THE EFFECTS OF THE PSYCHIATRIC DRUG CARBAMAZEPINE ON
FRESHWATER INVERTEBRATE COMMUNITIES AND ECOSYSTEM DYNAMICS

A THESIS

SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF SCIENCE

BY

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BALL STATE UNIVERSITY

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ABSTRACT

THESIS: The Effects of the Psychiatric Drug Carbamazepine on Freshwater Invertebrate Communities and Ecosystem Dynamics

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Carbamazepine has become a compound of concern due to its ubiquity, potential toxicity and persistence in surface waters around the world. Carbamazepine is a psychiatric drug used to treat epilepsy, depression, addiction and bipolar disorder. Currently, understanding of how carbamazepine affects freshwater organisms, populations, communities and ecosystems is limited. Descriptive assessments coupled with *in vitro* and *in situ* experiments were conducted to assess how freshwater ecosystems respond to carbamazepine at environmentally relevant concentrations. Carbamazepine was detected in central Indiana streams (1 – 88 ng/L) and potentially altered macroinvertebrate species composition and food resources in the Upper White and Mississinewa River watersheds. Additionally, results from an *in vitro* experiment indicate that carbamazepine may increase abnormal behavior and retard development of mayfly nymphs at concentrations found in surface waters around the world. Lastly, an outdoor mesocosm experiment demonstrated that carbamazepine increased invertebrate biodiversity, altered species composition and decreased decomposition. This study provides insight into how environmentally relevant concentrations of carbamazepine may adversely influence freshwater ecosystems on the population, community and ecosystem level.

INTRODUCTION

A number of pharmaceuticals and personal care products (PPCPs) have become compounds of particular concern due to their potential toxicity to aquatic organisms, recalcitrance and ubiquity. Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) is one of these compounds of concern (Hughes et al. 2013). Carbamazepine primarily reduces rapid firing of neurons by blocking sodium channels and is an anti-epilepsy drug used to treat a number of psychiatric disorders such as bipolar disorder and depression (Porter and Meldrum 2012). Global concentrations of carbamazepine detected in surface waters range from 0.5 to 11,561 ng/L (Loos et al. 2009, Ferguson et al. 2013) with a median of 174 ng/L and detection frequency of 85% among study sites (Hughes et al. 2013). With minimal removal from wastewater treatment processes (5-26%; Miao et al. 2005), high usage rates (1,014 tons annually; Zhang et al. 2008) and its moderate affinity for binding to sediments ($\log K_{OW} = 2.25$; Löffler et al. 2005), aquatic organisms are persistently exposed to carbamazepine.

Carbamazepine is one of the most frequently detected and studied PPCPs in North American, Asia and Europe (Hughes et al. 2013). However, currently there is little understanding of how carbamazepine influences freshwater ecosystems and its chemical mode of action in aquatic organisms (Oetken et al. 2005). Environmentally relevant concentrations of carbamazepine do not appear to be acutely toxic to freshwater organisms ($LC_{50} > 40$ mg/L in *Chironomus tentans*; Dussault et al. 2008). However, sub-lethal effects have been observed at environmentally relevant concentrations. Oetken et al. (2005) observed a negative effect on emergence of *Chironomus riparius* in sediment spiked with 70 µg/kg carbamazepine. Additionally, Lamichhane et al. (2013) found that *Ceriodaphnia dubia* exposed to carbamazepine experienced decreased fecundity at 196.7 µg/L. Recent research has highlighted

the potential for adverse effects of carbamazepine on aquatic organisms and ecosystems but are limited in their scope and transferability to natural ecosystems. Further research to quantify how carbamazepine alters aquatic environments is needed, which will aid in regulatory assessments (Rosi-Marshall and Royer 2012).

The purpose of this study was to determine how environmentally relevant concentrations of carbamazepine influence freshwater ecosystems. First, we determined how concentrations of carbamazepine found in the Upper White and Mississinewa River watersheds of central Indiana influenced the community structure of freshwater macroinvertebrates through descriptive sampling. We hypothesized that species richness would decline with increasing concentrations of carbamazepine and that abundances of pollution sensitive taxa (Ephemeroptera and Trichoptera) would decrease (Beketov et al. 2013). Second, we quantified how globally-relevant concentrations of carbamazepine influenced interactions between a primary consumer (*Stenonema* mayfly nymph) and producer (*Chaetophora* algae) and how mayfly development and behavior were affected by carbamazepine via *in vitro* experimental manipulations. We hypothesized that carbamazepine would directly alter the behavior, growth, development and food resource depletion of flat-headed mayflies and therefore indirectly influence the growth of algae (Oetken et al. 2005). Third, we demonstrated how carbamazepine influences invertebrate biodiversity and ecosystem dynamics of freshwater habitats using an *in situ* experimental manipulation. We hypothesized that elevated carbamazepine concentrations would decrease biodiversity, alter invertebrate species composition through habitat degradation and therefore indirectly affect ecosystem dynamics such as decomposition and primary production (McMahon et al. 2012).

Changes in the invertebrate community have the potential to alter predator prey interactions and food resource availability in freshwater ecosystems (Covich et al. 1999, Bernot and Turner 2001). Therefore, exposure to carbamazepine may have profound impacts on freshwater ecosystems.

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CHAPTER 1: The influence of the psychiatric drug carbamazepine on freshwater macroinvertebrate community structure

Abstract: Pharmaceutical pollutants are commonly detected in surface waters and have the potential to affect non-target organisms. However, there is limited understanding of how these emerging contaminants may affect macroinvertebrate communities. The pharmaceutical carbamazepine is ubiquitous in surface waters around the world and is a pollutant of particular concern due to its recalcitrance and toxicity. To better understand the potential effects of carbamazepine on natural macroinvertebrate communities, we related stream macroinvertebrate abundance to carbamazepine concentrations. Macroinvertebrate and water samples were collected from 19 streams in central Indiana in conjunction with other stream physiochemical characteristics. Structural equation modeling (SEM) was used to relate macroinvertebrate richness to carbamazepine concentrations. Macroinvertebrate richness was positively correlated with increasing concentrations of carbamazepine. From the SEM we infer that carbamazepine influences macroinvertebrate richness through indirect pathways linked to Baetidae abundance. Baetidae abundance influenced ephemeropteran abundance and FBOM percent organic matter, both of which altered macroinvertebrate richness. The pharmaceutical carbamazepine may alter freshwater macroinvertebrate species composition, which could have significant consequences to ecosystem processes.

INTRODUCTION

The integrity of freshwater ecosystems is dependent on the biodiversity of macroinvertebrates. Macroinvertebrates play key roles in freshwater ecosystems, by cycling nutrients, aerating sediments, and serving as conduits of energy flow in food webs (Covich et al. 1999, Clements and Rohr 2009). Anthropogenic stressors associated with an increasing human population threaten biodiversity and have diminished services provided by freshwater ecosystems (Vörösmarty et al. 2010, Dodds et al. 2013). Freshwater pollutants degrade habitat quality (Schulz et al. 2002, Clements et al. 2013), alter species composition (Muñoz et al. 2009, Beketov et al. 2013), and reduce macroinvertebrate richness, which is related to the pollution tolerance of taxa (Fig. 1; Wogram and Liess 2001). With the global human population anticipated to reach 9.6 billion in 2050, freshwater ecosystems will experience continued and elevated stressors, mostly from nutrient and organic pollution (Vörösmarty et al. 2010, UN 2013).

Pollutants such as heavy metals, nutrients and organic contaminants have been detected in freshwater ecosystems for decades (Murray et al. 2010). Research has illuminated the source, fate and effects of some of these emerging contaminants (e.g. nutrients; Carpenter et al. 1998, pesticides; Relyea 2005, heavy metals; Runck 2007); however, less is understood about personal care products and pharmaceutical (PPCPs) pollutants (Rosi-Marshall and Royer 2012, Hughes et al. 2013). Abiotic factors such as pH, dissolved oxygen and temperature have an effect on the fate of PPCPs and macroinvertebrate communities (Muñoz et al. 2009, Ferguson et al. 2013). Additionally, contaminants such as PPCPs influence macroinvertebrate community structure through changes in the habitat quality of freshwater ecosystems (Schulz et al. 2002, Muñoz et al. 2009, Clements et al. 2013,). Therefore macroinvertebrate richness and diversity is dependent on

the tolerance of the taxa present (Wogram and Liess 2001). An increase in concentrations of PPCPs, leads to habitat degradation, which alters the abundance of pollution sensitive (Ephemeroptera and Trichoptera) and tolerant taxa (Chironomidae and Oligochaeta) and changes macroinvertebrate community (Fig. 1).

Pharmaceuticals continuously enter freshwater ecosystems most commonly through effluent from wastewater treatment plants (WWTP; Rosi-Marshall and Royer 2012). However, septic tank leaching and agricultural runoff are also substantial contributors (Bunch and Bernot 2011, Bernot et al. 2013). This chronic exposure to pharmaceuticals has the potential to influence non-target organisms in unintended ways throughout life cycles (Hughes et al. 2013). Thus, ecosystem-level assessments investigating the influence of pharmaceuticals on aquatic systems are critically needed (Rosi-Marshall and Royer 2012).

Hundreds of pharmaceutical compounds ranging from antibiotics to hormones are commonly found in surface waters. Recent reviews have highlighted specific pharmaceutical compounds of concern due to their abundance, recalcitrance, and potential for toxicity (Murray et al. 2010). Among these is carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide), which is one of the most commonly detected contaminants globally (Hughes et al. 2013). Worldwide concentrations of carbamazepine range from 0.5 to 11,561 ng/L (Loos et al. 2009, Ferguson et al. 2013) with a global median of 174 ng/L and a detection frequency of 85% (Hughes et al. 2013). Carbamazepine is a psychiatric drug that blocks sodium channels and reduces the firing of neurons and therefore is used to treat epilepsy, bipolar disorder, chronic nerve pain and addiction (Porter and Meldrum 2012). Global human consumption of carbamazepine is estimated to be 1,014 tons per year, with lower usage occurring in the U.S compared to other countries (35 tons; Zhang et al. 2008). Carbamazepine is recalcitrant in freshwater (half-life = 82 d; Lam et al. 2004)

and minimally removed during wastewater treatment (5 - 26% removal; Miao et al. 2005). Further, carbamazepine has a moderate affinity for binding to sediments ($\log K_{OW} = 2.25$; Löffler et al. 2005). The high usage rates, limited removal from wastewater treatment processes and chemical properties suggest that freshwater ecosystems are persistently exposed to carbamazepine.

Carbamazepine has limited acute effects on freshwater organisms due to high lethal concentrations ($LC_{50} > 4$ mg/L in *Lumbriculus variegatus* and *Chironomus riparius*), above environmental-relevance (Nentwig et al. 2004). However, carbamazepine can have chronic effects on aquatic organisms. Specifically, Oetken et al. (2005) found that sediments with carbamazepine reduced the emergence of *Chironomus riparius* at 0.16 mg/kg dry weight and yielded no emergence at 20 mg/kg dry weight. Further, reduced feeding and hydranth attachment of *Hydra attenuate* has been observed at carbamazepine concentrations of 50 and 25 mg/L, respectively (Quinn et al. (2008). While previous studies have assessed the effects of carbamazepine at concentrations higher than those measured *in situ*, these studies indicate that exposure to carbamazepine may influence the emergence, feeding, reproductive success and behavior of freshwater invertebrates potentially through altering physiological functions. Therefore, carbamazepine could adversely affect freshwater macroinvertebrates in natural ecosystems.

The objectives of this study were to quantify the concentrations of pharmaceuticals in the Upper White and Mississinewa River watersheds and to determine the influence of carbamazepine on macroinvertebrate community structure. We hypothesized that carbamazepine would reduce macroinvertebrate richness and therefore change the community structure. Specifically, sites with high concentrations of carbamazepine were expected to have lower

Ephemeroptera and Trichoptera abundance and higher Oligochaeta and Chironomidae abundance.

METHODS

Nineteen sites, encompassing a gradient of land use types, were sampled along the Upper White and Mississinewa River watersheds over two weeks in July 2012 (Fig. 2). The Upper White River flows through central Indiana and is 104 km in length (USGS 2013). The Mississinewa River is 190 km in length and a tributary of the Wabash River running through western Ohio and eastern Indiana. The Upper White and Mississinewa River watersheds are dominated by agricultural land use (75% and 88% of the area, respectively) with relatively low urban development (15% and 1.9% of the area, respectively; IDEM 2001, Lanthrop et al. 2011). At each site, stream physiochemical characteristics were measured as well as primary producer and benthic organic matter biomass and macroinvertebrate diversity and abundance. Additionally, dissolved nutrient and pharmaceutical concentrations (i.e. acetaminophen, caffeine, carbamazepine, cotinine, DEET, gemfibrozil, ibuprofen, lincomycin, naproxen, paraxanthine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfathiazole, triclocarban, triclosan, trimethoprim and tylosin) were measured. Due to the ubiquity and potential toxicity of carbamazepine, only this pharmaceutical was studied further.

For pharmaceutical analyses, composite water samples were filtered in the field, using a 60 mL syringe fitted with a glass fiber filter (pore size = 0.7 μ m) into a 1 L amber glass bottle containing the dechlorinating sodium thiosulfate preservative. All samples were immediately placed on ice for transport to the laboratory. Individuals collecting samples did not ingest or apply any of the target pharmaceutical analytes for a minimum of 24 h prior to sampling and

individuals wore latex gloves during sample collection. At each sampling event, field blanks and matrix samples were collected to ensure robust chemical analyses. All water samples were transported on ice to the Indiana State Department of Health (ISDH) Chemical Laboratories in Indianapolis, Indiana within 6 h of collection for measurement of pharmaceutical concentrations via solid-phase extraction liquid chromatography mass spectrophotometry (SPE/LC/MS/MS) using an Applied Biosystems triple quad API 4000 equipped with an Agilent 1200 high performance liquid chromatograph. Detection limits varied for each tested compound and ranged from 0.5 - 25 ng/L (Ferguson et al. 2013, Bernot et al. 2013). A calibration curve was constructed from the peak area response ratio of each compound to a corresponding labeled internal standard and was used to determine all pharmaceutical concentrations. No contamination of analytes was detected in field blank samples during any of the sampling events.

Dissolved nutrient concentrations were measured from 60 mL filtered water samples collected from the thalweg of the stream channel. Nutrient samples were frozen within 24 h of collection until subsequent analysis. To quantify nitrate (NO_3^-), phosphate (PO_4^{3-}) and ammonium (NH_4^+) concentrations, water samples were analyzed by ion chromatography (DIONEX-ICS-3000) using standard protocols (APHA 2012).

Physiochemical measurements including flow, width, pH, turbidity, dissolved oxygen (DO), temperature and depth were measured at each site in the stream thalweg using a Hydrolab[®] MiniSonde with an LDO sensor and Marsh-McBirney[®] flow meter. Cross-sectional depth data from 10 equidistant locations were multiplied by measured stream width and velocity to calculate discharge. Sediment percent organic matter was quantified by collecting a homogenized sample of the top 5 - 10 cm of sediment at a minimum of 5 locations at each site.

