Published in final edited form as:

J Am Water Resour Assoc. 2014 April 1; 50(2): 420–433. doi:10.1111/jawr.12166.

ASSESSING THE POTENTIAL EFFECTS OF FUNGICIDES ON NONTARGET GUT FUNGI (TRICHOMYCETES) AND THEIR ASSOCIATED LARVAL BLACK FLY HOSTS

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Abstract

Fungicides are moderately hydrophobic and have been detected in water and sediment, particularly in agricultural watersheds, but typically are not included in routine water quality monitoring efforts. This is despite their widespread use and frequent application to combat fungal pathogens. Whereas the efficacy of these compounds on fungal pathogens is well documented, little is known about their effects on nontarget fungi. This pilot study, a field survey in southwestern Idaho from April to December 2010 on four streams with varying pesticide inputs (two agricultural and two reference sites), was conducted to assess nontarget impact of fungicides on gut fungi, or trichomycetes. Tissues of larval black flies (Diptera: Simuliidae), hosts of gut fungi, were analyzed for pesticide accumulation. Fungicides were detected in hosts from streams within agricultural watersheds but were not detected in hosts from reference streams. Gut fungi from agricultural sites exhibited decreased percent infestation, density within the gut, and sporulation, and black fly tissues had elevated pesticide concentrations. Differences observed between the sites demonstrate a potential effect on this symbiotic system. Future research is needed to parse out the details of the complex biotic and abiotic relationships; however, these preliminary results indicate that impacts to nontarget organisms could have far-reaching consequences within aquatic ecosystems.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article. The supporting information contains detailed information on the methods used to measure pesticides in black fly larval samples. These include the following: instrument parameters, quality assurance, and detection limits of pesticides. Tables S1–S6 and Figure S1 are also included in the supporting information.

Keywords

agriculture; aquatic fungi; black fly; fungicides; nontarget

INTRODUCTION

Modern agricultural practices throughout the world rely on the use of pesticides to combat the effects of an array of pathogens on economically important crops. Inevitably, some of these pesticides are transported to nearby streams via drift, runoff, and infiltration into the groundwater (Rasmussen *et al.*, 2012; Reilly *et al.*, 2012a; Relyea and Hoverman, 2006). In addition, many agricultural areas require field side streams for irrigation purposes, making them susceptible to contamination by pesticides, which are often found at high concentrations (Gilliom, 2007). Indeed, pesticide application to agricultural fields and resulting runoff may be one of the greatest stressors to aquatic ecosystems, with entire communities inadvertently exposed to pesticides (Kolpin *et al.*, 2002; Rasmussen *et al.*, 2012; Relyea and Hoverman, 2006).

Pesticides, at both acute and chronic levels, can impact nontarget organisms, including arthropods. Stream macroinvertebrates can be negatively affected by decreased population densities (Hurd *et al.*, 1996) when exposed to nonpoint source pesticide pollution. Sorption and accumulation of pesticides has been documented in macroinvertebrates, including in the silk of black fly larvae (Brereton *et al.*, 1999). Effects of pesticides vary depending on whether they are acting singularly or interactively (Elskus, 2012). For instance, in the presence of a pyrethroid insecticide, fungicide toxicity to the aquatic crustacean *Daphnia magna* is significantly increased (Norgaard and Cedergreen, 2010).

Fungicides, a group of pesticides that target fungal pathogens, are of particular concern because of the increased tendency for their reapplication during the growing season – in some cases, as much as 10 times per season (Reilly *et al.*, 2012a). In agricultural areas in the United States and Europe, fungicides have been detected 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; Smalling *et al.*, 2013) at concentrations that have the potential to cause adverse effects on a range of 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 nontarget organisms, such as macroinvertebrates (Elskus, 2012; Gustafsson *et al.*, 2010). Thus, there has been an increased awareness, interest, and concern about how fungicides may be affecting nonpathogenic fungi (Dijksterhuis *et al.*, 2011; Maltby *et al.*, 2009). Whereas several studies have focused on aquatic 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), trichomycetes, or gut fungi, are a widespread group of fungi that have not yet been studied in this regard.

Gut fungi are a cosmopolitan group of symbiotic arthropod associates. As endosymbionts, they live in the digestive tracts 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). Studies have shown their mutualistic potential (Horn and Lichtwardt, 1981) and demonstrated parasitism (Sweeney, 1981), although the fungi are more generally 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 short- and long-term evolutionary time scales (Hibbett *et al.*, 2007; McCreadie *et al.*, 2011; White, 2006). Beyond being excellent hosts of gut fungi, black fly larvae play important roles in stream ecosystems. They are ecosystem engineers that not only turn over resources in food webs but also serve as a dominant food source for fish and other predators (Wallace and Webster, 1996).

The primary goal of this first assessment was to evaluate the seasonal impact of exposure to broad-spectrum fungicides and other pesticides on gut fungi and their aquatic insect hosts (black fly larvae). The specific objectives of the study were to 1) survey surface waters known to contain fungicides to determine percent infestation and density of gut fungi of black fly larvae residing in those sites and to assess dissolved concentrations of fungicides in the water column, 2) measure fungicide and other pesticide concentrations in black fly host tissue, and 3) compare these metrics with those collected from reference streams. Larval black flies from agriculturally dominated streams containing fungicides were predicted to have a lower percent infestation and density of gut fungi compared to hosts from reference streams.

METHODS

Site descriptions and sample collection

The four selected study sites in Idaho (Figure 1, Table 1) included two agricultural streams (Sand Run Gulch and Wanstad Ditch) and two reference streams (Cottonwood Creek and Dry Creek). The former were selected because they drain high use agricultural areas (Table 1) and fungicides have been detected in each stream throughout the growing season (Reilly *et al.*, 2012a; Reilly *et al.*, 2012b). The latter were selected because they are known to contain insect larvae with robust and well-characterized populations of gut fungi (Bench and White, 2012; Kandel and White, 2012). The reference locations were also chosen based on proximity, accessibility, and, as much as possible, similarity to each other in terms of riparian vegetation and streambed substrate. The agricultural streams were also sampled prior to the start of the survey to ensure there were appropriate hosts for gut fungi.

