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Chemical cocktails in aquatic systems: Pesticide effects on the response and recovery of >20 animal taxa

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ABSTRACT

Natural systems are often exposed to individual insecticides or combinations of multiple insecticides. Using an additive and substitutive design, we examined how populations and communities containing >20 animal taxa are affected by four insecticides applied individually and as a mixture for 18 wks in aquatic mesocosms. The four insecticides had distinct lethal effects on the response and recovery of cladocerans, copepods, amphipods, isopods, and amphibians but not snails. The lethal effect on cladocerans and copepods induced trophic cascades that facilitated algal blooms and abiotic changes (higher pH and dissolved oxygen, but lower light transmission). Exposure to endosulfan resulted in a lag effect reducing cladocerans and spring-breeding amphibian abundance. The reduction in spring-breeding amphibian abundance led to cascading indirect effects on summer-breeding amphibians. Finally, the mixture treatment had lethal effects throughout the community that led to long-term effects on amphibian mass and unique indirect consequences on phytoplankton and abiotic variables.

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1. Introduction

Natural systems are exposed to a number of disturbances that shape species abundance and diversity. In particular, insecticides represent a common anthropogenic disturbance to ecological systems (Grube et al., 2011). The consequences of insecticides are wide reaching, spanning all biological organization levels and broad temporal scales (Picket and White, 1985). To understand the relative contribution of insecticides in shaping natural systems, we not only need to identify generalities across different insecticides (both within and across insecticide classes) but also across time and ecological levels. Further complicating this issue is that natural systems are often exposed to multiple insecticides that can lead to unanticipated additive, antagonistic, or synergistic interactions (Chèvre et al., 2005; Daly et al., 2007; Smalling et al., 2012). As insecticide use continues to increase, developing generalizations about how natural systems respond to individual insecticides or combinations of multiple insecticides is an important contemporary challenge (Pimentel, 2005; Puccinelli, 2012; Turner, 2010).

Given the large number of different insecticides and their mixtures in natural systems, toxicologists have traditionally relied on short-term, single-species laboratory tests to determine the lethal concentration of an insecticide that causes 50% of a population to die (i.e. LC50 values; Faust, 2000; Hammond et al., 2012; Jones et al., 2009). While this reductionist approach has been helpful in understanding the direct consequences of insecticide on individual species, a growing number of studies (chronic tests; community mesocosm studies) have demonstrated that insecticides can also have indirect cascading effects at the population and community levels (Fleeger et al., 2003; Peters et al., 2013; Relyea and Hoverman, 2006). Focusing solely on single-species, direct toxicity values over short time periods (1–4 d) can lead to limited or misleading conclusions about the effects of insecticides. Thus, we need studies that track the direct and indirect consequences of insecticides across multiple levels of biological organizations to develop generalizations about how these chemicals can alter aquatic systems (Kefford et al., 2005).

Despite the growing number of studies examining the short-term direct and indirect consequences of insecticides, our understanding still lacks much of the complexity of natural systems. Since a large number of chemicals are applied, we need to determine the generalizability of the direct and indirect consequences of different insecticides at different levels of ecological complexity. Moreover, most insecticides are designed to act immediately and degrade quickly (Newman, 1992), but these short-term consequences can potentially lead to unanticipated lethal or sublethal effects on communities that may last long after the insecticide has degraded (i.e. lag effects); to address the long-term effects of

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insecticides, we need to temporally extend our monitoring efforts. Finally, aquatic systems are commonly exposed to complex mixtures of insecticides that can interact in unpredictable, non-additive ways (Belden et al., 2007). Determining the prevalence of these non-additive interactions has significant conservation and ecological implications.

To address these issues, we created complex aquatic mesocosms containing over 20 animal taxa. Using one-time insecticide applications at low concentrations, we exposed these mesoscosms to four common insecticides applied as a mixture and applied individually at additive and substitutive concentrations. We then tracked the direct and indirect population and community responses for 18 wks by measuring the response and recovery of animal taxa and the associated changes in several abiotic variables.

