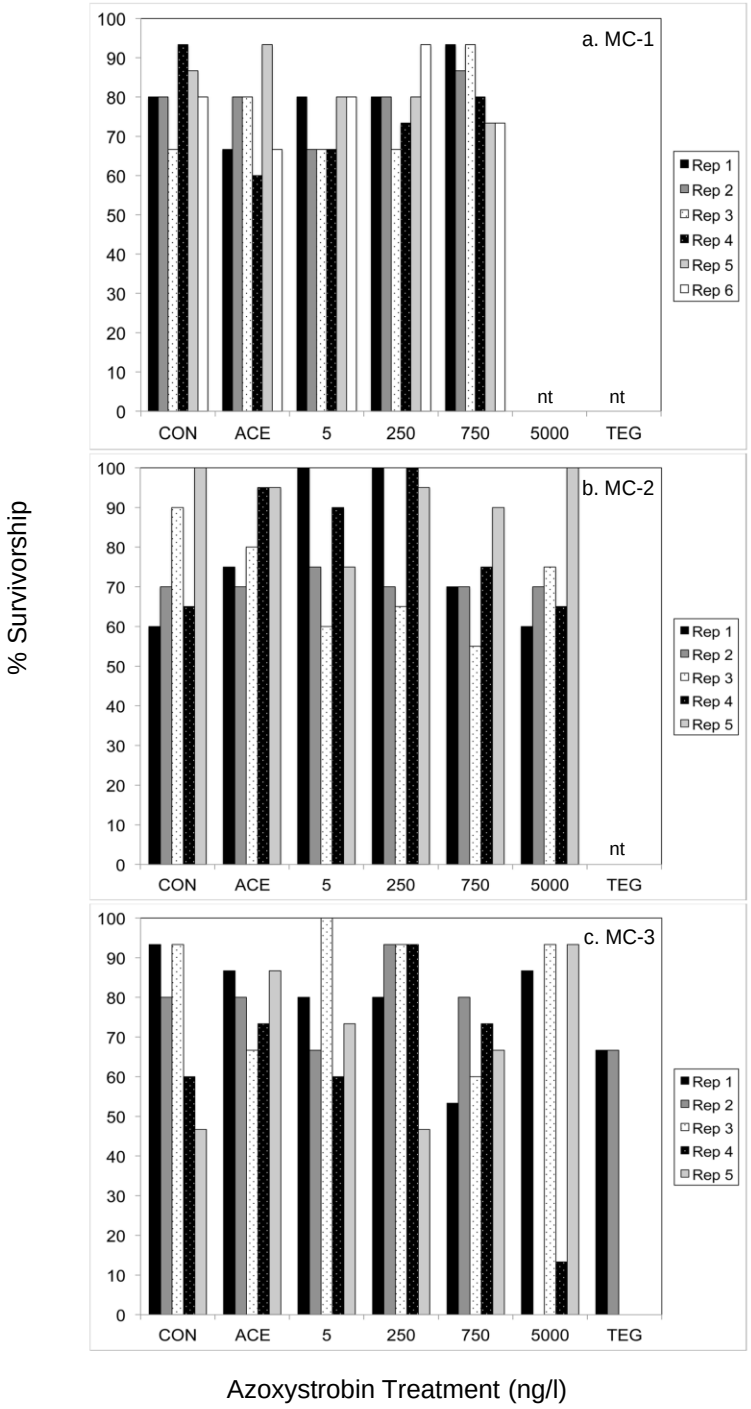

Figure 8. Percent survivorship of black fly larvae in each experimental unit at the end of the microcosm experiment (a) MC-1, (b) MC-2, and (c) MC-3. CON: control treatment; ACE: acetone vehicle treatment (8.3 µl/L); TEG: triethylene glycol treatment (10 ml/L).

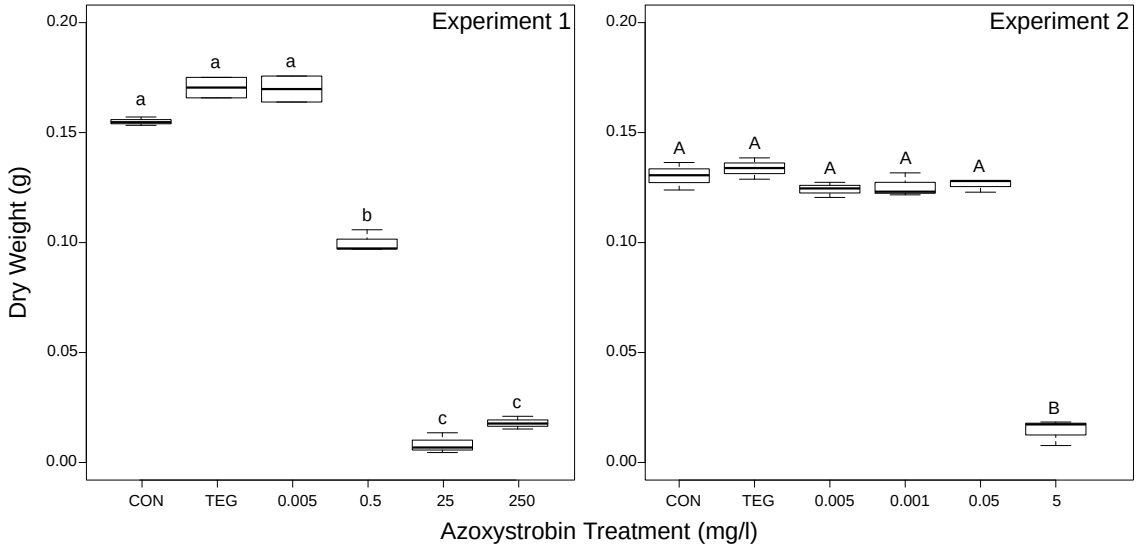

Figure 9. Dry weight (g) of *Smittium simulii* exposed to different concentrations of azoxystrobin in two *in vitro* trials (Experiment 1 and Experiment 2). Letters indicate significant differences. CON: control treatment; TEG: triethylene glycol vehicle treatment (50 ml/L in Experiment 1, 10 ml/L in Experiment 2).

