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Effects of Individual and Combined Pesticide Commercial Formulations Exposure to Egestion and Movement of Common Freshwater Snails, Physa acuta and Helisoma anceps

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ABSTRACT.—Pesticides are detected in streams at concentrations that might have adverse effects on aquatic organisms. These agrochemicals typically occur in streams as combinations, yet research has focused on the effects of individual pesticides. We studied the effects of commercial formulations of atrazine, metolachlor, carbaryl, and chlorothalonil on aquatic gastropods *Physa acuta* and *Helisoma anceps* egestion and movement. We observed an eightfold reduction in *P. acuta* egestion rates when exposed to individual (atrazine: 200 μg/L; metolachlor, carbaryl, and chlorothalonil: 100 μg/L) and combined (atrazine x metolachlor: 200 μg/L x 100 μg/L and carbaryl x chlorothalonil: 100 μg/L x 100 μg/L) pesticide treatments relative to controls. For *H. anceps* individual and combined pesticide treatments had no significant effects on egestion, highlighting differential species response. *Helisoma anceps* movement declined when exposed to atrazine, carbaryl, and chlorothalonil individually, though responses varied with exposure time. When combined atrazine + metolachlor and carbaryl + chlorothalonil reduced *H. anceps* movement relative to the control. In addition to pesticide physicochemical characteristics, it is important to consider exposure durations to better understand the effects of pesticides on aquatic organisms. Furthermore, future risk assessments should incorporate multiple species to better represent response diversity.

INTRODUCTION

Increased pesticide use as a result of increasing crop protection practices and global human population growth (Population Reference Bureau, 2015) has resulted in pesticide concentrations in freshwater ecosystems that might have adverse effects on aquatic organisms. At elevated concentrations, such as following runoff events, mortality of diverse species including bullfrogs (Relyea, 2006), cladocerans (Munn *et al.*, 2001), and caddisflies (Liess and Ohe, 2005) has been observed. Furthermore, at lower concentrations (*i.e.*, concentrations below mean concentrations and <1 ppm; Gilliom *et al.*, 2006), sublethal effects might result in changes of physiological processes, including growth in amphibians (Relyea, 2008), lower fecundity in cladocerans (Kashian and Dodson, 2002), reduction of decomposition rates in aquatic communities (McMahon *et al.*, 2012), and lower nutrient uptake of sediment microbes (Elias and Bernot, 2014). Though previous research has documented adverse effects of individual compounds on aquatic organisms, there is a need for more studies that evaluate the effect of multiple-stressors (*e.g.*, Rohr *et al.*, 2003; Boone *et al.*, 2005; Relyea *et al.*, 2009).

Pesticides are rarely applied individually in agriculture. Rather, pesticides are used in combination at specific times during crop production (Gilliom *et al.*, 2006). In the U.S. atrazine (triazine), metolachlor (chloroacetanilide), carbaryl (carbamate), and chlorothalonil (chloronitrile) have high usage rates (Solomon *et al.*, 1996), are prevalent in streams (Larson *et al.*, 1999), and co-occur more often as mixtures than as individual pesticides (Gilliom, 2007). Therefore, pesticide occurrence overlaps in space and time as a

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result of usage and application (Smiley *et al.*, 2014). Pre-emergent herbicides (atrazine and metolachlor) are usually detected following spring applications and associated with corn production (Thurman *et al.*, 1991; Giddings, 2005; Byer *et al.*, 2011; USEPA, 2016) and are commonly applied in Midwestern U.S. (Kolpin *et al.*, 1998). Insecticides (carbaryl) and fungicides (chlorothalonil) are applied multiple times throughout the year to control pest outbreaks and are concurrently sprayed on crops (*e.g.*, asparagus, snap beans, sweet corn; NASS – USDA, 2005). Carbaryl is the second most frequently detected insecticide in water and is found in 50% of urban streams (USEPA, 2012). Since 2001 there has been an increase in the use of chlorothalonil (USGS – NAWQA, 2016), and in channelized streams, it is detected at concentrations that could affect downstream sources of drinking water (Smiley *et al.*, 2010). Atrazine half-life ranges from 60 to >100 d depending on environmental conditions (University of Hertfordshire, 2015), with less than a 9% reduction (312.86 μg/L) of maximum average concentration (344.26 μg/L) after 7 d (USEPA, 2016) and ∼22% reduction (270.13 μg/L) after 14 d in flowing waterbodies. Changes in maximum concentration (45 μg/L) in static water bodies are less pronounced after 7 d (0%; 45 μg/L) and 14 d (12.5%; 39 μg/L; USEPA, 2016). Similarly, metolachlor is fairly stable in water bodies with a half-life of 88 d (University of Hertfordshire, 2015). Metolachlor concentration in runoff decreased by 5% (25 μg/L to 23.7 μg/L) after 7 d following a rain event (Caron *et al.*, 2012). Finally, chlorothalonil half-life in water ranges from 16 d to 38 d (University of Hertfordshire, 2015) and is likely to be present at high concentrations near golf courses runoff (Shuman *et al.*, 2000).