To assess seasonal differences in pesticide concentrations and gut fungi density over time, 12 water samples were collected from each of the agricultural streams, and 10 and 11 samples were collected from Dry Creek and Cottonwood Creek, respectively, between April and December of 2010. Samples were collected approximately biweekly starting in April for the two agricultural streams and Cottonwood Creek and starting in June for Dry Creek. This spans the typical growing season in Idaho (Reilly *et al.*, 2012a; Reilly *et al.*, 2012b). Sample collection did not target runoff events or other specific hydrologic conditions.

Water quality sampling followed the same procedures at all sites. Samples were collected by immersing pre-cleaned, amber glass bottles (one liter for pesticides and 125 ml for dissolved

organic carbon (DOC)) once at each site to a depth of not less than 0.1 m below the water surface. A one liter polyurethane bottle was rinsed three times with stream water, and then filled for determination of basic water quality characteristics (specific conductance in microseimens per centimeter at 25°C ($\mu\text{S}/\text{cm}$), pH in standard units, and turbidity in nephelometric turbidity units (NTU)). Samples were then packed on ice and shipped overnight from Idaho to the USGS Organic Chemistry Laboratory in Sacramento, California, for extraction and analysis. Site descriptions, watershed characteristics, and water sampling techniques are further detailed in Reilly *et al.* (2012b).

Host sampling and dissection

During each sampling event, a 10–20 m long stream section was sampled for immature stages of black flies using kick-nets and/or methanol-cleaned forceps to pick specimens from dangling vegetation and rocks within riffle zones. A minimum of 15–20 black fly larvae were targeted for assessment of gut fungi and tissue pesticide analysis. These were transported in stream water held on ice to Boise State University and stored at 4°C for up to 48 hours prior to dissection. If 15–20 hosts were not available within 90 minutes of searching, all collected insects were used exclusively for fungal metrics. When greater than 20 individuals were recovered in the field, additional subsamples were placed in glass vials using methanol-cleaned forceps and stored at –20°C prior to extraction and analysis of tissue for currently used pesticides.

Hosts were dissected and gut fungi prepared per Bench and White (2012). 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 and stained in lactophenol cotton blue to later identify the gut fungi to genus. 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 each sampling occasion.

Fungal density and sporulation in larval black fly midguts

Digital images of dissected, slide-mounted larval black fly peritrophic matrices (the chitinous lining of the midgut, referred to hereafter as PMs) were used to assess the density of thalli (individual fungal hyphae) and trichospores (asexual sporangiospores) of *Harpella* spp. (Figure S1). Before fixation in lactophenol cotton blue, the PM, freshly mounted in distilled water, was viewed and digital images were captured according to Bench and White (2012). Thalli and trichospores were counted directly. Previous studies (Beard and Adler, 2002; McCreadie and Beard, 2003) have used grids and ratios to estimate the amount of fungi within the gut. In this case, an exact count was possible by focusing on and enumerating the holdfasts (the structure that attaches the fungus to the PM), even when unbranched but overlapping thalli were present as dense masses in some of the PMs. 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 .

Extraction and analysis of surface water and tissue for pesticides

Surface water samples (one liter) were filtered using 0.7 μm glass fiber filters, extracted onto Oasis HLB solid phase extraction cartridges, dried, eluted with ethyl acetate, reduced, and analyzed for a suite of 90 pesticides by gas chromatography-mass spectrometry operating in electron ionization mode (GC-EIMS) (Hladik *et al.*, 2008; Reilly *et al.*, 2012b). Information on GC-EIMS settings, quality assurance (QA) parameters, and method detection limits are outlined in the Supplemental Information (SI).

Thawed composite black fly larvae samples (0.12 to 2.5 g), containing between 70 to 1500 individuals from each site, were analyzed for a suite of 12 current use pesticides. In total, seventeen composite larval tissue samples were analyzed based on the compounds historically detected in the water column (Reilly *et al.*, 2012b). Prior to extraction, tissue samples were spiked with trifluralin-d10, ring- ^{13}C -*p,p'*-DDE and phenoxy- ^{13}C -*cis*-permethrin (Cambridge Isotopes, Andover, Massachusetts) as recovery surrogates. All samples were homogenized with Na_2SO_4 and extracted two times with dichloromethane using a sonic water bath at 30° C for 25 min. 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 nondetectable amounts of lipid, no cleanup was necessary. Samples were exchanged to ethyl acetate and further reduced to 200 μl , and an internal standard was added to each sample prior to analysis.

Chromatographic analyses were performed using an Agilent 7890 gas chromatograph coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies, Folsom, California, USA) operating in multiple reaction monitoring mode (MRM). Details of MRM transition, collision energy, and limits of detection are described in Table S1 (see SI), where a complete description of the method, along with more information on QA parameters and body burden calculations, are also outlined.

Statistical analysis

Percent infestation was calculated as the number of black flies whose guts contained thalli relative to the total number of black flies in each sample, by site and collection date. To compare thalli and spore density at each site, a mixed linear model on the log transformed data (observed density plus an insubstantial number to allow a log transform when no thalli or spores were found) was employed. These models were used to compare paired sampling dates between Sand Run Gulch and each of the reference sites, and to compare change over sampling dates within each location. Each set of comparisons (for thalli and spore densities) were adjusted for multiple corrections using a Bonferoni correction. The final model included fixed effects for site, date, and their interaction, and separate residuals for each site/date combination to accommodate unequal variance in the observations. Initially, more complex covariance structures were considered to account for within-site correlation and within-sampling date correlation, but these models either did not converge or, if they did, would not allow the specific comparisons needed. Additionally, any models considered with these estimated correlations had higher Akaike's Information Criterion (AICC, adjusted for small sample size, Burnham and Anderson, 2002) and poor fit based on residual plots;

hence, the simpler model was used. Wanstad Ditch was not included in either model due to insufficient or empty (devoid of gut fungi) black fly larvae recovered for density calculations. The spore density model did not converge with the addition of Dry Creek data from June 22 as none of the black flies sampled had any gut fungi present; therefore, this site/date combination was not included in that model.

