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Effects of a common insecticide on wetland communities with varying quality of leaf litter inputs☆

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ABSTRACT

Chemical contamination of aquatic systems often co-occurs with dramatic changes in surrounding terrestrial vegetation. Plant leaf litter serves as a crucial resource input to many freshwater systems, and changes in litter species composition can alter the attributes of freshwater communities. However, little is known how variation in litter inputs interacts with chemical contaminants. We investigated the ecological effects resulting from changes in tree leaf litter inputs to freshwater communities, and how those changes might interact with the timing of insecticide contamination. Using the common insecticide malathion, we hypothesized that inputs of nutrient-rich and labile leaf litter (e.g., elm [*Ulmus* spp.] or maple [*Acer* spp.]) would reduce the negative effects of insecticides on wetland communities relative to inputs of recalcitrant litter (e.g., oak [*Quercus* spp.]). We exposed artificial wetland communities to a factorial combination of three litter species treatments (elm, maple, and oak) and four insecticide treatments (no insecticide, small weekly doses of 10 μg L^{-1}, and either early or late large doses of 50 μg L^{-1}). Communities consisted of microbes, algae, snails, amphipods, zooplankton, and two species of tadpoles. After two months, we found that maple and elm litter generally induced greater primary and secondary production. Insecticides induced a reduction in the abundance of amphipods and some zooplankton species, and increased phytoplankton. In addition, we found interactive effects of litter species and insecticide treatments on amphibian responses, although specific effects depended on application regime. Specifically, with the addition of insecticide, elm and maple litter induced a reduction in gray tree frog survival, oak and elm litter delayed tree frog metamorphosis, and oak and maple litter reduced green frog tadpole mass. Our results suggest that attention to local forest composition, as well as the timing of pesticide application might help ameliorate the harmful effects of pesticides observed in freshwater systems.

1. Introduction

Understanding the function of ecosystems requires attention to the activity and turnover of species, as well as the physical and chemical changes in the environment. For example, temperate ecosystems have experienced dramatic changes in species composition owing to fire suppression, logging, habitat fragmentation, and hunting of top predators (Abrams, 2003). At the same time, humans have dramatically amended the landscape to increase agricultural output leading to the increased use of pesticides to control pest species and disease vectors (Grube et al., 2011). Despite efforts to control unwanted dispersal of pesticides, contamination of non-target communities and ecosystems remains a widespread concern (Gilliom et al., 2006). Given that such chemical contamination is co-occurring with changes in the species composition of remaining forest fragments, it is imperative that we understand how the two factors interact.

In temperate forests, variation in tree species composition results in numerous changes in terrestrial and aquatic food webs. For example, tree leaf litter serves as a prominent organic subsidy of energy and nutrients in streams and wetlands (Webster and Benfield, 1986; Wallace et al., 1997; Moore et al., 2004). The energy and nutrients within litter are released through processes that include leaching, fragmentation, and microbial enzymatic activity (Moore et al., 2004). These processes promote aquatic primary production, and consumers subsequently utilize litter fragments,

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microbes, and algae as resources for growth and development (Moore et al., 2004; Holgerson et al., 2016). Interspecific variation in the chemical quality of leaf litter can lead to substantial changes in the structure and function of detrital food webs (Webster and Benfield, 1986; Leroy and Marks, 2006). For example, recalcitrant litter (e.g., litter rich in lignin and cellulose) can reduce nutrient and energy availability for consumers, but might also promote stable population sizes of primary producers and consumers (Melillo et al., 1982; Geddes, 2015). In contrast, highly labile litter substrates can rapidly leach soluble carbon and promote algal and consumer growth (Cottingham and Narayan, 2013). However, excess amounts of leached carbon can darken the water column, reduce primary productivity, increase aerobic respiration, and subsequently generate inhospitable conditions for many aquatic organisms (Stephens et al., 2013; Cottingham and Narayan, 2013; Fey et al., 2015; Stoler et al., 2016). Similarly, leaching of phenolic acids can inhibit litter decomposition, reduce rates of nutrient cycling, and directly harm consumers by hindering the functionality of cell processes (Melillo et al., 1982; Webster and Benfield, 1986; Hättenschwiler et al., 2005; Maerz et al., 2005).

Changes in the chemical composition of aquatic environments might interact with pesticide contamination. Although most systems are not contaminated at concentrations that are likely to directly harm non-target organisms, pesticides can become more toxic when combined with other chemical or biological stressors (Relyea, 2003, Relyea and Diecks, 2008). For example, subtle changes in environmental chemistry, such as variation in pH due to the addition of acidic humic compounds might increase the duration of pesticide exposure by slowing rates of chemical breakdown (Wolfe et al., 1977). Resource stress imposed by litter species of low nutritional quality might also interact antagonistically with the presence of chemical contaminants. In contrast, the presence of dissolved organic carbon from rapidly decaying litter might bind to hydrophobic pesticide contaminants and alleviate toxic effects on aquatic consumers (Benson and Long, 1991; Wershaw et al., 1969; Haitzer et al., 1998). Although no studies have explored the interaction between pesticide contamination and qualitative variation in leaf litter inputs, elucidating these potential effects would greatly improve management and mitigation efforts.

Among the myriad chemicals applied to the landscape, organophosphates accounted for 35% of all insecticides (as of 2007; Grube et al., 2011). Malathion is among the most commonly applied organophosphates in the US (Grube et al., 2011). The hydrophobic chemical works by inhibiting acetylcholinesterase and typically slowing nerve cell signal transmission. Malathion is commonly found in aquatic systems due to misuse, overspray, and aerial drift from applications that frequently occur at multiple times during a growing season (Grube et al., 2011). Consequently, non-target organisms might be subjected to prolonged insecticide exposure. Previous work has found that low concentrations of the insecticide can be highly toxic to several species of zooplankton grazers, leading to trophic cascades and systemic effects on wetland communities (Relyea and Diecks, 2008; Relyea, 2009). However, studies have also shown that malathion can serve as a source of phosphorus for nutrient-limited microbial species (Rosenberg and Alexander, 1979). In turn, this fertilization effect might have positive consequences for organisms less sensitive to insecticide exposure. Hence, it is possible for the chemical to have both detrimental and beneficial effects on different parts of the aquatic community.

In this study, we explored the interaction between malathion contamination and variation in tree leaf litter inputs. Based on previous studies, we hypothesized that the presence of nutrient-rich litter (i.e. American elm) would increase microbial, algal, and consumer growth relative to nutrient-poor labile litter (i.e. red maple) or recalcitrant litter (i.e. oak). In addition, we hypothesized that malathion contamination would result in trophic cascades in which zooplankton die, phytoplankton bloom, and periphyton declines leading to lower consumer growth. Moreover, the effects caused by malathion exposure would be most severe under conditions of repeated insecticide application. Lastly, we hypothesized that inputs of nutrient-rich litter (i.e., elm) – which can promote periphyton growth and also leach carbon compounds (e.g., tannins) that bind to hydrophobic insecticides (Haitzer et al., 1998) – would ameliorate the negative effects of insecticides on wetland communities relative to inputs of recalcitrant litter.