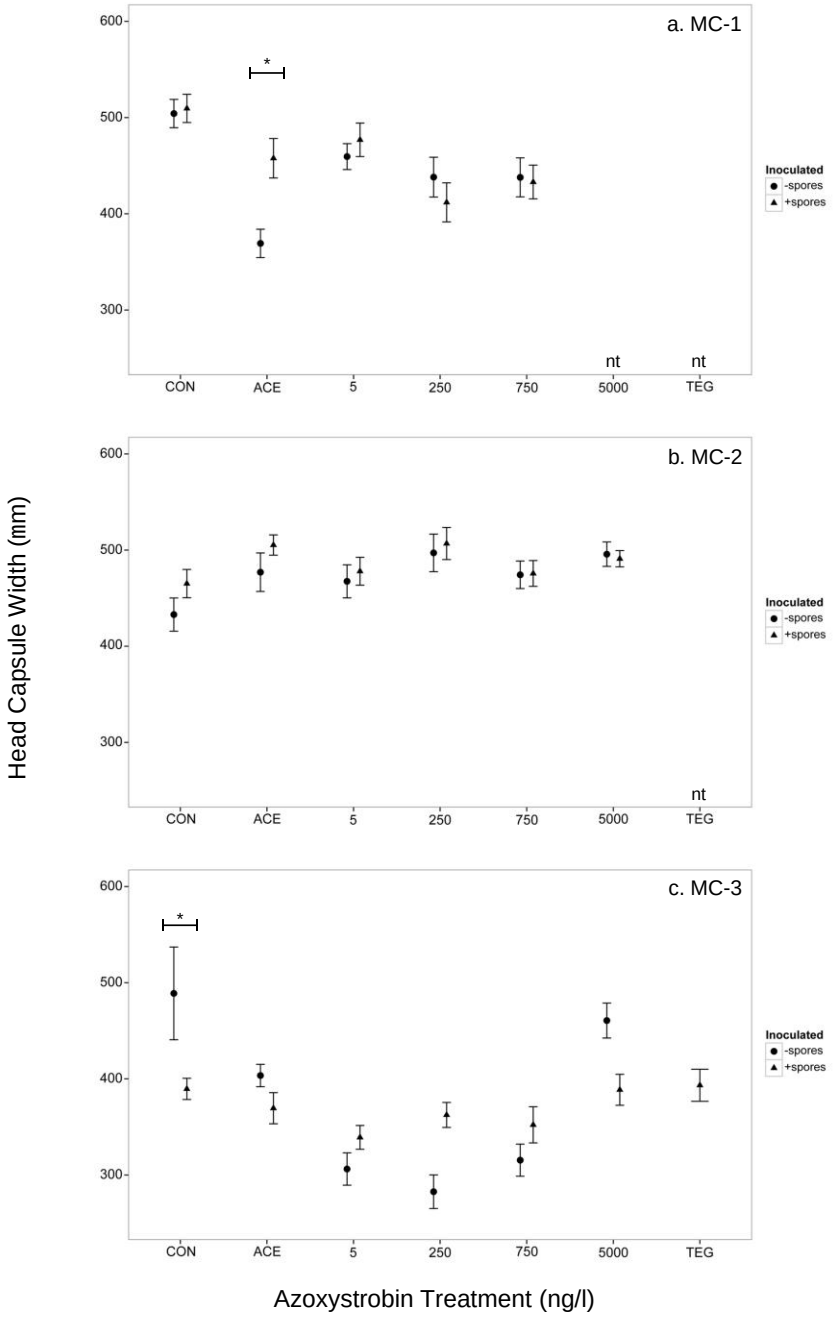

Figure 10. Larval black fly head capsule width (µm) mean (± standard error) for (a) MC-1, (b) MC-2, and (c) MC-3. Comparison of black fly larvae inoculated (+sp) or not inoculated (-sp) with *Smittium simulii*. Bars with * indicate significant differences within fungicide dosage treatments. See Table 4 for means comparisons and all sample sizes. CON: control treatment; ACE: acetone vehicle treatment (8.3 µl/L); TEG: triethylene glycol vehicle treatment (10 ml/L).

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APPENDIX A

Pesticide Analysis, Detection Limits, and Quality Control

Pesticide Analysis, Detection Limits, and Quality Control

Note: All of the water and tissue analyses were conducted and written by Kelly Smalling and others at the US Geological Survey California Water Science Center in Sacramento, CA. Many of these methods were developed by Smalling and have been submitted with manuscript version of this master's thesis.

Analysis of Pesticides in Surface Water

Surface water samples (1 L) were filtered using 0.7 µm glass fiber filters (GF/F) (Whatman, Florham Park, New Jersey), extracted onto Oasis HLB solid-phase extraction (SPE) cartridges (6 cc, 500 mg, 60 µm, Waters Corporation, Milford, Massachusetts), dried, eluted with ethyl acetate, reduced to 200 µL and analyzed for a suite of 90 pesticides by gas chromatography–mass spectrometry operating in electron ionization mode (GC-EIMS). Prior to extraction, samples were spiked with $^{13}C_3$-atrazine and diazinon diethyl-d_{10} (Cambridge Isotopes, Andover, Massachusetts) as recovery surrogates (Hladik et al., 2008). A complete list of target compounds can be seen in Table A.1.

The surface water extracts (1 µL injection volume) were then analyzed on an Agilent 5975 gas chromatograph (GC)/electron ionization mass spectrometer (EI-MS) (Folsom, CA, USA). Analyte separation on the GC was achieved using a 30 m x 0.25 mm i.d., 0.25 µm DB-5ms fused silica column (Agilent Technologies, Folsom, California) with helium as the carrier gas. The temperature of the splitless injector was held constant at 275°C. The temperature program for all herbicides and insecticides was 80°C (hold 0.5 min), increase to 120°C at 10°C/min, increase to 200°C at 3°C/min (hold 5 min), followed by a third increase to 219°C at 3°C/min, and a final increase to 300°C at

10°C/min (hold 10 min). The temperature program for the fungicides was 80°C (hold 0.5 min), increase to 180°C at 10°C/min, increase to 220°C at 5°C/min (hold 1 min), increase to 280°C at 4°C/min (hold 1 min), and a final increase to 300°C at 10°C/min (hold 10 min). The transfer line, quadrupole and source temperatures were 280°C, 150°C and 230°C, respectively. Data for all pesticides were collected in selective ion monitoring mode (SIM) with each compound having one quantifier ion and 1-2 qualifier ions (Table A.1).

Tissue Analysis in Black Fly Larvae

Thawed composite black fly larvae samples (0.12 to 2.5 g) from each site were analyzed for a suite of 12 pesticides. Prior to extraction, sediment samples were spiked with trifluralin-d_{10}, ring-^{13}C-*p,p'*-DDE and phenoxy-^{13}C-*cis*-permethrin (Cambridge Isotopes, Andover, Massachusetts) as recovery surrogates. Composite larval samples were homogenized with Na_2SO_4 and extracted using a sonic water bath at 30°C for 25 min. After two extractions with dichloromethane (DCM), samples were reduced to 1 mL using a Turbo Vap II (Zymark) operating at 25°C with high purity (>99.99 %) N_2. Ten percent by volume of each raw extract was allowed to evaporate to a constant weight in a fume hood for gravimetric lipid determination to the nearest 0.001 g using a microbalance. Due to the small sample mass and non-detectable amounts of lipid, no cleanup was necessary. Samples were exchanged to ethyl acetate, further reduced to 200 µL and acenaphthene-d_{10} was added to each sample prior to analysis as an internal standard. Chromatographic analyses were performed on an Agilent 7890 gas chromatograph coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies, Folsom, California, USA) operating in multiple reaction-monitoring

(MRM) mode. Details of MRM transition, collision energy and limits of detection (LOD) are listed in Table A.2.

Analyte separation on the GC was achieved using a 30 m x 0.25 mm i.d., 0.25 µm DB-5ms fused silica column (Agilent Technologies, Folsom, California). The temperature of the splitless injector was held constant at 275°C. The temperature program was 80°C (hold 1 min), increase to 220°C at 20°C/min (hold 1 min), and a final increase to 300°C at 20°C/min (hold 5 min). The transfer line and electron ionization source temperatures were 250°C. Electron ionization energy of 70 eV was used with a filament-multiplier delay of 5 min. The filament current was 35 µA, N_2 was used as the collision gas with a flow of 1.5 mL/min and the He flow was 2.5 mL/min. The temperatures of the quadrupoles were 150°C and 300°C. The detector voltage was automatically set by the instrument after automated MS/MS tuning, which was typically 1300 V. A full autotune of the mass spectrometer using the default parameters was performed prior to each sequence. Agilent MassHunter was used for instrument control and data acquisition/processing.

The final MRM acquisition method consisted of 2 ion transitions at the experimentally optimized collision energy (CE) for each analyte and dwell time of 2.5 ms was set for all transitions (inter dwell delay of 1 ms). The “wide” MS resolution setting of 1.2 amu full width at half maximum was entered into the MRM method for all transitions. Information on MRM transitions and CE for each compound can be found in Table A.2.

Instrument calibrations were achieved using concentration standards that spanned the linear range of instrument response. Calibration curves were considered acceptable if

the R^2 for each individual compound was greater than 0.995. The responses of the instrument were monitored every 6-8 samples with mid-level check standards. The instruments were considered to be stable if the recovery of the check standards fell within the range of 80-115% of the nominal standard concentration. If environmental sample concentrations fell outside the linear range of the instrument, the samples were diluted appropriately and re-analyzed.

Detection Limits

Surface Water

Surface-water method detection limits (MDLs) were previously validated for the majority of the pesticides (Hladik et al., 2008) using the EPA procedure described in 40 CFR Part 136 (EPA 1992). Water samples used to determine MDLs for insecticides and herbicides were collected in 2005 from the Sacramento River at Miller Park and water samples for fungicide MDLs were collected in 2008 from the American River near the California State University Campus. MDLs for all compounds in water ranged from 0.9 to 10.5 ng/L and instrumental LOD ranged from 0.5 to 1.0 ng/L (Table A.1). Analytes detected at concentrations greater than the instrumental LOD but less than the MDL were reported as estimates.

Larval Tissue Samples

Instrumental LOD were calculated for the 12 pesticides included in the method (Table A.2). LODs ranged from 0.001 to 0.004 µg/g wet weight and were based on the lowest measurable calibration standard divided by an average wet mass of tissue (1 g)

Quality Assurance

Pesticide concentrations in water and black fly larvae were validated against a comprehensive set of performance based quality assurance/quality control (QA/QC) criteria including laboratory blanks, matrix spikes, and surrogate recovery.

Surface Water

Eight laboratory blanks were processed to test the cleanliness of the laboratory procedures. No pesticides were detected in any of the blank samples. Ring-$^{13}C_3$-atrazine and diethyl-d_{10} diazinon were used as recovery surrogates to assess the efficiency of sample extraction. Percent recovery of surrogates for all samples analyzed (including QC samples) ranged from 73 to 118% with a mean (± standard deviation) of ring-$^{13}C_3$-atrazine and diethyl-d_{10} diazinon of 89 ± 9% and 93 ± 12%, respectively. Six samples were spiked in the laboratory with a suite of 90 pesticides and the percent recovery ranged from 78-110% with a median of 92%.