Pesticide effects on aquatic organisms have been observed in response to both individual and combined pesticide exposure. Individually, atrazine (3 μg/L) increases susceptibility of amphibians to trematode infection (Kohler and Triebskorn, 2013). Metolachlor (40 μg/L to 52 μg/L) interfered with crayfish chemosensory stimuli (Wolf and Moore, 2002; Cook and Moore, 2008) and reduced biomass and growth of *Lemna gibba* (University of Hertfordshire, 2015). Carbaryl (0.5 μg/L) increased refuge time of *Ambystoma barbouri* (Rohr *et al.*, 2003). Chlorothalonil (0.01 μg/L to 0.5 μg/L) increased nitrate remineralization of benthic microbes (Elias and Bernot, 2014). Additionally, chlorothalonil was used to control aquatic snails, *Pomacea* sp. in lowland rice (Stevens, 2003) and the intermediate host of *Schistosoma* sp., the freshwater snail *Oncomelania hupensis* (Quanbin *et al.*, 1992). Although a mode of action has not been established for chlorothalonil and gastropods, chlorothalonil reduces available glutathione in cells by reduced-substitution (Tillman *et al.*, 1993) and likely affecting normal cellular function in gastropods (Baturo and Lagadic, 1996). As mixtures atrazine (10 μg/L) and metolachlor (10 μg/L) increased time to initiate metamorphosis in *Rana pipiens* (Hayes *et al.*, 2006) and frequency of amphibians with thymic plaques (Hayes *et al.*, 2006). To our knowledge no studies have been conducted on aquatic organism response to carbaryl and chlorothalonil mixtures. Therefore, due to their spraying schedule, target crops (NASS – USDA, 2005), and potential effects on aquatic organisms (*e.g.*, Rohr *et al.*, 2003; Elias and Bernot, 2014), there is a need to assess the ecological effects of pesticides that co-occur in streams.

Aquatic gastropods are commonly used in ecotoxicological studies to address the effects of pesticide exposure (*see Pseudosuccinea columella,* Tate *et al.*, 1997; *Stagnicola elodes,* Koprovnikar and Walker, 2011; *Physella* spp., Baxter *et al.*, 2011; *Potamopyrgus antipodarum,* Hock and Poulin, 2012). However, few studies are conducted on *Physa acuta* or *Helisoma anceps*, particularly with respect to pesticides mixtures (but *see*, Basopo *et al.*, 2014; Hua and Relyea, 2014). *Physa acuta* and *H. anceps* are ubiquitous aquatic gastropods in North America and are common in freshwater habitats across a range of human influence (Dillon *et al.*,

2002; Thorp and Covich, 2009). These snails mature quickly (McCarthy *et al.*, 2000), lay eggs in masses (Dillon *et al.*, 2006), and graze on biofilm (algae, bacteria, fungus) growing on substrates (Hawkins *et al.*, 1987), as well as detritus (Brady and Turner, 2010). Consequently, snails fulfill an important role as primary consumers and decomposers (Newman *et al.*, 1996). *Physa acuta* and *H. anceps* are also prey for diverse vertebrate and invertebrate organisms including crayfish (*Orconectes juvenilis*; Dickey and McCarthy, 2007) and pumpkinseed sunfish (*Lepomis gibbosus*; Justice and Bernot, 2014). Furthermore, *P. acuta* and *H. anceps* are important intermediate hosts of parasites, including *Halipegus eccentricus, H. occidualis, Echinostoma trivolvis, Megalodiscus temperatus,* and *Fasciola hepatica* (Sapp and Esch, 1994) which can cause disease in wildlife (Gustafson and Bolek, 2015), livestock (Case, 1953), and humans (Graczyk and Fried, 1998). Therefore, research on the ecological effects of pesticides on *P. acuta* and *H. anceps* is essential, due to their key role in nutrient cycling, functional link between primary producers and secondary consumers, parasite and disease transmission, and as model organisms (Dillon *et al.*, 2011) for reproductive (*e.g.*, Wethington and Dillon, 1993; Jordaens *et al.*, 2007) and ecotoxicological studies (*e.g.*, Bernot *et al.*, 2005; Relyea, 2006; Bakry *et al.*, 2011; Maredza and Naik, 2013; Basopo *et al.*, 2014).