2. Methods

2.1. Malathion in the environment

Models of drift and atmospheric deposition indicate expected environmental concentrations (EECs) of malathion in the range of 0.6–89.8 μg L^{-1} (Mastrota et al., 2010), although wetland surveys have found up to 600 μg L^{-1} (California Department of Fish and Game, 1982). Based on a USEPA risk assessment on the California red-legged frog (*Rana aurora draytonii*), average EEC of malathion in water is 9 ± 27 μg L^{-1} (95% CI) for application frequencies of 2–14 d (Odenkirchen and Wente, 2007). Although the half-life of the chemical under neutral conditions is relatively short, it varies with changes in pH, with half-lives at pH of 6 and 8 equal to 26 and 2 d, respectively (Guerrant et al., 1970; Wang, 1991). Moreover, multiple applications of the insecticide typically occur in a single season; manufacturer recommendations typically suggest applying the chemical two to four times per season at 4- to 7-d intervals (Bonide Products, Inc, Oriskany, NY). Although concentrations in non-target aquatic systems have dropped slightly in recent years (Stone et al., 2014), malathion remains among the most commonly used organophosphate insecticides on the current market.

2.2. Experimental design

To test our hypotheses, we conducted an experiment in outdoor mesocosms containing a diverse community of microbes, algae, zooplankton, amphipods, snails, and amphibians. We conducted our experiment during summer 2015 at the Rensselaer Aquatic Lab in Troy, New York. Our experiment consisted of a full-factorial design including three leaf litter treatments crossed with four insecticide treatments. Leaf litter treatments consisted of three common tree species: elm (*Ulmus americana*), red maple (*Acer rubrum*), and black oak (*Quercus velutina*). Insecticide treatments consisted of a no-insecticide control, 10 μg malathion L^{-1} delivered weekly, 50 μg malathion L^{-1} delivered once at the beginning of the experiment, and 50 μg malathion L^{-1} delivered once three wks after the start of the experiment (treatments are henceforth referred to as 10-weekly, 50-early, and 50-late, respectively). We selected these insecticide concentrations and application frequencies to correspond with a range of environmentally relevant values and to simulate realistic application regimes for agricultural use (Relyea and Diecks, 2008). We replicated each of the 12 treatment combinations four times for a total of 48 experimental units. Experimental units consisted of 900-L, black, polyethylene cylindrical tanks. We covered each mesocosm with a 60% shade cloth to prevent the escape or entry of any organism and to simulate moderate canopy cover (Schiesari, 2006).

We filled tanks with 550 L of chlorinated tap water between 1 June and 3 June, and allowed chlorine to off-gas before adding leaf litter. On 12 June, we added leaf litter to all tanks. We collected leaf litter from the ground of local forests during spring 2015 and allowed all litter to air-dry prior to adding it to the experiment. The

three species that we collected are common throughout much of temperate North American forests. Red maple has relatively high phenolic and nutrient content, and is increasing in abundance as a result of fire suppression and a reduction in the density of competing species (e.g., oak; Ostrofsky, 1993; Abrams, 1998). In contrast, black oak has relatively low phenolic and nutrient content, and is declining across much of its range due to over-browsing by white-tailed deer and selective logging (Abrams, 2003). Elm has moderate amounts of phenolic acids and nutrients, and is also declining in abundance as a consequence of Dutch elm disease (Moser et al., 2009). We added 190 g of dried leaf litter to each mesocosm. This biomass of litter is within the range of natural litter subsidies to temperate wetlands and represents a density that is common to mesocosm experiments (Rubbo et al., 2008).

On the same day as leaf litter addition, we inoculated bacteria, fungi, phytoplankton, and zooplankton into each mesocosm. We collected water from three local ponds as a source of bacteria, fungi, and phytoplankton. We also collected zooplankton from these three ponds using a 64-μm zooplankton tow and transported them back to the lab in the water from their respective pond. In the lab, we removed all zooplankton predators, homogenized all pond water, and added equal aliquots of the slurry to each mesocosm.

To measure litter decay rate, we added two coarse-mesh bags (10-mm mesh size) filled with 5 g of litter to each mesocosm on 16 June. Bags only contained litter corresponding to the treatment in which they were placed. The mesh size of the bags allowed entry by all consumers except for some late-stage tadpoles. In addition to providing a method of measuring litter decay rate, litter bags also served as a standardized substrate to sample benthic grazers. On the same day of litter bag introduction, we also positioned two 15-cm by 15-cm ceramic tiles leaning upright against the east-facing wall of each mesocosm. These tiles served as a standardized substrate to sample periphyton biomass.

Starting on 24 June, we began adding macroconsumers to all mesocosms. On 24 June, we collected amphipods (*Hyalella* sp.) from the shallow, vegetated area of a single lake. Because the amphipods varied in their size and development stage, we pooled all individuals into a single bucket and added equal aliquots to each mesocosm. This procedure effectively added ~120 individuals to each mesocosm. On 26 June and 14 July we added gray treefrogs and green frogs, respectively (Fig. 1a). For gray treefrogs, we collected 10 amplexed pairs from a local pond on 17 May and allowed them to lay eggs overnight in aged tap water. We collected three green frog egg masses from the same local pond on 26 June. After hatching, we fed tadpoles rabbit chow *ad libitum* until they reached a safe handling stage (stage 25, Gosner, 1960). Upon reaching this stage, we homogenized tadpoles from all clutches and added 30 individuals of each species to each mesocosm. Initial mass was 94.7 ± 5 mg SE for gray treefrog tadpoles and 14.3 ± 1.4 mg SE for green frog tadpoles.

We applied the first insecticide treatments on 30 June (defined as day 0), which was 4 d after the introduction of gray treefrog tadpoles and 14 d prior to the introduction of green frog tadpoles (day 14; Fig. 1a). We dosed the 50-early and 50-late treatments on days 0 and 22, respectively, and the 10-weekly treatments on days 0, 7, 14, 22, and 29. To dose a mesocosm with malathion, we first created a concentrated stock solution of technical grade malathion (Cerilliant Corporation, Round Rock, TX) dissolved in 100% ethanol. For all mesocosms receiving malathion, we diluted aliquots of the stock solution in 200 mL of mesocosm water and spread the diluted stock evenly across the surface of the water. We also dosed no-pesticide controls with an amount of ethanol equivalent to the amount added to pesticide treatments. Previous work has demonstrated that ethanol has no effect on amphibians or aquatic communities (Hua and Relyea, 2014). After each insecticide application, we gently agitated the surface water of all mesocosms to mix the insecticide throughout the water column. We also agitated the surface water of all mesocosms not receiving insecticides to homogenize the disturbance among mesocosms.

Within 1 h after applying the insecticide treatments on day 0 and day 22, we collected 40 mL of water from just below the surface at the center of each mesocosm and pooled the water from each insecticide treatment (i.e. 12 total replicates per treatment) into a single sample (i.e. 4 total samples). On the same day as collection, we preserved samples with 2 mL dimethylene chloride and transported them on ice to the University of Connecticut's Center for Environmental Sciences and Engineering. Samples were analyzed by high-pressure liquid chromatography. Results indicated that actual concentrations of malathion in the 10-weekly treatments were 8.5 μg L^{-1} and 6.1 μg L^{-1} on days 0 and 22, respectively. The actual concentrations of malathion in the 50-early and 50-late treatments were 34.2 μg L^{-1} and 24.1 μg L^{-1}, respectively. Analysis confirmed the absence of malathion in control treatments.

2.3. Abiotic response variables

We measured several abiotic variables throughout the study (Fig. 1b). On days 7 and 22, we measured temperature, pH, dissolved oxygen, and conductivity with a calibrated, handheld multimeter (YSI, Yellow Springs OH, USA) ~ 10 cm below the water surface. On days 20 and 45, we measured light attenuation across 20 cm of the water column using a submersible quantum sensor (Li-Cor Instruments, Nebraska, USA; see appendix for details on calculation).

