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EFFECTS OF TWO FUNGICIDE FORMULATIONS ON MICROBIAL AND MACROINVERTEBRATE LEAF DECOMPOSITION UNDER LABORATORY CONDITIONS

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Abstract: Aquatic fungi contribute significantly to the decomposition of leaves in streams, a key ecosystem service. Little is known, however, about the effects of fungicides on aquatic fungi and macroinvertebrates involved with leaf decomposition. Red maple (*Acer rubrum*) leaves were conditioned in a stream to acquire microbes (bacteria and fungi) or leached in tap water (unconditioned) to simulate potential reduction of microbial biomass by fungicides. Conditioned leaves were exposed to fungicide formulations QUILT (azoxystrobin + propiconazole) or PRISTINE (boscalid + pyraclostrobin) in the presence and absence of the leaf shredder, *Hyalella azteca* (amphipods; 7-d old at start of exposures) for 14 d at 23 °C. The QUILT formulations (~0.3 μg/L, 1.8 μg/L, and 8 μg/L) tended to increase leaf decomposition by amphipods (not significant) without a concomitant increase in amphipod biomass, indicating potential increased consumption of leaves with reduced nutritional value. The PRISTINE formulation (~33 μg/L) significantly reduced amphipod growth and biomass ($p < 0.05$), effects similar to those observed with unconditioned controls. The significant suppressive effects of PRISTINE on amphipod growth and the trend toward increased leaf decomposition with increasing QUILT concentration indicate the potential for altered leaf decay in streams exposed to fungicides. Further work is needed to evaluate fungicide effects on leaf decomposition under conditions relevant to stream ecosystems, including temperature shifts and pulsed exposures to pesticide mixtures. *Environ Toxicol Chem* 2016;9999:1–11. Published 2016 Wiley Periodicals Inc. on behalf of SETAC. This article is a US Government work and, as such, is in the public domain in the United States of America.

Keywords: Aquatic toxicology Aquatic invertebrates Microbial toxicology Pesticide formulation *Hyalella azteca*

INTRODUCTION

There is growing evidence showing that pesticides, including fungicides, can alter leaf degradation [1–3], a key ecosystem function that drives energy and nutrient cycling in lotic systems [4]. Leaf degradation is initiated by aquatic fungi, which produce extracellular enzymes that degrade plant constituents and transform leaf materials into highly nutritive substrates. Such substrates encourage microbial growth and subsequent ingestion by aquatic invertebrates, such as leaf-shredding amphipods [4].

Fungicides can suppress the biomass and activity of aquatic fungi, shift fungal species diversity [5,6], and alter the feeding behavior of leaf shredders [6,7]. Laboratory studies of single fungicides (propiconazole, tebuconazole) report effects on aquatic fungi and leaf decay at concentrations of 1 μg/L to 50 μg/L [7–9]. Effective concentrations of fungicides are likely to be similar or lower in field situations, where exposures are chronic and a multitude of stressors are present. For example, strong negative relationships have been found between the abundance of invertebrate species in European streams, whose species traits identify them as pesticide-sensitive [2] and concentrations of the most toxic pesticides [1]. Moreover, fungicide concentrations in the 0.5 μg/L to 1 μg/L range have been shown to contribute significantly to the predicted toxicity of such pesticide mixtures [2,10]. Alterations in the composition of stream macroinvertebrates occur on both short- and long-term timeframes [1,11,12] at pesticide concentrations currently considered protective by European regulatory standards [2,13]. With the global use of fungicides expected to increase with climate change and increased use in the United States already evident [14,15], it is imperative that we understand and document the impacts fungicide mixtures have on stream ecosystem functions.

Exposure to fungicides may affect rates of leaf decomposition by shredders by altering food quality or by directly affecting macroinvertebrates. Reductions in the nutritive value of the leaves due to fungicide-mediated changes in the biomass or composition of the microbial community might be expected to increase leaf consumption by the amphipod shredder *Hyalella azteca* [7,16]. Alternatively, if fungicides have narcotic or toxic effects on the macroinvertebrate shredder, fungicide exposure may reduce leaf consumption [17].

To date, only a few studies have examined the combined effects of microbes and shredders on leaf decomposition under realistic fungicide exposure scenarios [1,6,10,18], and very few have examined the effects of fungicides on ecosystem function [19,20]. There has been a recent call for controlled studies of pesticide effects on microbial- and macroinvertebrate-mediated leaf decomposition, using environmentally realistic pesticide concentrations [10].

The objective of the present study was to test the effects of 2 fungicide formulations on leaf decomposition by microbes and by *H. azteca*. Numerous studies use *H. azteca* to study leaf decomposition [21], including studies examining the effects of leaf fungal composition on leaf consumption [22]. The active ingredients in the test formulations, QUILT (azoxystrobin and

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propiconazole) and PRISTINE (boscalid and pyraclostrobin), have been detected frequently in streams throughout the United States and Europe, at maximal concentrations of approximately 30 μg/L, 1 μg/L, 36 μg/L, and 7 μg/L for these 4 fungicides, respectively [23–25]. Three of these fungicides, azoxystrobin, boscalid, and pyraclostrobin, were among the pesticides implicated in altering the macroinvertebrate communities in pesticide-impacted streams [2]. For the present study, we tested concentrations similar to the highest concentrations reported in streams for azoxystrobin, propiconazole, boscalid, and pyraclostrobin and concentrations 10 and 100 times lower than these maximum concentrations reported in the environment. Maple leaves (conditioned in a stream or unconditioned) were exposed to QUILT, PRISTINE, or control water, in the presence and absence of *H. azteca* for 14 d. We hypothesized that exposure to fungicide formulations at environmentally relevant concentrations would alter leaf decomposition by affecting microbes on the leaves or the growth and leaf shredding activity of amphipods.

MATERIALS AND METHODS

Preparation of leaf discs

Leaf preparation protocols for use in the fungicide experiments are outlined in Figure 1. The partial decomposition and colonization of leaves with bacteria and aquatic hyphomycetes (fungi that grow on dead leaves and sporulate underwater) is known as conditioning [26]. To prepare stream-conditioned leaves, dry red maple leaves (*Acer rubrum*, collected in September 2013 as newly fallen leaves and kept dry until used in May and July 2014) were placed in fine mesh (500 μm) nylon bags and incubated in Little Birch stream (Sunkhaze Wildlife Refuge, Milford, ME, USA), a waterway considered to be free of contaminants, including pesticides. Leaves were conditioned in the stream for 14 d to 15 d in May (8–12 °C for QUILT experiment) and July (20–23 °C for PRISTINE experiment) in 2014. This length of time has been used to inoculate the leaves of various tree species with stream microbes for studies of leaf decomposition [27–29]. Pilot studies indicated that a 2-wk incubation of red maple leaves in stream water provided sufficient microbial colonization to support high survival and rapid growth of 7-d-old *H. azteca* (Supplemental Data, Figure S1). After retrieving the leaves from the stream, discs were cut from leaves using a 2 cm diameter cork borer and maintained in a nutrient conditioning medium [30] with gentle aeration at 23 °C and 16:8-h light:dark for 5 d to acclimate leaf microbial communities to laboratory conditions (Supplemental Data, Figure S2).

The nutritional value of unconditioned leaves can be 4 to 10 times lower than that of conditioned leaves and lead to altered consumption by shredders [31,32]. Two sets of unconditioned red maple leaves were prepared (unconditioned controls): 1 to accompany the QUILT experiment, and 1 to accompany the PRISTINE experiment. The unconditioned control leaves were exposed only to experimental water; they were not exposed to fungicides. The unconditioned control leaves served as "positive controls" and were used to distinguish effects on leaf decomposition, amphipod survival, and amphipod growth due to reduced microbial biomass (unconditioned control leaves vs stream-conditioned control leaves) from effects on these parameters due to fungicides (stream-conditioned leaves exposed to QUILT or PRISTINE vs stream-conditioned control leaves).

To prepare the unconditioned control leaves, leaves were first leached in the laboratory to achieve leaf mass loss similar to that of stream-conditioned leaves. Stream-conditioned leaves lose considerable amounts of leaf mass in the first few days due to the leaching of soluble organic and inorganic constituents [33]. To leach leaves for unconditioned control, dry leaves were placed in tubs of chlorinated tap water at room temperature with 90% water changes twice daily for 4 d (QUILT experiment) and 6 d (PRISTINE experiment) to remove soluble organic matter, similar to tap-water leaching techniques others have used [34].

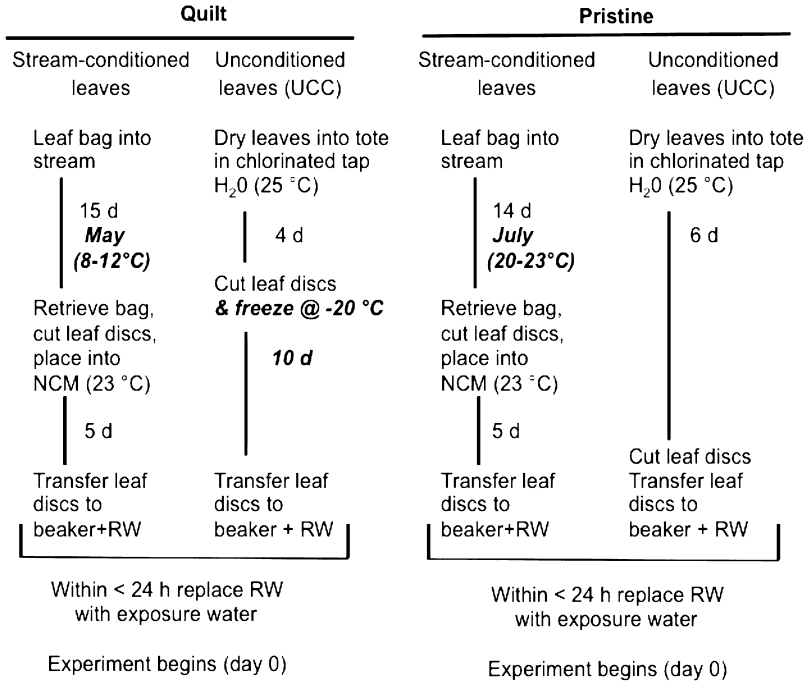

Figure 1. Schematic of leaf preparation for stream-conditioned and unconditioned red maple leaves used in the QUILT and PRISTINE experiments. NCM = nutrient conditioning medium; RW = reconstituted water. See *Materials and Methods* for details.

After leaching, unconditioned control discs (2 cm diameter) were cut from the leaves using gloved hands to reduce the chance of contamination with microbes. For the PRISTINE experiment, unconditioned control discs were cut and immediately placed into exposure water at 23 °C under 16:8-h light:dark conditions. For the QUILT experiment, unconditioned control discs were prepared 10 d ahead of time. To prevent excessive leaching, these unconditioned control discs for the QUILT experiment were removed from the leaching tubs after the 4 d leaching period and frozen at –20° C until used 10 d later. For both fungicide formulations, experiments were begun within 24 h of the unconditioned control leaf discs being placed into the exposure water. The exposure water used for the present studies was a reconstituted water prepared by adding $CaCl_2$, $MgSO_4$, KCl, $NaHCO_3$, and NaBr to deionized water at pH 7.8 to 8.2. It has been shown to support optimal amphipod survival, growth, and reproduction [35].

At the start of each experiment (day 0), 3 leaf discs were lightly blotted on paper towels, the wet weight recorded, and the discs placed into exposure beakers. Extra conditioned and unconditioned control leaf discs were used to determine the initial dry weight of discs in the exposure beakers. For the extra leaf discs, wet weights were measured for 10 replicates of 3 stream-conditioned leaf discs each and 10 replicates of 3 unconditioned leaf discs each. These leaf discs were then dried in an oven at 47 °C for 48 h to 72 h and dry weights taken. All weights were measured to the nearest 0.0001 g on a Phoenix GH-202 analytical balance (A&D Weighing).

Test conditions

The amphipods, *H. azteca*, were provided by Christopher Ingersoll from stocks cultured at the US Geological Survey, Columbia Environmental Research Center in Columbia, MO, USA in well water diluted to a hardness of 100 mg/L (as $CaCO_3$). Amphipods that were 2 d to 3 d old were shipped overnight to the University of Maine where they were maintained at 23 °C under 16:8-h light:dark conditions in reconstituted water optimized for maintaining this species [35]. They were fed stream-conditioned red maple leaves prior to use at 7 d of age. Standard protocols for using *H. azteca* in toxicity tests recommend that amphipods be acclimated to and tested at 23 °C [36]. Acclimation of the amphipods to test water that differs from culture water is routinely done [37] but is not required [36].

At the start of each experiment, 20 amphipods (7 d old) were preserved in a sugar-formalin solution (12 g sugar, 8 mL 37% formaldehyde, 92 mL deionized water) and photographed for determining initial length. At the conclusion of each experiment, amphipods were preserved in sugar-formalin and final lengths were determined. Digital photographs of preserved amphipods were taken with a ZEISS Axiocam ERc5s camera mounted to an Olympus SZX16 stereomicroscope (Micro Video Instruments). Amphipod length was measured from the base of the third uropod to the base of the first antennae [36] using Image J (Ver 1.47v, National Institutes of Health) and the ImageJ Plug-in for Cochlear Frequency Mapping in Whole Mounts.

Fungicide formulations were obtained from David Yarborough (University of Maine; QUILT, Syngenta) and directly from the manufacturer (PRISTINE, BASF). Stock solutions were made up fresh in deionized water for each water change: 0.05 mL QUILT/23.75 mL (~2.23 g QUILT/L) and 93.2 mg PRISTINE/L. Test concentrations were achieved through serial dilution of stock solutions in reconstituted water. Nominal concentrations of QUILT were: azoxystrobin + propiconazole: 0.06 μg/L + 0.05 μg/L, 0.6 μg/L + 0.52 μg/L, and 6 μg/L + 5.2 μg/L. Nominal concentrations of PRISTINE were: boscalid + pyraclostrobin: 0.23 μg/L + 0.12 μg/L, 2.3 μg/L + 1.2 μg/L, and 23 μg/L + 12 μg/L.

Exposures were conducted in 300-mL beakers containing 200 mL of exposure water, 3 leaf discs (2 cm diameter), with or without 4 juvenile *H. azteca*; these conditions are similar to those others have used with *H. azteca* of this age [16]. To evaluate the percentage of survival and growth rate for amphipods fed different masses of leaves, we conducted 14 d pilot studies in which the leaf disc to amphipod ratio was varied. The results of these studies indicated amphipod survival and growth rate were highest for 3 discs (2 cm diameter) fed to 4 juvenile amphipods (Supplemental Data, Figure S2). *Hyalella azteca* used in the present study were approximately 7 d old at the beginning of the exposure period, the standard age of *H. azteca* used in US Environmental Protection Agency toxicity testing protocols [38]. A power analysis indicated that 4 replicates per treatment would allow us to detect a 15% difference among treatments in amphipod growth rate over 14 d. Five replicates were used to ensure sufficient statistical power. The test was run for 14 d with exposure beakers held at 23 °C under a 16:8-h light:dark cycle with gentle aeration (glass pipet delivering ~1 bubble per second). Exposures were conducted under semistatic conditions; every 3 d 50% of the exposure water in each beaker was removed and replaced with fresh exposure water. This water exchange regime was based on preliminary studies indicating that azoxystrobin is stable for 3 d at 23 °C under a 16:8-h light:dark cycle in the exposure water.

The fungicide formulations were tested separately. In each test, there were 7 treatments, with 5 replicate beakers per treatment. Six of these treatments contained stream-conditioned leaves: 3 concentrations of the fungicide formulation with *H. azteca* (low, medium, high), 1 concentration of the formulation without *H. azteca* (high QUILT [–] or high PRISTINE [–]), and exposure water alone with and without (–) *H. azteca* (controls, and controls [–], respectively). The seventh treatment consisted of *H. azteca* fed unconditioned leaves in exposure water without the addition of fungicide formulations (unconditioned control) and served as the positive control to provide data on how low microbial biomass would affect the endpoints measured.

Analytical methods

Water chemistry (pH, alkalinity, dissolved oxygen (DO), temperature, conductivity, ammonia, nitrate, and nitrite) was measured every 3 d prior to the 50% water change, and varied little over the 14 d experiment, with no differences between treatments. Water chemistry values, and the methods used to measure them, are provided in the Supplemental Data and Table S1. Fungicide concentrations were measured periodically throughout the exposure period and were within 65% to 408% (QUILT) and 66% to 118% (PRISTINE) of target concentrations. Information on the methods used and the results of these analyses can be found in the Supplemental Data and Table S2.

Microbial respiration rate of leaf discs is an indirect measure of microbial biomass [10]. Microbial respiration was measured at the start (day 0) and termination (day 14) of each experiment. Initial rates (day 0) were determined with extra leaf discs for both stream-conditioned and unconditioned control discs ($n = 5$ replicates of 3 leaf discs each). To measure respiration, 3 leaf

discs were placed into a 37.5 mL serum bottle. The bottle was filled to overflowing with oxygen-saturated reconstituted water, sealed, capped, and maintained at 23 °C in the dark for 6 h. Time point trials indicated that the respiration rate is linear between 3 h and 7 h. Dissolved oxygen was measured optically with a ProODO (YSI). Respiration rates were calculated from declines in DO over time in bottles containing leaf discs in reconstituted water compared with bottles containing reconstituted water only. Respiration rates were normalized to the dry weight of the leaf discs.

Calculations and statistical analyses

Instantaneous growth rates for amphipods were calculated using Equation 1 as described by Willming et al. [16]

$$\text{Instantaneous growth rate} = \frac{\left[\text{In}\left(\frac{g_f}{g_i}\right)\right]}{d} \quad (1)$$

where g_f and g_i are the final and initial lengths of the amphipods, respectively, and d is the length of time (in days) that amphipod growth was tracked in the experiment. The biomass of surviving amphipods from each replicate was calculated as the sum of individual amphipod weights using the empirical relationship: weight (mg) = ((0.177 × length (mm))–0.0292)^3, as described by Kemble et al. [39].

Leaf loss was calculated using Equation 2 as

$$Dry\ weight\ loss = DWi - DWf \quad (2)$$

where *DWi* is the dry weight of the leaf discs in exposure beaker$_i$ on day 0 and *DWf* is the dry weight of the leaf discs in beaker$_i$ on day 14.

Toxic units have been used to compare the relative toxicity of pesticides for the standard aquatic crustacean test species, *Daphnia magna*, to the relative toxic contribution of pesticides for macroinvertebrates in streams in Europe, Siberia, and Australia [1,2], and to toxicant effects on freshwater ecosystem function, including leaf decomposition [3]. To compare the toxicity of the fungicides in the present study to the toxicity of pesticides in laboratory and stream studies by other researchers, the toxic unit (TU) for *D. magna* ($TU_{(D.\ magna)}$) was calculated for the fungicides in the 2 test formulations, QUILT and PRISTINE. To calculate $TU_{(D.\ magna)}$, the concentration of each fungicide measured in the water sample was compared with the 48 h median acute lethal concentration (LC50, concentration killing 50% of the organisms) or median effect concentration (EC50, concentration affecting 50% of the organisms) of that fungicide for *D. magna* ($TU_{(D.\ magna)}$) using Equation 3 [1,40]

$$TU_{(D.\ magna)} = \max^{n}_{i=1} (\log \times (Ci/LC50i)) \quad (3)$$

where $TU_{(D.\ magna)}$ is the maximum toxic unit of the single-most toxic fungicide in the water sample, *Ci* is the concentration (μg/L) of the most toxic fungicide *i* in the water sample, and *LC50i* is the 48 h LC50 of fungicide *i* for *D. magna* (μg/L) [41]. Although $TU_{(D.\ magna)}$ is not as robust a predictor of toxicity as species sensitivity distributions or the most "sensitive species" metric [40], the vast majority of toxicity data available are for standard test organisms such as *D. magna*. For this reason, $TU_{(D.\ magna)}$ remains a widely used metric for evaluating the relative toxicity of chemical mixtures in contaminant-impacted streams [3]. Moreover, TU values measured in simple microcosms provide estimates of what effects might be observed in complex stream systems with mixed contaminants, seasonal concentration shifts, and multiple stressors (e.g., temperature, pH, oxygen, predator/prey compositions).

All data were tested for normality (Shapiro–Wilk test) and equal variance (*F*-test) prior to statistical analysis. Tests of biological effects of treatment ("untreated" vs "treated") on dependent variables (survival, biomass, instantaneous growth rate, microbial respiration, dry wt leaf loss) were conducted using analysis of variance (ANOVA) if the data were normally distributed and Kruskal–Wallis or Mann–Whitney tests on ranks if the data were not normally distributed; Dunnett's was used as the post hoc test (Systat Software, SigmaPlot for Windows). Two-way ANOVA was used to test for the effects of fungicide, amphipod presence, and their interaction on microbial respiration and leaf dry weight loss. Analysis of covariance was conducted with treatment as the fixed factor, leaf loss as the dependent variable, and amphipod biomass and leaf respiration as the covariates. Two-tailed *t*-tests were used to determine if leaf preparation affected the endpoints measured; stream-conditioned controls were compared with unconditioned control. Percentage of survival data were transformed using arcsine-square root [arcsine (sqrt (percentage survival/100))×2]. One outlier was identified for PRISTINE instantaneous growth rate by Grubb's test using QuickCalcs (GraphPad) and was removed prior to statistical analyses.

RESULTS

Control leaves: Stream-conditioned and unconditioned

Amphipod survival and growth, and leaf microbial respiration rates, were similar for stream-conditioned control leaves in the QUILT and PRISTINE experiments. The 14-d survival of amphipods fed stream-conditioned control leaves was 100% in both the QUILT and PRISTINE experiments. Amphipods fed stream-conditioned control leaves grew at nearly the same rate (0.054 day^{-1} QUILT; 0.056 day^{-1} PRISTINE; Figure 2A), and attained nearly the same biomass weights (0.40 mg amphipod biomass/beaker QUILT; 0.48 mg amphipod biomass/beaker PRISTINE, Figure 2B). Mean microbial respiration rates were also similar for stream-conditioned control leaves in both experiments (1.25 mg DO/g dry wt/h, QUILT; 1.47 mg DO/g dry wt/h, PRISTINE, Figure 2C).

Leaves preleached in chlorinated tap water (unconditioned control leaves) were expected to have reduced microbial biomass relative to the microbial biomass of stream-conditioned leaves, as indicated by relative microbial respiration rates (Figure 2C). For the QUILT experiment, mean leaf microbial respiration rates tended to be lower for unconditioned control leaves than stream-conditioned control leaves, but the difference was not significant (0.88 mg DO/g dry wt/h and 1.25 mg DO/g dry wt/h, respectively, *t*-test, $p = 0.10$). For the PRISTINE experiment, mean leaf microbial respiration rates were significantly lower for unconditioned control leaves relative to stream-conditioned control leaves (0.88 mg DO/g dry wt/h and 1.47 mg DO/g dry wt/h, respectively, *t*-test, $p = 0.006$).

The survival of amphipods fed unconditioned control leaves differed considerably between the QUILT and PRISTINE experiments. Amphipods fed unconditioned control leaves in the QUILT experiment (unconditioned control leaves were frozen for 10 d before use) had much higher survival rates (85 ± 22.4%; 2–4 amphipods survived in each of 5 replicate beakers) than amphipods fed unconditioned control leaves in the

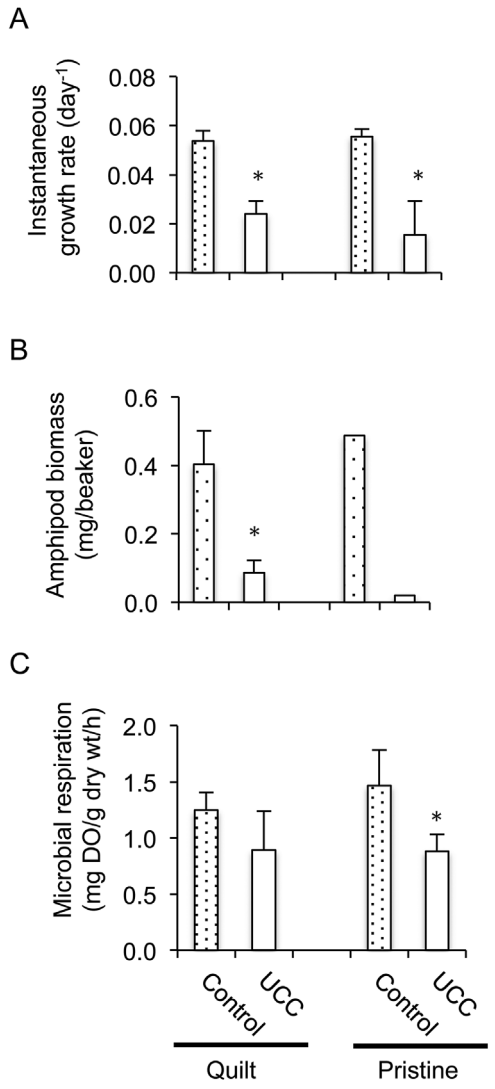

Figure 2. The effects on amphipods and microbes of a 14-d exposure to stream-conditioned control (control) and to unconditioned control (UCC) leaves in the QUILT and PRISTINE experiments. (**A**) instantaneous growth rate (day^{-1}) of amphipods, (**B**) amphipod biomass (mg biomass/beaker), and (**C**) microbial respiration of leaves (mg dissolved oxygen [DO]/g dry wt/h). Values are mean ± standard deviation except for biomass of amphipods for PRISTINE control and unconditioned control, where median values were calculated. There were $n = 5$ replicates for all treatment groups except PRISTINE unconditioned control amphipod growth ($n = 2$) and PRISTINE control microbial respiration ($n = 4$). *Significantly different from respective stream-conditioned control ($p < 0.05$).

PRISTINE experiment (unconditioned control were leaves never frozen; 10 ± 13.7%; 1 amphipod survived in each of 2 beakers out of 5 replicate beakers). The effect on amphipod survival of freezing versus not freezing the unconditioned control leaves was significant (Kruskal–Wallis ANOVA on ranks, Tukey test, $p < 0.001$). Amphipods fed unconditioned control leaves in the QUILT experiment grew 1.6 times as fast as the unconditioned control-fed amphipods in the PRISTINE experiment (means of 0.024 d^{-1} QUILT; 0.015 d^{-1} PRISTINE, Figure 2A), although the difference was not significant ($F = 1.68$; degree of freedom [df] = 1,16; $p = 0.21$). Amphipod biomass totals also tended to be higher in unconditioned control QUILT beakers (mean 0.09 mg biomass amphipods/beaker ± 0.03) than in unconditioned control PRISTINE beakers (median 0.02 mg biomass amphipods/beaker, 0.01 mg/beaker 25th percentile and 0.03 mg/beaker 75th percentiles), but the difference was not quite significant ($F = 6.485$; $df = 1,5$; $p = 0.051$; Figure 2B).

Comparing unconditioned control leaves to stream-conditioned control leaves demonstrates that leaf conditioning had significant effects on amphipod growth and variable effects on leaf respiration. In the QUILT experiment, amphipods fed unconditioned control leaves grew half as fast (means of 0.024 d^{-1} vs 0.054 d^{-1}, $p < 0.001$, Figure 2A), and attained amphipod biomass weights that were 34% lower ($p < 0.001$, Figure 2B), than amphipods fed stream-conditioned control leaves. However, leaf respiration rates were similar for unconditioned control (0.89 mg DO/g dry wt/h ± 0.35) and stream-conditioned control leaves (1.25 mg DO/g dry wt/h ± 0.16, Figure 2C). In the PRISTINE experiment, amphipods fed unconditioned control leaves grew 72% more slowly ($p < 0.01$, Figure 2A), and amphipod biomass tended to be smaller (median 0.02 mg amphipod biomass/beaker, 0.01 mg/beaker 25th percentile and 0.03 mg/beaker 75th percentile) than those fed stream-conditioned control leaves (median 0.48 mg amphipod biomass/beaker, 0.46 mg/beaker 25th percentile and 0.50 mg/beaker 75th percentile, $p = 0.10$, Figure 2B). Leaf respiration rates in the PRISTINE experiment were 40% lower in unconditioned control leaves compared to stream-conditioned controls ($p = 0.006$, Figure 2C).

In both studies, leaf decomposition rates did not differ between stream-conditioned control and unconditioned control leaves. Mean leaf loss rates for QUILT were 0.26 mg dry weight/day ± 0.31 for control and 0.61 mg dry weight/day ± 0.54 for unconditioned control ($p = 0.24$), and median loss rates for PRISTINE were 0.26 mg dry weight/day (0.15, 25th and 0.69, 75th percentiles) for control and 0.14 mg dry weight/day (0.01, 25th and 0.27, 75th percentiles) for unconditioned control ($p = 0.22$).

Effects of QUILT

Exposure to QUILT at tested concentrations was not lethal to *H. azteca* over the 14-d exposure period, with 100% survival in all treatment groups. There were also no significant effects of QUILT on amphipod growth. The mean instantaneous growth rate of amphipods fed stream-conditioned control leaves (0.054 d^{-1}) was similar to mean instantaneous growth rates of amphipods in beakers containing QUILT formulation (0.055 d^{-1} – 0.057 d^{-1}; Figure 3A). There were no significant effects of QUILT formulation on mean amphipod biomass, which ranged from 0.40 mg to 0.44 mg amphipod biomass/beaker for all amphipods fed stream-conditioned leaves exposed to the QUILT formulation (Figure 3B).

Analysis of covariance indicated that neither amphipod biomass nor leaf respiration significantly contributed to leaf loss in the QUILT experiment. Based on single-factor ANOVA, there were no statistical differences among treatments; however, leaf decomposition tended to increase with increasing QUILT concentration in beakers containing amphipods (Figure 4A). Median leaf loss was –0.18 mg dry weight lost/d (low), 0.23 mg dry weight lost/d (medium), 0.39 mg dry weight lost/d (high) QUILT; 0.27 mg dry weight lost/d (controls; $F = 1.19$; $df = 3,16$; $p = 0.35$, Figure 4A). Leaf loss tended to be higher when amphipods were present (0.39 mg dry wt lost/d high QUILT vs –0.17 mg dry wt lost/d high QUILT [–]; 0.27 mg dry wt lost/d control vs –0.08 mg dry wt lost/d control [–]; Figure 4A). However, the differences between beakers with and without amphipods were not significant ($F = 3.2$; $df = 1,1,1$;

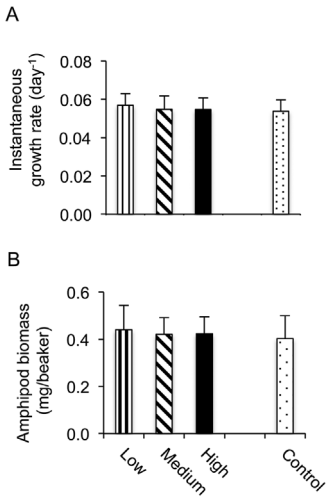

Figure 3. The effects on amphipods of a 14-d exposure to stream-conditioned leaves alone (control) or to stream-conditioned leaves in the presence of QUILT at 3 concentrations. (**A**) instantaneous growth rate of amphipods (day^{-1}), (**B**) amphipod biomass (mg biomass/beaker). Means ± standard deviation ($n = 5$).

$p = 0.09$), and there was no significant interaction between treatment and presence/absence of amphipods ($F = 0.19$; $df = 1,1,1$; $p = 0.67$).

Exposure to QUILT did not significantly affect microbial respiration of the leaves. Mean respiration rates of QUILT-exposed leaves and of control leaves fell within the same range (~1.2 mg–1.3 mg O_2/g dry wt/h; Figure 4B).

Effects of PRISTINE

Over the 14-d exposure period, PRISTINE was not lethal to amphipods with 95% to 100% survival in control, low, and medium PRISTINE treatments. There was a slight, but not significant, reduction in amphipod survival at the high PRISTINE concentration (85 ± 33.5%). However, exposure to PRISTINE did affect amphipod growth. Amphipods exposed to the highest concentration of PRISTINE grew at a rate that was 12% slower than controls ($F = 14.4$; $df = 4,77$; $p = 0.02$, Figure 5A), with a resulting biomass per beaker that was 26% lower than controls ($p < 0.05$, Kruskal–Wallis ANOVA on ranks, Figure 5B).

Leaf decomposition in the PRISTINE experiment was variable. Analysis of covariance indicated that neither amphipod biomass nor leaf respiration significantly contributed to leaf loss in the PRISTINE experiment. A one-way ANOVA indicated no significant treatment differences. Median dry weight leaf loss was 0.009 mg dry weight lost/d (low), 0.19 mg dry weight lost/d (medium), 0.16 mg dry weight lost/d (high) PRISTINE; 0.26 mg dry weight lost/d (controls; $F = 0.69$; $df = 3,15$; $p = 0.58$; Figure 6A). A two-way ANOVA followed by Dunnett's post hoc test was used to compare 4 of the treatments, those that contained leaves and amphipods (high PRISTINE, control) and those that contained only leaves (high PRISTINE[–], control[–]). The results indicated that there was no significant effect of amphipod presence/absence on leaf decomposition, and no significant interaction between treatment and amphipod presence/absence ($F = 1.17$; $df = 1,1,1$; $p = 0.30$). However, this analysis did indicate an effect of treatment, with leaf decomposition significantly suppressed by high PRISTINE compared to controls (Dunnett's, $F = 9.3$; $df = 1,1,1$; $p = 0.01$; Figure 6A).

The mean microbial respiration of stream-conditioned leaves ranged from approximately 1.5 mg to 1.7 mg DO/g dry weight/h for PRISTINE-containing beakers. Although there was a tendency for respiration to increase with increasing PRISTINE concentration, there was no significant effect of PRISTINE treatment relative to stream-conditioned control leaves, with or without amphipods (Figure 6B).

DISCUSSION

Effects of leaf preparation

The unconditioned control leaves were used to evaluate the effects of reduced microbial biomass on amphipods and on leaf decomposition in the absence of fungicides. It was

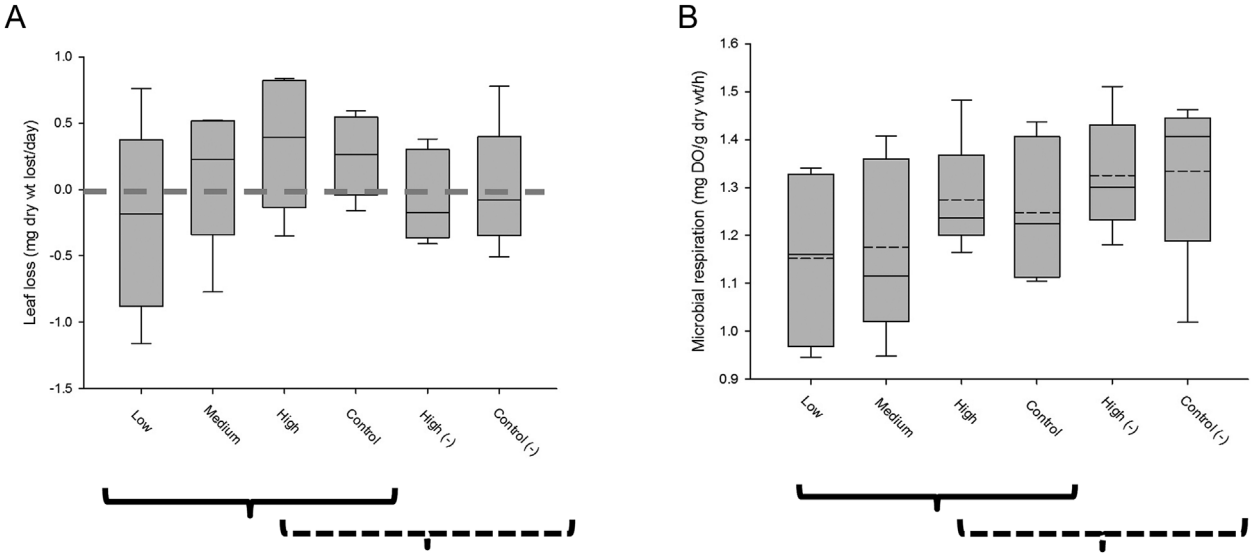

Figure 4. Box plots of (**A**) leaf loss (mg dry wt leaf lost/day) and (**B**) microbial respiration (mg dissolved oxygen [DO]/g dry wt/h) for the 14-d QUILT experiment. Plots indicate median, 10th, 25th, 75th and 90th percentiles; dashed lines inside boxes indicate mean. Treatments without amphipods are labeled (–); $n = 4$ to 5. Gray dotted line in (**A**) marks 0 loss rate. One-way analysis of variance (ANOVA; solid bracket), two-way ANOVA (dotted bracket).

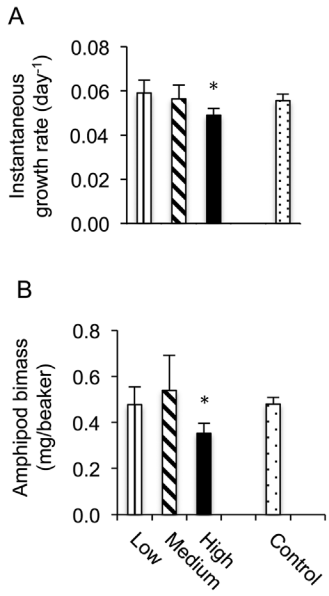

Figure 5. The effects on amphipods of a 14-d exposure to stream-conditioned leaves alone (control) or to stream-conditioned leaves in the presence of PRISTINE at 3 concentrations. (**A**) instantaneous growth rate (day^{-1}) of amphipods, (**B**) amphipod biomass (mg biomass/beaker). Means ± standard deviation ($n = 5$). * Significantly different from controls ($p < 0.05$).

hypothesized that unconditioned control leaves would have lower microbial biomass, and thus lower nutritional value, than stream-conditioned control leaves. Lower nutritional value can either reduce [34] or increase [7] leaf consumption by amphipods. In the PRISTINE experiment, microbial biomass (as measured by microbial respiration) was significantly lower, amphipod growth rate was slower, and amphipod biomass tended to be lower for unconditioned control leaves than for stream-conditioned controls. Unexpectedly, however, leaf decomposition rates were similar for unconditioned control and control leaves. In contrast, in the QUILT experiment, microbial respiration rate and leaf decomposition rate were similar for unconditioned control and stream-conditioned controls; yet, similar to the PRISTINE experiment, amphipod growth rate and biomass were significantly lower on unconditioned control leaves than on stream-conditioned control leaves. Together, these results indicate that amphipod consumption of leaves was not directly correlated with microbial respiration, and that factors other than leaf microbial biomass are likely responsible for the low amphipod growth on unconditioned control leaves in both experiments. For example, leaf parameters (e.g., protein and polyphenols) are affected by fungal and oomycete strains and by leaf conditioning strategies (stream, tap-water, autoclaving) and are strongly correlated with leaf consumption by amphipods [34].

Freezing the unconditioned control leaves may have affected leaf quality, resulting in differences in the nutritive value and palatability of the unconditioned control leaves. The survival of amphipods fed unconditioned control leaves in the QUILT experiment (~85% survival) was dramatically higher than that of amphipods fed unconditioned control leaves in the PRISTINE experiment (~10% survival). For both experiments, red maple leaves were drawn from the same leaf collection and unconditioned control leaves were prepared by leaching in chlorinated tap water for 4 d (QUILT) or 6 d (PRISTINE). However, the unconditioned control leaves for the QUILT experiment were frozen at –20° C for 10 d prior to use, whereas the unconditioned control leaves for the PRISTINE experiment were used immediately. The microbial activity of the unconditioned control leaves on day 14 was identical in the QUILT and PRISTINE experiments (~0.9 mg DO/g dry wt/h), indicating the microbial biomass was likely similar. However, it is possible that freezing the unconditioned control leaves in the QUILT experiment altered the composition of the microbial community on the leaves and softened the leaf tissue [42,43], potentially increasing leaf nutritive value and palatability.

Despite the likely difference in nutritive quality of the unconditioned control leaves of the QUILT and PRISTINE experiments, surviving amphipods in the unconditioned control beakers reached similar sizes. This likely reflects differences in

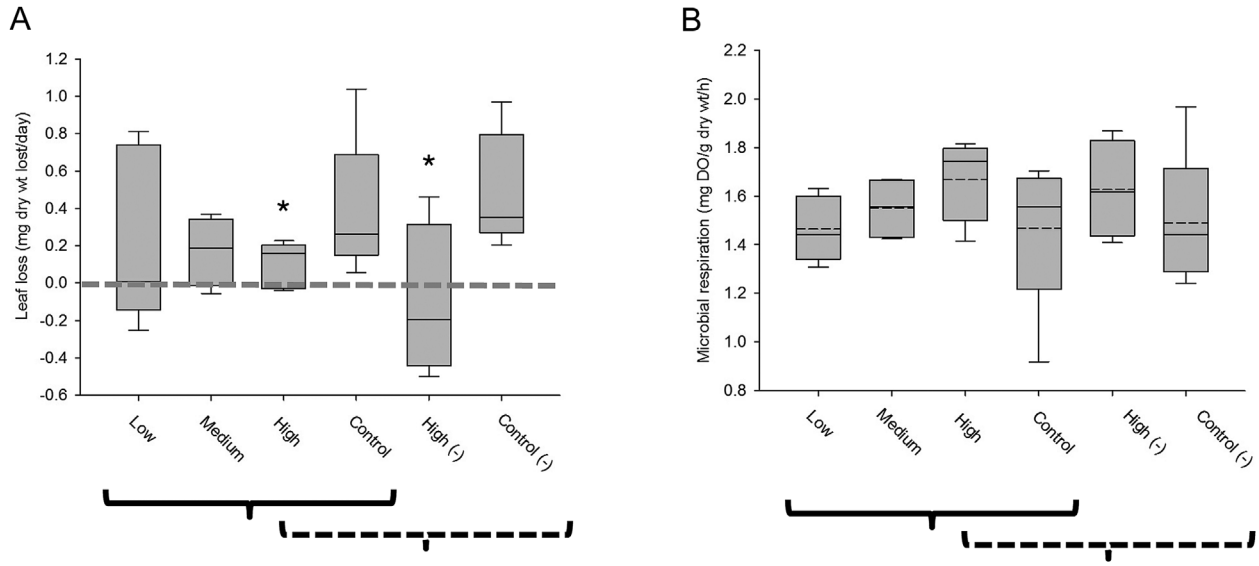

Figure 6. Box plots of (**A**) leaf loss (mg dry wt leaf lost/day) and (**B**) microbial respiration (mg dissolved oxygen [DO]/g dry wt/h) for the 14-d PRISTINE experiment. Plots indicate median, 10th, 25th, 75th and 90th percentiles; dashed lines inside boxes indicate mean. Treatments without amphipods are labeled (–); $n = 4$ to 5. Gray dotted line in (**A**) marks 0 loss. One-way analysis of variance (ANOVA; solid bracket), two-way ANOVA (dotted bracket). *Significantly different from corresponding control at $p = 0.01$ (two-way ANOVA).

the relative amount of leaf material available to surviving amphipods of the 2 experiments. More leaf material was available to surviving amphipods in the unconditioned control beakers of the PRISTINE experiment (3 leaf discs: 1 surviving amphipod) than in the unconditioned control beakers of the QUILT experiment (3 discs: 2–4 amphipods). This difference in leaf disc: surviving amphipods also accounts for the much higher median leaf loss rate in the unconditioned control beakers in the QUILT experiment (0.75 mg dry wt leaf lost/d, 0.18 mg dry wt lost/d 25th percentile and 0.97 mg dry wt lost/d 75th percentile) relative to the unconditioned control beakers in the PRISTINE experiment (0.14 mg dry wt lost/d, 0.13 mg dry wt lost/d 25th percentile, and 0.27 mg dry wt lost/d 75th percentile).

There was no apparent effect of season or temperature on the nutritive value of control leaves conditioned in May and July. Amphipods fed control leaves that had been stream-conditioned in May (8–12 °C, QUILT experiment) grew at similar rates and achieved similar size as amphipods fed control leaves that had been stream-conditioned in July (20–23 °C, PRISTINE experiment). This was somewhat unexpected because the composition of aquatic microbial communities (fungi, bacteria, and fungus-like eukaryotes known as oomycetes), and hence food quality, can change with temperature and season [4,34,44,45]. It is likely that other environmental variables (e.g., stream flow rate) over the 14-d incubation period of the leaf bags in Little Birch stream masked any effects that season (May vs July) may have had on community composition and biomass of the conditioned leaves.

Stream-conditioned control leaves were expected to have higher microbial respiration rates than unconditioned control leaves. This was observed in the PRISTINE experiment but not the QUILT experiment. The different outcome for the PRISTINE and QUILT experiments is not due to mean unconditioned control respiration rates, which were identical (~0.9 mg DO/g dry wt/h) and indicate that the effects of unconditioned control preparation on microbial biomass were consistent from experiment to experiment. However, the variability in the respiration of unconditioned control leaves was higher in the QUILT experiment (0.89 mg DO/g dry wt/h $\pm$ 0.35) than in the PRISTINE experiment (0.88 mg DO/g dry wt/h $\pm$ 0.15). The respiration of stream-conditioned control leaves also tended to be lower for the QUILT leaves (1.25 mg DO/g dry wt/h $\pm$ 0.16) relative to the PRISTINE leaves (1.47 mg DO/g dry wt/h $\pm$ 0.32), which may reflect seasonal differences in microbial biomass for leaves conditioned in May (8–12 °C, QUILT experiment) relative to those conditioned in July (20–23 °C, PRISTINE experiment). The difference, however, was not significant ($F = 1.6$; $df = 1,7$; $p = 0.25$).

Fungicide effects on leaf decomposition by amphipods

There were differences in how QUILT and PRISTINE affected leaf decomposition. QUILT tended to increase leaf decomposition (Figure 4A) but did not influence the growth or biomass of the amphipods (Figure 3). If QUILT reduced the leaves' nutritional quality (e.g., by altering the microbial biomass or community composition), shredders may have increased leaf consumption in an attempt to compensate [7] but were unable to gain significant weight from doing so. In contrast, PRISTINE tended to decrease leaf decomposition relative to controls (Figure 6A) and significantly reduced amphipod growth and biomass (Figure 5). This may reflect a fungicide-mediated reduction in leaf palatability and may also reflect a repellant property of the fungicides [9]. It is also possible that PRISTINE was directly toxic to the amphipods (see *Fungicide effects on* H. azteca *survival and growth*). The suppressive effects on amphipod growth of PRISTINE at the highest concentration are similar to the suppressive effects on growth observed with amphipods fed unconditioned control leaves. At least some portion of the suppressive effects of PRISTINE on amphipod growth may be due to potential alterations in the microbial biomass or composition of stream-conditioned leaves exposed to PRISTINE.

The tendency of the active ingredients in QUILT, azoxystrobin and propiconazole, to increase consumption of leaves by shredders without shredder growth has not been reported previously. Indeed, exposure to azoxystrobin tended to decrease leaf consumption by the shredder *Gammarus fossarum*, although the effect was not significant, even at 2500 μg/L [9]; amphipod growth was not measured. Exposure to tebuconazole, which, like propiconazole, is an azole fungicide, had no significant effect on leaf consumption by *G. fossarum* except at very high concentrations (500 μg/L), which suppressed leaf consumption [9]. It is possible that low QUILT concentrations altered microbial communities resulting in altered feeding by *H. azteca*. The effects of fungal species composition on amphipod feeding preference have not been consistent, with some researchers reporting that changes in fungal composition altered feeding by amphipods [6], whereas others found no consistent effect [46]. Further studies are needed on the effects of QUILT on microbial composition, leaf nutritional value, and food choice by *H. azteca*, as well as *H. azteca* growth as related to fungal densities.

The low loss of leaf biomass (3–11% loss) in QUILT and PRISTINE beakers containing amphipods over the 14-d exposure period may be a function of *H. azteca*'s life stage. The juvenile amphipods used in the present study (7 d old at day 0) may have gained sufficient nutrients by grazing the biofilm on the leaves, with little degradation of the leaves. Future experiments using longer exposure periods and higher ratios of amphipod mass to leaf mass may be needed to measure significant leaf biomass loss with this life stage.

Fungicide effects on H. azteca survival and growth

There were no significant effects of QUILT on the survival or growth of amphipods after 14 d, even at the highest concentration tested (3.9 μg /L azoxystrobin + 4.1 μg /L propiconazole). This indicates it is likely that, in this timeframe, waterborne exposures to QUILT at this concentration do not have direct toxic effects on these endpoints in *H. azteca*. However, adverse effects have been reported at similar concentrations for 1 component of this formulation, azoxystrobin, when exposure time is extended. Exposures of *H. azteca* for 28 d to 42 d to azoxystrobin at concentrations approximately 6 to 20 times lower than concentrations considered safe (aquatic life benchmarks, Table 1) affected reproduction (EC20 6.5 μg/L) and survival (EC20 of 8.5–8.7 μg/L) of *H. azteca* (J. Kunz et al., US Geological Survey, Columbia, MO, unpublished data). In 42-d studies with outdoor microcosms, azoxystrobin had population-level effects on *Copepoda* (no observed effect concentration [NOEC] of 1 μg/L) and community level effects on zooplankton (NOEC of 10 μg/L) [47]. Although these NOEC concentrations bracket the highest azoxystrobin concentration tested in the present study (3.9 μg/L), it should be noted that higher NOEC concentrations have been reported for this fungicide for *D. magna* (21 d NOEC 44 μg/L) [48]. Based on the findings of these studies, which used 1 component of this formulation, azoxystrobin, it is possible that longer exposures to the QUILT formulation may have measurable effects on

Table 1. Measured water concentrations, toxic units, surface water data, and published toxicity values for the active ingredients of QUILT and PRISTINE

Formulation	Fungicide	Measured concentration (mg/L)	Log $TU_{(D.\ magna)}$[b]	Maximum concentration in surface water[c]	Toxicity value: 48-h $LC50_{(D.\ magna)}$[d]	Toxicity value: 21-d chronic $NOEC_{(D.\ magna)}$[d]	Aquatic life benchmark[a] invertebrates: Acute[e]	Aquatic life benchmark[a] invertebrates: Chronic[e]
QUILT	Azoxystrobin	0.25	–2.96	29.7	230	44	130	44
		1.06	–2.34					
		3.91	–1.77					
	Propiconazole	0.11	–4.97	1.15	10 200	310	650	260
		0.72	–4.15					
		4.11	–3.39					
PRISTINE	Boscalid	0.27	–4.29	36	5300	1300	>533	298
		2.71	–3.29					
		24.1	–2.34					
	Pyraclostrobin	0.08	–2.30	7.11	16	4	7.85	4
		0.81	–1.30					
		8.41	–0.28					

[a]US Environmental Protection Agency [48].
[b]Log TU = log (concentration in beakers/48-h$LC50_{(D.magna)}$).
[c]Azoxystrobin [23]; propiconazole [24]; boscalid, pyraclostrobin [25].
[d]Pesticide Properties Database [41].
[e]Benchmark = toxicity value × level of concern. For details, see USEPA [48].
TU= toxic units; LC50 = median lethal concentration; NOEC = no observed effect concentration

H. azteca or that effects may not be manifested until a later life stage (e.g., reproduction, as noted by J. Kunz et al., US Geological Survey, Columbia, MO, unpublished data). It seems unlikely that the toxic effects of azoxystrobin on amphipods reported by others could have been dampened by the presence of the second active ingredient in QUILT, propiconazole, a sterol demethylase inhibiting fungicide. Studies with prochloraz, another demethylase inhibiting fungicide, demonstrated that at concentrations of azoxystrobin and prochloraz that were 100 to 1000 times higher than those used in the present study, prochloraz synergistically increased, rather than decreased, the toxicity of azoxystrobin to *D. magna* [49].

Of the 2 active ingredients in PRISTINE, pyraclostrobin, rather than boscalid, is likely responsible for the reduced growth rate of *H. azteca* exposed to the highest PRISTINE concentration compared with controls. Although boscalid is considered moderately to highly toxic to aquatic invertebrates [50], the high concentration in the present study (24 μg/L) falls well below the 48-h LC50 (5300 μg/L) and the 21-d chronic NOEC (1300 μg/L) of boscalid for *D. magna* (Table 1). However, the environmentally realistic pyraclostrobin concentration used in the high PRISTINE treatment (8.4 μg/L, Table 1), falls within the range of 4 μg/L, the 21-d chronic NOEC for *D. magna* [41]; 12 μg/L, the concentration killing 10% of *H. azteca* in 96 h (96-h LC10) [14]; and 25.1 μg/L, the 96-h LC50 for *H. azteca* [14]. Indeed, based on the 96-h LC10 of 12 μg/L pyraclostrobin for *H. azteca* [14], we might expect to see reduced survival of *H. azteca* exposed to 8.4 μg/L pyraclostrobin for 14 d in the PRISTINE treatment. Whether co-exposure to boscalid altered the effects of pyraclostrobin on the survival of *H. azteca* is not known, but interactive effects on shredders and other organisms have been reported for pesticides, including fungicides [7,17,51,52].

In addition to direct toxic effects on the growth of *H. azteca*, PRISTINE may also exert its toxic effects on growth by increasing amphipod metabolic energy demands. In the present laboratory study, exposure to the PRISTINE formulation (~33 μg/L) significantly reduced amphipod growth. Similarly, Zubrod et al. [18] reported that the fungicide formulation FOLICUR (active ingredient tebuconazole, concentrations of ~63–73 μg/L) had significant effects on the amphipod shredder, *G. fossarum*, reducing lipid levels and decreasing fecal production. The authors speculate that the decline in fungal biomass, and thus nutritional value, of the tebuconazole-exposed leaves likely led amphipods to mobilize their energy reserves to meet increased metabolic demands [18].

Fungicide effects on macroinvertebrates: TUs

Toxic units are considered a strong predictor of pesticide effects on leaf decomposition in streams [10]. Log TU ≥ –3.0 has been suggested as a threshold value for acute effects observed on the aquatic macroinvertebrate community structure in the field [2,53]. Indeed, researchers have reported reductions in the abundance of pesticide-sensitive macroinvertebrates in pesticide-contaminated streams that have a log $TU_{(D.\ magna)}$ of –3 [2,12], corresponding to pesticide concentrations 1/1000th of the 48-h $EC50_{(D.\ magna)}$. Significant effects have been reported in streams with even lower TUs, corresponding to pesticide concentrations of 1/10 000th (log TU –4) the 48-h $EC50_{(D.\ magna)}$ [2]. Although azoxystrobin and pyraclostrobin are among the pesticides identified as predominantly responsible for the maximum TUs measured in some stream systems [2], this is purely correlative and not predictive of what might be observed at similar TUs in laboratory studies. Thus, whereas the maximum log TUs in the present laboratory study (–1.8 for azoxystrobin in QUILT and –0.3 for pyraclostrobin in PRISTINE, Table 1) fall in the moderately contaminated (–2.5 < log TU ≤ –1.5) to highly contaminated (log TU > –1.5) categories, as modeled by Schäfer et al. for streams [2], they cannot be directly compared with field TUs.

Unlike herbicides and insecticides whose application is timed to meet a specific event (e.g., emerging plants or certain insect life-stages), fungicides are applied in response to dampness, which can lead to multiple applications over a season. It has been suggested that repeated fungicide applications increase the likelihood of chronic exposure to low concentrations of fungicides in streams [54]. In addition, the effects of fungicides on macroinvertebrates and microbes likely

differ for communities exposed to mixtures of several types of pesticides in the field [1,24], relative to communities exposed to only 2 fungicides under laboratory conditions. It is also important to keep in mind that the sensitivity of nontarget aquatic fungi and oomycetes to fungicides varies among fungal species and with the type of fungicide [5] and that the use and types of fungicide formulations vary with season. Thus, when conducting laboratory or in-stream evaluations of fungicides, effects on leaf decomposition may vary with seasonal differences in the microbial community present on stream-conditioned leaves and the relative sensitivity of those different communities to different fungicide formulations.

In the present study, a 2-wk exposure to PRISTINE, but not QUILT, at concentrations similar to the maximal environmental concentrations reported for boscalid and pyraclostrobin had significant adverse effects on amphipod growth and tended to reduce leaf decomposition. These findings, and the work of others, indicate that the increased use of fungicides in the United States [14,15], and the expected increase in use globally [55], are likely to pose significant risks to leaf-derived nutrient cycling in streams. Further work is needed to determine whether the fungicide concentrations currently associated with toxic effects in streams produce those effects in the laboratory. Specifically, there is a need to evaluate pesticides' effects on leaf decomposition over periods that mimic the repeated application rates of fungicides [54], using pesticide mixtures and concentrations reported in pesticide-affected streams [1,25,54], under conditions more reflective of stream environments (e.g., diurnal temperature shifts [16]). Under field conditions with multiple stressors (e.g., contaminant mixtures, predators, and fluctuations in temperature, oxygen, and flow-rate), and the potential for chronic exposure, fungicide concentrations currently detected in surface waters are likely to affect leaf decomposition by microbes and macroinvertebrate shredders in streams, as suggested by others conducting correlative field-studies [1,10–12]. In the present study, the suppressive effects of PRISTINE on leaf decomposition and amphipod growth, and the trend toward increased leaf decomposition with increasing QUILT concentration, indicate the potential for altered leaf decay in stream systems exposed to the fungicides in these formulations (boscalid, pyraclostrobin, azoxystsrobin, propiconazole) and for fungicide formulations to have differential effects on leaf decay. The present study adds to the increasing body of literature on the effects of realistic concentrations of current-use fungicide formulations on leaf decomposition by microbes and macroinvertebrate shredders.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI:10.1002/etc.3465.

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Data availability—Data, associated metadata, and calculation tools are available on request from the authors (aelskus@usgs.gov).

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