Larval Tissue Samples

Two laboratory blanks were processed with the 17 environmental samples and no pesticides were detected in the blank samples. Trifluralin-d_{10} was used as a recovery surrogate and the percent recovery for all samples analyzed (including QA) ranged from 81 to 126% with a mean (± standard deviation) of 108 ± 12%. Four samples were spiked in the laboratory with the 12 pesticides and the percent recovery of the spiked samples ranged from 73 to 126% with a median of 97%.

Table A.1 List of compounds analyzed in surface water, GC-EIMS quantifier and qualifier ions, method detection limits (ng/L) and instrumental limits of detection (ng/L). (D, degradate; F, fungicide; H, herbicide; I, insecticide; S, synergist).

Compound	Type	Quantifier ion	Qualifier ion (s)	MDL (ng/L)	LOD (ng/L)
3,4-dichloroaniline (DCA)	D	161	163	8.3	1.0
3,5-dichloroaniline (DCA)	D	161	163	7.6	1.0
Alachlor	H	188	160, 238	1.7	1.0
Allethrin	I	123	136, 124	6.0	1.0
Atrazine	H	200	215, 173	2.3	0.5
Azoxystrobin	F	344	388	3.1	0.5
Bifenthrin	I	181	166	4.7	0.5
Boscalid	F	140	112, 342	2.8	0.5
Butylate	H	146	174, 156	1.8	1.0
Carbaryl	I	144	115, 116	6.5	1.0
Carbofuran	I	164	149	3.1	1.0
Chlorothalonil	F	266	264, 268	4.1	0.5
Chlorpyrifos	I	314	197, 258	2.1	0.5
Clomazome	H	204	125, 240	2.5	1.0
Cycloate	H	154	155	1.1	1.0
Cyfluthrin	I	163	127, 199	5.2	1.0
Cyhalothrin	I	181	197, 161	2.0	0.5
Cypermethrin	I	163	127, 181	5.6	1.0
Cyproconazole	F	222	139, 125	4.7	0.5
Cyprodinil	F	224	225	7.4	1.0
Chlorthal-dimethyl (DCPA)	H	301	299, 332	2.0	0.5
Deltamethrin	I	253	172, 181	3.5	1.0
Diazinon	I	179	199, 304	0.9	1.0
Difenoconazole	F	323	265, 267	10.5	1.0
Dimethomorph	F	301	303	6.0	1.0
s-ethyl dipropylthiocarbamate (EPTC)	H	128	132	1.5	0.5
Esfenvalerate	I	225	125, 167	3.9	0.5
Ethalfluralin	H	276	316, 292	3.0	0.5
Etofenprox	I	163	164	2.2	1.0

Table A.1 (cont.) List of compounds analyzed in surface water, GC-EIMS quantifier and qualifier ions, method detection limits (ng/L) and instrumental limits of detection (ng/L). (D, degradate; F, fungicide; H, herbicide; I, insecticide; S, synergist).

Compound	Type	Quantifier ion	Qualifier ion (s)	MDL (ng/L)	LOD (ng/L)
Famoxadone	F	330	196	2.5	1.0
Fenarimol	F	139	107, 251	6.5	1.0
Fenbuconazole	F	129	198	5.2	1.0
Fenhexamide	F	97	177	7.6	1.0
Fenpropathrin	I	181	265, 125	4.1	1.0
Fipronil	I	367	369, 351	2.9	0.5
Fipronil desulfinyl	D	388	333, 390	1.6	0.5
Fipronil sulfide	D	351	353, 255	1.8	0.5
Fipronil sulfone	D	383	385, 255	3.5	0.5
Fluazinam	F	387	389, 417	4.4	1.0
Fludioxinil	F	248	127, 154	7.3	1.0
Fluoxastrobin	F	188	219	5.1	1.0
Flusilazole	F	233	206	4.5	1.0
Flutriafol	F	123	164	4.2	1.0
Hexazinone	H	171	128	8.4	1.0
Imazalil	F	215	173, 217	10.5	1.0
Iprodione	F	314	316, 187	4.4	1.0
Kresoxim-methyl	F	116	131, 206	4.0	1.0
Malathion	I	123	173, 158	3.7	1.0
Metconazole	F	125	250	5.2	0.5
Methidathion	I	145	125	7.2	1.0
Methoprene	I	111	107, 191	6.4	1.0
Methylparathion	I	263	109, 246	3.4	1.0
Metolachlor	H	162	238, 240	1.5	1.0
Molinate	H	126	98	3.2	1.0
Myclobutanil	F	179	150, 206	6.0	0.5
Napropamide	H	100	115, 128	8.2	1.0
Oxyfluorfen	H	252	300, 317	3.1	1.0
p,p'-DDD	D	235	237, 165	4.1	0.5
p,p'-DDE	D	318	246, 316	3.6	0.5
p,p'-DDT	I	235	237, 165	4.0	0.5

Table A.1 (cont.) List of compounds analyzed in surface water, GC-EIMS quantifier and qualifier ions, method detection limits (ng/L) and instrumental limits of detection (ng/L). (D, degradate; F, fungicide; H, herbicide; I, insecticide; S, synergist).

Compound	Type	Quantifier ion	Qualifier ion (s)	MDL (ng/L)	LOD (ng/L)
Pebulate	H	128	132	2.3	0.5
Pendimethalin	H	252	191, 162	2.3	0.5
Pentachloroanisole (PCA)	D	265	280, 267	4.7	0.5
Pentachloronitrobenzene (PCNB)	F	295	293, 265	3.1	0.5
Permethrin	I	183	165, 127	3.4	0.5
Phenothrin	I	123	183	5.1	1.0
Phosmet	I	160	133	4.4	1.0
Piperonyl butoxide	S	176	177	2.3	1.0
Prometon	H	226	225	2.5	1.0
Prometryn	H	241	184, 226	1.8	1.0
Propanil	H	161	163, 317	10.1	1.0
Propiconazole	F	173	259, 175	5.0	0.5
Propyzamide	H	256	173, 254	5.0	1.0
Pyraclostrobin	F	132	164	2.9	0.5
Pyrimethanil	F	198	199	4.1	0.5
Remethrin	I	123	128, 171	5.7	1.0
Simazine	H	201	186, 188	5.0	1.0
Tebuconazole	F	125	250, 127	3.7	0.5
Tefluthrin	I	177	197, 161	4.2	1.0
Terbuthylazine	H	214	230, 173	1.6	1.0
Tetraconazole	F	336	338	5.6	0.5
Tetramethrin	I	164	123, 165	2.9	1.0
t-Fluvalinate	I	250	252	5.3	1.0
Thiobencarb	H	100	125, 257	1.9	0.5
Triadimefon	F	208	181, 210	8.9	1.0
Triadimenol	F	112	168, 128	8.0	1.0
Trifloxystrobin	F	116	131, 222	4.7	0.5
Triflumizole	F	178	206, 179	6.1	1.0
Trifluralin	H	306	264	2.1	0.5
Triticonazole	F	235	237, 217	6.9	1.0
Zoxamide	F	187	189, 258	3.5	1.0

Table A.2 Retention time, MRM conditions, average (± standard deviation) percent recovery of matrix spikes (n = 4) and instrumental limits of detection (LOD) for pesticides analyzed in larval black fly tissue.

		MRM transitions					
	RT	**Quantifier**	**CE (V)**	**Qualifier**	**CE (V)**	**Average Recovery (%)**	**LOD (µg/g)**
s-ethyl dipropylthiocarbamate (EPTC)	6.06	189 > 86	10	189 > 128	10	103 (12)	0.001
Ethalfluralin	7.94	276 > 202	20	276 > 105	20	99 (11)	0.001
Trifluralin	8.02	306 > 264	14	264 > 160	20	97 (10)	0.001
Simazine	8.45	201 > 173	25	201 > 158	10	104 (10)	0.001
Atrazine	8.51	200 > 104	25	200 > 94	25	96 (10)	0.001
Metolachlor	10.12	162 > 132.1	30	162 > 91	30	101 (10)	0.001
Pendimethalin	10.57	252 > 162	16	252 > 191.1	14	103 (8)	0.001
Imazalil	11.23	173 > 109	38	173 > 73.9	38	79 (6)	0.002
Hexazinone	12.32	171 > 71	16	171 > 85	14	102 (12)	0.001
Pyraclostrobin	13.67	132 > 77	30	132 > 51	34	101 (20)	0.001
Boscalid	14.51	140 > 112.1	20	140 > 76	30	95 (13)	0.001
Azoxystrobin	16.29	344 > 329	10	344 > 156	34	91 (9)	0.004

CE: collision energy in volts.