These samples were combined in 125 mL specimen cups, dried and ashed for calculation of percent organic matter.

Habitat surveys of each stream were based on stratified random sampling using standard techniques (Bernot et al. 2010). Briefly, to calculate organic matter abundance, samples of each organic matter type were collected and means of stock density (g/m^2) for each organic matter type were weighted by the fractional contribution of the organic matter type to the total stream area. Fractional contribution was determined by presence or absence of each organic matter type at 10 points along each of 10 equidistant transects ($N = 100$). The relative abundance of the organic matter type determined the number of samples collected for biomass estimates. Three samples were collected for types that comprised $< 10\%$ total stream cover and 6 - 8 samples were collected for types that comprised $>10\%$ stream cover.

Biomass of each organic matter type was determined through replicate sampling of known areas with 100% cover. A 20 cm diameter metal cylinder was placed into the stream for collection of fine (FBOM) and coarse (CBOM) benthic organic matter samples. CBOM was removed and placed into a container. FBOM was then collected by agitating surface sediment to approximately 10 cm depth and collecting a sample of the suspension. To calculate the ash free dry mass (AFDM), a volume of FBOM suspension was filtered through a glass fiber filter (Whatman GFF, 49 mm) and dried (60°C), weighed, combusted at 500°C , and re-weighed in the laboratory. The collected CBOM was separated into wood and leaf categories then dried, weighed, combusted and re-weighed. Standing stock (g/m^2) was then calculated by multiplying the total cylinder volume by the mass divided by the suspension volume and then dividing by the area of the cylinder.

For epilithon biomass, a cylinder (4.5 cm diameter) equipped with a foam gasket was pressed firmly on a rock substrate and the sequestered epilithon was scraped with a wire brush and the material was then suctioned from the area (3 replicates per site). For sites with smaller rock substrates, 3 - 5 rocks were collected and rock surfaces were scrubbed with a wire brush and epilithic slurry was collected. Less than 6 h after collection, the epilithon slurry was returned to the laboratory to be filtered, dried, weighed, combusted and re-weighed as above. The scrubbed rocks were returned to the laboratory and the planar area was measured using tracing paper (in cm) to relate mass to the total area. The biomass of filamentous algae and macrophytes was calculated for each site by collecting all organic material within a known area (20 cm diameter cylinder; 3 replicates per site) characterized by 100% cover of the target organic matter type. Collected material was then dried, weighed, combusted and re-weighed as above for calculation of biomass. The epilithon, filamentous green algae and macrophyte biomass was summed for total autotrophic biomass.

For macroinvertebrate sampling, a surber sampler was placed in 10 - 30 cm of water, perpendicular to flow. An area of 1 m² was disturbed for 1 min. and contents were collected by the sampler and placed into a plastic container after rinsing. Any large debris was removed and the remaining contents were poured into a sieve (pore size < 125 µm). The sample was then placed into a glass jar containing 95% ethanol and 2 - 3 drops of rose bengal dye (5 mL of rose bengal powder to 100 mL of tap water). In the laboratory, macroinvertebrates were separated from debris and identified to genus, with the exception of Chironomidae and Oligochaeta, which were characterized to family and subclass, respectively. Additionally, the functional feeding group diversity (number of different functional feeding groups per site) was determined based on feeding ecology and food type preference of each taxon. These groups were scrapers

(Heptageniidae, Helicopsychidae, Psephenidae and Elmidae), shredders (*Micrasema spp.*, *Caecidotea spp.*) predators (Odonata, Hirudinea, Megaloptera, Tabanidae, Empididae, *Hydrotus spp.*, *Gyrinus spp.*, *Berosus spp.* and *Peltodytes spp.*), omnivores (Decapoda) and filter feeders (*Ephemera spp.*, *Caenis spp.*, *Tricorythodes spp.*, Baetidae, Leptoceridae, Polycentropodidae, Hydroptilidae, Chironomidae and Oligochaeta; Hershey and Lamberti 2001, Merritt and Cummins 2006).

Data were analyzed for bivariate relationships between pharmaceutical concentrations and abiotic factors as well as taxa abundance and species richness using correlation analysis (Pearson's r). A significance level of 0.05 was used for all analyses. Additionally, Hilsenoff Biotic Index (HBI) was calculated for each site. A conceptual model (Fig. 1) guided these analyses. The linear relationships from these analyses informed an *a priori* model for testing factors controlling carbamazepine and the macroinvertebrate community using structural equation modeling (SEM; Muñoz et al. 2009). The SEM was evaluated using the model chi-square and associated P value. Additionally, carbamazepine, water quality parameters and macroinvertebrate community characteristics were analyzed between the watersheds with an independent t- test. Statistical analyses were performed using IBM SPSS 21.0 and AMOS statistical software.

RESULTS

Detected pharmaceuticals

Five of the 19 pharmaceutical compounds analyzed were detected among study sites including caffeine (24.5 - 134.5 ng/L), carbamazepine (1 - 88 ng/L), cotinine (4 - 33.5 ng/L), naproxen (4.85 - 15 ng/L) and paraxanthine (48.5 - 62 ng/L). No other compounds measured (N

= 14) were above detection thresholds. Due to the ubiquity of carbamazepine in study streams (100% detection frequency), and variability in concentrations measured (1 – 88 ng/L), this compound was further assessed for potential influences on macroinvertebrate communities. Carbamazepine was measured at higher concentrations in the Upper White River watershed (mean = 26.73 ng/L) compared to the Mississinewa River watershed (mean = 8.66 ng/L); however, this difference was not statistically significant ($P = 0.067$).

Water quality parameters

Across sites, pH (7.86 - 8.64) and temperature (22.1 – 29.8 °C) varied < 30% while, salinity (0.14 – 0.34 ppt) and dissolved oxygen (4.00 – 8.90 mg/L) varied >100%. In contrast, discharge varied by 3 orders of magnitude (5.1 – 5,695 L/s; Table 1). Between watersheds, pH ($P = 0.004$) and temperature ($P = 0.005$) differed with the Mississinewa characterized by 4% higher pH (mean = 8.36), and 13% higher temperature (mean = 27.94°C) relative to the Upper White (mean = 8.06 and 24.73°C, respectively). However, there was no difference in salinity ($P = 0.946$) or dissolved oxygen concentrations ($P = 0.743$) between the two watersheds. Additionally, there was no difference in stream discharge between sites sampled in the two watersheds ($P = 0.067$).

Dissolved nutrient concentrations varied 1 – 2 orders of magnitude across sites. Specifically, nitrate ranged from 0.11 to 11.83 mg/L (mean = 3.4 mg/L), phosphate ranged from 0.07 to 1.17 mg/L (mean = 0.43 mg/L) and ammonium ranged from 0.04 to 0.34 mg/L (mean = 0.13 mg/L; Table 1). Nitrate differed among watersheds and was 94% lower (mean = 0.3 mg/L) in the Mississinewa relative to the Upper White (mean = 5.21 mg/L; $P = 0.001$). There were no

differences in phosphate or ammonium concentrations between study sites in the two watersheds ($P = 0.388$ and 0.052 , respectively).

Biological community structure

The macroinvertebrate communities observed in this study were consistent with a moderately perturbed lotic ecosystem with a fairly significant degree of organic pollution (mean HBI = 6.02, Hilsenoff 1988). There were no differences in the Hilsenoff Biotic Index (HBI), richness or total abundance of macroinvertebrates found in the Upper White and Mississinewa River watersheds ($P > 0.05$). Overall, 62 macroinvertebrate taxa were identified across the study sites. Macroinvertebrate richness across the sites ranged from 4 to 27 taxa present. Additionally, total abundance ranged from 55 to 802 individuals per site (Table 2). Total macroinvertebrate abundance was positively correlated with overall ephemeropteran and chironomid abundance ($r = 0.72$, $P = 0.001$ and $r = 0.05$, $P = 0.029$, respectively). Overall ephemeropteran (2 – 221 individuals) and chironomid (12 – 265 individuals) abundance varied $> 95\%$ among sites (Table 2). Macroinvertebrate richness was correlated with overall ephemeropteran abundance ($r = 0.48$, $P = 0.037$, Fig. 4) and the number of functional feeding groups ($r = 0.458$, $P = 0.049$; Fig. 6).

The ephemeropterans identified across study sites belonged to 13 genera (predominately *Ephemera*, *Caenis* and *Baetis*) as well as the Heptageniidae and Trichorythidae. The trichopterans belonged to 14 genera, a majority of which were members of the Hydropsychidae and Hydroptillidae. However, no further analyses of any Trichoptera abundance were done due to a lack of correlation with FBOM percent organic matter ($r = -0.432$, $P = 0.065$), macroinvertebrate richness ($r = 0.373$, $P = 0.116$) or carbamazepine ($r = 0.071$, $P = 0.773$).

Bivariate relationships

Temperature was positively correlated with pH ($r = 0.643$, $P = 0.003$) and negatively correlated with discharge ($r = -0.488$, $P = 0.034$) across all study sites. Discharge was positively correlated with ammonium concentrations across sites ($r = 0.801$, $P < 0.001$). Nitrate concentrations were negatively correlated with pH across study sites ($r = -0.492$, $P = 0.032$). No other physiochemical parameters were correlated with dissolved nutrient concentrations. Phosphate was positively correlated with nitrate concentrations ($r = 0.7$, $P = 0.001$); however, there was no correlation between phosphate and ammonium concentrations ($r = 0.389$, $P = 0.1$) nor nitrate and ammonium concentrations ($r = 0.332$, $P = 0.164$) across sites.

Carbamazepine was positively correlated with dissolved inorganic nitrogen (DIN; sum of NH_4 and NO_3) concentrations ($r = 0.713$, $P = 0.001$) and salinity ($r = 0.51$, $P = 0.027$) and negatively correlated with temperature ($r = -0.49$, $P = 0.033$; Fig. 3). Additionally, carbamazepine was correlated with nitrate (*data not shown*; $r = 0.711$, $P = 0.001$) and phosphate concentrations (*data not shown*; $r = 0.456$, $P = 0.05$); however, there was no correlation with ammonium concentrations (*data not shown*; $r = 0.364$, $P = 0.126$). Carbamazepine was also not significantly correlated with discharge ($r = 0.374$, $P = 0.115$), pH ($r = -0.326$, $P = 0.174$) or dissolved oxygen ($r = -0.217$, $P = 0.373$).

Macroinvertebrate richness was positively correlated with discharge, salinity and DIN and negatively correlated with temperature and FBOM percent organic matter (Fig. 4). Specifically, richness was greater where salinity ($r = 0.49$, $P = 0.034$), DIN ($r = 0.463$, $P = 0.046$) and discharge ($r = 0.47$, $P = 0.042$) were high as well as where temperature ($r = -0.403$, $P = 0.087$) and FBOM percent organic matter ($r = 0.62$, $P = 0.005$) were low. Macroinvertebrate richness was not significantly correlated with dissolved oxygen ($r = 0.228$, $P = 0.348$), pH ($r = -$

0.426, $P = 0.069$) Chironomidae ($r = -0.123$, $P = 0.617$) or Oligochaeta abundance ($r = 0.061$, $P = 0.804$). Additionally, positive correlations were found between macroinvertebrate richness, Baetidae abundance and carbamazepine (Fig. 5). Macroinvertebrate richness ($r = 0.48$, $P = 0.037$) and Baetidae abundance ($r = 0.52$, $P = 0.022$) increased with carbamazepine concentrations. Carbamazepine was not significantly correlated with ephemeropteran ($r = 0.17$, $P = 0.48$), chironomid ($r = -0.311$, $P = 0.194$) or oligochaete abundance ($r = -0.054$, $P = 0.827$).

SEM results

Structural equation models (SEM) identified several significant causal relationships between carbamazepine and the macroinvertebrate community with a significant fit to the covariance matrix (Fig. 7). However, the initial and intermediate models (Fig. 7A and B) did not account for a significant proportion of variability in the macroinvertebrate community ($r < 0.05$). In the initial model, pathways linking carbamazepine to Trichoptera, Chironomidae and Oligochaeta abundance were eliminated due to non-significant pathways ($P > 0.05$). The inclusion of Baetidae abundance and FBOM percent organic matter in the final SEM (Fig. 7C) accounted for a substantial portion of the variation in macroinvertebrate richness ($r = 0.78$), with a significant fit to the covariance matrix ($\chi^2 = 8.954$, $df = 18$, $P = 0.961$).

Significant pathways in the final model included the effects of DIN (standardized path coefficient = 0.52) on carbamazepine; the effects of carbamazepine on Baetidae abundance (0.52); and salinity, overall ephemeropteran abundance and FBOM percent organic matter effects on macroinvertebrate richness (0.39, 0.27 and -0.49, respectively). However, the pathways linking temperature (-0.2), salinity (0.28) and discharge (0.07) to carbamazepine (-0.2) were not significant. Additionally, pathways linking temperature (-0.24), discharge (0.23), carbamazepine (0.02) and DIN (0.07) to macroinvertebrate richness were not significant.

The SEM showed that study sites with elevated DIN had higher carbamazepine concentrations. Additionally, sites with elevated carbamazepine had higher Baetidae abundance. And lastly, ephemeropteran abundance increased and FBOM percent organic matter decreased macroinvertebrate richness. Therefore, while the pathway linking carbamazepine directly to macroinvertebrate richness was not significant (0.02), there were significant indirect pathways from carbamazepine to macroinvertebrate richness through Baetidae abundance.

Further, the model described a considerable amount of variability in both carbamazepine concentrations ($r = 0.63$) and Baetidae abundance ($r = 0.27$) across study sites. However, the model does not describe a substantial portion of variability in ephemeropteran abundance ($r = 0.13$) or FBOM percent organic matter ($r = 0.07$).

DISCUSSION

Carbamazepine influenced the macroinvertebrate community (Fig. 7). However, contrary to our hypotheses, carbamazepine was positively related to macroinvertebrate richness (Fig. 4). Previous research on other freshwater anthropogenic inputs suggests that macroinvertebrate richness would decline with higher concentrations of carbamazepine (Muñoz et al. 2009, Beketov et al. 2013). This inconsistency may be due to a lack of toxicity to freshwater organisms at environmentally relevant concentrations of carbamazepine (Dussault et al. 2008, Quinn et al. 2008, Oetken et al. 2005). Additionally, concentrations of carbamazepine detected in central Indiana were relatively low (median = 9 ng/L) compared to the global median (174 ng/L). The positive correlation between carbamazepine and macroinvertebrate richness may have been facilitated by changes in species composition potentially induced by sub-lethal effects in the community (Quinn et al. 2008, Nentwig et al. 2004, Oetken et al. 2005, Lamichhane et al. 2013).