In this study we measured the effects of individual and combined pesticides commercial formulations at concentrations expected after a runoff event or near golf courses throughout the U.S. Ecological risk assessments, such as those conducted by the U.S. Environmental Protection Agency (USEPA) and European Commission (EEC), that are typically performed on pure active ingredients (Pereira *et al.*, 2009). Therefore, ecotoxicological studies conducted with commercial formulations would provide more realistic results on the negative impacts of pesticides to nontarget biota. We observed the effects of exposure of two herbicides (atrazine and metolachlor), one insecticide (carbaryl), and one fungicide (chlorothalonil) on aquatic gastropods. We used egestion rates and movement rates of *P. acuta* and *H. anceps* as measures of relevant physiological and behavioral effects. We predicted similar effects of pesticide exposure on *P. acuta* and *H. anceps* egestion due to similar feeding behavior (grazers), available food (biofilms and detritus), and habitats. We also hypothesized pesticides targeting primary producers (atrazine: photosynthesis; metolachlor: gibberellins and mitosis) would have no effect on snail egestion or movement, whereas pesticides that target invertebrates (carbaryl) and a molluscicidal (chlorothalonil) would reduce snail egestion rate and movement.

METHODS

Physa acuta was collected from the White River (Muncie, Indiana; 40°18′05″N 85′432″W). This area is surrounded by urban and forest landscape (oaks, maples, white ash, elm, sycamore). *Helisoma anceps* was purchased through a commercial vendor (Meijer, Inc.). The shell length of snails (Range: 4.70–5.41 mm; Mean: 4.95 mm) was measured using digital calipers prior to the start of the experiments. These snails were maintained in synthetic spring water filled aquaria at 20 C ± 3 C. Synthetic spring water was prepared placing 20 L of MILL-Q water, 1.2 g of $CaSO_4 \bullet 2H_2O$, 1.2 g of $MgSO_4$, 1.92 g $NaHCO_3$, and 0.080g KCl into a carboy (USEPA, 2002). Snails were fed boiled spinach ad lib. and supplemented with Topfin tropical flakes fish food. Snails were maintained under a 16:8 h light:dark photoperiod for the duration of the experiments. Experimental units consisted of glass jars (150 mL) filled with 120 mL of synthetic spring water. One snail was placed into each jar at experiment start. Treatments were randomly assigned with four replicates each across seven treatments (n = 28) including: control, atrazine (200 μg/L), metolachlor (100 μg/L), carbaryl (100 μg/L),

TABLE 1.—Mean and maximum pesticide concentrations reported in U.S streams. Treatment concentrations were comparable to peak concentrations detected throughout the U.S. Atrazine and metolachlor concentrations correspond to monthly median concentration from integrator sites throughout the U.S. Maximum concentration of atrazine represents a range of all monitoring sites-years with detection of atrazine in the U.S. Maximum concentration of metolachlor was taken from 22 sampling sites in five states (Alabama, Florida, Georgia, Oklahoma). Maximum concentration of chlorothalonil was detected in runoff from golf courses

Compound	Mean concentration (μg/L)	Maximum concentration (μg/L)	Treatment concentration (μg/L)	References
Atrazine	0.01–1[1]	0.0035–344.26[2]	200	[1]Larson *et al.*, 1999; [2]EPA, 2016
Metolachlor	0.01–1[1]	143[3]	100	[1]Larson *et al.*, 1999; [3]Battaglin *et al.*, 2000
Carbaryl	0.058[4]	33.5[4]	100	[4]EPA, 2012
Chlorothalonil	0.15[5]	372–699[6]	100	[5]Scribner *et al.*, 2006; [6]Shuman *et al.*, 2000, and Haith and Rossi, 2003

chlorothalonil (100 μg/L), atrazine + metolachlor (200 μg/L + 100 μg/L), and carbaryl + chlorothalonil (100 μg/L + 100 μg/L).