RT: retention time

Table A.3 Quality assurance for azoxystrobin concentrations in microcosms (MC-) 1 and 2. Experiment Day refers to whether the water was tested before or after a water change; Day 0 = at water change, Day 2 = 48 hours from last water change; Rep = replicate number for Day 2 water change.

MC-1			
Treatment	**Experiment Day**	**Conc Detected (ng/L)**	**Difference From Expected (%)**
CON	Day 0	nd	-
ACE	Day 0	nd	-
5 ng/l	Day 0	3.1	-38.1
250 ng/l	Day 0	393.7	57.5
750 ng/l	Day 0	878.2	17.1
CON*	Day 0	-	-
ACE	Day 0	nd	-
5 ng/l	Day 0	6.6	32.1
250 ng/l	Day 0	365.3	46.1
750 ng/l	Day 0	894.0	19.2
CON	Day 2-rep1	nd	-
ACE	Day 2-rep1	nd	-
5 ng/l	Day 2-rep1	6.0	19.6
5 ng/l	Day 2-rep2	6.7	33.3
250 ng/l	Day 2-rep1	372.5	49.0
250 ng/l	Day 2-rep2	348.2	39.3
750 ng/l	Day 2-rep1	1017.5	35.7
750 ng/l	Day 2-rep2	1152.8	53.7
CON	Day 2-rep1	nd	-
ACE	Day 2-rep1	nd	-
5 ng/l	Day 2-rep1	3.5	-29.7
5 ng/l	Day 2-rep2	4.2	-15.0
250 ng/l	Day 2-rep1	331.4	32.6
250 ng/l	Day 2-rep2	289.5	15.8
750 ng/l	Day 2-rep1	1093.2	45.8
750 ng/l	Day 2-rep2	1008.9	34.5

nd: not detected

* sample broke in shipment

Table A.3 (cont.) Quality assurance for azoxystrobin concentrations in microcosms (MC-) 1 and 2. Experiment Day refers to whether the water was tested before or after a water change; Day 0 = at water change, Day 2 = 48 hours from last water change; Rep = replicate number for Day 2 water change.

MC-2			
Treatment	**Experiment Day**	**Conc Detected (ng/l)**	**Difference From Expected (%)**
CON	Day 0	nd	-
ACE	Day 0	nd	-
5 ng/l	Day 0	4.4	-12.4
250 ng/l	Day 0	144.8	-42.1
750 ng/l	Day 0	857.1	14.3
5000 ng/l	Day 0	4552.5	-8.9
CON	Day 0	nd	-
ACE	Day 0	nd	-
5 ng/l	Day 0	5.6	11.8
250 ng/l	Day 0	243.6	-2.6
750 ng/l	Day 0	649.4	-13.4
5000 ng/l	Day 0	5067.6	1.4
CON	Day 2-rep1	nd	-
ACE	Day 2-rep1	nd	-
5 ng/l	Day 2-rep1	6.6	32.5
5 ng/l	Day 2-rep2	6.1	22.6
250 ng/l	Day 2-rep1	243.8	-2.5
250 ng/l	Day 2-rep2	247.9	-0.9
750 ng/l	Day 2-rep1	756.7	0.9
750 ng/l	Day 2-rep2	786.4	4.8
5000 ng/l	Day 2-rep1	4589.1	-8.2
5000 ng/l	Day 2-rep2	5187.9	3.8

nd: not detected

* sample broke in shipment

Table A.4 All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Date	Collection ID	Temp (°C)	DOC (mg/L)	TDN (mg/L)	Conductance (uS/cm)	Turbidity (NTUs)	pH
Cottonwood Creek	2010-06-02	ID076	11.0	3.93	1.00	136	12.3	7.66
	2010-06-22	ID084	na	2.82	na	132	5.0	7.49
	2010-07-12	ID089	14.0	3.05	0.14	211	5.6	7.58
	2010-08-02	ID091	12.0	2.97	na	339	12.4	7.30
	2010-10-04	ID111	13.0	na	na	na	na	na
	2010-08-23	ID099	12.7	2.87	0.01	350	10.2	7.40
	2010-09-15	ID104	15.0	2.98	na	378	1.1	7.98
	2010-10-26	ID113*	7.0	3.07	na	na	na	na
	2010-11-22	ID115	6.0	3.26	na	401	0.6	7.37
	2010-12-06	ID117	7.0	4.32	0.36	374	0.6	7.49
	2010-04-20	ID070	na	3.71	0.61	121	16.7	7.32
	2010-05-13	ID072	8.0	2.78	0.15	169	5.8	7.45
Dry Creek	2010-06-02	ID075	8.4	3.91	0.28	112	9.4	7.81
	2010-06-22	ID083	na	3.00	na	120	5.0	7.75
	2010-07-12	ID088	13.0	2.61	0.18	141	5.3	7.92
	2010-08-02	ID092	16.0	2.35	na	152	5.0	7.88
	2010-08-23	ID098	13.3	2.58	0.06	155	4.2	7.71
	2010-09-15	ID103	12.0	2.06	na	168	2.4	7.57
	2010-10-04	ID110	14.0	2.36	na	na	na	na
	2010-10-26	ID112	7.0	3.91	0.12	166	2.5	8.43
	2010-11-22	ID114	2.5	1.67	na	167	1.1	7.66
	2010-12-06	ID116	2.0	2.61	0.34	161	0.3	7.65

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Date	Collection ID	Temp (°C)	DOC (mg/L)	TDN (mg/L)	Conductance (uS/cm)	Turbidity (NTUs)	pH
Sand Run Gulch	2010-04-13	IDFP33	11.5	2.69	4.3	854	28.4	7.82
	2010-05-03	IDFP35	11.0	3.13	3.19	465	12.4	8.06
	2010-05-24	IDFP37	13.5	3.51	2.08	370	17.6	7.71
	2010-06-16	IDFP41	15.0	3.40	2.68	405	28.2	7.52
	2010-07-06	IDFP43	16.0	3.69	2.70	420	44.9	7.59
	2010-07-26	IDFP45	19.0	3.79	3.11	289	134.0	7.79
	2010-08-16	IDFP47	18.0	3.22	3.18	307	91.0	7.77
	2010-09-09	IDFP49	16.0	3.47	2.96	451	11.5	7.77
	2010-09-29	IDFP50	16.0	2.98	3.10	494	15.7	7.74
	2010-10-18	IDFP52	na	2.01	6.47	614	108.0	7.68
	2010-11-08	IDFP54	9.5	2.67	5.44	669	7.8	7.95
	2010-11-29	IDFP57	4.0	2.78	7.03	713	61.3	7.67
Wanstad Ditch	2010-04-13	IDFP32	na	2.52	4.73	695	44.7	7.65
	2010-05-03	IDFP34	12.0	3.19	3.15	323	88.1	8.00
	2010-05-24	IDFP36	13.5	3.37	1.88	237	50.1	7.93
	2010-06-16	IDFP40	14.5	2.68	1.81	250	75.1	7.85
	2010-07-06	IDFP42	17.0	3.10	2.99	292	87.1	7.39
	2010-07-26	IDFP44	na	3.06	4.57	464	66.0	7.72
	2010-08-16	IDFP46	18.0	3.91	2.46	443	32.6	7.75
	2010-09-09	IDFP48	16.0	2.97	2.98	300	71.8	7.97
	2010-09-29	IDFP51	18.0	2.76	3.56	370	14.5	7.91
	2010-10-18	IDFP53	na	4.23	4.54	540	24.0	7.43
	2010-11-08	IDFP55	na	1.9	15.1	951	4.7	7.65
	2010-11-29	IDFP56	8.0	1.65	17.2	947	0.5	7.13