Carbamazepine concentrations in central Indiana may have altered macroinvertebrate species composition through sub-lethal effects, such as changes in behavior, molting or reproductive patterns (Quinn et al. 2008, Nentwig et al. 2004, Oetken et al. 2005). For instance, Oetken et al. (2005) found that sediments spiked with carbamazepine reduced the emergence of *Chironomus riparius* at sediment concentrations > 70 µg/kg. Additionally, Lamichhane et al. (2013) determined that *Ceriodaphnia dubia* exposed to carbamazepine had reduced fecundity at 196.7 µg/L. While many studies have observed lethal and sub-lethal effects of carbamazepine at concentrations that were not environmentally relevant and proposed that carbamazepine poses little risk to freshwater ecosystems, these toxicity data may underestimate the sensitivity of freshwater organisms to carbamazepine (Dussault et al. 2008, Lamichhane et al. 2013, Cleuvers 2003, Quinn et al. 2008). Studies focused on heavy metal contaminants have demonstrated how results obtained from laboratory experiments may not reflect the actual sensitivity of freshwater organisms (Buchwalter et al. 2007, Clements et al. 2013). Therefore, based on these results, macroinvertebrates may be more sensitive to lower concentrations of carbamazepine than previously hypothesized and sub-lethal effects may explain the observed changes in community structure.

Muñoz et al. (2009) found a negative causal association between the concentrations of pharmaceutical mixtures in the Llobregat River basin and the abundance and biomass of benthic macroinvertebrates. Higher concentrations of pharmaceuticals (> 10,000 ng/L) reduced macroinvertebrate richness and increased abundance of tolerant taxa (*Chironomus spp.* and *Oligochaeta*). While this particular study did not focus on carbamazepine specifically, concentrations among these study sites were higher (80 – 3,090 ng/L) than those found in central Indiana (1 – 88 ng/L), suggesting that different relationships may be observed at higher

concentrations of carbamazepine. Alternatively, in the Llobregat River basin a number of sites had higher total pharmaceutical concentrations (roughly 1,000 – 10,000 ng/L) and had increased macroinvertebrate richness. Additionally, these communities were characterized by the presence of mayflies. Thus, macroinvertebrate richness and community structure may be dependent on the additive and synergistic or antagonistic effects of the total pharmaceutical concentrations as well as the presence of other anthropogenic stressors.

Variability in carbamazepine

Carbamazepine was greater at sites below the Muncie wastewater treatment plant (WWTP), with concentrations rising from 9 ng/L above to 70 ng/L below the WWTP (Fig. 2). This trend suggests that carbamazepine varied based on WWTP effluent input into streams, which is consistent with previous studies (Conley et al. 2008). However, carbamazepine was not directly associated with wastewater since the SEM did not yield a significant model fit with the inclusion of ammonium nor was there a significant correlation between carbamazepine and ammonium concentrations. Additionally, there was no significant difference in carbamazepine concentrations detected between the Upper White and Mississinewa River watersheds, which would be expected due to the difference in land use (Fig. 2).

Alternatively, carbamazepine may be associated with pollution from both point and non-point sources since the SEM yielded a good model fit with a significant path and a correlation between DIN ($\text{NH}_4 + \text{NO}_3$) and carbamazepine (Fig. 3 and 7). The physiochemical parameters of the study sites are consistent with agricultural streams characterized by relatively high concentrations of nitrate and ammonium (Bernot et al. 2013). Therefore, carbamazepine may be associated with other anthropogenic stressors in freshwater ecosystems, which is consistent with

other pharmaceutical pollutants (Rosi-Marshall et al. 2013, Muñoz et al. 2009). The spatiotemporal variability of carbamazepine in surface waters is complex and likely depends on a number of factors including differences in land use, usage rates among the population, wastewater treatment, water chemistry and the physiochemical characteristics of the ecosystem (Veatch and Bernot 2011).

Changes in community structure

The SEM demonstrated the pathways linking carbamazepine to macroinvertebrate community structure (Fig. 7) that were not seen with bivariate correlations alone (Fig. 5). Carbamazepine had little direct effect on macroinvertebrate richness, but did have indirect effects on richness through two pathways both initiating with Baetidae abundance (Fig. 7). Specifically, sites with elevated carbamazepine had higher Baetidae abundance, which influenced overall ephemeropteran abundance and FBOM percent organic matter, both of which were significantly linked to macroinvertebrate richness.

Carbamazepine may induce changes in species composition by facilitating the dominance of baetid mayflies in the community if this family of ephemeropterans is better able to withstand the concentrations of carbamazepine and outcompete other taxa. This family is commonly found in lotic systems with moderately high levels of anthropogenic stress (tolerance value = 4; Hilsenoff 1988). The moderate tolerance of baetid mayflies could be the reason they had high abundance among the study sites, and may have increased the abundance of Ephemeroptera overall. Additionally, baetids are filter-feeding macroinvertebrates, which collect fine particulate organic matter from the water column. Therefore, this taxon may influence FBOM percent organic matter in ecosystems (Moulton et al. 2004). Both ephemeropteran abundance and FBOM

percent organic matter were linked to macroinvertebrate richness in the SEM, yielding a positive relationship between carbamazepine and macroinvertebrate richness (Fig. 4).

Potential ramifications for ecological processes

Species composition can have an equal or larger effect on ecosystem processes than those produced by richness alone (O'Connor and Crowe 2005, Downing and Leibold 2002, Cardinale et al. 2002, Hooper and Vitousek 1997, Tilman et al. 1997). Downing and Leibold (2002) demonstrated in a mesocosm experiment the importance of composition and diversity within functional groups, particularly to productivity, respiration and decomposition rates. Additionally, O'Connor and Crowe (2005) found a relationship between ecosystem functioning and taxa present wherein algal cover varied according to the identity of the taxa present rather than overall invertebrate richness. Further, Jonsson et al. (2002) found through an outdoor mesocosm experiment that detrital breakdown rates were dependent on complementary resource use, which was determined by macroinvertebrate species composition. These studies have consistently illustrated the importance of species composition to ecosystem dynamics. Thus, future research is needed to understand the functional role of species composition in ecosystem processes and how anthropogenic stressors may alter these functions.

In this study, higher abundance of baetid mayflies may have altered how the resource of fine particulate organic matter was divided among filter feeding organisms, thereby influencing the macroinvertebrate community through complementary resource use, believed to be a key mechanism linking biodiversity to ecosystem function (Cardinale et al. 2002, Hooper 1998). Cardinale and Palmer (2002) demonstrated that simulated natural disturbances (flood and mortality) reduced the probability of dominance by a single taxon and therefore altered the effect

of filter feeder richness (net-spinning caddisflies) on resource use. Alternatively, the anthropogenic stress of carbamazepine may have facilitated the dominance of baetid mayflies in the community potentially through the tolerance level of this family and altered resource use in the ecosystem (Fig. 7).

Additionally, the observed change in community structure may have facilitated compositional changes within functional groups, which potentially altered the competitive ability of taxa present (Loreau et al. 2001). Predicting how ecosystem processes will respond to changes in diversity and community structure is complicated due to differing phenological and morphological characteristics of the taxa present (Hooper 1998). Therefore, the response of ecosystem processes to changes in community structure is likely a complex function of the present taxa and the interactions between them (Hooper et al. 2012). The changes in species composition potentially induced by the presence of carbamazepine may alter the interactions of the taxa present and make predicting the changes to ecological processes more difficult.

Conclusion

The SEM in this study illustrates how carbamazepine may alter the macroinvertebrate community structure of freshwater streams in central Indiana, which could potentially lead to alterations in resource availability (i.e., presence and use of FBOM; Fig. 7) and predator-prey interactions (i.e., altered functional feeding groups present; Fig. 6). However, more work is needed to fully understand the potential hazards of this anthropogenic stressor, particularly changes to the physiology of aquatic organisms and ecosystem processes need to be quantified. Additionally, in order to fully understand how humans affect freshwater ecosystems, studies need to be conducted which focus on mixtures of anthropogenic changes, instead of focusing on

a single stressor (Rosi-Marshall et al. 2013, Hooper et al. 2012). Ecosystems today are bombarded by multiple anthropogenic inputs, spanning a number of contaminant classes (pesticides, nutrients, heavy metals and pharmaceuticals; Murraray et al. 2010). The SEM demonstrated the relative importance of two anthropogenic stressors (carbamazepine and nutrients; Fig. 7) on freshwater communities. However, to protect freshwater ecosystems and the services they provide, a comprehensive understanding of anthropogenic-induced changes is needed.

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Table 1 Physiochemical parameters and nutrient concentrations (mg/L) from study sites in the Upper White and Mississinewa river watersheds

Site Name	Upper White river watershed							
	Discharge (L/s)	pH	Temperature (°C)	Salinity (ppt)	Dissolved Oxygen (mg/L)	NO ₃	PO ₄ ³	NH ₄
White River Chesterfield	5695.2	7.9	23.1	0.26	5.09	8.51	1.05	0.34
Buck Creek 1	420.2	7.92	22.9	0.27	7.67	3.00	0.04	0.04
Buck Creek 2	429.3	7.89	22.1	0.27	6.83	3.05	0.10	0.06
Up Muncie WWTP	813.8	7.96	26.5	0.16	6.17	1.12	0.15	0.11
Muncie WWTP	1577.2	7.91	24.6	0.31	7.16	1.92	0.41	0.11
Down Muncie WWTP	1144.0	7.86	23.8	0.33	6.17	11.83	1.17	0.12
Yorktown Park	370.1	8.1	23.5	0.33	5.86	10.60	0.64	0.14
White River down Buck Creek	2544.2	8.04	23.0	0.29	5.61	5.78	0.07	0.16
White River Daleville	3113.6	8.12	23.0	0.27	5.42	1.59	0.45	0.26
White River and Burlington	795.0	8.19	28.7	0.22	8.66	10.25	1.05	0.11
Muncie Creek	145.8	8.19	29.5	0.34	8.23	3.25	0.18	0.20
Jakes Creek	111.2	8.64	26.0	0.14	5.15	1.66	0.36	0.13
Site Name	Mississinewa river watershed							
	Discharge (L/s)	pH	Temperature (°C)	Salinity (ppt)	Dissolved Oxygen (mg/L)	NO ₃	PO ₄ ³	NH ₄
Campbell Creek 1	28.4	8.33	27.5	0.24	6.14	0.11	0.37	0.09
Campbell Creek 2	23.2	8.2	26.2	0.25	4.00	0.06	0.45	0.08
Mississinewa 1	230.9	8.38	28.5	0.28	8.35	0.25	0.14	0.08
Mississinewa 2	1010.1	8.47	29.0	0.28	6.91	1.12	0.45	0.09
Mississinewa 3	368.7	8.53	27.8	0.3	8.90	0.18	0.14	0.08
Mississinewa 4	1245.3	8.41	26.8	0.24	5.34	0.26	0.62	0.09
Walnut Creek	5.1	8.21	29.8	0.21	6.19	0.14	0.27	0.15

Table 2 Species richness and taxa abundance at each site

Site Name	Upper White river watershed						
	Richness	Total	Odanata	Ephemeroptera	Trichoptera	Chironomidae	Oligochaeta
White River Chesterfield	18	260	3	53	3	35	117
Buck Creek 1	17	360	0	24	64	82	60
Buck Creek 2	13	513	0	10	3	228	19
Up Muncie WWTP	14	498	5	98	2	46	196
Muncie WWTP	13	262	2	67	2	74	34
Down Muncie WWTP	21	464	7	92	20	67	75
Yorktown Park	11	147	1	22	2	39	36
White River down Buck Creek	27	429	2	104	14	104	31
White River Daleville	15	319	2	125	10	46	67
White River and Burlington	17	421	6	189	3	87	32
Muncie Creek	12	412	3	17	0	129	119
Jakes Creek	4	201	0	5	0	80	34
Site Name	Mississinewa river watershed						
	Richness	Total	Odanata	Ephemeroptera	Trichoptera	Chironomidae	Oligochaeta
Campbell Creek 1	8	55	1	2	1	12	25
Campbell Creek 2	8	411	2	14	0	107	173
Mississinewa 1	20	316	2	70	6	97	7
Mississinewa 2	13	363	0	112	50	26	7
Mississinewa 3	11	359	1	63	1	67	69
Mississinewa 4	15	802	1	221	13	265	23
Walnut Creek	5	155	0	2	0	66	16

Figure Legends

Fig. 1 Conceptual model of potential factors influencing macroinvertebrate community structure.

Fig. 2 Sampling sites in the Upper White and Mississinewa river watersheds of central Indiana.

Fig. 3 Abiotic factors correlated with carbamazepine (CBZ; ng/L) across study sites. N = 19.

Fig. 4 Correlations between macroinvertebrate richness (number of taxa per site) and abiotic and biotic factors measured. DIN = dissolved inorganic nitrogen ($\text{NH}_4 + \text{NO}_3$). N = 19.

Fig. 5 Bivariate correlations of carbamazepine (CBZ; ng/L), macroinvertebrate richness (# of taxa per site), and Baetidae and Ephemeroptera abundance (# of individuals per site). N = 19.

Fig. 6 Correlation between macroinvertebrate richness and functional diversity (# of functional feeding groups per site)

Fig. 7 Initial (A), intermediate (B) and final (C) structural equation models describing the relationship between carbamazepine and the macroinvertebrate community. The initial ($\chi^2 = 23.124$, $df = 24$, $P = 0.512$) and intermediate ($\chi^2 = 3.722$, $df = 6$, $P = 0.714$) SEM account for a relatively small portion ($r < 0.5$) of variability of macroinvertebrate species richness. The final model ($\chi^2 = 8.954$, $df = 18$, $P = 0.961$) accounts for a substantial portion of the variability in macroinvertebrate richness ($r = 0.78$). Numbers are standardized path coefficients. Solid lines indicate significant paths in the model ($P < 0.05$). Dashed lines are non-significant hypothesized pathways ($P > 0.05$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Chapter 2: The effects of the pharmaceutical carbamazepine on life history characteristics of flat-headed mayflies (Heptageniidae) and aquatic community interactions

Abstract

Pharmaceutical pollutants are commonly detected in freshwater ecosystems around the world and have biological effects on aquatic organisms. However, current understanding of the influence this contaminant class has on freshwater communities and ecosystems is lacking. Recently the scientific community has called for research focusing on certain pharmaceuticals due to their ubiquity and potential toxicity. Carbamazepine is one of these pharmaceuticals. To better understand the effect carbamazepine has on life history characteristics of aquatic organisms and consumer-resource interactions, we assessed the influence of carbamazepine on the development, growth and behavior of mayfly nymphs (*Stenonema* sp.) and the alterations in food consumer-resource interactions between *Stenonema* and algae (*Chaetophora*). Microcosms were assembled consisting of a factorial design containing algae and mayfly nymphs native to central Indiana and dosed with environmentally relevant concentrations of carbamazepine. From this ecotoxicology experiment, we were able to infer that carbamazepine influenced the development and behavior of *Stenonema* nymphs and the body dimensions of adult individuals. However, it appears that carbamazepine does not influence consumer-resource interactions at concentrations found in surface waters. The pharmaceutical carbamazepine may influence the behavior, growth and development of mayflies, which could have significant consequences at the population, community and ecosystem level.