2. Methods

2.1. Pesticide background

We chose to work with four commonly applied insecticides: chlorpyrifos, diazinon, endosulfan, and malathion (Aston and Seiber, 1997; Gilliom, 2007; Grube et al., 2011). Chlorpyrifos, diazinon, and malathion belong to the same chemical class (organophosphates) and have similar modes of action (acetylcholine esterase inhibitor; Brown, 2005). In contrast, endosulfan belongs to the organochlorine class and is a GABA inhibitor (Table A1). We chose these insecticides with the intention to make comparisons between insecticides that share and differ in their chemical properties. All four insecticides are used in agricultural, residential, and public pest-control and they occur in water bodies via direct application and via indirect accidental run-off (Gilliom, 2007).

2.2. Experimental design

To investigate the effects of separate and combined insecticides on aquatic systems, we carried out an 18-wk mesocosm study at the University of Pittsburgh's Pymatuning Laboratory of Ecology. We used a completely randomized design that contained 11 treatments: a negative control (water), a solvent control (ethanol), four insecticides (chlorpyrifos, diazinon, endosulfan, and malathion) applied separately at a nominal concentration of 10 ug/L (additive concentration), four insecticides applied separately at a nominal concentration of 40 ug/L (substitutive concentration), and a mixture treatment that combined 10 ug/L of all four insecticides for a total concentration of 40 ug/L. We replicated the 11 treatments four times for a total of 44 experimental units. For additional details on methods, see Supplementary Information (S.I.) 1.0.

The experimental units were plastic, 1200-L cattle watering tanks filled with ~825 L of well water on 8 to 12 April 2009. All tanks were covered using 60% shade cloth to prevent organisms from entering or leaving. This level of shade still allows high levels of primary productivity. Three days after the tanks were filled (15 April), we added 25 g of rabbit chow and 300 g of dry leaves (primarily *Quercus* spp.) to provide nutrients and additional substrate for periphyton. The following day (16 April), we collected pond water from three nearby ponds and added equal aliquots to each mesocosm to provide a natural source of algae and bacteria. We placed four ceramic tiles (15 × 15 cm) on the north side of each mesocosm to serve as periphyton samplers. On 20 April and 1 May, we collected zooplankton from four local ponds using a 30-micron zooplankton tow and added equal aliquots of the zooplankton/pond water mix to each mesocosm (For additional details see (S.I.) 1.1). After adding the algae and zooplankton, we let the mesocosms to sit for an additional 22 d to allow the algae and zooplankton to grow.

We then added two species of detritivores and three species of snails to the mesocosms. For the detritivores, we collected amphipods (*Crangonyx psesudocracilis*) and isopods (*Asellus aquaticus*) from two nearby wetlands and added 20 similar-sized individuals of each species to every mesocosm on 22 and 23 May. For snails we added 5 egg masses of each species to the mesocosms (for additional details see (S.I.) 1.2).

To mimic natural amphibian assemblages and densities (Werner et al., 2009), we added six species of amphibians to each mesocosm over time. We collected at least 11 newly oviposited egg masses for each species (Table A2). Egg masses were hatched in 200-L wading pools and fed rabbit chow *ad libitum* after hatching. We added 15 tadpoles of each species to the mesocosms. We began by adding four species of spring-breeding tadpoles 43 days after the tanks were filled (24 May): wood frogs (*Lithobates sylvaticus* [*Rana sylvatica*]), leopard frogs (*L.* [*R.*] *pipiens*), American toads (*Anaxyrus* [*Bufo*] *americanus*), and spring peepers (*Pseudacris crucifer*). All spring-breeding amphibians were selected from a mixture of all clutches, which were all early in development (Table A2). After adding the spring-breeding amphibians, we allowed the animals to acclimate for 9 d and then applied the insecticide treatments on 2 June. From this point on, we refer to June 2 as Day 1 of the experiment.

2.3. Application of the pesticide treatments

All pesticides were purchased as technical grade chemicals (Chem Service, West Chester, PA). On 2 June, to achieve nominal concentrations of 10 ug/L, we added 0.330 ml of a 0.025 g/ml stock solution of chlorpyrifos, 0.339 ml of a 0.023 g/ml stock solution of diazinon, 0.330 ml of a 0.025 stock solution of endosulfan, and 0.343 ml of a 0.024 g/ml stock solution of malathion to the mesocosms. From the same stock solutions, to achieve nominal concentrations of 40 ug/L, we added 1.32 ml of chlorpyrifos, 1.36 ml of diazinon, 1.32 ml of endosulfan, and 1.37 ml of malathion to the mesocosms. To create the mixture treatment, we combined 0.330 ml of chlorpyrifos, 0.339 ml of diazinon, 0.330 ml of endosulfan, and 0.343 ml of malathion to the mesocosms. For details regarding the confirmation of nominal insecticide concentrations see (S.I.) 1.3. For simplicity, we will refer to nominal concentration of 10 ug/L as the "low concentration" and 40 ug/L as the "high concentration."