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Number of Detects Per Sample	3,5 DCA	Alachlor	Atrazine	Azoxystrobin	Boscalid	Chlorothalonil
Cottonwood Creek	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	1	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	na	na	na	na	na	na	na
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
Dry Creek	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	2	nd	nd	21.4	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd
	0	nd	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Number of Detects Per Sample	3,5 DCA	Alachlor	Atrazine	Azoxystrobin	Boscalid	Chlorothalonil
Sand Run	7	nd	nd	9.6	1.8	8.2	nd
Gulch	7	nd	nd	5.8	2.2	10.2	nd
	6	nd	nd	nd	1.4	4.4	nd
	4	nd	nd	28.1	nd	nd	nd
	9	nd	nd	7.5	6.3	10.8	nd
	5	nd	nd	nd	11.4	16.0	nd
	3	nd	nd	nd	20.2	37.8	nd
	4	nd	nd	4.3	12.5	14.9	nd
	1	nd	nd	nd	nd	nd	nd
	6	nd	nd	nd	2.6	5.4	nd
	1	nd	nd	nd	nd	nd	nd
	2	nd	nd	6.6	nd	nd	nd
Wanstad	6	8.8	nd	nd	3.4	36.6	nd
Ditch	10	nd	14.1	4.6	4.0	13.3	nd
	11	nd	nd	nd	4.2	7.0	nd
	9	nd	nd	15.2	3.4	15.6	3.6
	6	nd	nd	nd	9.2	20.9	nd
	5	nd	nd	nd	31.0	11.6	nd
	4	nd	nd	nd	40.4	21.0	nd
	3	nd	nd	nd	3.4	11.7	nd
	2	nd	nd	5.0	nd	nd	nd
	4	nd	nd	nd	3.0	4.6	nd
	4	nd	nd	2.6	3.6	9.4	nd
	4	nd	nd	3.4	2.2	5.2	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Chlorpyrifos	Clomazone	Diazinon	EPTAM	Ethalfluralin	Fipronil
Cottonwood Creek	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	14.4
	nd	nd	nd	nd	nd	nd
	na	na	na	na	na	na
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
Dry Creek	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Chlorpyrifos	Clomazone	Diazinon	EPTAM	Ethalfluralin	Fipronil
Sand Run	nd	nd	nd	nd	nd	nd
Gulch	nd	nd	nd	nd	9.0	nd
	nd	nd	nd	23.3	nd	nd
	nd	nd	nd	22.9	nd	nd
	nd	nd	nd	9.5	5.9	nd
	nd	nd	nd	nd	3.2	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
Wanstad	nd	nd	nd	nd	nd	nd
Ditch	nd	nd	nd	nd	13.5	nd
	5.0	nd	10.6	25.2	14.4	nd
	nd	35.8	nd	21.1	nd	nd
	nd	nd	nd	nd	5.5	nd
	nd	nd	nd	nd	5.7	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Hexazinone	Imazalil	Metolachlor	p p' DDD	Pendimethalin	Propiconazole
Cottonwood Creek	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	na	na	na	na	na	na
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
Dry Creek	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Hexazinone	Imazalil	Metolachlor	p p' DDD	Pendimethalin	Propiconazole
Sand Run	771	205	21.6	nd	nd	nd
Gulch	96.6	nd	122	nd	34.2	nd
	137	nd	66.9	nd	28.5	nd
	nd	nd	171	nd	38.9	nd
	16.9	nd	137	nd	45.9	nd
	nd	nd	78.4	nd	nd	nd
	nd	nd	16.2	nd	nd	nd
	nd	nd	19.6	nd	nd	nd
	nd	nd	11.0	nd	nd	nd
	nd	nd	nd	nd	nd	4.8
	nd	nd	5.4	nd	nd	nd
	nd	nd	2.8	nd	nd	nd
Wanstad	nd	176	28.8	nd	nd	nd
Ditch	66.9	nd	259	17.0	51.3	nd
	46.2	nd	114	nd	154	nd
	nd	nd	113	nd	47.4	nd
	nd	nd	566	nd	31.9	nd
	nd	nd	84	nd	nd	nd
	nd	nd	321	nd	nd	nd
	nd	nd	71.3	nd	nd	nd
	nd	nd	40.4	nd	nd	nd
	nd	nd	36.4	nd	nd	nd
	nd	nd	37.0	nd	nd	nd
	nd	nd	20.4	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Pyrimethanil	Simazine	Tetraconazole	Trifluralin	Trilumizole
Cottonwood Creek	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	na	na	na	na	na
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
Dry Creek	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.4 (cont.) All collected water metrics and detected pesticides from 2010. All pesticide units are ng/L.

Site	Pyrimethanil	Simazine	Tetraconazole	Trifluralin	Trilumizole
Sand Run Gulch	nd	10.6	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	1.2	nd
	nd	nd	nd	2.0	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	5.2	nd	4.8	nd	150
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
Wanstad Ditch	nd	nd	nd	1.9	nd
	nd	13.9	nd	nd	nd
	nd	15.6	nd	2.2	nd
	nd	nd	nd	5.5	nd
	nd	nd	nd	40.9	nd
	nd	nd	nd	3.9	nd
	nd	nd	nd	5.2	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	68.8
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd

na: not available for that sample
nd: not detected
DOC: Dissolved Organic Carbon
TDN: Total Dissolved Nitrogen
* sample broke in shipment

Table A.5 Pesticides detected in agricultural sites in 2009. All units are ng/L.

Site	Date	Number of Detects Per Sample	Azoxystrobin	Boscalid	Chlorothalonil	Pyraclostrobin
Sand Run Gulch	2009-06-23	6	6.1	17.6	nd	nd
	2009-07-14	8	8.0	18.7	nd	10.1
	2009-08-04	10	16.3	27.2	0.4	15.2
	2009-08-07	7	15.9	109	4.1	60.1
	2009-08-25	4	38.2	73.2	0.2	nd
	2009-09-15	8	34.5	26.4	0.2	9.6
	2009-10-05	6	nd	23.9	nd	21.5
	2009-11-05	4	nd	16.6	nd	2.0
Wanstad Ditch	2009-06-23	7	18.6	94.2	nd	nd
	2009-07-14	10	34.6	100	nd	43.8
	2009-08-04	10	24.4	107	228	49.7
	2009-08-07	8	24.0	246	0.5	85.6
	2009-08-25	8	31.5	109	5.9	239
	2009-09-15	6	41.7	52.8	nd	64.1
	2009-10-05	4	nd	21.6	nd	21.4
	2009-11-05	4	nd	18.3	nd	2.2

nd: not detected

Table A.5 (cont.) Pesticides detected in agricultural sites in 2009. All units are ng/L.

Site	Pyrimethanil	Atrazine	Chlorpyrifos	EPTAM	Ethalfluralin	Hexazinone
	nd	nd	nd	nd	4.4	nd
Sand Run	nd	9.1	nd	39.8	nd	nd
Gulch	1.2	8.7	2.4	nd	1.6	nd
	0.9	12.7	nd	nd	nd	nd
	nd	nd	nd	nd	nd	nd
	1.3	6.0	nd	nd	nd	nd
	nd	4.0	nd	nd	nd	nd
	nd	9.1	nd	nd	nd	nd
	nd	nd	65.0	nd	2.6	nd
Wanstad	2.9	10.1	7.8	45.7	34.4	nd
Ditch	1.6	7.7	5.1	nd	1.8	nd
	nd	8.5	nd	nd	nd	54.9
	nd	4.3	3.3	nd	nd	nd
	nd	5.2	1.9	nd	nd	nd
	nd	2.0	nd	nd	nd	nd
	nd	2.4	nd	nd	nd	nd

nd: not detected

Table A.5 (cont.) Pesticides detected in agricultural sites in 2009. All units are ng/L.

Site	Metolachlor	Pendimethalin	Trifluralin
	234	38.3	2.1
Sand Run	146	12.1	0.8
Gulch	37.3	31.0	nd
	60.1	nd	nd
	25.1	nd	nd
	9.6	nd	0.2
	9.8	4.8	0.2
	2.4	nd	nd
	1746	54.2	0.8
Wanstad	664	57.4	nd
Ditch	120	42.1	nd
	141	31.9	nd
	49.1	27.7	nd
	52.9	nd	nd
	26.6	nd	nd
	14.8	nd	nd

nd: not detected

References

U.S. Environmental Protection Agency, 1992, Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11: Code of Federal Regulations 40, Protection of the Environment, CFR Part 136, Appendix B, p. 565–567

Hladik, M. L., Smalling, K. L. & K. M. Kuivila, 2008. A Multi-Residue Method for the Analysis of Pesticides and Pesticide Degradates in Water Using HLB Solid-Phase Extraction and Gas Chromatography-Ion Trap Mass Spectrometry. *Bulletin of Environmental Contamination and Toxicology* 80:139-144.