Introduction

Emerging contaminants such as nutrients, heavy metals and organic pollutants are continuously entering freshwater ecosystems at trace concentrations (Murray et al. 2010). While research has provided insight into the effects of nutrients (Carpenter et al. 1998), heavy metals (Runck 2007) and pesticides (Relyea 2005), there is little understanding of the effects of pharmaceutical pollutants (Hughes et al. 2013). Pharmaceuticals enter surface waters through effluent from wastewater treatment (WWTP; Rosi-Marshall and Royer 2012), septic tank leaching (Bunch and Bernot 2011) and agricultural runoff (Veatch and Bernot 2011, Bernot et al. 2013). Therefore, freshwater organisms are chronically exposed to the biological properties of pharmaceuticals and are influenced throughout life cycles (Hughes et al. 2013). Currently, there is a critical need for assessments investigating the effects of pharmaceuticals on freshwater ecosystems (Rosi-Marshall and Royer 2012).

While hundreds of pharmaceuticals including antibiotics and psychiatric drugs enter freshwater ecosystems, recent research has emphasized the need for assessments focused on certain pharmaceutical compounds due to their recalcitrance, potential toxicity and ubiquity. Among these is carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide), which is one of the most commonly detected pharmaceuticals globally (Hughes et al. 2013). Worldwide concentrations range from 0.5 to 11,561 ng/L (Loos et al. 2009, Ferguson et al. 2013) with a detection frequency of 85% across sites sampled (Hughes et al. 2013). Carbamazepine is a psychiatric drug used to treat epilepsy, bipolar disorder, chronic nerve and addiction by blocking sodium channels and reducing the firing of neurons (Porter and Meldrum 2012). Due to limited removal from wastewater treatment processes (5 – 25% removal; Miao et al. 2005), a moderate affinity for binding to sediments ($\log K_{OW} = 2.25$; Löffler et al. 2005) and long half-life (82 d;

Lam et al. 2004), carbamazepine is recalcitrant in freshwater ecosystems. Thus, freshwater organisms are persistently exposed to carbamazepine.

Exposure to carbamazepine is not likely to result in lethal toxicity due to high lethal concentrations ($LC_{50} > 4$ mg/L in *Chironomus riparius* and *Lumbriculus variegatus*), which are orders of magnitude higher than environmentally relevant concentrations (Nentwig et al. 2004). However, chronic exposure to carbamazepine can have sub-lethal effects on organisms, in which alterations in behavior (Quinn et al. 2008, Brandão et al. 2013), development (Nentwig et al. 2004, Oetken et al. 2005), reproductive success (Lürling et al. 2006, Lamichhane et al. 2013), and feeding rates (Quinn et al. 2008) have been observed. While many of these studies have focused on carbamazepine concentrations that were not environmentally relevant, the findings indicate that carbamazepine may influence freshwater organisms and adversely affect ecosystem dynamics.

Studies assessing the potential effects of carbamazepine in aquatic environments have primarily focused on single-species toxicity tests on organisms tolerant of organic pollution (Nentwig et al. 2004, Oetken et al. 2005). However, little has been done to determine the influence of carbamazepine on moderately sensitive aquatic organisms (e.g. stoneflies, mayflies and caddisflies), nor on community and ecosystem dynamics (Relyea and Hoverman 2006, Clements and Rohr 2009, Rosi-Marshall and Royer 2012). Aquatic insects such as mayflies are important members of the freshwater community, playing critical roles in nutrient cycling and decomposition, and are a fundamental link in freshwater food webs (McCafferty 1981). Therefore, alterations in the mayfly population have the potential to influence communities and ecosystems. In order to fully understand the effect of carbamazepine in freshwater ecosystems,

more extensive work assessing carbamazepine's influence on food web and community interactions is needed.

The objectives of this study were to: (1) determine the effects of environmentally realistic concentrations of carbamazepine on *Stenonema* nymphal (Family Heptageniidae) development, growth and behavior and, (2) assess the influence of carbamazepine on interactions between *Stenonema* nymphs and *Chaetophora* algae (Family Chaetophoraceae). *Stenonema* were selected for study due to their relative abundance in central Indiana and importance in North American streams (McCafferty 1981). We hypothesized that carbamazepine would directly alter the behavior, development, food resource depletion and growth of *Stenonema* and indirectly influence the growth of *Chaetophora*. Therefore, carbamazepine would have top-down effects on consumer-resource interactions.

Methods

Experimental Design

A 6 x 2 factorial design of a range of six carbamazepine concentrations and the presence or absence of a primary producer (*Chaetophora sp*) or a primary consumer (*Stenonema sp*; Fig. 1) was used to assess the effects of carbamazepine on consumer-resource interactions and the development and behavior of aquatic insects. Each combination of carbamazepine and organism treatment was replicated four times (N = 96 total microcosms). Microcosms (236 mL glass jars) were maintained in the laboratory under 16:8 lighting conditions for the experiment duration (9 d). Each microcosm contained 150 mL of stream water collected locally (mean physiochemical characteristics $\pm$ standard deviation: pH 7.3 ± 0.11 ; temperature $22 \pm 1.44^{\circ}\text{C}$; nitrate (NO_3) 0.3 ± 0.23 mg/L; phosphate (PO_4) 19.6 ± 2.85 $\mu\text{g/L}$). A glass stone-shaped substrate was added to each

microcosm and microcosms were continuously aerated with a bubble stone and covered with fiberglass screens. Water was replenished with stream water if there was > 10% loss (15 mL).

Experimental Treatments

Carbamazepine treatments represented globally environmentally relevant concentrations measured in freshwaters at 2, 20, 200 and 2000 ng/L in addition to water and methanol controls (Fig. 1). Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide; CAS no 298-46-4) was obtained from SigmaAldrich (Milwaukee, WI). Methanol was HPLC grade and obtained from SigmaAldrich. A stock solution of 2 mg/mL was prepared with pure methanol (> 99%), by dissolving 0.5 g carbamazepine in 250 mL of methanol, since carbamazepine is relatively insoluble in water (17.7 mg/L; Syracuse Physprop Database 2003). Working solutions were prepared by diluting the stock standard solution with water in each microcosm so that the total volume in each microcosm was < 0.1% standard stock solution (0.15 mL). Nominal concentrations (i.e. added quantity) of carbamazepine were added to 64 randomly selected microcosms as a single dose 24 h after experimental set-up and introduction of organisms. Monitoring of organisms occurred 24 h following the addition of carbamazepine stock solution. Methanol (0.79 mg/mL) was added to 16 randomly selected microcosms as methanol controls, which resulted in the total volume of these microcosms being < 0.1% methanol (0.15 mL), consistent with carbamazepine treatments. Water controls (N = 16) contained 150 mL stream water only.

Five mayfly nymphs (*Stenonema sp.*; hereafter “*Stenonema*”) were added to each of 48 microcosms containing primary consumers (organismal treatments: M only and A + M). *Stenonema* nymphs were collected from Cool Creek in central Indiana (40° 0' 26" N 86° 7' 21"

W) on 6 May 2013. The study organisms were transported to the laboratory in glass jars containing stream water and natural substrate. In the laboratory, mayflies were maintained in continuously aerated aquaria with stream water under a light:dark photoperiod of 16:8 at a constant temperature of $20\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The mayflies were acclimated to laboratory conditions for 2 d prior to experiment start and fed TetraMin fish food *ad libitum*. Only active, healthy nymphs were used in the experiment as determined by overall appearance. Each microcosm contained roughly equal total mass and length (mean = 0.04 ± 0.01 g and $\sim 11 \pm 0.8$ mm, respectively; Table 2). The mayfly nymphs used in the experiment were of variable instars ranging from early to final stages. Additionally, individuals had variable initial masses (wet mass range: 0.02 – 0.06 g) and lengths (mm from head to the end of the abdomen; 8.2 – 11.9 mm), which were measured prior to introduction into experimental microcosms. In the occasion of death or emergence of mayfly nymphs within 24 h of experimental set-up, individuals were replaced with another nymph of similar body dimensions (N = 16).

Algae (*Chaetophora* sp.; hereafter “*Chaetophora*”) were added to each of the 48 microcosms containing primary producers (organismal treatments: A only and A + M), also 24 h prior to the start of the experiment. *Chaetophora* was collected on 6 May 2013 from Cool Creek in central Indiana (40° 0' 26" N 86° 7' 21" W) and were transported to the laboratory in glass jars filled with stream water. In the laboratory, the algae were maintained under a light:dark photoperiod of 16:8 in continuously aerated aquaria with stream water at a constant temperature of $20\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The algae were acclimated to laboratory conditions for 2 d prior to experiment start. Initial mass (wet weight ranging from 1.5 - 2 g) was measured prior to introduction into experimental microcosms (Table 3).

Determination of mayfly response to carbamazepine

Stenonema were observed daily to determine the effects of carbamazepine on number of molting, adult emergences and mortality. Any emerged individuals (i.e., adults) were removed from the microcosm and gender was recorded along with measurements of mass and body length (as for initial dimensions). Dead nymphs and exuviae were also recorded and removed from microcosms daily. Because methods for determining specific instars of Ephemeroptera are unreliable (Fink 1982), instar classifications were not used for designating the stage of development. Rather, colorization of each nymph was evaluated daily to place it into one of three “molt categories”: 1 = a post-molt individual with white appearance and transparency; 2 = a post-molt individual with slightly darkened appearance or white appearance without transparency; and, 3 = an inter-molt individual determined by dark appearance and fully sclerotized cuticle (Fig. 2). For purposes of this study, a post-molt individual is a *Stenonema* nymph that recently molted and has not completely sclerotized and an inter-molt individual is fully sclerotized (Soluk 1990). *Stenonema* behavior was also recorded daily for each individual nymph. Behaviors included: running, free-swimming, clinging to substrate (glass or bubble stone), or clinging to algae. *Stenonema* typically cling to the underside of flat stones in moderately fast flowing waters (McCafferty 1981). Therefore, running and free-swimming behaviors were considered abnormal.

Determination of consumer-resource interaction response to carbamazepine

After daily monitoring for mayfly responses was performed on the last day (9 d), the remaining *Stenonema* nymphs and *Chaetophora* algae were removed and final mass and length measurements were collected. Additionally, each microcosm was drained to collect the

remaining algae, which was weighed for final mass. To determine how carbamazepine influenced consumer-resource interactions, final mass and length of mayflies and mass of algae were compared across organismal treatments.

Measurement of ancillary variables

Dissolved oxygen (DO), pH and temperature were measured every 2 d in each microcosm to ensure that physiochemical characteristics remained constant. These measurements were also made when the replenishment of water was necessary or when material was removed from a microcosm (such as dead nymphs and exuviae).

Statistical Analyses

Data were analyzed for effects of carbamazepine on growth of algae and behavior, molting patterns, adult emergence, and occurrence of nymphal mortality of mayflies with the use of Kruskal-Wallis test, due to non normal distribution of data, and correlation analyses. After determining there were no differences between water and methanol controls, these treatments were combined for future analyses. Mean molt category and exposure duration of mayflies were analyzed with factorial analysis of covariance (ANCOVA) with carbamazepine treatments (0 and 2000) as the covariate. Additionally, all response variables were analyzed for effects due to differences in temperature, dissolved oxygen and pH with the use of one-way ANOVA and correlation analyses (Bonferroni correction). A significance level of 0.05 was used for all analyses. Statistical analyses were performed using IBM SPSS 21.0 statistical software. Alpha was set to 0.05 for all tests.

Results

Temperature (mean = 22.88 ± 1.32 °C), pH (mean = 8.34 ± 0.22) and dissolved oxygen (DO; mean = 7.77 ± 0.53 mg/L) varied < 50% across microcosms, but did not differ among carbamazepine and organismal treatments (Table 1). None of the mayfly or consumer-resource interaction responses observed were correlated with temperature, pH or DO ($p > 0.05$), nor were there any differences in response variables due to alterations in temperature, pH or DO ($p > 0.05$).

Mayfly responses

Alterations in molting and mortality of nymphs and adult emergence

While carbamazepine had no effect on nymphal molting or adult emergence of *Stenonema* ($p > 0.05$; Table 4), the molting of mayfly nymphs was 41% higher in the carbamazepine treatments (0.43 number/microcosm) compared to the controls (0.25 occurrence/microcosm) and adult emergence were 27% higher in the carbamazepine treatments (0.27 number/microcosm) compared to the controls (0.2 number/microcosm). Additionally, mortality was 38% higher in the controls (0.14 number/microcosm) than in the carbamazepine treatments (0.089 occurrence/microcosm), yet this difference was not significant ($p = 0.65$; Fig. 3). Therefore, these environmentally relevant concentrations of carbamazepine did not influence *Stenonema* molting, adult emergence or mortality.

The molting of mayfly nymphs without the presence of *Chaetophora* (mean = 0.45 number/microcosm) was 6% higher than with *Chaetophora* (mean = 0.42 number). Adult emergences of *Stenonema* without the presence of *Chaetophora* (mean = 0.25 number/microcosm) were 11% lower than *Stenonema* with *Chaetophora* (mean = 0.28 number).

Nymph mortality without *Chaetophora* (mean = 0.06 total number/microcosm) was an order to magnitude higher than with *Chaetophora* (mean = 0.16 number). Again, none of these differences were significant (*data not shown*; $p > 0.05$).

Changes in mayfly nymph development

Stenonema individuals in carbamazepine treatments had up to a 7% lower molt category over the course of the experiment compared to the control (mean = 2.66 and 2.87 molt category of all individuals in 2000 and 0 ng/L, respectively). Molt category of mayfly nymphs differed between the controls and the 2000 ng/L carbamazepine treatment ($p < 0.001$; Fig. 4). It took 1 – 3 days for all individuals to complete an entire molt cycle in the control. However, in the 2000 ng/L carbamazepine treatment, it took > 9 d for all individuals in the treatment to complete a molt cycle (Fig. 5).

As the carbamazepine concentration increased, the molt category of mayfly nymphs decreased, suggesting that carbamazepine delayed the molting cycle of *Stenonema*. Additionally, there was a negative correlation between the molt category of individuals exposed to carbamazepine and the number of molts and adult emergences ($p = 0.002$, $r = -0.148$ and $p = 0.034$, $r = -0.104$, respectively).

Lastly, the molt category of mayfly nymphs without the presence of *Chaetophora* (mean = 2.8) was 1% higher than with *Chaetophora* (mean = 2.77). However, molt category was not affected by the organismal treatment ($p > 0.05$). Therefore, the carbamazepine treatment influenced the molt cycle of mayfly nymphs, not the abundance or assimilation of food.

Effects on mayfly behavior

Behaviors considered normal for flat-headed mayflies (clinging to substrate and algae) were observed most frequently (96% of all behaviors observed) across all carbamazepine treatments. While, other behaviors such as free-swimming and running were rare, 73% of these abnormal behaviors occurred in carbamazepine treatments. Free-swimming accounted for 0.8% of all behaviors observed and only occurred in treatments containing carbamazepine (mean = 0.03 individuals per treatment). Specifically, free-swimming *Stenonema* nymphs only occurred in 20 and 2000 ng/L carbamazepine treatments containing primary consumers and producers (A + M organismal treatments). The only significant difference in the occurrence of free-swimming was between the controls and 2000 ng/L carbamazepine treatment ($p = 0.043$; Fig. 6A).