Stock solutions were prepared for atrazine (Atrazine 4L, 42.2% purity, Loveland, Colorado), metolachlor (Me-too-lachlor II, 84.4% purity, Drexel Chemical Company, Memphis, Tennessee), carbaryl (Sevin XLR Plus, 44.1% purity, Bayer, North Carolina), and chlorothalonil (Bravo, 54% purity, Syngenta, North Carolina) to achieve final stock concentrations of 10,000 μg/L for atrazine and metolachlor, 5000 μg/L for carbaryl, and 8000 μg/L for chlorothalonil. Aliquots from each stock solution were added to test units to reach target nominal treatment concentrations. These exposure concentrations were not confirmed through analytical methods. Therefore, final exposure concentrations for each pesticide may have varied due to compound breakdown or application errors including miscalculations and equipment calibration. Pesticide treatment concentrations were selected to represent peak concentrations detected throughout the U.S. (Table 1), usually after storm events and recent pesticides application (Ng and Clegg, 1996; Southwick *et al.*, 2003; USEPA, 2011). Water changes for cultured snails as well as treatments concentration renewal were conducted twice weekly.

EXPERIMENTAL DESIGN

Pesticide effects on P. acuta and H. anceps egestion rates.—Effects of pesticides on *P. acuta* and *H. anceps* egestion rates were estimated by weighing feces. Snails were starved for 24 h before the start of the egestion experiment to fully empty their intestines of fecal matter (sensu Bernot *et al.*, 2005). Snails were then placed in individual glass jars with freshwater and treatment solutions for 24 h as well as 0.05 g of boiled spinach (wet mass) as a food source. After 24 h fecal matter was removed with micropipettes, filtered onto filter paper, dried (60 C for 24 h) using a Model 30 GC laboratory oven, and weighed to the nearest milligram (Autobalance AD6, Perkin Elmer). Snails were blotted dry using Kimwipes (Kimberly-Clark) and weighed (Mettler AE260, Delta Range). Egestion rates (mg/g/h) were calculated as the amount of feces produced (mg) divided by the mass of each snail (g) per hour (h).

Pesticide effects on H. anceps movement rates.—Snail movement was quantified for *H. anceps* in response to pesticide treatments (n = 7) after 24 h, 1 w, and 2 w exposure (*sensu* Bernot *et al.*, 2005) to atrazine, metolachlor, carbaryl, and chlorothalonil. Similar high concentrations of chlorothalonil (172 μg/L and 351 μg/L) and longer duration exposure (4 w) were used by McMahon *et al.* (2012) to determine the effects of chlorothalonil in biodiversity. In our study, at each time point, snails were removed from test units for movement measurement and subsequently returned to their corresponding test unit. For each movement measurement, individual snails were placed in glass aquaria (50.8 × 27.9 × 30.5 cm) with 1 cm^2 square grid paper beneath (Brown *et al.*, 2012). Synthetic spring water was added to the glass aquaria to a height of 5 cm. The snail was then placed with the aperture down on the bottom of the aquarium in the center of the grid paper. After 10 s of acclimation, grid lines crossed by individual snails within 2 min were counted (grid lines crossed during the first 10 s were not recorded). A total of four replicate snails from each treatment (n = 28) were assessed for movement at each time point (n = 3).

DATA ANALYSES

Physa acuta and H. anceps egestion rates.—The egestion experiment was set up as a factorial design consisting of two levels of species (*P. acuta* and *H. anceps*) and seven levels of pesticide treatments (control, atrazine, metolachlor, carbaryl, chlorothalonil, atrazine + metolachlor, and carbaryl + chlorothalonil) with four replicates for each treatment (n = 28). Each individual snail species was matched with a corresponding pesticide treatment. Two-way analysis of variance was used to analyze the effects of pesticide treatments on snail species (*P. acuta* and *H. anceps*) egestion rates. The carbaryl + chlorothalonil treatment had 100% mortality in *P. acuta*. Therefore, this treatment was not included in data analyses. Differences among treatments were assessed with Tukey multiple comparison tests. Analyses were conducted using SigmaPlot© 12.0 software.