APPENDIX B

Individual Larval Pesticide Accumulation Concentrations

Individual Larval Pesticide Accumulation Concentrations

Individual black fly larvae were composited in the field for pesticide analysis. Composite weights varied by site and date and ranged from 0.1 to 2.5 g with a median of 0.7 g (Table B.1). Individual larval body burden concentrations were estimated by counting the number of black fly larvae in each sample. An estimate of the mass of the individual larvae was then calculated based number of larvae in the composite sample and the known composite mass. The estimated mass of the individual larvae ranged from 0.8 to 2.8 mg with a median of 1.6 mg and varied by site and date (Table B.2). The mass of the individual larvae has the potential to vary within a composite depending on age and development; therefore, the calculation is only an estimate because it assumes that all larvae weigh the same within the composite. Larval instar stage of the collected samples for pesticide analysis was not determined in the field. Estimated individual larvae concentrations in µg/g wet weight were calculated based on the concentration of the pesticide in the composite sample and the estimated individual larvae mass.

$$C_{ind} = (C_{comp} \times m_{comp})/(m_{ind}/1000)$$

Where C_{ind} is the concentration of the pesticide in the individual black fly larvae (µg/g), C_{comp} is the concentration of the pesticide in the composite sample (µg/g), m_{comp} is the mass of the composite sample (g) and m_{ind} is the estimate mass of the individual larvae within the composite (mg).

Table B.1 Composite pesticide concentration (µg/g wet weight) in sampled black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Date	Larval composite mass (g)	Atrazine	Azoxystrobin	Boscalid
Cottonwood Creek	2010-05-13	1.913	nd	nd	nd
	2010-06-22	2.446	nd	nd	nd
	2010-07-13	1.300	nd	nd	nd
	2010-08-23	2.436	nd	nd	nd
	2010-09-15	0.698	nd	nd	nd
Dry Creek	2010-06-21	0.541	0.180	nd	nd
	2010-08-02	1.164	nd	nd	nd
	2010-08-23	1.312	nd	nd	nd
	2010-09-15	0.211	0.01	nd	nd
Sand Run Gulch	2010-05-03	1.192	nd	0.056	0.926
	2010-07-06	0.527	0.250	0.121	0.033
	2010-07-26	0.730	nd	0.377	0.112
	2010-09-09	0.226	0.064	0.428	0.090
	2010-09-29	1.499	0.097	0.136	0.106
	2010-10-18	0.118	0.058	0.035	0.029
	2010-11-08	0.150	nd	nd	nd
Wastad Ditch	2010-08-16	0.485	nd	0.360	0.876

nd: not detected

Table B.1 (cont.) Composite pesticide concentration (µg/g wet weight) in sampled black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Ethalfluralin	Hexazinone	Imazalil	Metolachlor
Cottonwood Creek	nd	nd	nd	nd
	nd	nd	nd	nd
	nd	nd	nd	nd
	nd	nd	nd	nd
	nd	nd	nd	nd
	nd	nd	nd	nd
Dry Creek	nd	nd	nd	nd
	nd	nd	nd	nd
	nd	nd	nd	nd
Sand Run Gulch	nd	0.083	0.181	nd
	nd	0.096	0.326	0.441
	0.035	0.060	0.374	nd
	0.009	nd	0.178	0.162
	0.016	nd	0.021	0.163
	0.073	nd	0.066	0.106
	nd	nd	nd	nd
Wastad Ditch	nd	0.100	0.277	0.294

nd: not detected

Table B.1 (cont.) Composite pesticide concentration (µg/g wet weight) in sampled black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Pendimethalin	Pyraclostrobin	Simazine	Trifluralin
Cottonwood Creek	nd	nd	0.043	0.032
	nd	nd	0.016	0.005
	nd	nd	nd	0.031
	nd	nd	0.010	nd
	nd	nd	nd	nd
	nd	nd	0.234	nd
Dry Creek	nd	nd	0.026	0.017
	nd	nd	nd	0.016
	nd	nd	0.008	0.019
Sand Run Gulch	0.046	nd	nd	0.001
	0.018	nd	0.023	nd
	0.252	0.43	0.062	0.043
	0.123	0.84	nd	0.013
	0.138	0.29	0.054	0.020
	0.088	0.16	0.078	nd
	nd	nd	nd	nd
Wastad Ditch	0.009	0.190	nd	0.001

nd: not detected

Table B.2 Estimated pesticide concentrations (µg/g wet weight) of individual black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Date	Estimated individual larval mass (mg)	Estimated number of lavae per composite	Atrazine	Azoxystrobin
Cottonwood Creek	2010-05-13	2.4	804	nd	nd
	2010-06-22	2.6	954	nd	nd
	2010-07-13	2.1	611	nd	nd
	2010-08-23	2.8	877	nd	nd
	2010-09-15	2.4	286	nd	nd
Dry Creek	2010-06-21	1.0	557	99.4	nd
	2010-08-02	1.0	1141	nd	nd
	2010-08-23	0.9	1509	nd	nd
	2010-09-15	1.0	203	1.1	nd
Sand Run Gulch	2010-05-03	1.6	751	nd	42.0
	2010-07-06	1.4	369	90.9	44.8
	2010-07-26	1.4	504	nd	190
	2010-09-09	1.6	161	9.1	60.8
	2010-09-29	1.6	944	86.6	129
	2010-10-18	1.7	71	4.1	2.5
	2010-11-08	1.9	80	nd	nd
Wanstad Ditch	2010-08-16	0.8	616	nd	222

nd: not detected

Table B.2 (cont.) Estimated pesticide concentrations (µg/g wet weight) of individual black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Boscalid	Ethalfluralin	Hexazinone	Imazalil	Metolachlor
Cottonwood Creek	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
Dry Creek	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
	nd	nd	nd	nd	nd
Sand Run Gulch	69.6	0.94	62.0	136	nd
	12.3	0.14	35.3	120	163
	56.2	17.5	30.1	188	nd
	12.8	1.3	nd	25.3	23.0
	100	14.9	nd	19.8	154
	2.0	5.1	nd	4.7	7.5
	nd	nd	nd	nd	nd
Wanstad Ditch	53.9	0.2	61.4	171	181

nd: not detected

Table B.2 (cont.) Estimated pesticide concentrations (µg/g wet weight) of individual black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Site	Pendimethalin	Pyraclostrobin	Simazine	Trifluralin
Cottonwood Creek	nd	nd	34.4	25.8
	nd	nd	15.3	4.8
	nd	nd	nd	18.8
	nd	nd	9.0	nd
	nd	nd	nd	nd
	nd	nd	131	36.5
Dry Creek	nd	nd	29.3	19.5
	nd	nd	nd	24.3
	nd	nd	1.6	3.9
Sand Run Gulch	34.5	nd	nd	0.85
	6.8	nd	8.6	nd
	127	215	31.2	21.7
	17.5	120	nd	1.8
	131	270	51.3	19.3
	6.2	11.5	5.5	6.7
	nd	nd	nd	nd
Wanstad Ditch	5.5	117	nd	0.53

nd: not detected

APPENDIX C

Calculations for Spore Enumeration and Fungicide Dosing

Calculations for Spore Enumeration and Fungicide Dosing

Spores of *Smittium simulii* (JAP-51-5) were enumerated using a C-Chip Neubauer Improved disposable hemocytometer (INCYTO, Korea). Each spore slurry was manually shaken or stirred with the pipette tip to resuspend the spores, and 10 µl were aliquoted onto the hemocytometer. The number of viable (refractive) spores was counted at 200x on each grid three times and averaged. The slide was imaged using the same software as described in the methods (Fig. C.1). This was repeated twice for each treatment to estimate the concentration of spores per milliliter (S_{conc}). A volume needed to inoculate each experimental unit with the same concentration of spores was then calculated. To determine S_{conc} the following equation was used:

$$S_{conc} = S_{num} \times 10^4$$

Where S_{num} is the average number of spores per large square (area = 1 mm^2) and 10^4 is the volume factor for the area. Since the spore slurry was fully concentrated, no dilution factor was included. For MC-1, the desired S_{conc} was 1200 and for MC-2 and -3, 500 spores/mL. Each treatment jar contained 300 mL of water, so the total number of spores per jar (S_{jar}) was 360,000 spores/mL for MC-1, and 15,000 spores/mL for MC-2 and -3 (Table C.1). The volume (mL) of spore slurry needed to add to each jar (S_{vol}) to achieve the desired S_{conc} was calculated as follows:

$$S_{vol} = S_{jar}/S_{conc}$$

From there, S_{vol} (mL) could be multiplied by the number of doses needed for the experiment to determine the total volume of spore slurry needed. If the volume of the spore slurry available was less than what was needed, then the slurry was supplemented with another that had more spores per milliliter (Table C.1).

Table C.1. Estimated pesticide concentrations (µg/g wet weight) of individual black fly larvae collected from reference sites (Cottonwood Creek, Dry Creek) and agricultural sites (Sand Run Gulch, Wanstad Ditch).