Sets of comparisons that were corrected for multiple testing included pairwise comparisons of dates within each stream (three sets) and all date comparisons that had a suitable match (i.e., that fell within 7 days) (one set). All analyses were done in SAS version 9.3 using PROC MIXED.

RESULTS

Percent infestation of gut fungi

Black fly larvae were present in sufficient numbers ($n = 15$) at both reference sites and for 11 of the 12 sampling events at the agricultural site, Sand Run Gulch. In contrast, hosts were absent for six of the twelve sampling events at the second agricultural site, Wanstad Ditch, even though the habitat appeared suitable for black flies (Adler *et al.* 2004). During three of the six sampling events at Wanstad Ditch where black fly larvae were present, only one to seven individuals were collected. Percent infestation of black fly larvae with gut fungi was almost always higher in the reference sites compared to the agricultural sites (Figure 2). At the reference sites, Cottonwood Creek had 100% infestation rates for all sampling events (Figure 2a), and Dry Creek had an average 93% infestation rate (Figure 2b). At the agricultural sites, Sand Run Gulch had an average infestation rate of 54% (Figure 2c) and Wanstad Ditch had an average 33% infestation rate (Figure 2d). The highest percent infestation rate at Sand Run Gulch was observed from July through September (100% for all dates) (Figure 2c). While these rates are comparable to the reference sites, this does not take into account the actual number of fungi within the gut. Therefore, the densities of thalli and spores in the gut were enumerated.

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. Of interest at the reference sites, thalli densities from the June and early July sampling dates were statistically lower than sampling dates during August through November (with the exception of 4 October at Cottonwood Creek and 15 September at Dry Creek) (Figure 3). Spore densities at the reference sites were more consistent over the sampling period showing spikes on 26 October at Cottonwood Creek and 4 October and 22 November at Dry Creek (Figure 4). At Sand Run Gulch, thalli and spore density both were significantly higher on 16 August than sampling dates from June and July (Figures 3 and 4). Due to insufficient numbers of black flies and *Harpella* thalli recovered, no statistical analysis was conducted for the agricultural site Wanstad Ditch.

Densities were compared between Sand Run Gulch and each of the reference sites at six paired sampling dates. Thallial densities at Dry Creek were statistically significantly higher on five of the six dates, with the magnitude of the difference increasing throughout the

sampling period (Table 2). At Cottonwood Creek, densities were significantly higher on three of the sampling dates in mid June, late August, and early September (Table 2). Spore density was not significantly different from Sand Run Gulch at either reference site in June, early July, or August. However, spore density was significantly higher at both reference streams in the two September comparisons (Table 2).

For reference sites, the maximum number of thalli/ μm^2 was 2.9×10^{-4} and 2.6×10^{-4} at Cottonwood Creek and Dry Creek, respectively (Figure 3). Cottonwood Creek had a maximum number of spores/ μm^2 of 3.6×10^{-4} while Dry Creek had 3.2×10^{-4} spores/ μm^2 (Figure 4). At agricultural sites, the maximum number of thalli/ μm^2 was 0.8×10^{-4} and 0.04×10^{-4} at Sand Run Gulch and Wanstad Ditch, respectively (Figure 3). The maximum number of spores/ μm^2 at Sand Run Gulch was 0.4×10^{-4} . Wanstad Ditch had even fewer with 0.08×10^{-4} spores/ μm^2 (Figure 4).

Pesticides in surface water and black fly tissue

Fungicides were not detected in any surface water samples collected from the reference sites (Table S2). Only two pesticides were detected in samples from these reference sites during the course of this study. In surface water samples from the agricultural sites, twenty-two pesticides (eight fungicides, ten herbicides, two insecticides, and two degradates) were detected (Table S2). The fungicides azoxystrobin and boscalid were detected in eleven of the twelve (92%) water samples collected from Wanstad Road Ditch and in eight of the twelve (67%) sampling events at Sand Run Gulch.

Selected black fly larvae were analyzed directly for a suite of twelve pesticides (four fungicides and eight herbicides) based on the detection frequency and maximum concentrations observed in surface water samples during the study. A total of seventeen larval tissue samples were analyzed, five from Cottonwood Creek, four from Dry Creek, one from Wanstad Ditch, and seven from Sand Run Gulch. No fungicides were detected in any larval tissue samples from the reference sites. The herbicides atrazine, simazine, and trifluralin were detected in 22 %, 67 %, and 67 %, respectively, of the larval tissues from reference sites with composite concentrations ranging from 5–234 $\mu\text{g/kg}$ wet weight (Table S3). A total of eleven pesticides (four fungicides and seven herbicides) were detected in larval tissue samples from agricultural sites with detection frequencies ranging from 50–88 % and composite concentrations ranging from 21 to 840 $\mu\text{g/kg}$ wet weight (Table 3). Azoxystrobin, one of the most frequently detected fungicides in the United States (Battaglin *et al.*, 2011), and boscalid, the most frequently detected fungicide in the study areas (Reilly *et al.*, 2012a), were detected in 85% of the tissue samples and 71% of the water samples, respectively.

Within the agricultural sites, fungicides were detected frequently in surface water and larval tissue from Sand Run Gulch throughout the sampling period (Table 3). However, Wanstad Ditch only yielded enough larval black flies for one tissue sample (Tables S3, S4). All four of the fungicides analyzed were detected in larval black fly tissue at Sand Run Gulch. Of those detected, only azoxystrobin and boscalid were detected in the surface water samples that corresponded with the tissue samples; indeed, pyraclostrobin had the highest composite tissue concentration of any of the fungicides (Table 3). Composite tissue concentrations of

all fungicides detected were highest in the July and early September samples, which also corresponded to the highest surface water concentrations (Table 3).