Helisoma anceps movement rate.—The movement experiment was set up as a factorial design with seven levels of pesticide treatments (control, atrazine, metolachlor, carbaryl, chlorothalonil, atrazine + metolachlor, and carbaryl + chlorothalonil) and three levels of pesticide exposure period (1 d, 1 w, and 2 w) with four replicates for each treatment (n = 28). Each individual snail was matched with its corresponding pesticide treatment. Two-way repeated measures analysis of variance was used to analyze the effects of pesticide exposure period and pesticide treatments on *H. anceps* movement rate. Differences among treatments were assessed with Tukey multiple comparison tests (Gravetter and Wallnau, 1999), similar to approaches used by Kerr *et al.*, 2001; Lu *et al.*, 2010; Furusawa *et al.*, 2013). Analyses were conducted using SigmaPlot© 12.0 software.

RESULTS

Physa acuta and H. anceps egestion rates.—Snails egestion rate was different among species ($F_{1,42} = 79.2$, $P < 0.001$) and pesticide treatments ($F_{6,42} = 21.6$, $P < 0.001$). There was a significant interaction between the effects of species and pesticide treatments ($F_{6,42} = 14.6$, $P < 0.001$). *Physa acuta* egestion rates were higher than *H. anceps* across treatments (Fig. 1). For both species egestion rates varied between 0.04–0.5 mg feces/g snail/h (*P. acuta*) and 0.01–0.07 mg feces/g snail/h (*H. anceps*). Control egestion rate for *P. acuta* was seven times higher than control egestion rate for *H. anceps* ($F_{1,42} = 79.2$, $P < 0.001$). A similar trend was observed for *P. acuta* and *H. anceps* in response to pesticide exposure; where *P. acuta* egestion rates was eight times higher than *H. anceps* with metolachlor ($F_{1,42}$

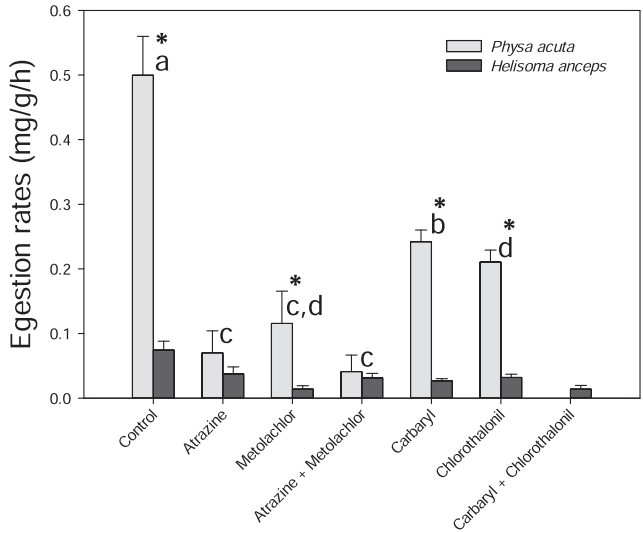

FIG. 1.—*Physa acuta* and *H. anceps* egestion rates (mean ± SE) exposed to atrazine, metolachlor, carbaryl, chlorothalonil, atrazine + metolachlor, and carbaryl + chlorothalonil concentrations. Different letters represent significant differences between treatments for *P. acuta*. * Represents significant differences between egestion rates of *P. acuta* and *H. anceps* ($P < 0.05$)

$= 79.2$, $P = 0.016$), nine times higher with carbaryl ($F_{1,42} = 79.2$, $P < 0.001$), and seven times higher with chlorothalonil ($F_{1,42} = 79.2$, $P < 0.001$). Therefore, when comparing *P. acuta* and *H. anceps*, there were no significant differences between egestion rates when exposed to atrazine and atrazine + metolachlor ($P > 0.05$). As individual species *P. acuta* egestion was differentially influenced by herbicide (atrazine and metolachlor), insecticide (carbaryl), or fungicide (chlorothalonil) exposure. For the individual pesticides, *P. acuta* egestion rates were seven times lower with atrazine, four times lower with metolachlor, two times lower with carbaryl, and two times lower with chlorothalonil relative to control ($F_{6,42} = 21.6$, $P < 0.001$; Fig. 1). For the pesticide mixtures, atrazine + metolachlor exposure resulted in *P. acuta* egestion rates 12-fold lower than control ($F_{6,42} = 21.6$, $P < 0.001$). For *H. anceps* individual and mixtures of atrazine, metolachlor, carbaryl, and chlorothalonil had no significant effects on egestion rates ($P > 0.5$). The significant interaction of species and pesticide treatments indicates pesticide treatment effects on snail egestion rate are influenced by the snail species. Therefore, we observed no effect of pesticide exposure to *H. anceps* egestion and significant effect of pesticide exposure to *P. acuta* egestion rate.