Microcosm	Treatment	S_{num}	Slurry Tube Volume (mL)	Volume factor	S_{conc}	Desired # spores/ mL	S_{jar}	S_{conc}/S_{jar}	S_{vol} (mL)
MC-1	CON	33	9	10000	330000	1200	360000	0.9	1.1
	ACE	37	10	10000	370000	1200	360000	1.0	1.0
	5 ng/L	40	10	10000	400000	1200	360000	1.1	0.9
	250 ng/L	39	10	10000	390000	1200	360000	1.1	0.9
	750 ng/L	38	10	10000	380000	1200	360000	1.1	0.9
MC-2	CON	31	30	10000	312500	500	150000	2.1	0.5
	ACE	28	30	10000	276000	500	150000	1.8	0.5
	5 ng/L	43	30	10000	432000	500	150000	2.9	0.3
	250 ng/L	15	30	10000	152000	500	150000	1.0	1.0
	750 ng/L	35	30	10000	350000	500	150000	2.3	0.4
	5000 ng/L	20	30	10000	198000	500	150000	1.3	0.8
MC-3	CON	18	30	10000	177500	500	150000	1.2	0.8
	ACE	32	15	10000	322500	500	150000	2.2	0.5
	5 ng/L	29	30	10000	287000	500	150000	1.9	0.5
	250 ng/L	45	10	10000	450000	500	150000	3.0	0.3
	750 ng/L	32	15	10000	322500	500	150000	2.2	0.5
	5000 ng/L	45	10	10000	450000	500	150000	3.0	0.3
	TEG	45	10	10000	450000	500	150000	3.0	0.3

S_{num}: average number of spores per large square
S_{conc}: concentration of spores per milliliter
S_{jar}: total number of spores per jar
S_{vol}: volume (ml) of spore slurry needed to add to each jar

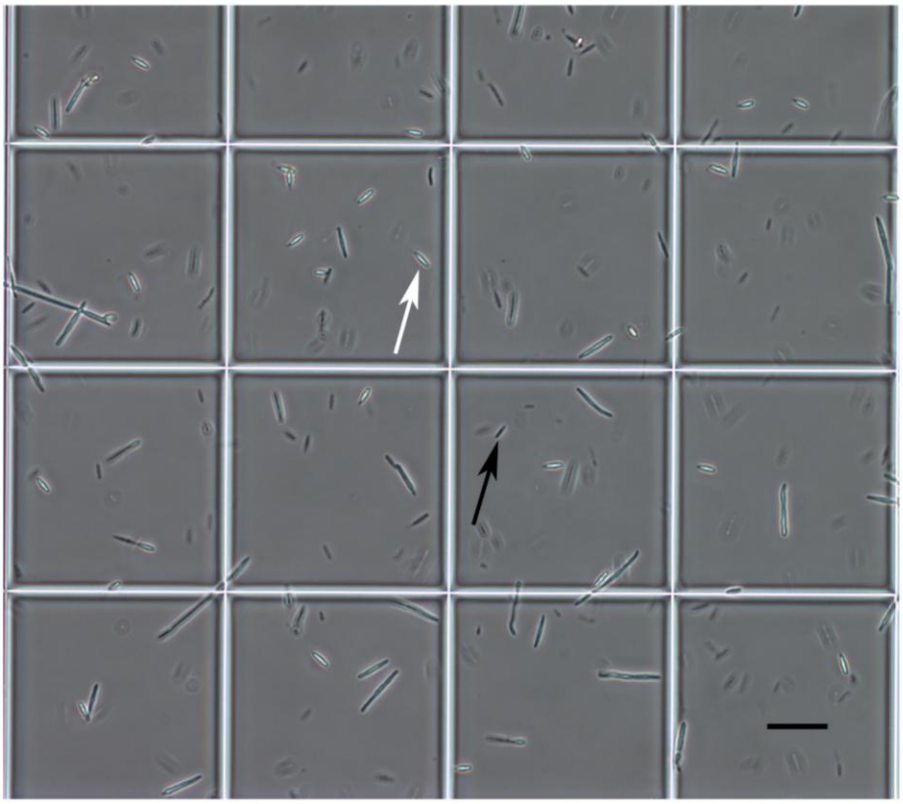

Figure C.1. Hemocytometer image of *Smittium simulii* (JAP-51-5) spores isolated from culture (100x). White arrow indicates a refractive spore, black arrow shows non-viable spore. Scale bar = 100 µm.

APPENDIX D

Images of Insect Guts and Microcosm Setup

Images of Insect Guts and Microcosm Setup

Table D.1. Identified black fly larvae from 2010 samples. N refers to the number of black fly larvae indentified from that site over the eight month sampling period (421 total).

Site	Species	Frequency	N
Cottonwood Creek	*Simulium piperi*	0.99	170
	Simulium canadense	0.01	
Dry Creek	*Simulium tuberosum*	0.07	135
	Simulium piperi	0.27	
	Simulium canadense	0.65	
Sand Run Gulch	*Simulium* sp.	0.88	101
	Simulium vittatum	0.12	
Wanstad Ditch	*Simulium* sp.	0.13	15
	Simulium vittatum	0.87	

Figure D.1. Composite image of black fly peritrophic matrix colonized by *Harpella* sp. from reference site Cottonwood Creek (slide no. ID-84-E1). Scale bar = 100 µm.

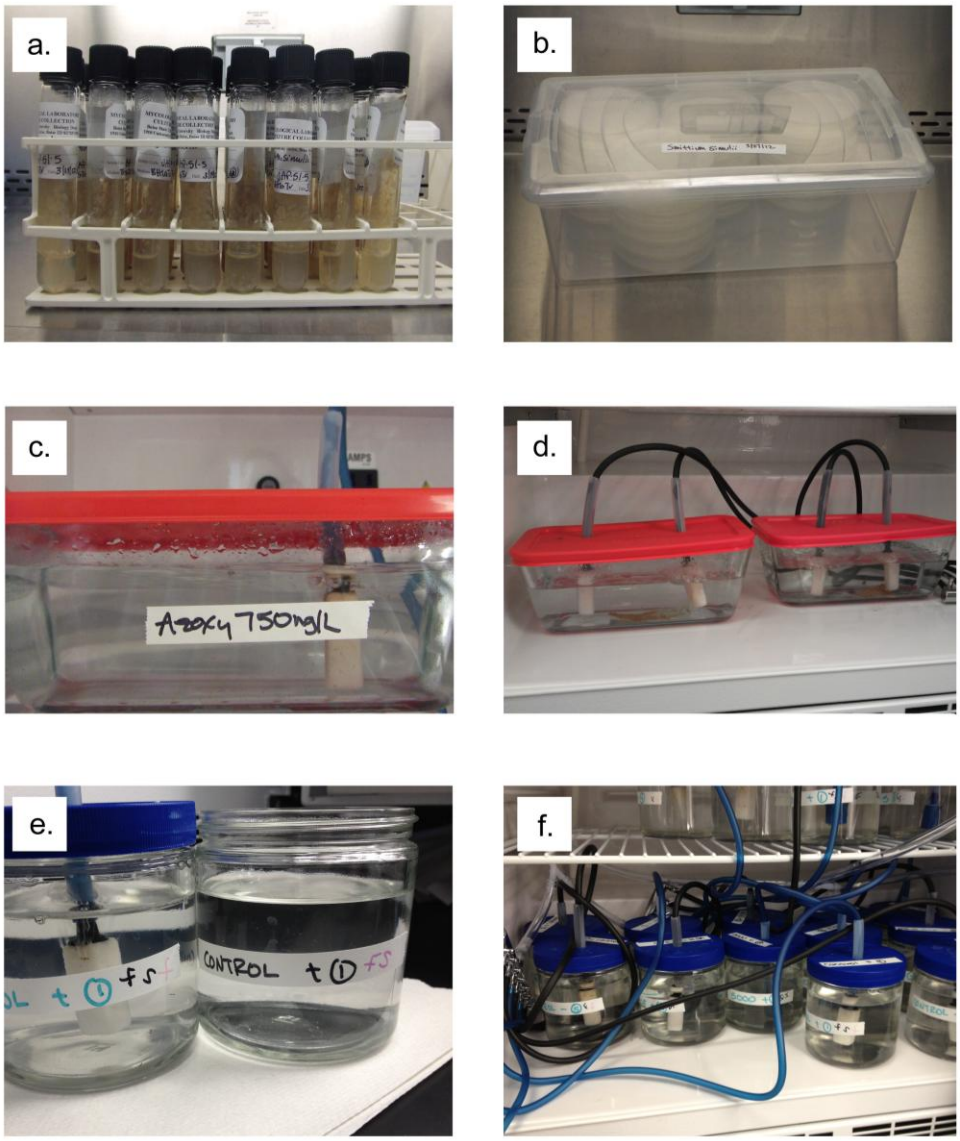

Figure D.2. Microcosm supplies and setup. *Smittium simulii* (JAP-51-5) growing in (a) slants and (b) 100mm petri dishes. (c, d) Rearing containers for black fly larvae showing air bubblers and tubing. (e,f) Experimental containers with air bubblers, tubing, and manifold setup (f).