Approximately 3 wks after adding the insecticides (19 and 23 June), we added two species of summer-breeding amphibians to the mesocosms: gray treefrogs (*Hyla versicolor*) and green frogs (*L.* [*R.*] *clamitans*). These tadpoles were selected from a mixture of egg masses and then added to the mesocosms (Table A2). Since summer breeding amphibians were introduced into the mesocosms after the insecticide perturbation, we re-sampled each mesocosm for insecticide concentrations on 20 June ((S.I.) 1.3).

2.4. Abiotic response variables

On weeks 2, 4, 9, and 18 we quantified temperature, pH, and dissolved oxygen using a calibrated digital water meter (WTW, Woburn, Massachusetts, USA) and quantified light attenuation using an underwater light meter (LI-COR, Lincoln, Nebraska, USA; for additional details see (S.I.) 1.4).

2.5. Biotic response variables

We sampled zooplankton assemblages during weeks 2, 4, 9, and 18 and identified all zooplankton to the level of species. We then pooled all zooplankton into either cladocerans or copepods. A justification for this decision is provided in (S.I.) 1.5.

Phytoplankton abundance was measured during weeks 2, 4, 9, and 18 by sampling 500 mL of water from each mesocosm. To assess the abundance of phytoplankton, we measured the concentration of chlorophyll-a in each sample. Additional details can be found in (S.I.) 1.6.

We measured periphyton abundance by removing a clay tile from each mesocosm during week 2, 4, 9, and 18. The periphyton on the tiles was scrubbed and rinsed with filtered well water. The periphyton-water mix was then filtered through a Whatman GF/C 7-cm filter that had been previously dried at 80 °C for ≥24 h. The filters containing periphyton were re-dried for 24 h and then re-weighed to determine periphyton biomass.

Snail abundance and diversity was not assessed early in the experiment because the hatchling snails were very small and difficult to accurately assess for abundance. As a result, snail abundance was only assessed at weeks 6 and 18. We did this assessment by counting the number of individuals occupying the sides of the mesocosms from the surface of the water down to a depth of 40 cm (i.e. at the top of the clay tiles that were used as periphyton samplers).

The abundance of detritivores remained low early in the study, so we did not sample their populations until week 13. To assess detritivore abundance, we added mesh bags, containing 15 g of oak leaf litter, to each mesocosm. We first soaked the bags for 3 wks in a wading pool containing natural pond water from three local ponds to allow natural colonization by algae and bacteria. On 14 August, we added the mesh bags to each mesocosm. One week later, we removed one bag from each mesocosm and counted the number of amphipods and isopods.

Over the course of the experiment, the amphibians began to metamorphose. Once the first metamorphs were observed, metamorph emergence was checked daily (for additional details, see (S.I.) 1.7). We recorded survival to metamorphosis, time to metamorphosis, and mass at metamorphosis. Since green frogs are an overwintering species, they did not undergo metamorphosis. Therefore, we assessed the individual mass of green frog tadpoles at week 18 by non-destructively sampling five individuals from each mesocosm.

2.6. Statistical analysis

Since the data included a large number of response variables that were measured once or more than once during the experiment, we used several different analyses of variance to examine the effects of our treatments. We conducted a repeated-measures, multivariate analysis of variance (rm-MANOVA) on the abiotic response variables that were measured at four time points (temperature, pH, dissolved oxygen, and light attenuation) and on the biotic response variables measured at four time points (cladocerans, copepods, phytoplankton, periphyton). We also used a rm-MANOVA for the snails that were quantified at two time points. For all significant insecticide by time interactions, we used targeted post-hoc tests that separately compared the effect of each insecticide treatment at the four time points. For the two species of detritivores that were measured at a single time point, we analyzed total abundance using an ANOVA and then analyzed amphipod abundance and isopod abundance using a MANOVA. For the five amphibian species that

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