Helisoma anceps movement rate.—Overall, *H. anceps* movement rate was different among pesticide treatments ($F_{6,42} = 6.9$, $P < 0.001$) and pesticide exposure period ($F_{2,42} = 17.3$, $P < 0.001$). There was not a significant interaction between the effects of pesticide treatments and pesticide exposure period ($P > 0.5$). Snail movement rate decreased when exposed to pesticide treatments (Fig. 2a, b). After pesticide exposure snail movement rate ranged from 0 to 0.75 cm/min. For individual pesticides (Fig. 2a) *H. anceps* movement was two-fold lower with atrazine ($P = 0.005$), metolachlor ($P = 0.026$), and chlorothalonil ($P = 0.005$) exposure and four times lower with carbaryl ($P < 0.001$) exposure relative to control. Similar trends were observed in snail movement in response to pesticide mixtures

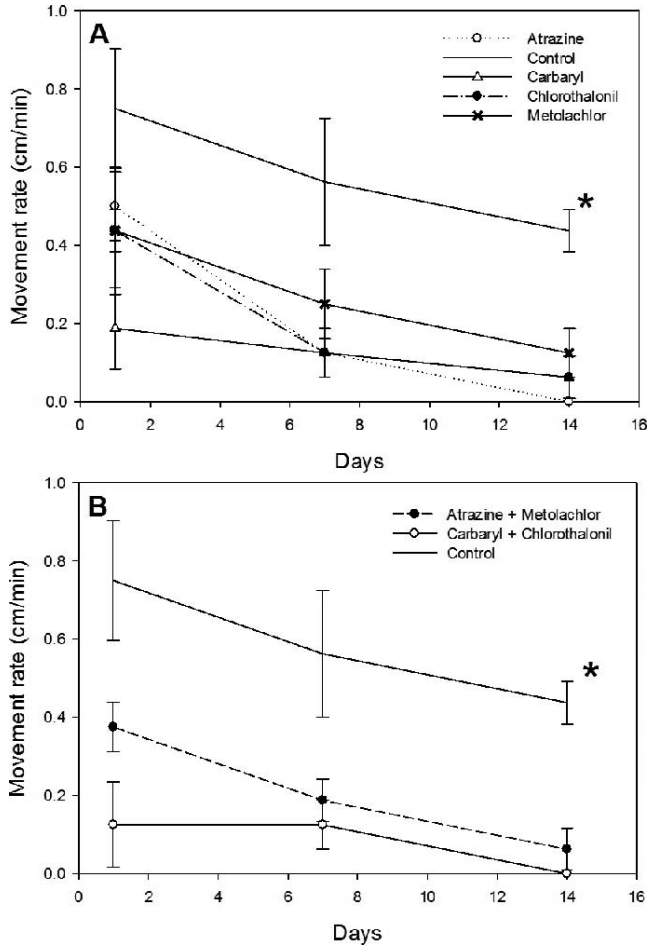

FIG. 2.—*H. anceps* movement rate (mean ± SE) after 24 h, 1 w, and 2 w exposure to (**a**) individual atrazine, metolachlor, carbaryl, and chlorothalonil, and combined (**b**) atrazine + metolachlor and carbaryl + chlorothalonil. * Represents significant differences between control treatment and pesticide treatments ($P < 0.05$)

(Fig. 2b). *Helisoma anceps* movement was three times lower with atrazine + metolachlor ($P = 0.005$) and seven times lower with carbaryl + chlorothalonil ($P < 0.001$) relative to control. Pesticide exposure period affected snail movement rate differently. *Helisoma anceps* movement rate decreased over time ($n = 14$ d) for both control and pesticide treatments with a more pronounced effect after 1 wk and 2 wk. Snail movement rate ranged from 0.13 to 0.75 cm/min after 24 h, from 0.13 to 0.56 cm/min after 1 wk, and from 0 to 0.44 cm/min after 2 wk. Snail movement rate was two-fold lower after 1 wk ($F_{2,42} = 17.3$, $P < 0.001$) and four times lower after 2 wk ($F_{2,42} = 17.3$, $P = 0.002$) relative to 24 h pesticide exposure.