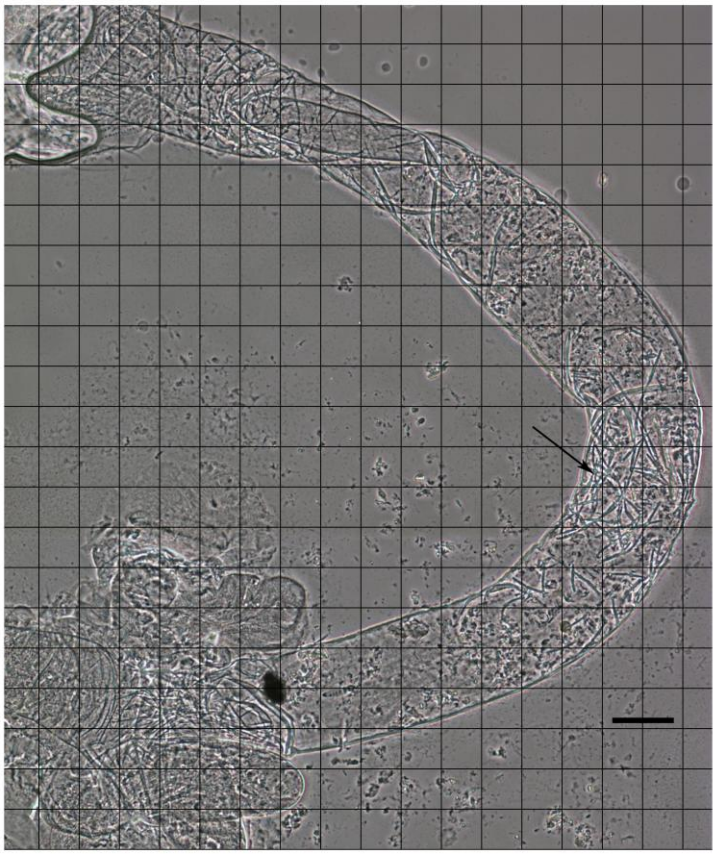

Figure D.3. Composite image of black fly hindgut with 1 mm^2 grid overlay from MC-2 (slide no. MC2-A5-2-G1). Scale bar = 100 µm. Arrow pointing to a grid with thalli.

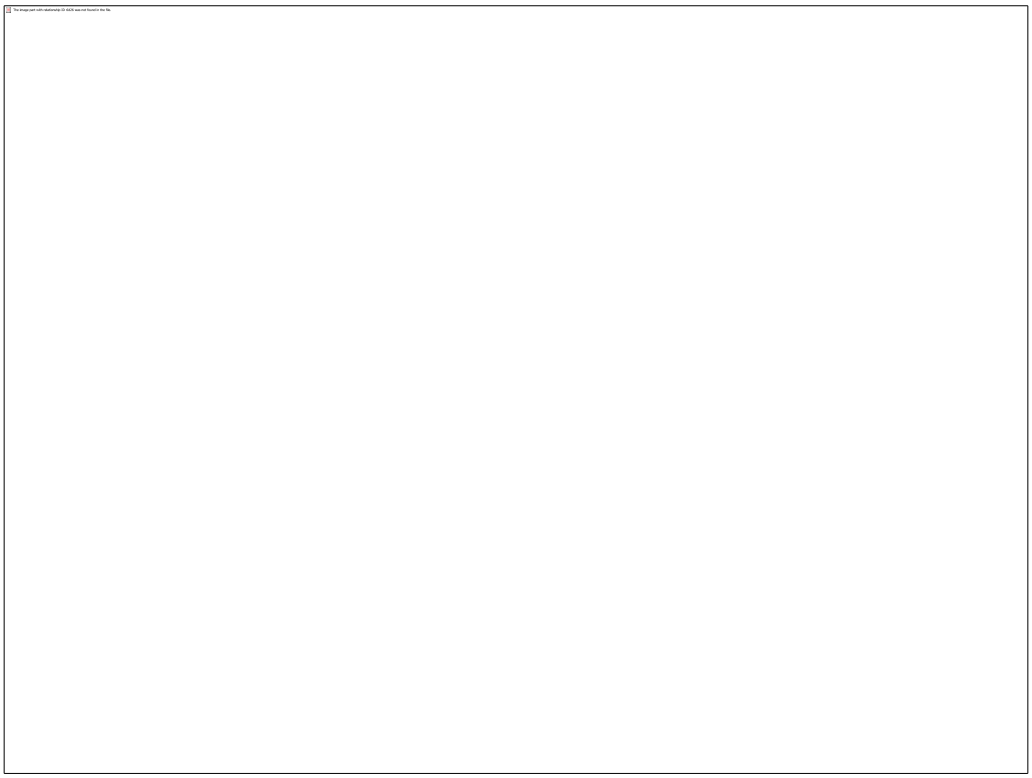

Figure D.4. Larval black fly head capsule, cleared with KOH and suspended in glycerin (100x). Arrows are pointing to antennal buttresses. Scale bar = 100 µm.

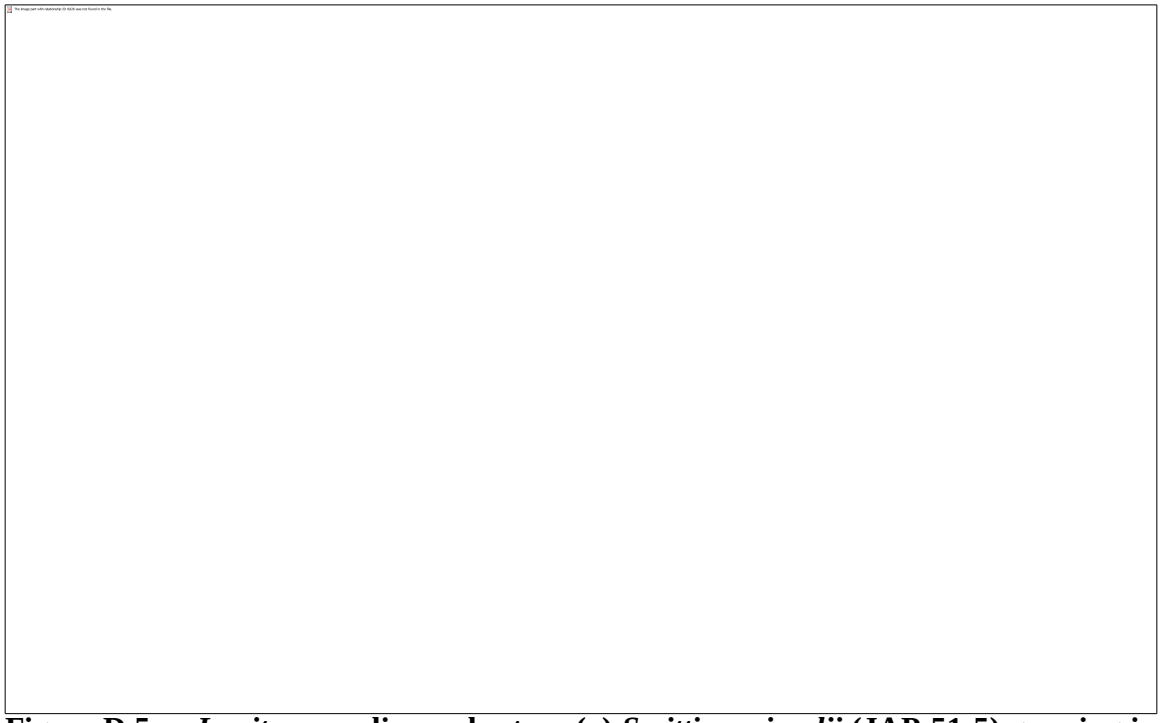

Figure D.5. ***In vitro*** **supplies and setup. (a)** ***Smittium simulii*** **(JAP-51-5) growing in 250 ml Erlenmeyer flasks with cotton stoppers in incubated shaker; (b)** ***S. simulii*** **after 4 days of growth (Experiment 1); (c–e) filtration process for** ***S. simulii*** **after growth on the shaker prior to drying.**