DISCUSSION

This is the first exploration of fungicide effects on gut fungi and these results contribute to a better understanding of pesticide impacts across trophic levels in aquatic systems. Current-use fungicides have the potential to effect nontarget organisms, and current test organisms may not be adequate in predicting the effects of fungicides on nontarget fungi (Maltby *et al.*, 2009). The organisms selected for this study are nontarget fungi that exist as endosymbionts of stream invertebrates, thus giving a potential glimpse into the trophic consequences that may be impacted from fungicide exposure.

The metrics used for assessing effects on insect hosts were abundance of black fly larvae and estimations of body burden of select pesticides in their tissue. Potential habitats for black flies are quite varied and include impacted areas such as irrigation channels and drainage ditches (Crosskey, 1990) similar to those sampled at the agricultural sites. However, black fly larvae can have a decreased abundance and species composition in agricultural streams (Pramual and Kuvangkadilok, 2009). Black fly larvae were absent for approximately half of the sampling events at the agricultural site Wanstad Ditch (Figure 2), which may have been due to the presence of contaminants, life history, or environmental conditions (Beard *et al.*, 2003). Pesticides have been detected in up to 97% of surface water near agricultural land and often at least two pesticides are present per water sample (Gilliom, 2007). In the current study, fungicides were detected in nearly 80% of the samples collected from the agricultural streams, which is likely related to repeated field application throughout the growing season (Reilly *et al.*, 2012a). With this high frequency of detection, the organisms living in these waterways are potentially receiving chronic exposure to a mixture of pesticides. In this instance, fungicides were detected at very high concentrations in their tissue: orders of magnitude higher than were detected in the surrounding water column (Table 3).

Fungal percent infestation, density, and spore production in sampled black fly midguts were used to assess the health and fitness of gut fungi. On average, black fly larvae from the reference sites were nearly 100% infested compared to 33–54% at agricultural sites (Figure 2). 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 even more rarely taken into account in other studies. Certain gut fungus colonization rates can differ between species, although PM-dwelling *Harpella* spp. typically have the highest colonization rates regardless of host taxa (Beard and Adler, 2002; Lichtwardt and Williams, 1988). In the current study, various larval *Simulium* spp. were collected (Table S5), but it was assumed that all species had an equal probability of being colonized by *Harpella* spp. Therefore, the low number of gut fungi in agricultural sites is more likely due to an effect of stream conditions, including fungicide exposure, than differences in host species.

The reference sites had at least an order of magnitude higher density and spore production in larval black fly PMs compared to those in agricultural sites, but there was a great deal of variation in both number of thalli and spores over time (Figures 3 and 4). 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). However, prior to this study, the influence of chronic nonpoint source pesticide exposure in a natural setting had not been explored. Future investigations modeling variables of season, environment, host species, and other factors (including pesticide concentrations) would offer a window into the forces driving fungal patterns observed in this symbiotic system.

Fungicides were detected frequently in the tissue of black flies collected from Sand Run Gulch throughout the sampling period (Table 3). It is difficult to put these data into context as there are few field studies available for the presence of current use fungicides in aquatic insect tissue, although several laboratory studies have documented the effects of organophosphate insecticides (chlorpyrifos and diazinon) on aquatic insects (Buchwalter *et al.*, 2004; Stuijzand *et al.*, 2002). It has been 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 (Stuijzand *et al.*, 2002). Several field studies have documented current use pesticides in other aquatic tissues including crab embryos (Smalling *et al.*, 2010), sand crabs (Anderson *et al.*, 2010; Dugan *et al.*, 2005), and fish (Dugan *et al.*, 2005; Sapozhnikova *et al.*, 2004). The precise effects of fungicides within the host tissue on fungal endosymbionts are unknown and are difficult to separate from the possible effects of dissolved fungicides, whether in the water itself, bound with particulates, or passing through the hosts.

Fungicides constituted 37–81 % of the pesticide mass detected in each individual tissue sample. Composite concentrations for individual fungicides ranged from 21–840 µg/kg wet weight (Table 3). The median body burdens of the detected fungicides and herbicides were 65.2 and 18.2 µg/g wet weight, respectively. Where pesticides were detected in the water column, pesticides in individual black fly larvae constituted between 0.01 and 0.1 % of the mass of an individual larva (Table S3). The estimated body burden on a black fly larva is substantially higher than what has been observed in other studies (Dugan *et al.*, 2005; Sapozhnikova *et al.*, 2004; Smalling *et al.*, 2010). Whereas organismal effects, including effects on fungi and exposure pathways in the field, are still unknown, future research is needed to determine not only how the accumulation directly affects the gut fungi within the host, but also, more broadly, how this could affect whole aquatic systems.

As it currently stands, the role and responsiveness of symbiotic gut fungi within their arthropod hosts is not completely understood. However, if fungicides in streams are impacting nontarget mutualistic, endosymbiotic fungi, the symbionts may not be available or sensitive to the needs of their host in times of stress (i.e., during exposure to other pesticides and toxicants). Gut fungi are also common in aquatic bioindicator taxa, including but not limited to, Ephemeroptera, Plecoptera, and Trichoptera (Lichtwardt, 1986), but to date there have not been any studies examining the relationship between host sensitivity to pesticides or other stressors and the prevalence of gut fungi in these taxa.

This pilot study shows a significant difference in gut fungus densities in agricultural compared to reference streams while taking into account change over time. Given the nature of this study, direct causation cannot be explicated; however, the lack of hosts and fungi at Wanstad Ditch, the increased detection frequency of fungicides, and the correspondingly high amount of accumulation within the black fly tissue is intriguing and clearly invites further effort. These findings all point toward environmental stressors possibly influencing the symbiotic system. Research is needed that distinguishes between, and ascertains the significance of, natural and varying environmental conditions and the presence of dissolved pesticides as potential stressors affecting gut fungi in nonpristine habitats.