DISCUSSION

In our study we measured the individual and combined effects of atrazine, metolachlor, carbaryl, and chlorothalonil on *P. acuta* and *H. anceps*. Snail species egestion (*P. acuta*) and movement (*H. anceps*) were negatively affected by individual and combined atrazine, metolachlor, carbaryl, and chlorothalonil exposure at peak concentrations detected following a rain event and/or pesticide application. Control egestion rate for *P. acuta* was greater than *H. anceps*, possibly influenced by species innate assimilation efficiency (Studier *et al.*, 1975; Barnese *et al.*, 1990) and species sensitivity (Hylleberg, 1975; Bernot *et al.*, 2005; Suski *et al.*, 2012). Traditionally, species sensitivity to toxicants has been predicted through a taxonomic-based approach (Rubach *et al.*, 2012), where related species are expected to have a similar response to toxicant exposure. However, species sensitivity traits are better predictors of the effects to toxicant exposure (Rubach *et al.*, 2012). For example macroinvertebrates with low sensitivity traits exposed to thiacloprid (3.3 μg/L) resulted in short term harmful effects (*i.e.*, reduction of abundance and richness). In contrast species with high sensitivity traits exposed to thiacloprid (0.1 μg/L) resulted in permanent adverse effects, *i.e.*, no significant recovery of abundance and taxa richness (Liess and Beketov, 2011). Although, *P. acuta* and *H. anceps* sensitivity traits to atrazine, metolachlor, carbaryl, and chlorothalonil have not been identified, we observed higher egestion rates of *P. acuta* than *H. anceps* when exposed to pesticide treatments, with no significant effect of pesticide exposure on *H. anceps* egestion. Therefore, consistent with a species trait approach, our results suggest dissimilar species sensitivity to environmental stressors (Hylleberg, 1975; Bernot *et al.*, 2005; Suski *et al.*, 2012).

Contrary to hypotheses we observed a decrease in *P. acuta* egestion rates when exposed to any pesticide. This might be partially explained by narcosis (Wezel and Opperhuizen, 1995; Ren, 2002; Roberts and Costello, 2003), which is a nonspecific mode of action where a chemical does not interact with a particular receptor in an organism (Verhaar *et al.*, 1992; Cleuvers, 2002). While no adverse outcome pathway (Ankley *et al.*, 2010; Vinken, 2013) has been developed for these pesticides on freshwater snails, a narcotic effect of pesticides on *P. acuta* egestion might be a result of disruption of Van der Waals interactions between lipid and protein components within the membrane (Frank and Lieb, 1990; Yamakura *et al.*, 2001). Damage of cell membrane could increase cell susceptibility to lysis due to abnormal ion fluxes and dysfunction of organelles (Kinter and Pritchard, 2011). This could lead to cardiac or hepatic failure, therefore impairing snail egestion. However, we observed no effect of individual or combined pesticide treatments in *H. anceps* egestion rates. Similarly, no significant effects on growth and fecundity were observed for *Physella* sp. exposed to atrazine concentrations of 0, 1, 10, 30, and 100 μg/L (Baxter *et al.*, 2011) or *Helisoma trivolvis* exposed to 0.51 mg/L of carbaryl (Relyea, 2005). Therefore, we presume pesticide exposure concentrations, as well as species sensitivity, played an important role in differential species response.