2.4. Community response variables

Because zooplankton respond relatively rapidly to insecticide contamination, we measured the abundance of zooplankton in all tanks shortly after dosing the 50-early and 50-late, on days 7 and 27. We also measured their abundance several weeks after the 50-late dosing to determine if populations rebounded or if there was any lagged competitive release of other zooplankton species following initial exposure to malathion, as documented by Relyea and Diecks (2008, Relyea, 2009). We enumerated rotifer, copepod, and cladoceran densities, as zooplankton species within these larger taxonomic groups respond similarly to insecticides (Relyea and Diecks, 2008; Rubach et al., 2010; Hua and Relyea, 2014).

To examine the resources available to consumers throughout the study, we measured phytoplankton density, periphyton biomass, and litter decay rate (Fig. 1b). We measured phytoplankton on days 14 and 34 as the concentration of chlorophyll *a* in 1 L of water collected just below the surface. On days 21 and 41, we measured periphyton biomass as the oven-dried biomass of material scraped from a single ceramic tile. We assessed litter decay rate by removing a single mesh bag from each mesocosm approximately halfway through and at the end of the experiment (specifically, on days 28 and 58). We rinsed leaf litter of all organisms, and recording the oven-dried mass of remaining leaf litter. We calculated a single value of decay rate as the slope of ln-transformed litter mass loss through time (*sensu* Petersen and Cummins, 1974).

During both leaf litter samples, we collected all benthic macroinvertebrates rinsed from the litter in a 250-μm sieve (Fig. 1b). We preserved all macroinvertebrates in 70% ethanol. Due to extremely low or non-existent densities of all species across all treatments in the first sample date, we have excluded this sample from our analysis. In addition to amphipods, we also found a high abundance of ostracods, as well as *Physa acuta* and *Gyraulus*

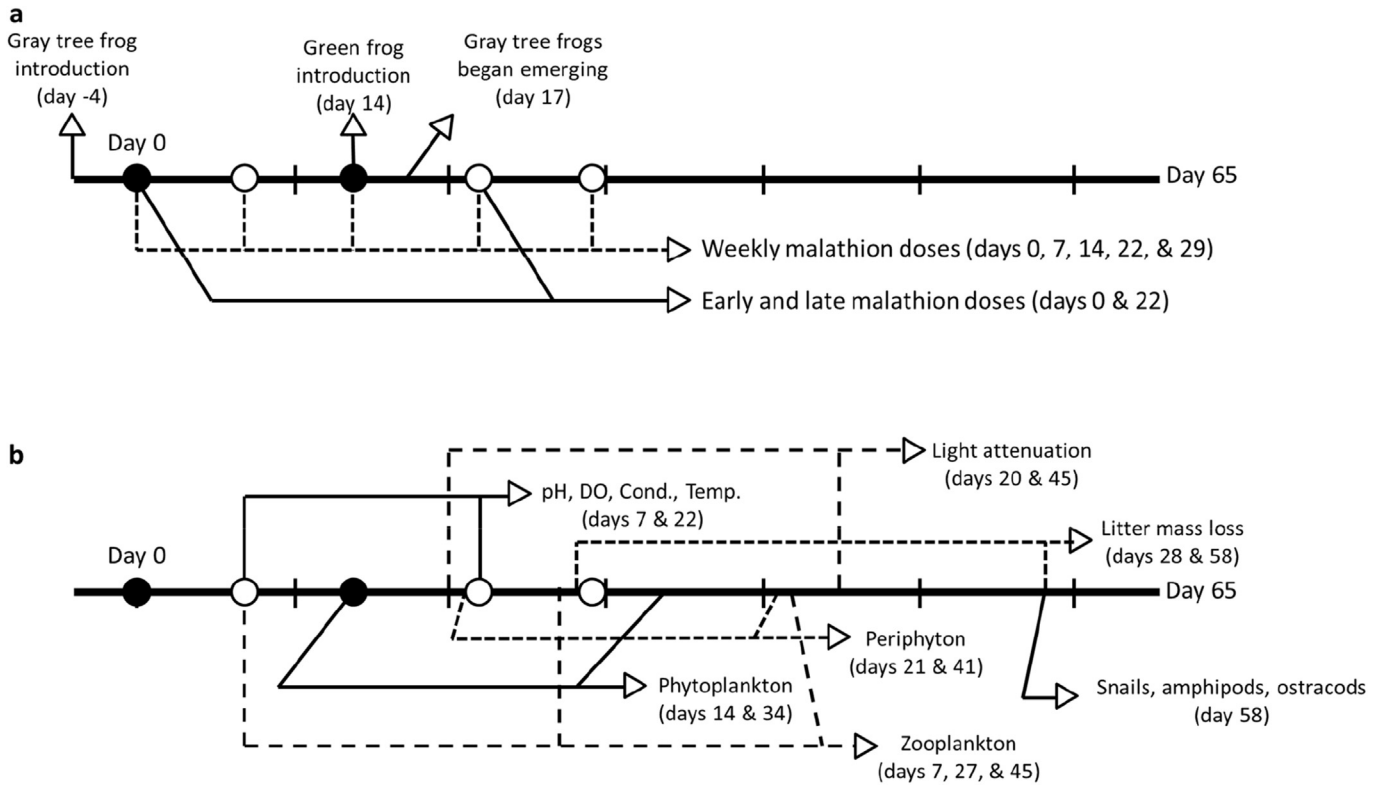

Fig. 1. Timeline of (a) tadpole introduction and insecticide applications, and (b) abiotic and biotic measurements, excluding tadpole responses. Tick marks on main axis are in 10 d intervals. Variation between dashed and solid lines is only for ease of distinguishing among lines. Black circles represent days of 50-early, 50-late, and 10-weekly treatment dosing; white circles represent days of only 10-weekly treatment dosing.

parvalus snails, which we introduced as eggs with zooplankton aliquots. Because our method of introducing zooplankton should have introduced similar numbers of snail eggs into all mesocosms, we also enumerated the abundance of both snail species.

Gray treefrogs began metamorphosing on day 17 (Fig. 1b). After this date, we checked mesocosms daily and removed any individual with four legs and a partially resorbed tail. We brought metamorphosing individuals into the lab and placed them in plastic containers containing a small amount of aged tap water. We considered metamorphosis complete once individuals resorbed tails to 2 mm. At this time, we noted the time to metamorphosis, euthanized individuals in a 2% MS-222 solution, and preserved them in 10% formalin. We recorded the survival and mean individual mass of preserved gray treefrog metamorphs from each mesocosm as our response variables. We concluded the experiment on day 65. At this time, none of the green frog tadpoles had metamorphosed. We collected remaining tadpoles and preserved them in formalin. We recorded the survival and mean individual mass of tadpoles in each mesocosm.

2.5. Statistical analyses

To analyze the effects of treatments on all responses, we employed protected analysis of variance (ANOVA; Scheiner and Gurevitch, 2001). We first analyzed the effects of treatments on all abiotic responses (i.e. pH, DO, temperature, conductivity, and light attenuation) using multivariate ANOVA (MANOVA) and a model that including both main effects and their interaction. Because we collected data for each of the abiotic responses on two sample dates, we employed a repeated-measures structure (rm-MANOVA) to examine the effects of time (within-subjects factor) and the interactions of time with all model terms (between-subject factors). Upon finding a significant multivariate effect, we conducted univariate rm-ANOVAs on each term and a model that included both main effects and their interaction.