Future studies should continue to explore 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. There is a need to monitor aquatic ecosystems for fungicides, as demonstrated by the potential impact on nontarget fungi, such as gut fungi and their black fly hosts. This is yet another example of the potential of pesticides to affect nontarget organisms, but this may represent only a small fraction of impacts amongst the myriad cascades and associations connecting organisms within watersheds. Such delineations, formed holistically, would pave the way for connecting the interface between host, symbiont, stream health, and environment in this complex web of interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Research was funded by the National Science Foundation DEB 098182 and generous support from the US Geological Survey Toxic Substances Hydrology Program. The authors would like to thank the following members of the USGS for their support: Kathryn Kuivila, Mark Hardy, William Battaglin, Adria Elskus, Megan McWayne-Holmes, Jim Orlando and Alexandria Ethridge; graduates and undergraduates at Boise State University: Sarah Oman, Korinne Wade, Donavon Carrie, Yan Wang, Eric Tretter, and Eric Johnson; Other personnel at BSU: Julie Heath, and Department of Biological Sciences; as well as Rusty Rodriguez (USGS), Mike Thornton and Bill Buhrig (University of Idaho), Eddie Beard and Peter Adler (Clemson University), Bruce Horn (USDA), and Robert Lichtwardt (University of Kansas). This work was supported in part by the National Institute of General Medical Sciences of the National Institutes of Health under Awards Number P20RR016454 and P20GM103408.

LITERATURE CITED

- Adler, PH.; Currie, DC.; Wood, DM. The Black Flies (Simuliidae) of North America. New York: Comstock Pub. Associates; 2004. ISBN-13:9780801424984
- Adler, J. R in a Nutshell. Sebastopol, California: O'Reilly Media, Inc.; 2010. ISBN: 978059680170-0
- Anderson B, Phillip B, Hunt J, Siegler K, Voorhees J, Smalling K, M K, Adams M. Watershed-scale evaluation of agricultural BMP effectiveness in protecting critical coastal habitats. Final Report. California State Water Resources Control Board. 2010
- Battaglin W, Fairchild J. Potential Toxicity of Pesticides Measured in Midwestern Streams to Aquatic Organisms. Water Science and Technology. 2002; 45(9):95–102. [PubMed: 12079130]
- Battaglin W, Sandstrom M, Kuivila K, Kolpin D, Meyer M. Occurrence of Azoxystrobin, Propiconazole, and Selected Other Fungicides in US Streams, 2005–2006. Water, Air, and Soil Pollution. 2011; 218:307–322.
- Beard CE, Adler PH. Seasonality of Trichomycetes in Larval Black Flies from South Carolina, USA. Mycologia. 2002; 94:200–209. [PubMed: 21156489]

- Beard CE, McCreadie JW, Adler PH. Prevalence of the Trichomycete Fungus *Harpella melusinae* (Harpellales: Harpellaceae) in Larval Black Flies (Diptera: Simuliidae) Across a Heterogeneous Environment. *Mycologia*. 2003; 95:577–583. [PubMed: 21148966]
- Bench, ME. New Species and Expanded Geographic Ranges of Gut Fungi and their Symbiotic Relationship with Insect Hosts. MS thesis. Boise, Idaho: Boise State University; 2009.
- Bench ME, White MM. New Species and First Records of Trichomycetes from Immature Aquatic Insects in Idaho. *Mycologia*. 2012; 104(1):295–312. [PubMed: 21933923]
- Brereton C, House WA, Armitage PD, Wotton RS. Sorption of Pesticides to Novel Materials: Snail Pedal Mucus Acid Blackfly Silk. *Environmental Pollution*. 1999; 105:55–65.
- Buchwalter D, Sandahl J, Jenkins J, Curtis L. Roles of Uptake, Biotransformation, and Target Site Sensitivity in Determining the Differential Toxicity of Chlorpyrifos to Second to Fourth Instar *Chironomus riparius* (Meigen). *Aquatic Toxicology*. 2004; 66(2):149–157. [PubMed: 15036870]
- Bundschuh M, Zubrod JP, Kosol S, Maltby L, Stang C, Duester L, Schulz R. Fungal Composition on Leaves Explains Pollutant-Mediated Indirect Effects on Amphipod Feeding. *Aquatic Toxicology*. 2011; 104(1–2):32–37. [PubMed: 21536010]
- Burnham, KP.; Anderson, DR. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. 2nd Ed.. New York: Springer; 2002. p. 488 ISBN 0-387-95364-7
- Crosskey, RW. The Natural History of Blackflies. New York: John Wiley & Sons, Inc.; 1990. ISBN-13:978-0471927556
- Cuppen JGM, Van den Brink PJ, Camps E, Uil KF, Brock TCM. Impact of the Fungicide Carbendazim in Freshwater Microcosms. I. Water Quality, Breakdown of Particulate Organic Matter and Responses of Macroinvertebrates. *Aquatic Toxicology*. 2000; 48(2–3):233–250. [PubMed: 10686329]
- Deb D, Engel B, Harbor J, Hahn L, Jae Lim K, Zhai T. Investigating Potential Water Quality Impacts of Fungicides Used to Combat Soybean Rust in Indiana. *Water, Air and Soil Pollution*. 2010; 207(1–4):273–288.
- Dijksterhuis J, van Doorn T, Samson R, Postma J. Effects of Seven Fungicides on Non-Target Aquatic Fungi. *Water Air and Soil Pollution*. 2011; 222:421–425.
- Dugan, JE.; Ichikawa, G.; Stephenson, M.; Crane, D.; McCall, J.; Regalado, K. Monitoring of Coastal Contaminants Using Sand Crabs. Central Coast Regional Water Quality Control Board. 2005. p. 38 http://www.swrcb.ca.gov/water_issues/programs/swamp/docs/sandcrab.pdf
- Elskus, AA. Toxicity, Sublethal Effects, and Potential Modes of Action of Select Fungicides on Freshwater Fish and Invertebrates. U.S. Geological Survey Open-file Report: 2012–1213. 2012. p. 49 <http://pubs.er.usgs.gov/publication/ofr20121213>
- Geissen V, Ramos FQ, Bastidas-Bastidas PD, Diaz-Gonzalez G, Bello-Mendoza R, Huerta-Lwanga E, Ruiz-Suarez LE. Soil and Water Pollution in a Banana Production Region in Tropical Mexico. *Bulletin of Environmental Contamination and Toxicology*. 2010; 85:407–413. [PubMed: 20734023]
- Gilliom RJ. Pesticides in U.S. Streams and Groundwater. *Environmental Science & Technology*. 2007; 41:3408–3414. [PubMed: 17547156]
- Gustafsson K, Blidberg E, Elfgrén IK, Hellström A, Kylin H, Gorokhova E. Direct and Indirect Effects of the Fungicide Azoxystrobin in Outdoor Brackish Water Microcosms. *Ecotoxicology*. 2010; 19:431–444. [PubMed: 19830551]
- Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson K-H, Lichtwardt R, Longcore J, Miadlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N. A Higher-Level Phylogenetic Classification of the Fungi. *Mycological Research*. 2007; 111:509–547. [PubMed: 17572334]