Pesticide effects on organism are likely influenced by compound mode of action (Elias and Bernot, 2014), species sensitivity to toxicants (Van Straalen and Denneman, 1989; DeLorenzo *et al.*, 2009), and pesticide exposure period (Ashauer *et al.*, 2009; Maltby *et al.*, 2009). We predicted pesticide mode of action and pesticide exposure period as the main factors influencing invertebrate response. However, *H. anceps* movement was not influenced by these factors. As in the egestion experiment, snail movement rate decreased when exposed to any pesticide (*i.e.*, narcosis). A similar reduction in movement (decreased avoidance behavior from predatory cues) was observed on the snail *Physa pomilia* exposed to 25 mg/L of malathion (Salice and Kimberly, 2012). In contrast to Salice and Kimberly,

(2012) in which reduction of movement occurred with increased exposure duration (48 h) at 25 mg/L of malathion, we observed that lower snail movement rate was not influenced by pesticide exposure duration (24h, 7 d, and 14 d) but likely due to decline in *H. anceps* fitness.

Snail fitness can be assessed by quantifying fitness traits, such as fecundity (Coutellec and Lagadic, 2006), survival (Wethington and Dillon, 1997), and movement (Bernot *et al.*, 2005). Snail fitness decreases in response to environmentally related stress (Coutellec and Lagadic, 2006), including physicochemical parameters (Hunter, 1990), predation (Dewitt, 1998), contaminants (Justice and Bernot, 2014), and food availability (Auld and Henkel, 2014). Our study provided steady room temperature (~20 C) and did not include predation stimulus or limited food. Further, contaminants (*i.e.,* pesticides) did not have a significant interaction effect with exposure period. However, optimal fitness (*e.g.,* growth, egg production) of *Helisoma duryi* occurs between 26 C to 28 C (El-Emam and Madsen, 1982). Therefore, the decline of snail movement through time might be a result of the lower temperature of our test units. In addition snail movement measured (number of grids crossed in 2 min) in the glass aquaria was conducted in treatment free water (no pesticides). There may be unaccounted effects from using treatment free water (Berrill *et al.,* 1998; Samson *et al.,* 2001; Jones *et al.,* 2009) rather than the corresponding pesticide concentration for each treatment.

In our study we cannot use "concentration addition" (mixture of pesticides with the same mode of action; Deneer, 2000) or "independent action" (mixture of pesticides with dissimilar mode of action; Cedergreen *et al.*, 2008) to classify the effects of combined atrazine and metolachlor or combined carbaryl and chlorothalonil. To address chemical interactions, multiple concentration treatments for each pesticide is required (Perez *et al.*, 2012; Abhishek *et al.*, 2014; Zhu *et al.*, 2014). While this study is not addressing synergistic, additive, and antagonistic interactions between atrazine and metolachlor and carbaryl and chlorothalonil, we focused on the ecological effects (changes in egestion and movement of snails) of these pesticides when they co-occur in water at peak concentrations. We observed atrazine + metolachlor had a greater effect on *P. acuta* egestion than individual atrazine and metolachlor. A similar result was observed in *Rana pipiens* when exposed to individual and combined atrazine and S-metolachlor. *Rana pipiens* tadpoles exposed to both atrazine and S-metolachlor had a greater reduction of larval development and growth than atrazine or S-metolachlor applied individually (Hayes *et al.*, 2006). Therefore, our results suggest changes in *P. acuta* egestion rates are greater when exposed to pesticide mixtures than individual pesticides, although the specific mechanisms (*e.g.*, chemical interaction, higher exposure level) cannot be determined from our experimental design.

Atrazine, metolachlor, carbaryl, and chlorothalonil not only affect *P. acuta* egestion and *H. anceps* movement, they can also indirectly affect food webs and prey-predator interactions. Lower egestion rates for *P. acuta* when exposed to pesticides might reduce nitrogen and carbon availability, thereby affecting nutrient fluxes and algal biomass (Conley *et al.*, 2009). Furthermore, decreasing movement of *H. anceps* might alter snail antipredator behavior including hiding or avoidance from predatory fish, amphibians, and insects (Covich *et al.*, 1994; Justice and Bernot, 2014), and grazing behavior (Turner and Montgomery, 2003; Bernot *et al.*, 2005). Therefore, in addition to pesticide mode of action, it is important to consider species sensitivity to pesticides coupled with different exposure periods to better understand the potential adverse ecological effects of co-occurring pesticides such as atrazine, metolachlor, carbaryl, and chlorothalonil to ecosystems. Similarly, future risk assessments and ecotoxicological studies should include more than one species of snails to

have a better representation of the freshwater gastropods community given their importance in nutrient cycling, foods webs, and parasite transmission.

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