Running accounted for 3% of all behaviors observed and occurred in both controls and carbamazepine treatments (mean = 0.1 individuals per treatment). This behavior was also observed across organismal treatments (organismal treatments A + M and M). The occurrence of running increased with carbamazepine concentrations regardless of *Chaetophora* presence (mean = 0.03 – 0.19 individuals per treatment). However, the only significant difference was between the controls and 2000 ng/L carbamazepine treatment ($p = 0.038$; Fig. 6B).

Changes in adult body dimensions

Overall, adult males (mass: 0.012 – 0.081 g, length: 5.9 – 13.7 mm) were ~ 44% smaller than adult females (mass: 0.012 – 0.119 g, length: 10 – 16 mm). Over the course of the experiment, adult mass (< 40%) and length (< 9%) decreased with significant differences occurring between initial (first 5 days) and final (last 4 days) measurements ($p < 0.05$) with the exception of male length, which was not different between periods ($p = 0.463$). Additionally, the

effects on adult mayfly dimensions were not dependent on the presence of a food resource (*Chaetophora*) since there was no difference between the two organismal treatments for mass or length of adult mayflies ($p > 0.05$). Carbamazepine decreased the mass of adult males and the length of females but did not influence the mass of adult females or the length of adult males ($p > 0.05$, Fig. 7).

Carbamazepine influenced the mass of adult male mayflies. Specifically, between the controls and 2000 ng/L carbamazepine treatment, the mass of adult males decreased 38% (Fig. 7B). The mass of adult *Stenonema* males was lower in carbamazepine concentrations of 2, 20 and 2000 ng/L (mean = 0.021, 0.029 and 0.021 g, respectively) than controls, but similar to controls in the 200 ng/L carbamazepine treatment (mean 0.034 g). The only significant difference between treatments and controls was the mass of adult male *Stenonema* at 2 ng/L carbamazepine ($p = 0.05$). Additionally, the mass of adult males differed between measurements (initial and final) at 200 ng/L carbamazepine ($p = 0.05$).

Carbamazepine influenced the length of adult female mayflies. Between the controls and 200 ng/L carbamazepine treatments, the length of adult females decreased 3% (Fig. 7C). The length of adult *Stenonema* females was lower than the controls in the 2, 20 and 200 ng/L carbamazepine treatments (mean = 12.67, 12.7 and 12.68 mm, respectively). The length of adult females was 6% higher in the 2000 ng/L carbamazepine treatment compared to the controls (mean = 13.88 and 13.07 mm, respectively). However, the only significant difference in the length of adult female mayflies was between the control and carbamazepine treatment at 20 ng/L ($p = 0.042$).

Alterations to consumer-resource interactions

Effects on primary consumers

Overall, the mass (mean = 0.04 g initial and 0.03 g final) of *Stenonema* nymphs decreased 25% throughout the course of the experiment, regardless of the organismal or carbamazepine treatments. The mass of mayfly nymphs varied 50% in both organismal treatments across nominal carbamazepine concentrations (Table 2). When algae and mayflies were combined, final *Stenonema* mass decreased 25% between the controls (0.04 g) and highest carbamazepine treatment (0.03 g). In contrast, in the mayfly-only treatment *Stenonema* nymph mass increased 33% between the controls (0.02 g) and carbamazepine treatment (0.03 g). However, none of these differences in final mayfly nymph mass were statistically significant ($p > 0.05$).

While, there were no differences in final length of *Stenonema* between organismal or carbamazepine treatments ($p > 0.05$) to suggest that carbamazepine affected the length of the primary consumer. Nymph length (mean = 10.3 mm initial and 8.2 mm final) decreased 20% over the course of the experiment, across all carbamazepine and organismal treatments. The length of *Stenonema* nymphs varied 13% in the mayfly-only treatment and 29% in the combined algae and mayfly treatment (A + M) across nominal carbamazepine concentrations (Table 2). When algae and mayflies were combined, final length also decreased 11% between the controls (9.6 mm) and carbamazepine treatments (8.5 mm). In the mayfly-only treatment, final length increased 1% between the controls (7.6 mm) and highest carbamazepine treatment (7.7 mm).

Effects on primary producers

While, there were no significant differences in final mass of *Chaetophora* to suggest that carbamazepine altered the primary producer abundance. Overall, the algal mass (mean = 1.7 g initial and 0.7 g final) decreased 59% over the course of the experiment, regardless of carbamazepine or organismal treatments. In the algae-only treatment, final mass varied 96% and in algae and mayfly combined treatment (A + M) final mass varied 11% across nominal concentrations of carbamazepine (Table 3). In the algae-only treatment, final mass increased 96% between the controls (0.51 g) and highest carbamazepine treatment (1.0 g). In the combined treatment (A + M), algal mass increased 3% in the controls (0.63 g) relative to the carbamazepine treatment (0.65 g).

Discussion

Carbamazepine had sub-lethal effects on *Stenonema* through changes in development and behavior, consistent with our hypotheses. However, in contrast to our expectations, carbamazepine did not influence consumer-resource interactions between *Chaetophora* and *Stenonema* (Tables 2 and 3). Previous research has determined that environmentally relevant concentrations of carbamazepine are not likely to result in lethal toxicity, which is consistent with results from this *in vitro* experiment (Nentwig et al. 2004, Oetken et al. 2005, Dussault et al. 2008; Fig. 3). It has been demonstrated, however, that carbamazepine has sub-lethal effects on freshwater organisms altering behavior (Quinn et al. 2008, Brandão et al. 2013), reproductive success (Lürling et al. 2006, Lamichhane et al. 2013), feeding rates (Quinn et al. 2008) and development (Nentwig 2004, Oetken et al. 2005). The observed sub-lethal effects of

carbamazepine on *Stenonema* may have been induced by the chemical mode of action of this pharmaceutical.

Possible chemical mode of action of carbamazepine

Carbamazepine is an anti-epilepsy drug, which is also used to treat a number of other psychiatric disorders. Carbamazepine primarily blocks sodium channels and therefore reduces the firing of neurons (Porter and Meldrum 2012). However, carbamazepine also binds to adenosine receptors (Van Calker et al. 1991, Biber et al. 2002, Porter and Meldrum 2012). Studies on mammalian tissues have determined that carbamazepine may antagonize certain adenosine receptors and could potentially inhibit the accumulation of cyclic AMP (Van Calker et al. 1991, Porter and Meldrum 2012). However, the significance of this secondary mode of action is unknown (Porter and Meldrum 2012).

Currently, there is little understanding of the physiological effects of carbamazepine in invertebrates (Nentwig et al. 2004, Oetken et al. 2005). However, assuming that this pharmaceutical pollutant has the same mode of action as in mammals, carbamazepine may be an antagonist to adenosine receptors in invertebrates and alter the accumulation of cyclic AMP (Van Calker et al. 1991). Martin-Diaz et al. (2009) demonstrated that carbamazepine reduced cyclic AMP levels and Protein Kinase A (PKA) activities in glands, gills and mantle in the mussel *Mytilus galloprovincialis*. In insects cyclic AMP as a secondary messenger for molting hormones including ecdysone, eclosion hormone and bursicon (Delachambre et al. 1979, Smith et al. 1984, Gilbert et al. 2002, Rewitz et al. 2009). Therefore, it has been suggested that carbamazepine influences the synthesis and bioavailability of ecdysone and alter ecdysis of aquatic insects as seen by Oetken et al. (2005) and Nentwig et al. (2004). Additionally, carbamazepine may alter

the bioavailability and synthesis of eclosion hormone and bursicon thereby altering behavior and sclerotization of aquatic insects, which could explain the results of our *in vitro* experiment (Fig. 4, 5 and 6).

The effects of carbamazepine on Stenonema and Chaetophora

Our *in vitro* experiment suggests that carbamazepine may influence *Stenonema* development with exposed individuals experiencing an altered molt cycle (Fig. 4 and 5). The alteration in development is consistent with previous research. Sediments enriched with carbamazepine negatively affected the emergence of *Chironomus riparius* by blocking pupation, which was thought to be due to physiological interference or endocrine disruption in *C. riparius* (Oetken et al. 2005). Nentwig et al. (2004) proposed that carbamazepine interferes with the binding receptors, synthesis or bioavailability of ecdysteroids or juvenile hormone. While the specific mode of action of carbamazepine in invertebrates is not fully understood, it appears that this pharmaceutical pollutant retards the development of aquatic insects.

Stenonema nymphs exposed to carbamazepine in our experiment displayed an increasing occurrence of abnormal behaviors compared to the controls (Fig. 6). Previous research on how carbamazepine influences behavior of aquatic organisms has reported conflicting results. For instance, De Lange et al. (2006) found that activity of *Gammarus pulex* was slightly reduced in the presence of 1 and 10 ng/L carbamazepine compared to controls. Additionally, Cleuvers (2003) found that carbamazepine immobilized *Daphnia magna* at concentrations > 100 mg/L. However, Brandão et al. (2013) found a positive correlation between exposure to carbamazepine and time *Lepomis gibbosus* spent in motion. The opposing effects on behavior are likely due to differing physiological modes of action of carbamazepine across organismal groups.

In this experiment, carbamazepine altered the body dimensions of adult mayflies (Fig. 7). *Stenonema* exposed to carbamazepine had decreased mass in adult males and decreased length in adult females. However, carbamazepine did not influence the growth of *Stenonema* nymphs (Table 2). Similarly, Lamichhane et al. (2013) found that carbamazepine decreased body length of *Ceriodaphnia dubia* at 264.6 µg/L in the F2 generation. Additionally, Lüring et al. (2006) determined that 200 µg/L of carbamazepine decreased somatic growth rate in *Daphnia pulex*. The results presented here and in previous research suggest that carbamazepine may interfere with growth of aquatic organisms across multiple life stages.

Research on organic pollutants has demonstrated that anthropogenic pollutants are known to alter feeding rates of freshwater organisms. For instance, chronic exposure of pharmaceutical pollutants, including carbamazepine, decreased the feeding response of *Hydra attenuate* (EC50 to carbamazepine = 3.76 mg/L; Quinn et al., 2008) and oxazepam altered the feeding rate of *Perca fluviatilis* (Brodin et al. 2013). Additionally, Alexander et al. (2007) found that a short exposure (24 h pulse) to 5 µg/L of imidacloprid reduced the feeding rate of *Epeorus longimanus*. However, at our environmentally relevant concentrations of carbamazepine we saw no significant influence on consumer-resource interactions between *Stenonema* and *Chaetophora*.

Potential ramifications from carbamazepine exposure

Carbamazepine is ubiquitous and recalcitrant in freshwater ecosystems and aquatic insects are likely exposed throughout life cycles (Pascoe et al. 2003, Veach and Bernot 2011, Hughes et al. 2013, Bernot et al. 2013, Ferguson et al. 2013). Therefore, it is likely that carbamazepine may have variable effects on exposed individuals. Because carbamazepine may hinder development (Nentwig et al. 2004, Oetken et al. 2005), change behavior (Cleuvers 2003,

De Lange et al. 2006, Quinn et al. 2008, Brandão et al. 2013), alter feeding rates (Quinn et al. 2008), reduce fecundity and decrease body dimensions of aquatic organisms (Lürling et al. 2006, Lamichhane et al. 2013) even at ng/L concentrations, continued discharge of this emerging contaminant may have potential ramifications on populations, communities and ecosystem dynamics (Maltby 1999).

In the case of *Stenonema*, nymphs exposed to carbamazepine had lower molt categories (Fig. 4) and took longer to complete molt cycles (Fig. 5). Additionally, in carbamazepine treatments there was a negative correlation between molt category and the total number of molts and emergences, suggesting that carbamazepine delayed development of *Stenonema*. Like other aquatic organisms, ephemeropterans must adjust life history characteristics to balance the conflicting fitness advantages of survival and future fecundity (Schluter et al. 1991, Abrams et al. 1996). Ephemeropterans accelerate development in response to unfavorable habitat conditions to attain the lowest possible ratio between mortality and fecundity (Peckarsky et al. 2001, Harper and Peckarsky 2006). So, while carbamazepine may ultimately contribute to habitat degradation, we saw delayed rather than accelerated development at the relevant level of exposure.

Additionally, the delay in development (Fig. 4 and 5) and increase in abnormal behavior (Fig. 6) may alter the predation risk of *Stenonema*. The vulnerability of mayfly nymphs to predation is dependent on the molting condition of an individual. Nymphs in a post-molt condition may be at a higher risk of predation than individuals in an inter-molt condition (Soluk 1990). Therefore, *Stenonema* exposed to carbamazepine may be at a higher risk of predation due to prolonged sclerotization. Also, the increase in abnormal behavior could potentially elevate the risk of predation of nymphs exposed to carbamazepine due to a decrease in predator avoidance behavior (Peckarsky 1980, Brandão et al. 2013). A heightened risk of predation could disturb

the predator-prey balance, leading to the overexploitation of *Stenonema* and ultimately impacting populations, community structure and ecosystem processes (Bernot and Turner 2001, De Lange et al. 2006).

Lastly, exposure to carbamazepine reduced the body dimensions of adult *Stenonema* (Fig. 7). The reduction in adult body dimensions may be due to acceleration of development to avoid unfavorable habitat conditions and maximize overall fitness. Exposure to anthropogenic contaminants can prompt nymphs to accelerate development at the cost of future reproductive success (Alexander et al. 2008, Palmquist et al. 2008, Conley et al. 2009). The result of this accelerated development may lead to smaller adults, reduced mating success and altering synchronous emergence of individuals. A reduction in female body length may result in a decrease in fecundity through smaller clutch sizes (Conely et al. 2009) as well as diminished egg quality (smaller egg mass and length; Scrimgeour and Culp 1994, Palmquist et al. 2008). Also, a reduction in male body mass may hinder an individual's ability to compete and lead to a loss in mating success (Flecker et al. 1988). Lastly, acceleration of development may influence the synchronous emergence of individuals. Emergence during the peak time of year results in higher mating success (more individuals to encounter) and fecundity (larger eggs and first instar nymphs; Corkum et al. 1997). Overall, exposure to carbamazepine may affect the fitness of future generations of *Stenonema*.

Conclusion

Carbamazepine is one of the most frequently detected compounds in North America, Asia and Europe. However, despite the ubiquity of this contaminant, previous research has not adequately addressed the potential risk to freshwater organisms by carbamazepine (Hughes et al.

2013). Global concentrations of carbamazepine range from 0.5 to 11,561 ng/L in surface waters (Loos et al. 2009, Ferguson et al. 2013) with a median of 174 ng/L (Hughes et al. 2013). Nevertheless, a majority of the research assessed responses to carbamazepine concentrations that were orders of magnitude higher than environmentally realistic concentrations (Cleuvers 2003, Nentwig et al. 2004, Oetken et al. 2005, De Lange et al. 2006, Lürling et al. 2006, DeLorenzo and Fleming 2008, Quinn et al. 2008, Brandão et al. 2013, Lamichhane et al. 2013). This *in vitro* experiment demonstrated that environmentally relevant concentrations of carbamazepine could have chronic effects on *Stenonema* and *Chaetophora*. While data obtained from these types of *in vitro* experiments are useful in understanding the effects of anthropogenic stressors, the results commonly underestimate the sensitivity of freshwater organisms *in situ* (Buchwalter et al. 2007, Clements et al. 2013). In order to fully understand the effects carbamazepine has on freshwater ecosystems, more research focusing on chronic exposures of this pharmaceutical pollutant at environmentally relevant concentrations is needed (Murray et al. 2010, Rosi-Marshall and Royer 2012, Hughes et al. 2013).