- Hladik ML, Smalling KL, Kuivila KM. 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*. 2008; 80:139–144. [PubMed: 18196191]
- Horn BW, Lichtwardt RW. Studies on the Nutritional Relationship of Larval *Aedes aegypti* (Diptera, Culicidae) with *Smittium culisetae* (Trichomycetes). *Mycologia*. 1981; 73:724–740.
- Hurd MK, Perry SA, Perry WB. Nontarget Effects of a Test Application of Diflubenzuron to the Forest Canopy on Stream Macroinvertebrates. *Environmental Toxicology and Chemistry*. 1996; 15:1344–1351.
- Kandel P, White MM. A New Species of *Ephemerellomyces* from North America Highlights its Morphological Plasticity and Possible Intergeneric Similarities with Other Harpellales. *Fungal Biology*. 2012; 116:171–184. [PubMed: 22289763]
- Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in US Streams, 1999–2000: A National Reconnaissance. *Environmental Science and Technology*. 2002; 36:1202–1211. [PubMed: 11944670]
- Kreutzweiser DP, Capell SS, Sousa BC. Hexazinone Effects on Stream Periphyton and Invertebrate Communities. *Environmental Toxicology and Chemistry*. 1995; 14:1521–1527.
- Lichtwardt, RW. *The trichomycetes, Fungal Associates Of Arthropods*. New York: Springer-Verlag; 1986. ISBN-13:978-1461293484
- Lichtwardt RW, Williams MC. Distribution and Species-Diversity of Trichomycete Gut Fungi in Aquatic Insect Larvae in 2 Rocky-Mountain Streams. *Canadian Journal of Botany*. 1988; 66:1259–1263.
- Maltby L, Brock TCM, van den Brink PJ. Fungicide Risk Assessment for Aquatic Ecosystems: Importance of Interspecific Variation, Toxic Mode of Action, and Exposure Regime. *Environmental Science and Technology*. 2009; 43:7556–7563. [PubMed: 19848176]
- McCreadie JW, Adler PH, Beard CE. Ecology of Symbiotes of Larval Black Flies (Diptera: Simuliidae): Distribution, Diversity, and Scale. *Environmental Entomology*. 2011; 40:289–302.
- McCreadie JW, Beard CE. The Microdistribution of the Trichomycete *Smittium culisetae* in the Hindgut of the Black Fly Host *Simulium vittatum*. *Mycologia*. 2003; 95:998–1003. [PubMed: 21149008]
- Montuelle B, Dorigo U, Berard A, Volat B, Bouchez A, Tlili A, Gouy V, Pesce S. The Periphyton as a Multimetric Bioindicator for Assessing the Impact of Land Use on Rivers: An Overview of the Ardisres-Morcille Experimental Watershed (France). *Hydrobiologia*. 2010; 657:123–141.
- Nelder MP, Beard CE, Adler PH, Kim SK, McCreadie JW. Harpellales (Zygomycota: Trichomycetes) Associated with Black Flies (Diptera: Simuliidae): World Review and Synthesis of their Ecology and Taxonomy. *Fungal Diversity*. 2006; 22:121–169.
- Nelder MP, McCreadie JW, Beard CE. Laboratory Investigations of Trichomycete Prevalence, Abundance and Fecundity in a *Smittium*-simuliid Model. *Mycologia*. 2005; 97:338–345. [PubMed: 16396341]
- Norgaard KB, Cedergreen N. Pesticide Cocktails can Interact Synergistically on Aquatic Crustaceans. *Environmental Science and Pollution Research*. 2010; 17:957–967. [PubMed: 20077025]
- Pramual P, Kuvangkadilok C. Agricultural Land Use and Black Fly (Diptera, Simuliidae) Species Richness and Species Assemblages in Tropical Streams, Northeastern Thailand. *Hydrobiologia*. 2009; 625:173–184.
- Rasmussen JJ, Wiberg-Larsen P, Baattrup-Pedersen A, Monberg RJ, Kronvang B. Impacts of Pesticides and Natural Stressors on Leaf Litter Decomposition in Agricultural Streams. *Science of the Total Environment*. 2012; 416:148–155. [PubMed: 22177030]
- Reilly TJ, Smalling KL, Orlando JL, Kuivila KM. Occurrence of Boscalid and Other Selected Fungicides in Surface Water and Groundwater in Three Targeted Use Areas in the United States. *Chemosphere*. 2012a; 89:228–234. [PubMed: 22564453]
- Reilly, TJ.; Smalling, KL.; Wilson, ER.; Battaglin, W. Dissolved Pesticides, Dissolved Organic Carbon, and Water-Quality Characteristics in Selected Idaho Streams, April–December 2010. U.S. Geological Survey Data Series 667. 2012b. p. 17 <http://pubs.usgs.gov/ds/667/>