Next, we analyzed the effects of treatments and time on resources and consumers. For all zooplankton responses (measured three times), we conducted another rm-MANOVA followed by univariate rm-ANOVAs. Similarly, we analyzed phytoplankton and periphyton responses in the same manner a rm-MANOVA followed by univariate rm-ANOVAs. Because we only had a single measurement for litter decay rate (i.e. the slope of mass loss over time), we analyzed this response using univariate ANOVA. For all organisms collected from leaf packs (measured once), including amphipods, ostracods, and snails, we conducted a single MANOVA followed by univariate ANOVAs. Because we only had a single measurement for amphibian responses (i.e. survival and mean individual mass for both green frogs and gray treefrogs, and mean time to metamorphosis for gray treefrogs), we analyzed these responses using MANOVA followed by univariate ANOVAs.

For all significant effects of litter, we conducted Tukey's HSD post-hoc comparisons to determine significant treatment differences. For all significant effects of insecticide, we conducted Dunnett's post-hoc comparisons of treatments with controls. When we detected an interaction of time with a model term, we conducted post-hoc comparisons within sample date. We considered all effects as significant when $P \leq 0.05$, but we also explored nearly significant effects when $P \leq 0.08$ (Murtaugh, 2014).

We transformed data when necessary to meet assumptions of ANOVA, which we verified by examining the linearity of residual

values and homoscedasticity of error variances within Q-Q and scale-location plots, respectively. Specifically, we ln-transformed pH, phytoplankton, all zooplankton, ostracod, and *H. trivolvis* responses, and square-root transformed periphyton responses. In addition, we rank-transformed count data for consumers taken from litter bags and arc-sin square root transformed amphibian survival data. Due to an accidental combination of individuals during the collection of green frog tadpoles, we were forced to remove one replicate of 10-weekly/oak litter and one replicate of 50-early/maple litter. Because there were no errors in the collection of any other response for these replicates, we only removed them from the MANOVA on amphibian responses. We conducted all analyses in R (Version 3.1.2, The R Foundation for Statistical Computing) using packages *car*, *vegan*, *agricolae*, and *multcomp* (Fox and Weisberg, 2010; Bretz et al., 2011).

3. Results

3.1. Abiotic measurements

For abiotic responses, we found multivariate effects of litter, insecticide, and a nearly significant interaction of litter and time, but no interaction of insecticide and litter treatments (Table 1; Fig. 2). We found main effects of insecticide treatment on pH, conductivity, and temperature (Table A1) that are detailed in the appendix. Univariate analyses revealed an interaction of litter and time for DO, light attenuation, and conductivity, so we explored effects of litter on these responses within each sample date (Table A1). On the first sample date, maple litter induced 0.74 times lower DO and 1.89 to 1.08 times greater light attenuation relative to elm and oak treatments, respectively. On the second sample date, maple litter induced 0.90 times lower DO relative to elm treatments and 1.50 to 1.63 greater attenuation than elm and oak treatments, respectively. Effects of litter on conductivity were minimal; elm litter generated 1.08 times higher conductivity than maple and oak treatments on both sample dates.

3.2. Zooplankton

Multivariate analysis of zooplankton densities revealed effects of litter, insecticide, and time, as well as two-way interactions of time with both litter and insecticides (Table 1; Fig. 3). Univariate analysis of cladocerans revealed an interaction of insecticide and time (Table A3). On the three sample dates, cladoceran densities in the 10-weekly treatment were suppressed to 0.21, 0.54, and 0.07 times the density in the no-insecticide control, respectively. Densities in the 50-early treatment were initially suppressed to less than 0.01 times the density in the no-insecticide control, but later rebounded to no-insecticide treatment levels as the insecticide rapidly broke down. In contrast, densities in the 50-late treatment were initially similar to densities found in the no-insecticide control, but the late insecticide addition suppressed densities to 0.20 times the density found in the control.

Univariate analysis of rotifer densities indicated effects of insecticides, time, as well as two-way interactions of time with both litter and insecticides (Table A3). Among litter treatments on the first sample date, densities with maple were 1.65–2.03 times higher than in elm and oak treatments, respectively. On the second sample date, densities with maple were 2.18 times higher than in oak treatments. We did not detect differences among litter treatments on the third sample date. Among insecticide treatments, densities inversely mirrored the patterns of cladocerans; densities in 10-weekly treatments increased by 16.84, 6.50, and 6.14 times more than densities in no-insecticide controls on the first, second, and third sample dates, respectively. Densities in the 50-early treatment were 25.80 and 9.86 times higher than densities in the no-insecticide control on the first and second sample dates, respectively, but then declined to similar densities as the no-insecticide control on the third sample date. In contrast, densities in the 50-late treatment were initially similar to the no-insecticide control but then increased to 4.91 and 9.46 times greater than densities in the no-insecticide control on the second and third sample dates, respectively.

For copepod densities, univariate analysis indicated effects of litter, insecticide, and time, as well as an interaction between insecticide and time (Table A3). Averaged across sample dates, densities in the maple treatment were 1.36 and 1.39 higher relative to other litter treatments. Among insecticide treatments, densities in the 10-weekly treatment were 3.23, 3.13, and 321 times higher than densities in the no-insecticide control on the first, second, and third sample dates, respectively. Densities in the 50-early treatment were also higher than the no-insecticide control (3.74 times higher), but only on the second sample date. Densities in the 50-late treatment were similar to levels in the no-insecticide control.

3.3. Resources: phytoplankton, periphyton, and litter decay rate

Multivariate analysis of phytoplankton and periphyton responses revealed effects of litter and time, as well as an interaction of insecticide and time, and a nearly significant three-way interaction between litter, insecticide, and time (Table 1).

Univariate analysis of phytoplankton densities revealed a significant three-way interaction, however analyses within each sample date failed to detect any insecticide-by-litter interactions (Table A2; Fig. 4a). Regarding the effects of litter, we found 1.14 times higher phytoplankton densities in the maple treatment

Table 1
MANOVA results for abiotic, zooplankton, resources (phytoplankton and periphyton), benthic invertebrate, and amphibian responses. For all responses measured more than once, statistics for repeated-measures MANOVAs are presented. Subscripts indicate degrees of freedom. Bolded values are significant ($P \leq 0.05$); italicized values are nearly significant ($P \leq 0.080$).

No. times sampled	Abiotics		Zooplankton		Periphyton & phytoplankton		Benthic invertebrates		Amphibians	
	2		3		2		1		1	
	F	P	F	P	F	P	F	P	F	P
Litter	**$9.7_{2,35}$**	**<0.001**	**$9.6_{2,36}$**	**<0.001**	**$9.4_{2,36}$**	**0.001**	**$2.6_{2,66}$**	**0.016**	**$4.2_{2,58}$**	**<0.001**
Insecticide	**$3.9_{3,35}$**	**0.019**	**$4.2_{3,36}$**	**0.012**	$1.4_{3,36}$	0.267	**$5.4_{3,88}$**	**<0.001**	**$3.0_{3,18}$**	**<0.001**
Litter x insecticide	$2.0_{6,35}$	0.094	$1.2_{6,36}$	0.332	$0.9_{6,36}$	0.533	**$2.1_{6,24}$**	**0.004**	**$2.2_{6,36}$**	**0.001**
Time	**$586.3_{1,35}$**	**<0.001**	**$26.2_{2,72}$**	**<0.001**	**$35.5_{1,36}$**	**<0.001**				
Litter x time	*$3.0_{2,35}$*	*0.062*	**$4.1_{4,72}$**	**0.005**	$2.0_{2,36}$	0.154				
Insecticide x time	$1.5_{3,35}$	0.241	**$5.1_{6,72}$**	**<0.001**	**$8.0_{3,36}$**	**<0.001**				
Litter x insecticide x time	$0.9_{6,35}$	0.517	$1.0_{12,72}$	0.448	*$2.3_{6,36}$*	*0.052*				

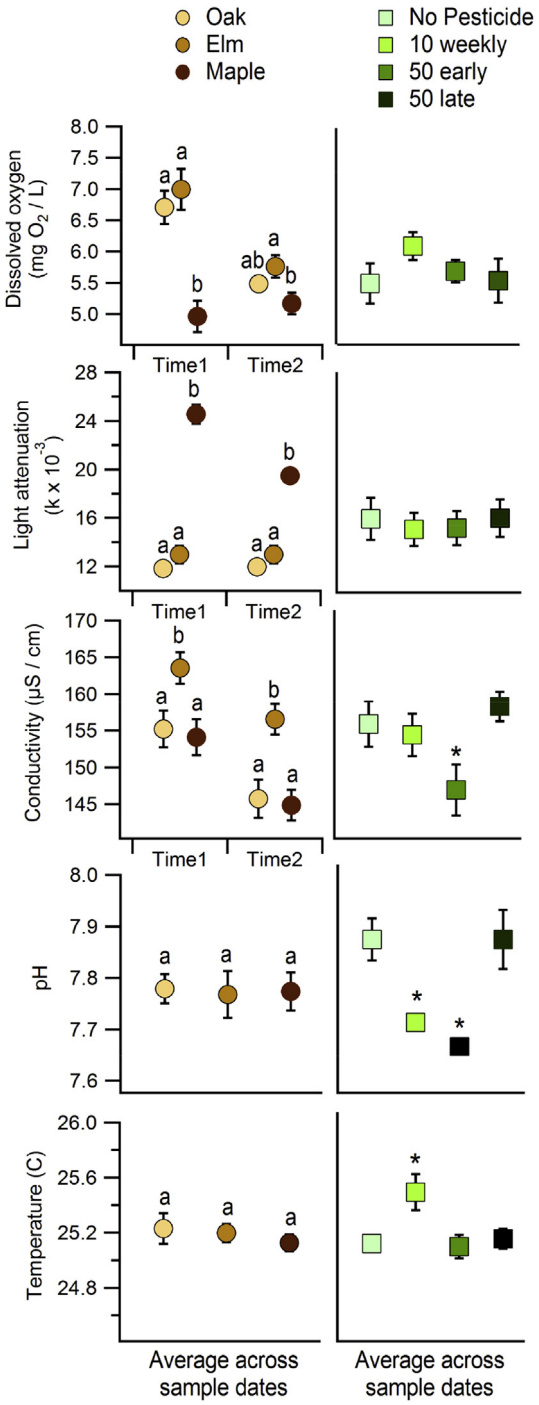

Fig. 2. Effects of litter and insecticide treatments on abiotic responses. Left and right panels depict effects on litter and insecticide treatments, respectively. Unless otherwise indicated, means represent averages across sample dates. Letters above litter treatment means indicate significant differences via Tukey's post-hoc comparisons; stars above insecticide treatment means indicate significant differences of means from no-insecticide controls as determined by Dunnet's post-hoc comparisons. Bars represent ±1 SE.

relative to the oak treatment on the first sample date, and 1.15 and 1.19 times higher densities in maple and elm treatments relative to the oak treatment on the second sample date. Regarding the effects of insecticides, we found 1.13 times higher densities in the no-insecticide control relative to the 10-weekly treatment on the first sample date and 1.15 times higher densities in the 50-early treatment relative to the no-insecticide control on the second sample date.

Univariate analysis of periphyton biomass revealed an effect of time, a nearly significant effect of litter, an interaction of insecticide and time, and a three-way interaction (Table A2; Fig. 4b). On the first sample date, we detected independent effects of litter and insecticides that included 1.56 times more periphyton biomass in the maple treatment relative to the oak treatment, and 1.60 times more biomass in the no-insecticide control than in the 10-weekly treatment. On the second sample date, we detected a nearly significant interaction of litter and insecticide treatments. Treatment comparisons found 4.73 times more periphyton biomass in the 50-late treatment relative to the no-insecticide control, but only in the presence of maple leaf litter.

Litter decay rates were affected by litter and insecticide treatments, but not by their interaction (litter: $F = 24.1_{2,36}$, $P < 0.001$; insecticide: $F = 6.5_{3,36}$, $P = 0.001$; litter x insecticide: $F = 1.0_{6,36}$, $P = 0.467$; Fig. 5). The decay rate of maple litter was 1.47 and 2.32 times greater than elm and oak litter, respectively. The decay rate of elm litter was 1.57 times greater than oak litter. Litter in the no-insecticide control was 1.74 times faster decay rate relative to litter in the 10-weekly treatment; the decay rate in the other insecticide treatments was similar to the control.

3.4. Benthic invertebrates

Multivariate analysis of benthic macroinvertebrate responses revealed effects of litter and insecticide, but not their interaction (Table 1; Fig. 6). For amphipods, univariate analysis revealed an effect of insecticides (Table A4); densities in 10-weekly, 50-early, and 50-late treatments were 0.27, <0.01, and 0.04 times lower, respectively, relative to the no-insecticide control. For ostracods, univariate analysis revealed an effect of litter (Table A4); densities in the maple treatment were 349 times higher than in the oak treatment. We did not find any treatment effects on the densities of the two snail species.

3.5. Amphibians

Multivariate analysis of amphibian responses revealed significant effects of litter, insecticide, and their interaction (Table 1; Fig. 7). Univariate analyses of gray treefrog responses revealed only an effect of litter on individual metamorph mass (Table A5). Mass in the oak litter treatment was 0.71 and 0.88 times lower than in elm and maple treatments, respectively. We also found an interaction of insecticide and litter treatments on survival. With oak litter, survival was unaffected by the insecticide treatments. With elm litter, we found 8% lower survival in the 50-early treatment relative to the no-insecticide control. With maple litter, we found 13% lower survival in the 10-weekly treatment relative to the no-insecticide control. In addition to effects on mass and survival, we also found a nearly significant interaction of insecticide and litter treatments on time to metamorphosis. Among both elm and oak treatments, we found that individuals in the 50-early treatment metamorphosed 3 d–4 d earlier relative to the no-insecticide control.

We also found an interaction of insecticide and litter treatments on green frog tadpole mass (Table A5). With oak litter, we found 1.31 and 1.37 times higher biomass in the no-insecticide control relative to 50-late and 50-early treatments, respectively. With maple litter, we found 1.36, 1.37, and 1.64 times greater biomass in the no-insecticide control relative to 50-late, 50-early, and 10-weekly treatments, respectively. With elm litter, the insecticide treatments had no effect. Univariate analyses also revealed an effect of litter on green frog survival, which was generated by 1.10% and 1.11% higher survival in the maple treatment relative to elm and oak treatments, respectively.

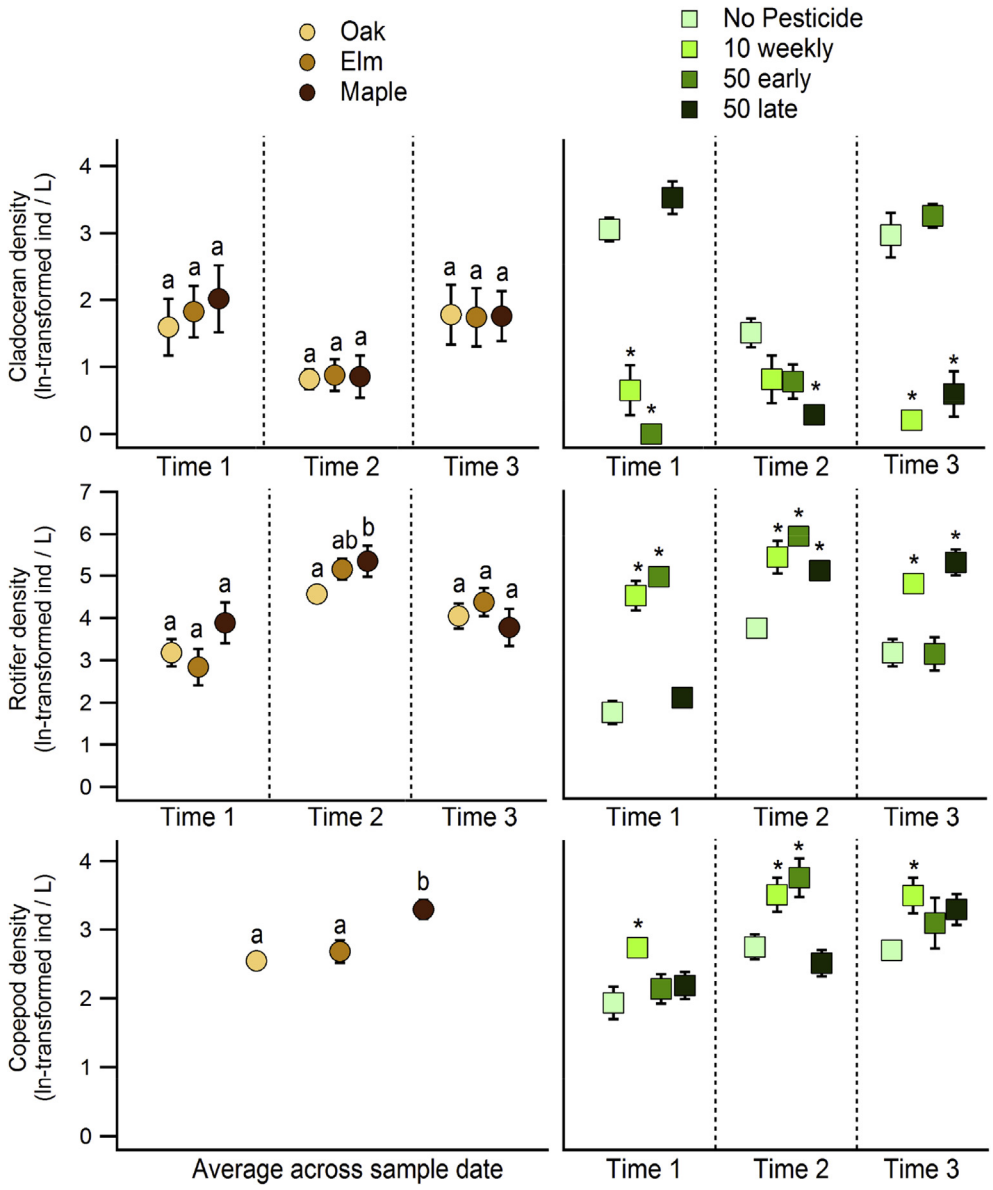

Fig. 3. Effects of litter and insecticide treatments on (a) cladocerans, (b) rotifers, and (c) copepods. Interpretation is as in Fig. 2.

4. Discussion

Our study examined the interactive effects of insecticide contamination with variation in the nutritional and chemical quality leaf litter inputs on a wetland community. As predicted, variation in interspecific leaf litter chemistry had pronounced effects on the community, with elm and maple litter most often associated with greater production of phytoplankton, periphyton, and consumer biomass. Also as predicted, insecticide contamination had numerous effects on the biological attributes of our communities, and led to substantial declines in the abundance of some zooplankton and benthic grazers. The effects of insecticide inputs depended on the timing and magnitude of dosing, although repeated insecticide application did not always cause the most severe effects as expected. In addition, we found several instances in which the effects of insecticide treatments were dependent on the species of leaf litter present, particularly with regard to the performance of the amphibians.

4.1. Effects of leaf litter inputs

Compared to oak litter, we found that elm leaf litter promoted growth of phytoplankton and periphyton, and was associated with an increased mass of gray treefrog metamorphs. These results largely agree with those of Stoler and Relyea (2016). Both elm and oak leaves typically contain high levels of lignin and cellulose, which can create a more difficult substrate for microbes to immediately colonize. However, elm also contains more nutrients and can be a more labile resource after some degredation of lignin and cellulose (Melillo et al., 1982). Hence, it is reasonable to expect that positive effects of elm on primary and secondary production will become more pronounced later in the experiment, particularly after significant leaf degradation has occurred. Indeed, we found that elm litter induced higher densities of phytoplankton than oak, but only on the second sample date. This suggests that elm litter can also became a more labile substrate for grazers, which might explain the observed increase in treefrog biomass.

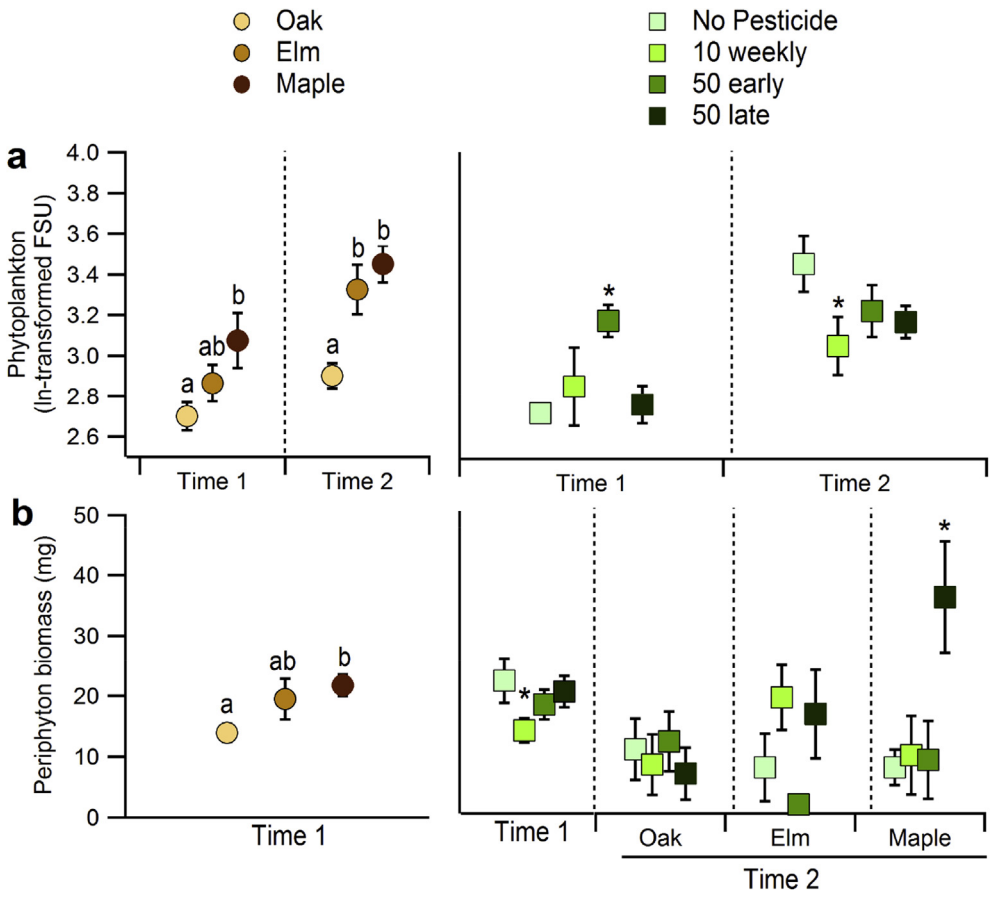

Fig. 4. Effects of litter and insecticide treatments on (a) phytoplankton density and (b) periphyton biomass. Interpretation is as in Fig. 2. Due to an interaction between insecticide and litter treatments on periphyton biomass during the second sample date, we present means and standard errors of insecticide treatments within litter treatments.

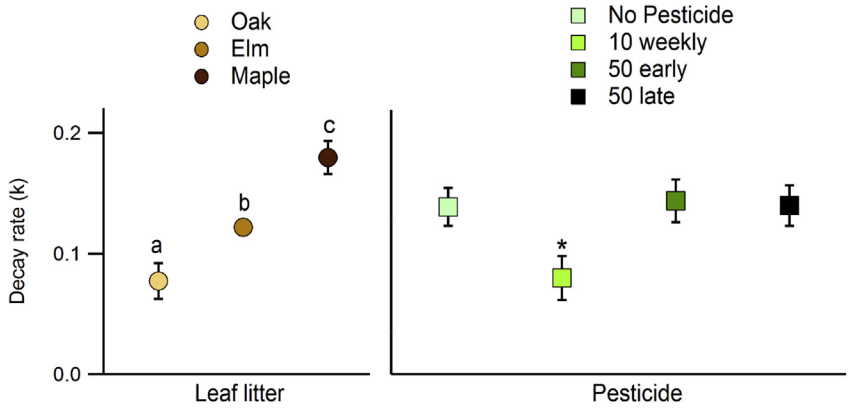

Fig. 5. Effects of litter and insecticide treatments on litter decay rate. Interpretation as in Fig. 2.

Compared to oak, we also found that maple litter decayed faster, induced lower levels of dissolved oxygen, and was associated with higher light attenuation than elm and oak inputs. These results are similar to past studies that have explored the effects of maple and other litter species with high levels of soluble carbon (Rubbo and Kiesecker, 2004; Stephens et al., 2013; Stoler and Relyea, 2016). However, those studies generally found that low levels of oxygen and light can lead to harsh conditions that result in high consumer mortality. In contrast, we found that the presence of maple litter generally increased phytoplankton and periphyton production, as well as survival, mass, and density of several consumer species. A likely explanation for this is that we employed spring-collected litter, whereas past studies have used freshly senesced litter collected in the autumn. Spring-collected litter has already been leached of many acidic compounds that can be harmful to aquatic consumers, inhibit microbial mineralization processes, and reduce the availability of nutrients in the litter. Consequently, spring-collected maple litter is likely to provide a more labile resource

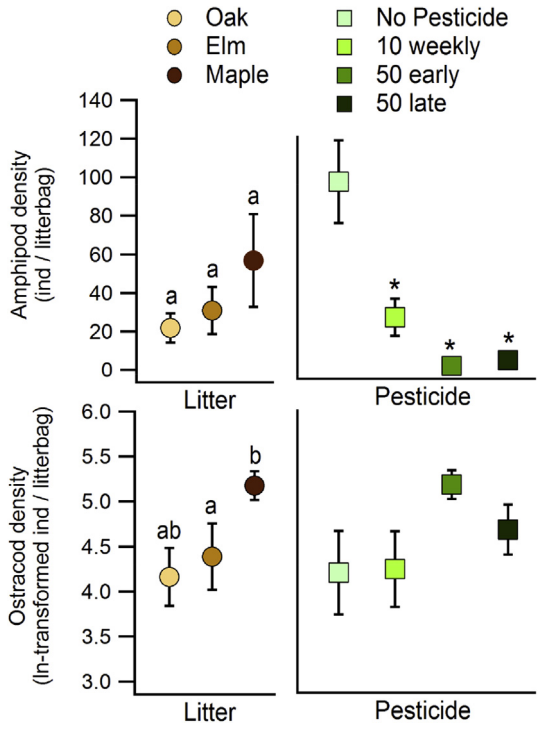

Fig. 6. Effects of litter and insecticide treatments on (a) amphipod and (b) ostracod densities as found in litter bag samples. Interpretation is as in Fig. 2.

than those used in previous studies.

4.2. Effects of insecticides

As expected from Relyea and Diecks (2008), malathion contamination led to a rapid reduction in cladoceran densities. Whereas densities were continually suppressed in the 10-weekly treatment, densities in the 50-early treatment recovered by the end of the experiment. These results are similar to those of Relyea and Diecks (2008) and are likely due to the rapid reproduction rate of most cladocerans and the fast breakdown rate of malathion. In our study, reductions in cladoceran abundance were associated with an increase in rotifers, which can compete with cladocerans for small phytoplankton species (Hanazato, 2001), and are far more tolerant to malathion (cladoceran LC_{50} value $\leq$ 2.3 μg malathion L^{-1} vs. rotifer LC_{50} value > 35,000 μg malathion L^{-1}; Siepman and Slater, 1998).

We also found that a rise in rotifer densities often coincided with a rise in copepod densities, which are both predators and competitors of rotifers. However, it is worth noting that copepod densities in 50-early treatments exhibited a lagged response to insecticide dose, suggesting that they might be moderately sensitive to malathion at 50 μg L^{-1}. Siepman and Slater (1998) indicate that several species of freshwater copepods share a similar level of intolerance to malathion with cladocerans. In addition, both Stoler et al. (2016) also found an initial depression in copepod abundance at 50 μg L^{-1} relative to a 5 μg L^{-1} treatment using a similar acetylcholine-esterase inhibiting insecticide (i.e., carbaryl; carbamate insecticide). In contrast, Relyea and Diecks (2008) found a consistent rise in copepod densities even after dosing mesocosms

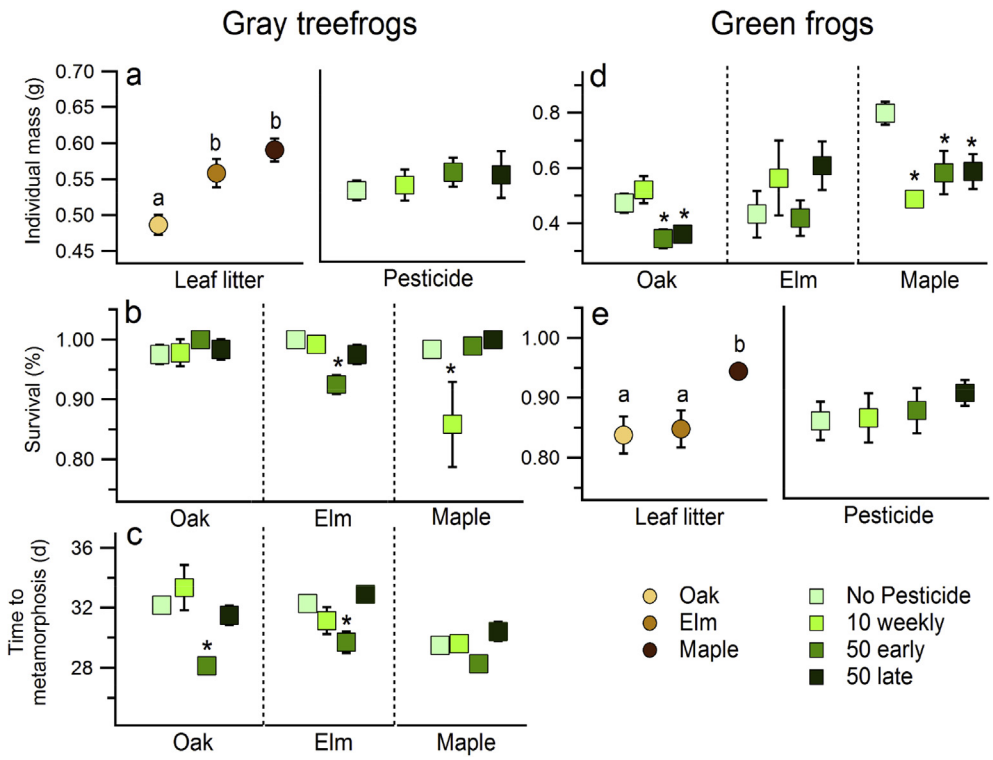

Fig. 7. Effects of litter and insecticide treatments on (a–c) gray treefrog responses and (d–e) green frog responses. Interpretation is as in Fig. 2. Due to interactions of insecticide and litter treatments on gray treefrog survival, gray treefrog time to metamorphosis, and individual mass of green frog tadpoles, we present means of insecticide treatments within litter treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with malathion at a concentration of 250 μg L^{-1}. Hence, another possible explanation is that copepod and rotifer densities exhibited a lagged predator-prey response. However, this is unlikely to be the only explanation, given that copepod densities were consistently high in the 10-weekly treatments. Nevertheless, it is possible that copepod communities in treatments with 50 μg malathion L^{-1} were primarily composed of predatory species whereas populations with 10 μg L^{-1} were primarily composed of primary consumers. This explanation coincides with the observed rise in phytoplankton densities in the 50-early treatment. Future work analyzing how copepod species composition changes with insecticide contamination might shed light on the underlying mechanisms.

In addition to effects of malathion on zooplankton, we found that 10-weekly treatments reduced rates of litter decomposition of all litter species. Previous studies have also found that pesticides can reduce leaf breakdown rate (Wallace et al., 1982; Schäfer et al., 2007) as a result of direct lethal effects on detritivorous organisms. Although we found that malathion contamination resulted in an acute reduction of amphipod density, we found no similar effect on other grazers. Moreover, we found similar reductions in amphipod density among all treatments contaminated with malathion, but we only detected reduced rates of litter decomposition in the 10-weekly treatments. A second possible explanation for this result is that malathion had a directly toxic effect on litter biofilms. However, there is no evidence that acetylcholinesterase inhibitors have any effect on microbes, and research has even shown some bacteria and fungi benefit from organophosphates as a nutrient source (Rosenberg and Alexander, 1979). Indeed, the addition of 50 μg malathion l^{-1} provides approximately 5 μg phosphorus l^{-1}. Although this quantity is not nearly enough to warrant concerns about eutrophication, it can still provide a stimulus for microbial activity. Hence, it is possible that litter breakdown rates were reduced in all insecticide treatments, yet this was offset in 50-early and 50-late treatments due to phosphorus fertilization and growth of biofilm. This explanation is validated by the observation that periphyton biomass was only reduced in the 10-weekly treatments, yet further work directly exploring the effects of organophosphates on biofilm growth must be conducted to fully verify this phenomenon.

4.3. Interaction of insecticides and litter

We hypothesized that inputs of litter rich in nutrients and soluble carbon (i.e., elm) would ameliorate the negative effects of insecticides on wetland communities by promoting periphyton growth and by leaching compounds that can bind to malathion (Haitzer et al., 1998). Although we did find an interactive effect of leaf litter and insecticide treatments on periphyton, we found little evidence to support our hypothesis. Instead, we found elevated periphyton biomass in the 50-late treatment, but only in the presence of maple litter. Instead of being a direct effect of malathion, this effect likely resulted from consumptive release following a reduction in green frog mass, which declined in the 50-early and 50-late insecticide treatments with oak litter and in all insecticide treatments with maple litter. It is unclear why periphyton biomass did not also increase among oak litter treatments, but this might be due to the lack of available nutrients inherent in oak litter chemistry. Indeed, the most likely explanation for the reduction in green frog mass is that the combination of nutrient deficiency from oak litter or phenolic leaching from maple litter acted synergistically with the stress of malathion to generate sublethal effects. This is not surprising, given that green frogs have been found to be highly sensitive to both phenolic-rich substrates and malathion (Relyea, 2004; Maerz et al., 2005). Hence, our results demonstrate that interactive effects of insecticide contamination and litter inputs might be most readily experienced by sensitive consumers, with cascading effects on consumer resources.

We also found reduced survival of gray treefrogs in the 10-weekly maple and 50-early elm treatments, but no effect of insecticide treatments with oak litter. Our results for oak litter treatments are similar to the responses of wood frogs in the study by Relyea and Diecks (2008), where oak litter was the primary substrate. Wood frogs and gray treefrogs have a similar and relatively short larval duration, and Relyea and Diecks (2008) noted that an insecticide-induced trophic cascade only became over a relatively longer period of time. Hence, our observation of reduced survival with maple and elm treatments indicates the presence of some additional stressor emanating from the litter species. For maple litter, this additional stress can likely be attributed to a high amount of phenolic acids. Elm is also known to have a relatively high concentration of condensed tannins (Ostrofsky, 1993), although this species did not have any observable negative effects of amphibians in Stoler and Relyea (2016). It is also unclear why the interaction of insecticides and litter existed for different application regimes. Further work that explores the effects of individual chemical components on amphibian performance will help to reveal underlying mechanisms.

However, we did find some evidence that maple litter reduced or eliminated the negative effects of pesticides on amphibians. Relative to no-pesticide controls, we found a shorter time to metamorphosis of grey treefrogs in 50-early treatments with elm and oak litter, whereas maple litter induced a relatively short time to metamorphosis regardless of insecticide treatment. This interaction might be due to the fertilization effects of malathion in the 50-early malathion treatment, and the lability of spring-collected maple litter as resource. An abundance of resources is generally associated with faster time to metamorphosis for this species (Relyea and Hoverman, 2003; Relyea and Diecks, 2008), and Stoler and Relyea (2016) found that gray treefrogs metamorphosed faster with maple litter than with elm litter. This outcome is indicative of potentially higher fitness for individuals in such an environment, given that larger metamorph size and earlier time to metamorphosis are both positively related to terrestrial adult fitness (Semlitsch et al., 1988).

5. Conclusion

Although many components of our community were only affected by either insecticide or litter treatment, we did find several important interactions between treatments. Specifically, we found that malathion contamination can have either neutral or negative effects on tadpole growth and development depending the species of leaf litter present. This finding presents an important part of the story concerning recent declines in amphibian populations around the world. These declines have been attributed to numerous factors, such as pesticides, habitat fragmentation, UV radiation, and disease. However, the current study adds to the growing literature suggesting that a variety of these factors have interactive effects on freshwater communities. For example, our study indicates that the recent declines in oak and increases in maple (Abrams, 2003) might result in reduced tadpole survival when chronically exposed to insecticides. Mechanisms underlying these interactions are still unclear, but are certainly related to the chemical characteristics of the litter, and further work is needed to elucidate the exact chemical components that alter wetland communities. Such work could afford an element of generality to understanding the interaction between aquatic environmental chemistry and contaminant exposure.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.04.019.

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