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Table 1 Mean mesocosm pH, temperature and dissolved oxygen (DO) across CBZ treatments. Standard deviation in parentheses. There were no significant differences among treatments ($P > 0.1$).

CBZ (ng/L)	pH	Temperature (°C)	DO (mg/L)
0	8.32 (0.25)	22.92 (0.86)	8.02 (0.55)
1	8.34 (0.20)	22.99 (0.96)	8.03 (0.52)
2	8.31 (0.24)	23.20 (1.38)	7.93 (0.64)
20	8.35 (0.19)	23.20 (0.76)	7.98 (0.40)
200	8.37 (0.17)	22.68 (0.91)	8.04 (0.49)
2000	8.31 (0.22)	22.96 (2.29)	7.99 (0.53)

Table 2 Mean mayfly nymph mass and length in mayfly (M) and algae + mayfly (A+ M) treatments across nominal carbamazepine (CBZ) concentrations. Standard deviation in parentheses. There were no significant differences in mayfly nymph mass between treatments ($P > 0.05$).

	Mayfly (M)		Algae + Mayfly (A+M)	
CBZ (ng/L)	Initial mass (g)	Final mass (g)	Initial mass (g)	Final mass (g)
Water	0.04 (0.016)	0.02 (0.002)	0.04 (0.008)	0.04 (0.036)
Methanol	0.04 (0.008)	0.03 (0.011)	0.04 (0.008)	0.02 (0.005)
2	0.04 (0.016)	0.02 (0.008)	0.04 (0.007)	0.03 (0.012)
20	0.05 (0.011)	0.03 (0.010)	0.04 (0.006)	0.03 (0.003)
200	0.04 (0.014)	0.03 (0.008)	0.04 (0.009)	0.03 (0.011)
2000	0.04 (0.004)	0.03 (0.005)	0.04 (0.005)	0.03 (0.013)

CBZ (ng/L)	Initial length (mm)	Final Length (mm)	Initial length (mm)	Final length (mm)
Water	9.8 (1.13)	7.6 (0.49)	10.1 (0.82)	9.6 (2.23)
Methanol	10.1 (0.46)	8.6 (1.77)	10.4 (0.72)	6.8 (0.18)
2	10.7(1.35)	8.4 (2.58)	10.5 (0.86)	8.2 (0.89)
20	11.0 (0.39)	8.4 (1.12)	9.9 (0.57)	7.6 (0.48)
200	10.1 (1.12)	8.0 (1.84)	10.5 (0.41)	8.4 (1.39)
2000	10.2 (0.45)	7.7 (0.67)	10.2 (0.58)	8.5 (0.91)

Table 3 Mean algal mass in algae (A) and algae + mayfly (A + M) treatments across nominal carbamazepine (CBZ) concentrations. Standard deviation in parentheses. There were no significant differences in algae mass between treatments ($P > 0.05$).

CBZ (ng/L)	Algae (A)		Algae + Mayfly (A+ M)	
	Initial mass (g)	Final mass (g)	Initial mass (g)	Final mass (g)
Water	2.87 (0.42)	0.51 (0.21)	1.54 (0.19)	0.63 (0.17)
Methanol	1.64 (0.07)	0.94 (0.35)	1.52 (0.17)	0.86 (0.35)
2	1.72 (0.13)	0.69 (0.27)	1.49 (0.33)	0.56 (0.35)
20	1.53 (0.20)	0.77 (0.68)	1.71 (0.29)	0.58 (0.28)
200	1.62 (0.34)	0.91 (0.39)	1.31 (0.36)	0.60 (0.21)
2000	1.63 (0.34)	1.00 (0.27)	1.78 (0.18)	0.65 (0.29)

Table 4 Mean number of molts and emergences in mayfly (M) and algae + mayfly (A + M) treatments over course of experiment across nominal carbamazepine (CBZ) concentrations. Standard deviation in parentheses. There were no significant differences in the # of molts or emergences between treatments and control ($P > 0.05$).

CBZ (ng/L)	Molts		Emergences	
	Mayfly (M)	Algae + Mayfly (A+M)	Mayfly (M)	Algae + Mayfly (A+M)
Water	0.39 (0.60)	0.41 (0.60)	0.22 (0.48)	0.25 (0.50)
Methanol	0.36 (0.83)	0.39 (0.56)	0.19 (0.58)	0.23 (0.43)
2	0.44 (0.65)	0.38 (0.74)	0.33 (0.59)	0.26 (0.67)
20	0.50 (0.74)	0.38 (0.61)	0.33 (0.68)	0.22 (0.49)
200	0.44 (0.65)	0.44 (0.69)	0.25 (0.50)	0.33 (0.63)
2000	0.45 (0.77)	0.50 (0.65)	0.36 (0.64)	0.22 (0.48)

Figure Legends

Fig. 1 Experimental set-up of a 6 x 2 factorial design of carbamazepine and the presence or absence of a primary producer (algae; *Chaetophora*) or a primary consumer (mayfly; *Stenonema*). Each combination of carbamazepine treatment and presence or absence of algae and mayfly was replicated four times (N = 96 total microcosms). Circles represent individual microcosms. Response variables for mayflies included nymph and adult body length and mass, behavior, molt category and the number of molts, emergences and mortalities. Response variables for algae included mass.

Fig. 2 Classification of molt categories of *Stenonema* nymphs. Categories from top to bottom were: 1 = post-molt individual with white or transparent appearance, 2 = post-molt individual with a white appearance with transparency and 3 = inter- molt individual dark or fully sclerotized color.

Fig. 3 Mean mortality of mayfly nymphs compared to nominal carbamazepine (CBZ) concentrations over 9 d. There were no significant differences between controls and CBZ treatments ($P > 0.05$) Numbers at bottom of bars are number of individuals for each CBZ treatment. Data are means $\pm$ 1 SE.

Fig. 4 Mean molt category for all mayfly nymphs in each carbamazepine (CBZ) treatment compared to nominal CBZ concentrations over 9 d. Categories ranged from 1-3, 1 being the newest molt and 3 the oldest. Numbers at bottom of bars are number of individuals for each CBZ treatment. Data are means $\pm$ 1 SE. ** $P < 0.01$

Fig. 5 Mean molt category of mayfly nymphs over exposure time (9 d) for the water control and 2000 ng/L CBZ treatment. Each data point represents the mean molt category for all *Stenonema* individuals in each treatment. Data are means $\pm$ 1 SE.

Fig. 6 Mean number of mayfly nymphs (A) free-swimming and (B) running during monitoring period compared to nominal carbamazepine (CBZ) concentrations over 9 d. Organismal treatments (A + M and M) combined. Numbers at bottom of bars are number of individuals for each CBZ treatment. Data are means $\pm$ 1 SE. * $P < 0.05$.

Fig. 7 Mean mass (top panels) and length (bottom panels) of adult female (left panels) and males (right panels) mayflies at experiment start (first 5 days exposed) and at the end of the experiment (last 4 days exposed) across nominal carbamazepine (CBZ) concentrations. Numbers at bottom of bars are number of individuals for each CBZ treatment. Data are means $\pm$ 1 SE. * $P < 0.05$.

		Carbamazepine treatments					
Organismal Treatment		Nominal Carbamazepine Concentration (ng/L)					
		Controls					
		Water	Solvent	2	20	200	2000
	Algae
Mayfly
Algae + Mayfly

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

CHAPTER 3: Influence of the psychiatric drug carbamazepine on freshwater invertebrate communities and ecosystem dynamics

Abstract: Freshwater ecosystems are persistently exposed to pharmaceutical pollutants, including carbamazepine. Despite the ubiquity and recalcitrance of carbamazepine, there is limited understanding of how this pharmaceutical may influence freshwater ecosystems and communities. To better understand how carbamazepine influences the invertebrate community and ecosystem dynamics in freshwaters, we conducted a mesocosm experiment utilizing environmentally relevant concentrations of carbamazepine. Mesocosms were populated with four gastropod taxa (*Elimia*, *Physa*, *Lymnaea* and *Helisoma*), zooplankton, filamentous algae and phytoplankton. After a 31 d experimental duration, structural equation modeling (SEM) was used to relate changes in the community structure and ecosystem dynamics to carbamazepine exposure. Invertebrate diversity increased in the presence of carbamazepine. Additionally, carbamazepine altered the biomass of *Helisoma* and *Elimia*, induced a decline in *Daphnia pulex* abundance and shifted the zooplankton community towards copepod dominance. Lastly, carbamazepine decreased decomposition and altered primary production and dissolved nutrient concentrations. Changes in the invertebrate community were through direct (i.e., exposure to carbamazepine) and indirect pathways (i.e., changes in food resource availability). These data show the psychiatric drug carbamazepine may alter freshwater community structure and ecosystem dynamics and could have profound effects on natural systems.

INTRODUCTION

Freshwater ecosystems are continually exposed to anthropogenic stressors including urban and sub-urban development, climate change and point and non-point source pollution. Globally, only a fraction of freshwater ecosystems remain relatively pristine (Palmer et al. 2009, Vörösmarty et al. 2010). Multiple studies have demonstrated how alterations in biodiversity and community structure influence ecosystem dynamics (e.g., Tilman et al. 1997, Hooper and Vitousek 1997, Downing and Leibold 2002, Steiner et al. 2005,). However, many of these studies assessed relatively pristine ecosystems, despite their rare occurrence. Anthropogenic stressors have the potential to profoundly alter both ecosystem dynamics and community structure (Jonsson et al. 2002, Relyea 2005, Muñoz et al. 2009, McMahon et al. 2012, Liess et al. 2013, Dolciotti et al. 2014,). Therefore, further research is needed to quantify how common anthropogenic stressors influence biodiversity and ecosystem dynamics (Relyea and Hoverman 2006, Clements and Rohr 2009, Rosi-Marshall and Royer 2012).

A wide range of pollutants regularly enter surface waters (Murray et al. 2010). Previous studies have identified the source, fate and effects of many of these pollutants, including pesticides (Relyea 2005), industrial compounds (Runck 2007), heavy metals (Clements et al. 2013) and nutrients (Bernot et al. 2006), yet little is known about pharmaceuticals (Rosi-Marshall and Royer 2012). Unlike other pollutants, pharmaceuticals are biologically active and elicit responses from organisms across multiple trophic levels (Brun et al. 2006). Pharmaceutical compounds are frequently detected in urban and agriculturally dominated ecosystems due to both human and veterinary use and subsequent movement into aquatic environments (Veatch and Bernot 2011, Bunch and Bernot 2011, Hughes et al. 2013). Currently, there is a need for studies

focused on the influence of pharmaceuticals on the ecosystem level (Rosi-Marshall and Royer 2012, Hughes et al. 2013).

Frequently detected pharmaceutical pollutants span a number of different chemical classes, including painkillers, psychiatric drugs and antibiotics. The psychiatric drug carbamazepine is one of the most frequently detected pharmaceutical compounds in freshwater ecosystems of North America, Europe and Asia (Hughes et al. 2013). Carbamazepine is an anti-epilepsy drug, which is additionally used to treat bipolar disorder, depression and addiction. Carbamazepine reduces the firing of neurons by blocking sodium channels (Porter and Meldrum 2012). Global concentrations of carbamazepine range from 0.5 ng/L to 11,561 ng/L (Loos et al. 2009, Ferguson et al. 2013) with a detection frequency of 85% among study sites (Hughes et al. 2013). Removal of carbamazepine through abiotic and biotic degradation pathways is minimal in natural ecosystems (5-26%; Moa et al. 2005). Thus, carbamazepine is considered recalcitrant in freshwater ecosystems (half-life 82 d; Lam et al. 2004). With high usage rates (1,014 tons per year; Zhang et al. 2008) and limited removal freshwater ecosystems are persistently exposed to this pollutant.

Carbamazepine is not acutely toxic to freshwater organisms at environmentally relevant concentrations as lethal concentrations are higher than those measured in surface waters ($LC_{50} > 4$ mg/L in *Lumbriculus variegatus* and *Chironomus riparius*; Nentwig et al. 2004). However, chronic effects from exposure to carbamazepine have been observed (Quinn et al. 2008, Gust et al. 2013, Brandão et al. 2013, Lamichhane et al. 2013). For example, Martin-Diaz et al. (2009) determined that environmentally relevant concentrations of carbamazepine altered biochemical pathways of *Mytilus galloprovincialis* (Mediterranean mussel), including a reduction in cyclic AMP (cAMP) levels and Protein Kinase A (PKA) activities. While many studies determined that

carbamazepine poses little risk to freshwater organisms at concentrations found in surface waters, these toxicity data may underestimate the sensitivity of freshwater organism. Evaluations of heavy metal contaminants have demonstrated that laboratory experiments may not reflect the actual sensitivity of freshwater organisms to pollutants (Buchwalter et al. 2007, Clements et al. 2013). Freshwater organisms may be more sensitive to environmentally relevant concentrations of carbamazepine and experience sub-lethal effects, such as changes in behavior, mating success, immuno-competence and development. These sub-lethal effects could alter community structure and diversity of freshwater ecosystems (Bernot and Turner 2001). Therefore, more information is needed to determine how carbamazepine influences freshwater community structure and ecosystem dynamics (Hughes et al. 2013).

The objectives of this study were to determine how environmentally relevant concentrations of carbamazepine influence the invertebrate community and freshwater ecosystem dynamics. We hypothesized that carbamazepine would reduce the diversity of freshwater invertebrates and change the invertebrate community, which would alter ecosystem characteristics such as primary production, decomposition and dissolved nutrient concentrations.

METHODS

Experimental Design

A mesocosm experiment was conducted to quantify the effects of carbamazepine on freshwater invertebrate biodiversity and ecosystem dynamics. Mesocosms (75 L HDPE circular containers) were maintained at a Ball State University field station (Hults farm) located in Albany, Indiana (40°18'12"N, 85°13'52"W) for the experiment duration (31 d) in 2013. Each mesocosm contained 41.5 L of well water (mean physiochemical characteristics $\pm$ standard

deviation: pH 8.2 ± 0.09 ; dissolved oxygen 6.5 ± 0.7 ; temperature $25.6 \pm 0.92^{\circ}\text{C}$; nitrate (NO_3) 0.15 ± 0.21 mg/L; phosphate (PO_4) 43.1 ± 57.5 $\mu\text{g/L}$, which was added 27 d prior to the introduction of organisms. Mesocosms were covered with a fiberglass screen (mesh size: 1 mm) and were exposed to natural elements and light cycles from 8 June 2013 – 11 July 2013. Mesocosms were incubated *in situ* for 31 d following completion of experimental set-up. Each mesocosm received one of four treatments (water and methanol controls and carbamazepine treatments of 200 and 2000 ng/L) with four replicates each ($N = 16$ total mesocosms; Fig. 2).

Mesocosm Substrates

Mesh bags (mesh size: 1 mm; dimensions: 14 x 10 cm) containing 20 g of leaf litter were added to each mesocosm to provide nutrition and refuge. Leaf litter was collected from a local pond (40°20'12"N, 85°13'41"W) then dried and weighed prior to addition to mesh bags. Additionally, homogenized sediment collected from a local pond (40°20'12"N, 85°13'41"W) and was equally distributed among mesocosms (~ 300 cm³ of sediment). Both the leaf litter and sediment were added to each mesocosm 27 d prior to introduction of organisms.

Experimental Treatments

Carbamazepine treatments reflected environmentally relevant concentrations measured in surface waters (Loos 2009, Hughes et al. 2013, Ferguson et al. 2013) at 200 and 2000 ng/L in addition to water and methanol controls (Fig. 1). Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide; CAS no 298-46-4) and methanol (HPLC grade) was obtained from SigmaAldrich (Milwaukee, WI). A stock solution of 2 mg/mL was prepared by dissolving 0.5 g carbamazepine

in 250 mL of pure methanol (> 99%), as pure carbamazepine is insoluble in water (17.7 mg/L; Syracuse Physprop Database 2003).

Working solutions for each mesocosm were prepared by diluting the stock standard solution with existing water in each experimental unit (i.e., mesocosm) so that the total volume in each mesocosm was < 0.1% standard stock solution (0.664 mL). Nominal concentrations of carbamazepine were added to 8 randomly selected mesocosms as a single dose 48 h after introduction of organisms. Sample collection started 2 d following the addition of carbamazepine stock solution. Pure methanol (12.7 mg/L) was added to 4 randomly selected mesocosms as methanol controls, which resulted in the total volume of these mesocosms being < 0.1% methanol (0.664 mL), consistent with carbamazepine treatments. Water controls (N = 4) contained 41.5 L well water only.

Organisms

Zooplankton and algae were collected and homogenized from a local pond (40°20'12"N, 85°13'41"W) and were introduced into the mesocosms immediately after collection and homogenization 27 d after experimental set-up and were allowed to incubate *in situ* for 31 d. Diverse aliquots of zooplankton (consisting of calanoid and cyclopoid copepods, ostracods, *Scapholeberis sp.*, *Pleuroxus sp.*, *Alona sp.*, *Daphnia pulex*, *Chydorus sp.*, *Ceriodaphnia sp.* and *Chaoborus*) were evenly distributed among mesocosms. Aliquots (20 mL) of algae (*Spirogyra sp.*) were also equally distributed among mesocosms.

Four snail species, *Lymnaea stagnalis*, *Physa acuta*, *Helisoma trivolvis* and *Elimia livescens* (hereafter referred to by generic name), were collected from a local pond (40°0'26"N, 86°7'21"W) and the White River (40°11'8"N, 86°26'21"W) on 6 June 2013 and were acclimated

to laboratory conditions for 48 h prior to introduction to mesocosms. Gastropods were added to each mesocosm on 8 June 2013 (27 d after experimental set-up) after measurements of length were collected to determine biomass. Gastropods were allowed to incubate for 31 d *in situ*. These gastropod taxa are common to central Indiana and are important to North American freshwater ecosystems (Wojdak 2005, Pyron et al. 2008). To ensure equal biomass across mesocosm treatments at the experiment start, the number of individuals added to each mesocosm varied from 3 – 5 individuals of per species due to differences in average body size between individuals (*Physa*, *Lymnaea* and *Helisoma* biomass ~ 30 mg for each taxa per mesocosm and *Elimia* biomass ~ 350mg per mesocosm.).

Determination of biotic response to carbamazepine

To quantify the effects of carbamazepine on gastropod biomass, richness and abundance, water was pumped from each mesocosm with the use of a diaphragm pump and drained through a sieve (mesh size: 1 mm) at the end of the experiment (31 d). The contents from the sieve were combed through for 3 minutes for collection of gastropods. Snails were preserved in 10% buffered formalin. All snails were counted and the shell length for each individual was measured. Length measurements were converted to dry mass of fleshy tissue using species-specific length-weight regressions derived locally (*data not shown*; Benke et al. 1999). Two measurements of biomass were calculated for gastropods, (1) change in biomass and (2) standing biomass. The change in biomass was the average loss or gain (unique to each taxon) of gastropod biomass over the course of the experiment. Total snail biomass change was calculated as the biomass of all live and dead snails (from the later being collected shells without soft tissue) at experiment end minus the initial biomass of snails. The standing biomass was calculated as only the snails living

at the end of the experiment (Wojdak 2005). Shannon diversity (H) was calculated for the gastropod community for each mesocosm with the following equation: $H = -\sum (p_i \ln(p_i))$.

To determine the influence of carbamazepine on zooplankton richness and abundance, samples were collected weeks 1, 3 and 5 post-organism introduction from each mesocosm using a PVC pipe (diameter: 10 cm, height 60 cm) that was arbitrarily placed upright in each mesocosm to ensure an equal area was sampled with 3 swipes of a dip net (mesh size: 20 μ m). Two samples were collected from each mesocosm during individual sampling events. The samples were homogenized and preserved with 90% ethanol. Zooplankton identity was determined with the use of Pennak's Freshwater invertebrates of the United States (Smith 2001) and abundance was quantified using a zooplankton counting wheel and a dissecting microscope. Shannon diversity (H) was calculated for the zooplankton and the total invertebrate communities (i.e., gastropod and zooplankton) for each mesocosm with the following equation: $H = -\sum (p_i \ln(p_i))$.

Grab water samples (50 mL) were collected from just below the water surface of each mesocosm to determine the effects of carbamazepine on phytoplankton biomass. These samples were collected weekly throughout the duration of the experiment. Each water sample was transported to the laboratory on ice, filtered immediately onto a glass fiber filter (pore size: 0.7 μ m) and frozen until subsequent analysis. Chlorophyll *a* concentrations of samples were determined via hot ethanol extraction under dim lighting conditions (APHA 2012). The frozen samples were thawed and submerged in 10 mL of 95% ethanol in a 60 mL falcon tube. The falcon tubes were placed in a water bath of 79° C for 5 min. The samples were then covered in foil to eliminate light exposure and extracted for 24 h in a refrigerator. The absorbance of the supernatant was measured with a spectrophotometer (UV-1700 PharmaSpec, Shimadzu) at 645,

665 and 750 nm. The chlorophyll *a* concentration was then calculated per standard methods (APHA 2012).

Additionally, at the end of the experiment, the leaf litter bags were collected from each mesocosm for measurement of mass. Algae were also collected from each mesocosm after the water was removed from the experimental units with a diaphragm pump. Both the algal samples and leaf litter bags were subsequently dried and weighed.

Determination of abiotic response to carbamazepine

Weekly grab samples of water were collected just below the surface of the water to measure fluctuations in dissolved nutrient concentrations among mesocosms. Dissolved nutrient concentrations were measured from 20 mL filtered water samples (Whatman glass fiber filter; pore size = 0.7 μm). Nutrient samples were frozen within 24 h of collection until subsequent analyses. Water samples were analyzed by ion chromatography (DIONEX-ICS-3000) using standard protocols (APHA 2012) to quantify nitrate (NO_3), phosphate (PO_4^{3-}), fluoride, chloride, bromide, sulfate (SO_4) and ammonium (NH_4) concentrations. Additionally, dissolved oxygen (DO), pH and temperature were measured twice weekly in each mesocosm.

At the end of the experiment, homogenized sediment samples were collected to determine differences in sediment ash free dry mass (AFDM) among mesocosm treatments. To calculate ash free dry mass, a sediment sub-sample was dried (60°C), weighed, combusted at 500°C and re-weighed in the laboratory. Percent organic matter was calculated for each mesocosm as the difference in ash and dry weight divided by dry weight (APHA 2012).

Statistical Analyses

Data were analyzed for effects of carbamazepine on diversity of zooplankton and gastropods as well as biotic and abiotic characteristics using Kruskal-Wallis tests, due to non normal distribution of data, and correlation analyses. After determining there were no differences between water and methanol controls, these treatments were combined for future analyses. A conceptual model (Fig. 1) guided these analyses. The linear relationships from these analyses informed an *a priori* model for testing the influence of carbamazepine on freshwater communities using structural equation modeling (SEM; McMahon et al. 2012). The SEM was evaluated using the model chi-square and associated *P* value. Additionally, all response variables were analyzed for effects due to differences in temperature, dissolved oxygen and pH with the use of one-way ANOVA and correlation analyses (Bonferroni correction). Statistical analyses were performed using IBM SPSS 21.0 and SPSS Amos statistical software. Alpha was set to 0.05 for all tests.

RESULTS

Physiochemical chemical characteristics

Temperature (mean = 26.43 ± 3.22 °C), pH (mean = 8.44 ± 0.31) and dissolved oxygen (DO; mean = 6.0 ± 0.89 mg/L) varied < 2% across mesocosms. Additionally, there were no statistically significant differences in temperature, pH or DO between carbamazepine treatments (Table 1). None of the biotic or abiotic response variables were correlated with pH or DO ($P > 0.05$). However, DO ($r = 0.43$, $P < 0.01$), pH ($r = -0.549$, $P < 0.01$), zooplankton richness ($r = 0.721$, $P < 0.01$), *Elimia* standing biomass ($r = 0.628$, $P = 0.009$) and change in *Elimia* biomass ($r = 0.682$, $P = 0.004$) were correlated with temperature (*data not shown*). Over the experiment

duration, temperature increased from 25.3° C in week 1 to 32.3° C in week 5 across all mesocosms consistent with the growing season.

Influence of carbamazepine on diversity

Invertebrate richness increased over the course of the experiment, due to increases in zooplankton richness over time ($r = 0.71$, $P < 0.01$). Invertebrate richness was 20% higher in the carbamazepine treatments (mean = 3.58 taxa per mesocosm) compared to the water control (mean = 2.83 taxa per mesocosm; Table 2). However, environmentally- relevant concentrations of carbamazepine did not influence invertebrate richness (*data not shown*, $P > 0.05$). Invertebrate diversity also increased throughout the experiment and in the carbamazepine treatments (Fig. 3). Shannon diversity of the invertebrate community was 16% higher in the 2000 ng/L carbamazepine treatment (mean diversity = 1.8 per mesocosm) compared to the controls (mean = 1.5 taxa per mesocosm; $P = 0.05$).

Response of gastropods to carbamazepine

Overall, the total gastropod biomass per mesocosm decreased over the course of the experiment. However, there was no significant difference in total gastropod biomass change between the carbamazepine treatments and controls (*data not shown*; $P = 0.258$). Biomass changes in the presence of carbamazepine were dependent on each gastropod taxon. Specifically, *Physa*, *Lymnaea* and *Elimia* lost and *Helisoma* gained biomass over the course of the experiment, regardless of the carbamazepine treatment (Fig. 4). However, carbamazepine did not change the biomass of *Physa*, *Lymnaea* or *Helisoma* ($P > 0.05$) but did influence *Elimia* biomass ($P = 0.043$; Fig. 4). The loss of biomass in *Elimia* was 100% lower in the carbamazepine

treatments (mean = -144.76 mg dry mass/ 31 d) compared to controls (mean = -303.8 mg dry mass/ 31 d).

Carbamazepine did not affect the standing biomass of the total gastropod community (i.e., living biomass of all gastropods in mesocosms; $P = 0.718$). The effect of carbamazepine on standing biomass was also unique to each gastropod taxon. Overall, *Physa*, *Lymnaea* and *Elimia* gained and *Helisoma* lost standing biomass over the course of the experiment in the carbamazepine treatments. However, there were no significant differences in standing biomass between the carbamazepine treatments and controls for any of the gastropod taxa ($P > 0.05$; Table 3).

Response of zooplankton to carbamazepine

Copepod and ostracod abundance increased in the presence of carbamazepine and cladoceran abundance decreased in the carbamazepine treatments (Fig. 5). However, there was no significant difference in abundance of copepods, ostracods or cladocerans between the carbamazepine treatments and controls (*data not shown*; $P = 0.51, 0.271$ and 0.241 respectively). However, the abundances of *Daphnia pulex*, *Chydorus* and *Ceriodaphnia* was $> 66\%$ lower in the carbamazepine treatments (mean = 4.5, 7.8 and 2 individuals per mesocosm, respectively) compared to the controls (mean = 10.5, 13 and 21.67 individuals per mesocosm). However, carbamazepine did not influence the abundances of *Chydorus* or *Ceriodaphnia* at environmentally relevant concentrations ($P > 0.05$). The abundance of *Daphnia pulex* was higher in the controls than in the 200 ng/L carbamazepine treatment ($P = 0.5$).

Response of ecosystem dynamics to carbamazepine

Despite variations in primary producers, carbamazepine did not influence algal mass or phytoplankton biomass found in mesocosms ($P = 0.718$ and 0.318 respectively; Fig.6). The percent organic matter in sediments was $> 29\%$ lower in the carbamazepine treatments (mean = 4.31%) compared to the controls (mean = 5.56% ; $P < 0.04$; Fig.6).

Influence of carbamazepine on dissolved nutrient concentrations

Carbamazepine treatments only negatively affected bromide concentrations ($P = 0.04$). Fluoride, chloride, nitrate (NO_3), phosphate (PO_4) and sulfate (SO_4) concentrations were not affected by carbamazepine (Table 4).

SEM results

Structural equation models (SEM) identified several significant causal relationships between carbamazepine and the freshwater ecosystem with a significant fit to the covariance matrix (Fig. 7C). However, the initial and intermediate models (Fig. 7A and B) did not account for a significant proportion of variability in the abiotic components of the ecosystem ($r < 0.05$) and did not have a significant fit to the covariance matrix. Therefore, they were rejected in favor of the final SEM. The final SEM identified several significant causal relationships between carbamazepine and the biotic and abiotic characteristics of the mesocosms (Fig. 7C) with a significant fit to the covariance matrix ($\chi^2 = 8.126$, $df = 14$, $P = 0.883$). Significant pathways included the effects of carbamazepine on percent organic matter in sediment (standardized path coefficient = -0.47); the effects of copepod abundance and the effects of *Elimia* and *Helisoma*

standing biomass on nitrate concentrations (0.87, -0.33 and -0.45, respectively); the effects of *Helisoma* standing biomass on phosphate concentrations (-0.91); and the effects of percent organic matter in sediment on cladoceran abundance (0.72). However, the pathways linking carbamazepine to cladoceran abundance (-0.07) and *Helisoma* standing biomass (-0.3) were not significant. Additionally, the pathway linking *Elimia* standing biomass to algal mass (0.13) was not significant. The model accounted for a substantial portion of the variation in algal mass ($r = 0.56$), sediment percent organic matter ($r = 0.63$) and concentrations of nitrate ($r = 0.65$) and phosphate ($r = 0.58$), therefore providing insight into how environmentally relevant concentrations of carbamazepine affect freshwater ecosystems.

Discussion

Results from this mesocosm experiment indicate that environmentally relevant concentrations of carbamazepine influenced biodiversity of freshwater invertebrates and ecosystem dynamics. Ecosystem processes were altered in the presence of carbamazepine as hypothesized (McMahon et al. 2012). However, contrary to our predictions, carbamazepine induced an increase in diversity. Research focused on other freshwater pollutants suggests that biodiversity declines in the presence of anthropogenic stressors (Beketov et al. 2013, McMahon et al. 2012, Muñoz et al. 2009, Vörösmarty et al. 2010). However, this inconsistency can be understood given that environmentally relevant concentrations of carbamazepine are not likely acutely toxic to freshwater organisms (Nieto et al. 2013, Oetken et al. 2005, Dussault et al. 2008). Despite limited toxicity, freshwater organisms exposed to carbamazepine experience sub-lethal effects, which appeared to alter the community structure. These alterations may have led to

an increase in biodiversity through community interactions such as competition and changed ecosystem dynamics (Fig. 7).

Sub-lethal effects, such as changes in behavior (De Lange et al. 2006) development (Nentwig et al. 2004, Oetken et al. 2005), mating success (Lürding et al. 2006) and immune response (Martin-Diaz et al. 2009, Gust et al. 2013, Gillis et al. 2014), have been observed after exposure to carbamazepine. For instance, Gust et al. (2013) demonstrated that mixtures of psychiatric drugs, consisting of venlafaxine (200 ng/L), carbamazepine (200 ng/L) and diazepam (10 ng/L), influenced the immune response of pond snails (*Lymnaea stagnalis*). While this mixture of pharmaceuticals did not impair immune-competence, significant changes in gene expression were observed in which Toll-like receptor (TLR4), heat-shock proteins (HSP70) and selenium-dependent glutathione peroxidase (Se-GPx) were up-regulated and allograft inflammatory factor-1 (AIF-1), catalase (CAT) and glutathione reductase (GR) were down-regulated. Additionally, Lamichhane et al. (2013) found that *Ceriodaphnia dubia* exposed to carbamazepine experienced decreased fecundity at 196.7 µg/L in the F0 and F1 generations and decreased adult body length at 264.6 µg/L in the F2 generation. In the present study, *Daphnia pulex* abundance declined in carbamazepine treatments (Fig. 5). Sub-lethal effects, such as changes in behavior, may have caused this decline (Cleuvers 2003, Lamichhane et al. 2013). However, it is not likely that the carbamazepine treatments were acutely toxic to *D. pulex* (LC50 > 100 mg/L; Han et al. 2006). Similar studies focused on pesticides have observed an increase in zooplankton diversity due to declines in *Daphnia* abundance; an organism that commonly depresses populations of small zooplankton taxa (Hanazato 1994). Therefore, changes in community structure potentially brought about by sub-lethal effects and the decline in *D. pulex* abundance may explain the observed increase in invertebrate diversity.

Changes in diversity and species composition can profoundly alter ecosystem dynamics (Chaplin et al. 1997, Hooper and Vitousek 1997, Tilman et al. 1997, Downing and Leibold 2002, Cardinale et al. 2002, Steiner et al. 2005, Wojdak 2005, Hooper et al. 2012). Downing and Leibold (2002) utilized a mesocosm experiment to demonstrate the importance of both species richness and composition to productivity, respiration and decomposition in freshwater ecosystems. Additionally, McMahon et al. (2012) showed that declines in species richness induced by exposure to chlorothalonil reduced decomposition and water clarity and elevated primary production and dissolved oxygen in a mesocosm experiment. Further, zooplankton diversity can be critical to the algal community with high zooplankton diversity resulting in an increase in large, grazer resistant algae ($> 35 \mu\text{m}$ chlorophyll *a*; Steiner et al. 2005). In this study, carbamazepine increased *Elimia* and decreased *Helisoma* standing biomass, increased invertebrate diversity and decreased *Daphnia pulex* abundance, which in turn affected ecosystem characteristics such as dissolved nutrient concentrations, primary production and decomposition.

Carbamazepine effects on the invertebrate community

Carbamazepine did not influence biomass or standing biomass of *Physa* or *Lymnaea*. Both taxa exhibited changes in biomass likely due to seasonal variability, not from exposure to carbamazepine (Brown 2001,). However, carbamazepine influenced biomass measurements of *Elimia* and *Helisoma*. Across treatments, *Elimia* lost biomass over the course of the experiment; however, biomass loss was less in the carbamazepine treatments. *Helisoma* biomass increased over the course of the experiment but this increase was lower in the carbamazepine treatments. Additionally, the SEM indicated the potential direct effect of carbamazepine on the standing biomass of *Helisoma* and indirect effect of carbamazepine on *Elimia* standing biomass. Exposure

to carbamazepine may have induced physiological stress in *Helisoma* and affected interspecific competition between *Elimia* and *Helisoma* (Gillis et al. 2014, Martin-Diaz et al. 2009, Gust et al. 2013, Brown 2001). Other studies suggest the influence of anthropogenic pollutants on freshwater organisms may depend on the relative strength of interspecific competition (Wojdak 2005, Liess et al. 2013, Dolciotti et al. 2014). Pleurocerids (i.e., *Elimia*) are better competitors when compared to pulmonates (i.e. *Helisoma*; Brown et al. 1998). Therefore, exposure to carbamazepine may have negatively influenced the immune-competence of *Helisoma* and further impaired the competitive ability of this taxon and hindered any potential recovery, leading higher standing biomass of *Elimia* and decreased biomass loss in carbamazepine treatments.

Carbamazepine altered the community structure by reducing the abundance of cladocerans and increasing ostracod and copepod abundance. This shift in the zooplankton community may have been influenced by the abundance of *Daphnia pulex*. *Daphnia* depresses population growth of small zooplankton taxa through competition (Hanazato 1994, Hanazato 2001). Exposure to carbamazepine potentially reduced the abundance of *D. pulex*, which may have increased the abundance of copepods and small cladocerans (Fig. 5). Moreover, the SEM suggests that carbamazepine had indirect effects on cladoceran abundance through sediment organic matter and indirect effects on copepod abundance through a reduction in cladocerans. The observed shift to copepod dominance is similar to other studies focused on anthropogenic pollutants (Havens 1994, Relyea 2005). Relyea (2005) observed a decrease in cladoceran and an increase in copepod abundance after exposure to insecticides in a mesocosm experiment. The effects of carbamazepine on the zooplankton community may also be explained by alterations in food resource availability within the mesocosm (Smith 2001). Carbamazepine reduced the

abundance of *D. pulex* and sediment organic matter but had no effect on algae, which may have also affected changes in the zooplankton community toward copepod dominance.

Carbamazepine influence on ecosystem dynamics

Environmentally relevant concentrations of carbamazepine negatively affected sediment organic matter. Though not measured in this study, sediment organic matter is a critical characteristic governing microbial activity in freshwater (Palmer et al. 2000). Thus, carbamazepine may have influenced microbial activity as well as invertebrate activity affecting decomposition (Ferrari et al. 2003, McMahon et al. 2012). Decomposition of organic matter is a main source of energy in aquatic habitats and alterations in decomposition can have profound effects on freshwater ecosystems (Covich et al. 1999, Palmer et al. 2000).

In contrast, carbamazepine did not directly influence primary production. Zhang et al. (2012) found carbamazepine could inhibit growth of *Scenedesmus obliquus* and *Chlorella pyrenoidosa*. However, the effective concentrations (EC50) were orders of magnitude higher than those found in surface waters (EC50 > 0.8 and 7 mg/L; respectively) and assessed in this study. At environmentally relevant concentrations, carbamazepine may be more likely to have indirect effects on primary production through changes in the invertebrate community. The dominance of copepods in the zooplankton community may explain the observed changes in algal and phytoplankton biomass. Copepods typically do not consume the smallest primary producers, which are commonly ingested by cladocerans (Smith 2001). Therefore, phytoplankton biomass may increase and algal mass may decrease over prolonged periods of carbamazepine exposure. While a small increase in phytoplankton biomass was observed in this experiment,

there was no relationship to carbamazepine exposure. Future research should be conducted to quantify potential effects on primary production.

The SEM indicated that carbamazepine influenced nitrate and phosphate concentrations through alterations in the invertebrate community. Specifically, carbamazepine's effects on copepod abundance and standing biomass of *Elimia* and *Helisoma* influenced nitrate and phosphate concentrations; therefore this pharmaceutical pollutant may have indirectly altered nutrient cycling. Aquatic invertebrates, particularly those that bioturbate sediments, alter the flux of nutrients into water (Covich et al. 1999, Palmer et al. 2000,). Changes in nutrient concentrations may lead to changes in the efficiency of energy transfer through food chains (Dickman et al. 2008). Additionally, changes in nutrient concentrations may influence other ecosystem functions such as primary production and decomposition, as identified in this study (Palmer et al. 2000).

Conclusion

Despite the ubiquity of carbamazepine in surface waters, previous studies have not adequately addressed how chronic exposure at environmentally relevant concentrations may influence freshwater ecosystems (Rosi-Marshall and Royer 2012, Hughes et al. 2013). Results from this *in situ* mesocosm experiment demonstrate how environmentally relevant concentrations of carbamazepine alter the communities and processes of freshwater ecosystems. The SEM illustrates that carbamazepine altered the biomass of gastropods and shifted zooplankton abundances to favor copepods. These changes affected primary production and decomposition. Additionally, carbamazepine altered dissolved nutrient concentrations, which along with the decline in *Daphnia pulex* abundance potentially influenced the transfer of energy

though food chains (Dodson and Hanazato 1995, Hanazato 2001, Dickman et al. 2008). However, more research is needed to fully understand how carbamazepine affects freshwater ecosystems. Specifically, additional research should be conducted to determine how ecosystem dynamics (i.e., decomposition and primary production) respond to carbamazepine exposure. Furthermore, future research should integrate ecological principles into ecotoxicology experiments to develop mechanisms for how carbamazepine influences population dynamics (i.e., intra- and interspecific competition and predation) and ecosystem processes (Relyea and Hoverman 2006, Clements and Rohr 2009).

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Table 1 Mean mesocosm dissolved oxygen (DO), pH and temperature across CBZ treatments. Standard deviations in parentheses. There were no significant differences among treatments ($P > 0.05$, $df = 3$).

Treatment	Dissolved Oxygen (DO)	pH	Temperature (°C)
Water Control	5.89 (1.25)	8.47 (0.54)	25.43 (3.70)
MeOH Control	6.02 (0.96)	8.53 (0.66)	25.57 (3.93)
200 ng CBZ/L	6.07 (0.98)	8.55 (0.38)	25.84 (4.01)
2000 ng CBZ/L	5.91 (1.00)	8.55 (0.41)	25.63 (3.82)

Table 2 Mean species richness of gastropods and zooplankton across CBZ treatments. Standard deviations in parenthesis. There were no significant differences in richness of gastropods and zooplankton between treatments and control ($P > 0.05$).

CBZ (ng/L)	Gastropods	Zooplankton
Water Control	0.75 (0.50)	0.82 (0.50)
MeOH Control	1.11 (0.12)	0.86 (0.32)
200	1.08 (0.13)	0.65 (0.20)
2000	1.05 (0.05)	0.90 (0.65)

Table 3 Mean standing biomass (mg dry mass/ 31 days) of all four taxa of gastropods across CBZ treatments. Standard deviations in parenthesis. There were no significant differences in standing biomass of any taxa between treatments and control ($P > 0.05$).

CBZ (ng/L)	<i>Physa</i>	<i>Lymnaea</i>	<i>Elimia</i>	<i>Helisoma</i>
Water Control	5.72 (5.89)	0.62 (1.24)	91.53 (99.26)	199.56 (71.71)
MeOH Control	13.94 (14.60)	2.88 (3.33)	179.00 (45.00)	160.23 (21.37)
200	16.94 (13.81)	2.39 (1.61)	123.03 (53.78)	190.73 (25.53)
2000	6.07 (6.36)	1.74 (2.14)	165.36 (35.52)	133.55 (50.63)

Table 4 Mean dissolved nutrient concentrations (mg/L) across CBZ treatments. Standard deviations in parenthesis. * denotes significant difference ($P < 0.05$)

CBZ (ng/L)	Fluoride	Chloride	Bromide	Nitrate	Phosphate	Sulfate
Water		31.27				
Control	0.29 (0.08)	(12.83)	0.03 (0.02)	0.01 (0.02)	37.79 (16.73)	1.92 (2.27)
MeOH						
Control	0.29 (0.09)	24.92 (7.38)	0.03 (0.013)	0.01 (0.02)	48.19 (16.07)	1.94 (2.74)
200	0.29 (0.05)	26.41 (3.62)	0.025 (0.01)*	0.03 (0.07)	43.59 (9.10)	1.97 (2.03)
2000	0.30 (0.88)	26.99 (3.68)	0.03 (0.01)	0.07 (0.19)	45.36 (14.82)	2.46 (2.73)

Figure Legends

Fig. 1 Conceptual model of potential carbamazepine effects on freshwater ecosystems

Fig. 2 Mesocosm experimental design with all carbamazepine treatments (CBZ) and replicates (N = 16).

Fig. 3 Shannon-Wiener diversity for all invertebrates across carbamazepine (CBZ) treatments. N = 4. Data are mean $\pm$ 1 SE. * denotes significant difference ($P < 0.05$).

Fig. 4 Biomass change (mg dry mass/31 d) of *Physa*, *Lymnaea*, *Elimia*, and *Helisoma* among carbamazepine (CBZ) treatments. N = 4. Data are mean $\pm$ 1 SE. * denotes significant difference ($P < 0.05$).

Fig. 5 Abundance of zooplankton taxa including Calanoida, Cyclopoida, Ostracoda, *Daphnia pulex*, *Chydorus sp.*, and *Ceriodaphnia sp* over course of experiment among carbamazepine (CBZ) treatments. N = 4. Data are mean $\pm$ 1 SE. * denotes significant difference ($P < 0.05$).

Fig. 6 Algal dry mass, chlorophyll *a*, and sediment organic matter (%) in mesocosms at experiment termination among carbamazepine (CBZ) treatments. N = 4. Data are mean $\pm$ 1 SE. * denotes significant difference ($P < 0.05$).

Fig. 7 Initial (A), intermediate (B) and final (C) structural equation models describing the relationship between carbamazepine and the freshwater ecosystem. The initial ($\chi^2 = 69.791$, $df = 19$, $P = 0.00$) and intermediate ($\chi^2 = 30.279$, $df = 13$, $P = 0.04$) SEM did not have significant fit to the covariance matrix. The final model ($\chi^2 = 8.126$, $df = 14$, $P = 0.883$) accounts for a substantial portion of the variability in the abiotic components of the ecosystem ($r > 0.50$). Numbers are standardized path coefficients. Solid lines indicate

significant paths in the model ($P < 0.05$). Dashed lines are non-significant hypothesized pathways ($P > 0.05$).

Fig. 1

Carbamazepine treatments				
Replicates	Controls		<i>Carbamazepine Treatments (ng/L)</i>	
	Water	Solvent	200	2000

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7