- Relyea R, Hoverman J. Assessing The Ecology In Ecotoxicology: A Review And Synthesis In Freshwater Systems. *Ecology Letters*. 2006; 9:1157–1171. [PubMed: 16972879]
- Sapozhnikova Y, Bawardi O, Schlenk D. Pesticides and PCBs in Sediments and Fish from the Salton Sea, California, USA. *Chemosphere*. 2004; 55:797–809. [PubMed: 15041284]
- Schummer C, Mothiron E, Appenzeller BMR, Rizet AL, Wennig R, Millet M. Temporal Variations of Concentrations of Currently Used Pesticides in the Atmosphere of Strasbourg, France. *Environmental Pollution*. 2010; 158:576–584. [PubMed: 19775790]
- Smalling, K.; Orlando, J. Occurrence of Pesticides in Water and Sediment from three Central California Coastal Watersheds, 2008–2009. U.S. Geological Survey Data Series 600. 2011. p. 70 <http://pubs.usgs.gov/ds/600/>
- Smalling KL, Morgan S, Kuivila K. Accumulation of Current-Use and Organochlorine Pesticides in Crab Embryos from Northern California, USA. *Environmental Toxicology and Chemistry*. 2010; 29:2593–2599. [PubMed: 20853457]
- Smalling KL, Reilly TJ, Sandstrom MW, Kuivila KM. Occurrence and Persistence of Fungicides in Bed Sediments and Suspended Solids from Three Targeted Uses Areas in The United States. *Science of the Total Environment*. 2013; 447:179–185. [PubMed: 23380566]
- Stuijzand S, Poort L, Greve G. Variables Determining the Impact of Diazinon on Aquatic Insects: Taxon, Developmental Stage, and Exposure Time. *Environmental Toxicology and Chemistry*. 2000; 19:582–587.
- Sweeney AW. An Undescribed Species of *Smittium* (Trichomycetes) Pathogenic to Mosquito Larvae in Australia. *Transactions of the British Mycological Society*. 1981; 77:55–60.
- Wallace JB, Webster JR. The Role of Macroinvertebrates in Stream Ecosystem Function. *Annual Review of Entomology*. 1996; 41:115–139.
- Warming TP, Mulderij G, Christoffersen KS. Clonal Variation in Physiological Responses of *Daphnia magna* to the Strobilurin Fungicide Azoxystrobin. *Environmental Toxicology and Chemistry*. 2009; 28:374–380. [PubMed: 18808216]
- White MM. Evolutionary Implications of a rRNA-Based Phylogeny of Harpellales. *Mycological Research*. 2006; 110:1011–1024. [PubMed: 16930972]
- Zubrod JP, Bundschuh M, Feckler A, Englert D, Schulz R. Ecotoxicological Impact of the Fungicide Tebuconazole on an Aquatic Decomposer-Detritivore System. *Environmental Toxicology and Chemistry*. 2011; 30:2718–2724. [PubMed: 21919044]

FIGURE 1.

Map of study area depicting the four surface-water sampling sites. Reference sites: Cottonwood Creek and Dry Creek. Agricultural sites: Sand Run Gulch and Wanstad Ditch. For watershed characteristics refer to Table 1 (Reilly *et al.*, 2012b).

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 are n = 15 unless otherwise noted; ns: not sampled.

FIGURE 3.

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. Boxes on plots represent median (thick bar) and interquartile range (25–75%).

Whiskers represent adjacent values: upper = largest observation that is upper quartile plus 1.5 times length of interquartile range; lower = smallest observation that is lower quartile less 1.5 times length of interquartile range (Adler, 2010).

FIGURE 4.

Number of spores per μm^2 of peritrophic matrix (PM) in black fly larvae from reference sites (a) Cottonwood Creek, (b) Dry Creek, and agricultural sites (c) Sand Run Gulch, (d) Wanstad Ditch. Boxes on plots represent median (thick bar) and interquartile range (25–75%). Whiskers represent adjacent values: upper = largest observation that is upper quartile plus 1.5 times length of interquartile range; lower = smallest observation that is lower quartile less 1.5 times length of interquartile range (Adler, 2010).

TABLE 1

Description of the four surface-water sampling sites and associated watershed characteristics. Reference sites: Cottonwood Creek and Dry Creek. Agricultural sites: Sand Run Gulch and Wanstad Ditch (Reilly et al., 2012b).

Sampling Site	Latitude	Longitude	Watershed Area (Acres)	Percent of Watershed in Agriculture	Dominant Crops (Percentage of agricultural acreage)
Cottonwood Creek	43.61972	-116.18525	10,613	0.1%	NA
Dry Creek	43.66830	-116.18906	6,171	<0.1%	NA
Sand Run Gulch	43.76405	-116.91210	50,877	37.1%	Alfalfa (25.0), Corn (15.9), Winter Wheat (14.3)
WanstadDitch	43.80303	-116.95663	760	90.5%	Winter wheat (27.5), Hay (18.1), Corn (17.7)

NA: Not applicable.

TABLE 2

Model-based means of thalli and spores per μm^2 ($\pm$ 95% Confidence Intervals). P-value tests null hypotheses that mean number of thalli and spores, respectively, are equivalent between reference sites and Sand Run Gulch, the agricultural site where there were enough fungi for comparisons. Due to insufficient numbers of black flies Wanstad Ditch was omitted from the model. Dates are staggered to represent alternation of sampling weeks. P-values have been adjusted for multiple testing with Bonferroni correction. See table S6 for mixed model results.

REFERENCE SITES					AGRICULTURAL SITES					P-VALUES*	
Date	Site**	No. samples	Average (+/- CI) Thalli/mm^2	Average (+/- CI) Spores/mm^2	Date	Site**	No. samples SRG / WD	SRG Average (+/- CI) Thalli/mm^2	SRG Average(+/- CI) Spores/mm^2	Thalli p-value	Spores p-value
2-Jun-10	CC	18	7.4E-06	6.4E-07 (1.6E-07, 2.5E-06)	16-Jun-10	SRG/WD	18 / 18	4.0E-06 (2.2E-06, 7.2E-06)	5.3E-07 (1.2E-07, 2.3E-06)	p<0.0001	1.000 nt
	DC	23	(6.3E-06, 8.8E-06) 1.0E-05 (7.8E-06, 1.3E-05)	3.1E-06 (1.3E-06, 7.3E-06)							
22-Jun-10	CC	18	3.2E-05	2.0E-06 (4.4E-07, 9.4E-06)	6-Jul-10	SRG/WD	20 / 29	9.2E-06 (7.3E-06, 1.2E-05)	2.3E-07 (4.9E-08, 1.1E-06)	0.412	1.000
	DC	18	(2.8E-05, 3.7E-05) 9.9E-07 (5.8E-07, 1.7E-06)	0 (all observations)							
12-Jul-10	CC	35	7.0E-06	4.1E-07 (1.3E-07, 1.3E-06)	26-Jul-10	SRG/WD	20 / 0	9.1E-06 (6.4E-06, 1.3E-05)	6.5E-07 (1.6E-07, 2.6E-06)	1.000	0.754
	DC	25	(6.4E-06, 7.8E-06) 9.0E-06 (5.5E-06, 1.5E-05)	9.7E-07 (2.8E-07, 3.4E-06)							
2-Aug-10	CC	20	1.6E-05	3.6E-06 (9.7E-07, 1.4E-05)	16-Aug-10	SRG/WD	20 / 0	2.4E-05 (1.7E-05, 3.5E-05)	1.0E-05 (4.5E-06, 2.3E-05)	p<0.0001	0.001
	DC	19	(7.8E-06, 3.4E-05) 4.6E-05 (3.6E-05, 5.8E-05)	1.7E-05 (1.2E-05, 2.6E-05)							
23-Aug-10	CC	20	1.0E-04	2.3E-05 (1.7E-05, 3.3E-05)						p<0.0001	0.646
	DC	19	(8.6E-05, 1.3E-04)	5.8E-06 (1.4E-06, 2.3E-05)							

REFERENCE SITES				AGRICULTURAL SITES				P-VALUES*			
Date	Site**	No. samples	Average (+/- CI) Thalli/mm^2	Average (+/- CI) Spores/mm^2	Date	Site**	No. samples SRG / WD	SRG Average (+/- CI) Thalli/mm^2	SRG Average(+/- CI) Spores/mm^2	Thalli p-value	Spores p-value
15-Sep-10	CC DC	20 20	1.2E-04 (1.1E-04, 1.4E-04)		9-Sep-10	SRG/WD	20 / 0	1.3E-05 (1.0E-05, 1.6E-05)	2.3E-06 (7.4E-07, 7.4E-06)	p<0.0001 p<0.0001	0.001 0.003
			1.2E-04 (1.1E-04, 1.4E-04)								
			1.2E-04 (1.1E-04, 1.4E-04)								
4-Oct-10	CC DC	20 20	7.8E-05 (7.0E-05, 8.7E-05)	3.6E-05 (2.9E-05, 4.5E-05)	29-Sep-10	SRG/WD	18 / 0	6.2E-06 (3.9E-06, 1.0E-05)	1.3E-06 (3.3E-07, 5.4E-06)	1.000 p<0.0001	0.027 0.033
			8.7E-05 (2.3E-05, 6.7E-05)	2.8E-05 (2.3E-05, 3.4E-05)							
			(5.4E-05, 8.3E-05)								
26-Oct-10	CC DC	20 20	1.1E-05 (6.7E-06, 1.9E-05)	1.3E-05 (8.2E-06, 2.1E-05)	18-Oct-10	SRG/WD	20 / 2	All zero except two observations	All zero but one observation	nt nt	nt nt
			1.9E-05 (6.8E-06, 1.0E-04)	1.9E-05 (6.8E-06, 5.3E-05)							
			(8.8E-05, 1.2E-04)								
22-Nov-10	CC DC	15 20	1.5E-04 (1.2E-04, 1.8E-04)	7.7E-05 (2.7E-05, 2.2E-04)	8-Nov-10	SRG/WD	20 / 1	All zero except one observation	All zero except 1 observation	nt nt	nt nt
			1.8E-04 (1.3E-04, 1.7E-04)	6.6E-06 (1.6E-06, 2.7E-05)							
6-Dec-10	CC DC	20 19	9.9E-05 (7.8E-05, 1.3E-04)	6.0E-05 (4.4E-05, 8.2E-05)	29-Nov-10	SRG/WD	20 / 7	All zero	All zero	nt nt	nt nt
			1.4E-04 (1.3E-04, 1.6E-04)	3.4E-05 (2.2E-05, 5.2E-05)							
			4.4E-05 (3.9E-05, 5.1E-05)	6.2E-06 (2.2E-06, 1.8E-05)							
			8.2E-05 (7.1E-05, 9.4E-05)	1.1E-05 (4.4E-06, 2.5E-05)							

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p-value is Bonferroni-corrected to test null hypothesis that the density of thalli between CC or DC and SRG are equal; nt = not tested
*
** CC: Cottonwood Creek, DC: Dry Creek, SRG: Sand Run Gulch, WD: Wanstad Ditch.

TABLE 3

Composite fungicide concentration ($\mu\text{g/kg}$ wet weight) in sampled black fly larvae and surface water ($\mu\text{g/l}$) collected from Sand Run Gulch (Agricultural Site). Only water data with corresponding tissue data is presented.

Date	Azoxystrobin		Boscalid		Imazalil		Pyraclostrobin	
	Tissue	Water	Tissue	Water	Tissue	Water	Tissue	Water
2010-05-03	56.0	0.00220	93.0	0.01020	181.0	nd	nd	nd
2010-07-06	121.0	0.00629	33.4	0.01076	326.0	nd	nd	nd
2010-07-26	377.0	0.01140	112.0	0.01600	374.0	nd	426.0	nd
2010-09-09	428.0	0.01250	90.0	0.01490	178.0	nd	842.0	nd
2010-09-29	136.0	nd	106.0	nd	21.0	nd	285.0	nd
2010-10-18	35.0	0.00259	29.0	0.00541	66.0	nd	163.0	nd
2010-11-08	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected