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RESEARCH ARTICLE

Seed treatments containing neonicotinoids and fungicides reduce aquatic insect richness and abundance in midwestern USA–managed floodplain wetlands

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Abstract

Agrochemicals including neonicotinoid insecticides and fungicides are frequently applied as seed treatments on corn, soybeans, and other common row crops. Crops grown from pesticide-treated seed are often directly planted in managed floodplain wetlands and used as a soil disturbance or food resource for wildlife. We quantified invertebrate communities within mid-latitude floodplain wetlands and assessed their response to use of pesticide-treated seeds within the floodplain. We collected and tested aqueous and sediment samples for pesticides in addition to sampling aquatic invertebrates from 22 paired wetlands. Samples were collected twice in 2016 (spring [pre-water level drawdown] and autumn [post-water level flood-up]) followed by a third sampling period (spring 2017). Meanwhile, during the summer of 2016, a portion of study wetlands were planted with either pesticide-treated or untreated corn seed. Neonicotinoid toxic equivalencies (NI-EQs) for sediment ($\overline{X}$=0.58 μg/kg), water ($\overline{X}$=0.02 μg/L), and sediment fungicide concentrations ($\overline{X}$=0.10 μg/kg) were used to assess potential effects on wetland invertebrates. An overall decrease in aquatic insect richness and abundance was associated with greater NI-EQs in wetland water and sediments, as well as with sediment fungicide concentration. Post-treatment, treated wetlands displayed a decrease in insect taxa-richness and abundance before recovering by the spring of 2017. Information on timing and magnitude of aquatic insect declines will be useful when considering the use of seed treatments for wildlife management. More broadly, this study brings attention to how agriculture is used in wetland management and conservation planning.

Keywords Neonicotinoid · Thiamethoxam · Aquatic invertebrate · Surface water · Ecotoxicology · Fungicide

Introduction

Globally, extensive land conversion to agricultural production coupled with increased fertilizer and pesticide use has improved agricultural yields to meet the worldwide demands of a growing human population (Liu et al., 2015; Tilman et al., 2001). Despite increased pesticide use, insect pests have not declined and future climate scenarios may make pests more abundant and less predictable in their response to pesticides (Birch et al. 2011). Agricultural demand, insect pest pressure, and the increased prevalence of pesticides have all contributed to a shift away from integrated pest management (IPM), a philosophy which focuses on minimizing agricultural chemical use by applying pesticides only after monitoring indicates pest populations have reached a critical threshold (Douglas and Tooker, 2015; Kogan, 1998). The shift away from IPM has been accelerated by the introduction of chemical seed treatments including neonicotinoids, a class of systemic insecticides which offer flexibility

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in application methods (Jeschke et al 2011; Goulson 2013). Neonicotinoid-treated seeds provide agricultural producers with early season pest protection and are generally applied whether or not a pest issue exists. The prophylactic application of neonicotinoids has resulted in a substantial increase in global pesticide use, with neonicotinoids representing 25% of the worldwide insecticide market (Bass et al., 2015). Neonicotinoids are currently used more than any other class of insecticide, with approximately 50% of soybean and 80–100% of corn seed planted in the United States (US) treated with a neonicotinoid active ingredient (Douglas and Tooker, 2015; Sur et al., 2003). As a protective strategy, pesticide seed treatments are not unique to insecticides. Prior to the adoption of neonicotinoid seed treatments, fungicides were commonly applied as seed coatings to protect against fungal disease (Atwood et al., 2018). In conjunction with neonicotinoids, fungicides are often applied to the seed as a cocktail of active ingredients to provide protection from a broad range of disease and pests (Hitaj et al., 2020).

Increasing pesticide use has led to the widespread contamination of surface waters by insecticides, which exceed regulatory levels in 50% of US water bodies, resulting in a threat to non-target ecosystems (Stehle and Schulz, 2015; Wolfram et al., 2019). Neonicotinoids are an environmentally persistent class of chemicals meaning active ingredients can remain in agricultural soils from days to years and, given their water soluble characteristics, make them susceptible to leaching into groundwater and adjacent water bodies (Goulson, 2013). Thus, although neonicotinoids are applied in terrestrial systems, they have been detected in US surface waters including rivers, streams, lakes, and freshwater wetlands of the US and Canada (Hladik et al., 2014, 2018; Kuechle et al., 2019; Main et al., 2014). In surveys of US streams, neonicotinoids were detected at broad spatial (63% of streams sampled) and temporal (year-round presence in US tributaries of the Great Lakes) scales (Goulson, 2013; Hladik et al., 2018; Hladik and Kolpin, 2015). Furthermore, neonicotinoids were detected in > 90% of prairie pothole wetlands sampled throughout Saskatchewan, Canada (Main et al., 2014), and in 100% of streams sampled in the Midwestern US (Hladik et al. 2014).

Widespread declines of insect species have been recently documented and are often, at least partially, attributed to the intensification of agriculture and the pervasive use of synthetic pesticides among other drivers (e.g., climate change and habitat loss) (Sánchez-Bayo and Wyckhuys, 2019). Although neonicotinoids are targeted towards crop pests, their mode of action, namely binding and activating postsynaptic nicotinic acetylcholine receptors (nAChR), means that non-target invertebrates are also susceptible (Goulson, 2013; Jeschke and Nauen, 2008). Neonicotinoids can have substantial lethal and sub-lethal effects on non-target aquatic invertebrates, with members of class Insecta generally among the most sensitive (Alexander et al., 2008; Morrissey et al., 2015). In fact, neonicotinoids have been found to decrease overall aquatic invertebrate abundance and diversity in outdoor mesocosms (Pestana et al., 2009). Sub-lethal effects on invertebrates have also been reported, including feeding inhibition and reduced motility, which subsequently led to altered predator–prey interactions and decreased leaf decomposition (Englert et al., 2012). Chronic imidacloprid exposure has been linked to physiological differences and community composition of aquatic invertebrates in the shredder functional feeding group, potentially reducing ecosystem functions related to decomposition, nutrient cycling, and water quality (Agatz et al., 2014; Chagnon et al., 2014; Kreutzweiser et al., 2009).

Impacts of neonicotinoids on aquatic insects are of great concern as aquatic invertebrates play critical roles in aquatic and terrestrial food webs. Although direct neonicotinoid toxicity is not often cited as a major threat to vertebrate wildlife (but see Berheim et al., 2019), neonicotinoids may indirectly impact vertebrates by disrupting complex food webs (Pisa et al., 2014). In mid-latitude wetlands, aquatic invertebrates are a temporally abundant and important food resource for resident and migratory wildlife (Fredrickson and Taylor 1982; Tidwell et al. 2013) and reductions in the aquatic invertebrate portion of the food web may have implications for higher trophic level organisms. Additionally, imidacloprid and fipronil (two systemic insecticides) reduced benthic arthropods and ultimately growth rates of the fish whose diets relied on them for a food source (Hayasaka et al., 2012). Beyond reductions in abundance, neonicotinoids can alter the phenology and physiology of emerging adult mayflies (*Ephemeroptera*) and non-biting midges (*Chironomidae*) through changes in emergence timing, adult body size, and sex ratios (Alexander et al. 2008; Mohr et al. 2012; Cavallaro et al. 2017a, 2017b). Many communities of vertebrates (e.g., insectivorous birds) and invertebrates (spiders) rely on aquatic insects as a food resource when adult insects emerge and populate riparian and upland habitats (Epanchin et al., 2010; Nakano and Murakami, 2001; Paetzold et al., 2005). In the Netherlands, negative population trends in insectivorous birds were correlated with increased neonicotinoid concentrations in surface waters indicating a potential disruption between aquatic and terrestrial food webs (Hallmann et al., 2014).

Numerous studies in laboratory and mesocosm settings have demonstrated detrimental effects of neonicotinoid insecticides to aquatic invertebrate communities (Beketov et al., 2008; Cavallaro et al., 2017a; Miles et al., 2017; Williams and Sweetman, 2018). However, while surveys of neonicotinoid concentrations in a range of ecosystems are becoming increasingly prevalent (Hladik and Kolpin, 2015; Kuechle et al., 2019; Main et al., 2014), studies of the impacts of neonicotinoids to aquatic invertebrates in field settings are more limited. Recent efforts have shown environmental impacts of neonicotinoids can occur at concentrations previously considered below effect thresholds, suggesting complex ecological systems (e.g., wetland food webs) may be adversely impacted through chronic neonicotinoid exposure (Cavallaro et al., 2019; Schepker et al., 2020). Here, we use an experimental approach to expand on previous mesocosm and semi-field studies by evaluating impacts to aquatic invertebrate communities after the direct application of neonicotinoid- and fungicide-treated seed in wetlands. A common management practice in mid-latitude floodplain wetlands involves planting agricultural crops to meet wildlife habitat objectives, which in recent years, have often contained neonicotinoid insecticides as a component of seed treatments (Gray et al., 2013; Kuechle et al., 2019). Planting agricultural crops treated with neonicotinoid insecticides and fungicides in hydrologically independent wetlands enabled us to experimentally manipulate neonicotinoid and fungicide application in a field setting and monitor the aquatic invertebrate response. We planted treated wetlands using a commercially available corn (*Zea mays*) seed treatment containing a neonicotinoid and a combination of fungicides and evaluated the effects on aquatic invertebrate abundance and richness.

Methods

Study sites

We selected 22 wetlands on nine conservation areas (CAs) managed by the Missouri Department of Conservation (MDC) based on similarity in landscape position (i.e., within a flood-plain) and the capacity to control wetland inundation. First, we evaluated whether study CAs had at least two hydrologically independent wetlands that had the capacity to be planted to similar proportions of corn and could potentially serve as study wetlands (Fig. 1). Then, we evaluated

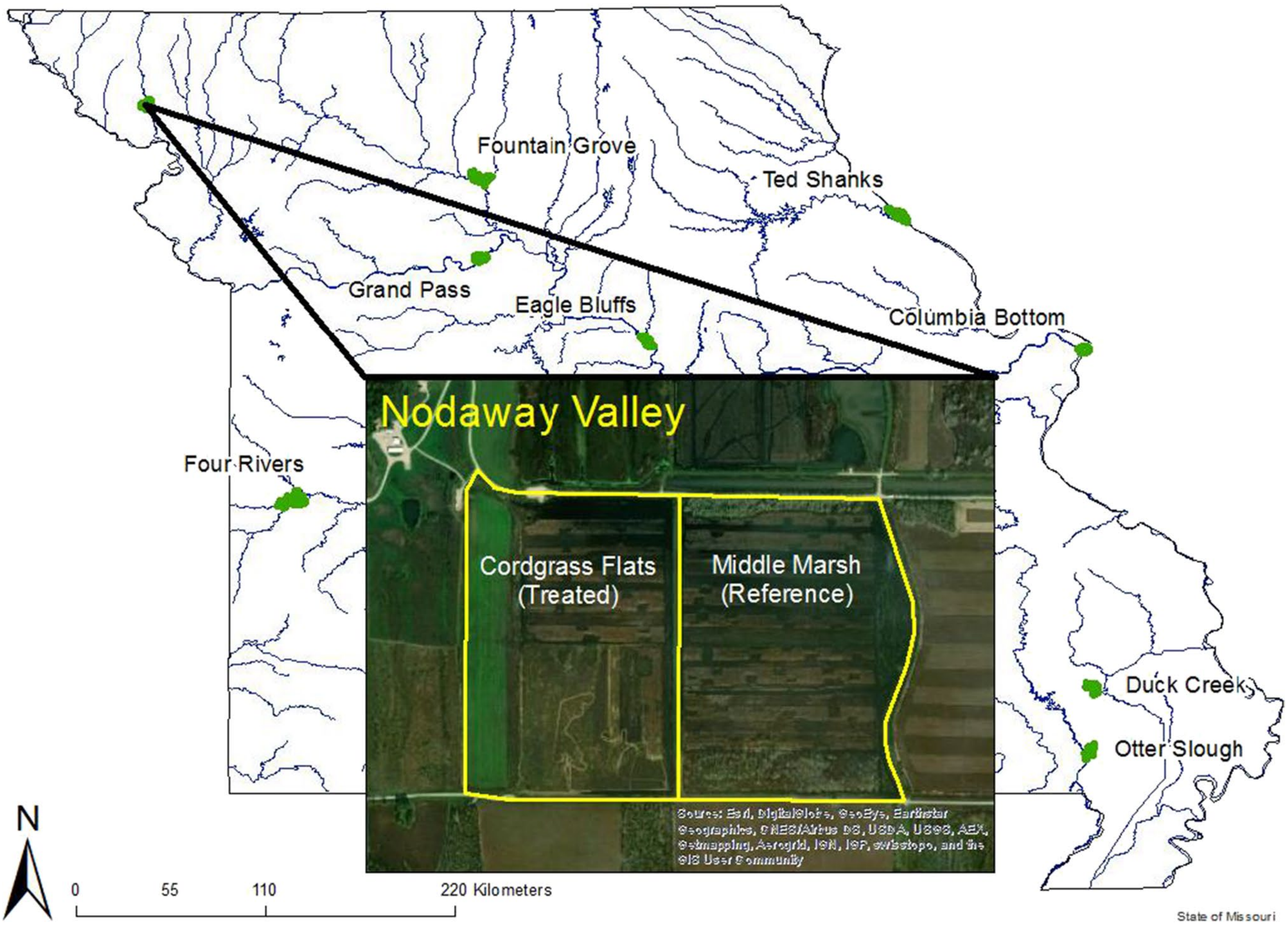

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Fig. 1 Map of study conservation areas ($n=9$) including an inset of study wetlands ($n=22$) at Nodaway Valley Conservation Area. Treated wetlands were planted with thiamethoxam-treated seed corn where control wetlands received the same proportion of untreated corn. Map image is the intellectual property of Esri and is used herein under license. Copyright © 2020 Esri and its licensors. All rights reserved the potential study wetlands for comparable plant communities, water source, and management to ensure wetlands could be paired in our study design with one serving as a control wetland and the other a treated wetland (Online Resource; Table S1). Seven CAs had one pair (i.e., 2 wetlands) each while two CAs had two paired wetlands each. Wherever possible, treated wetlands were randomly assigned; however, it was occasionally necessary to designate treated wetlands outside the random framework to minimize the potential draining of neonicotinoid contaminated water into other study wetlands during spring draw down. In spring of 2016, water in study wetlands was removed via water control structures as would occur under normal management scenarios. Following water level draw down, ~ 15% of total wetland area was planted with seed corn treated with Syngenta® CruiserMaxx corn 250, which contains the neonicotinoid thiamethoxam and the fungicides fludioxonil, mefenoxam, azoxystrobin, and thiabendazole. All treated fields were planted following the CruiserMaxx corn 250 label seeding rate to ensure we did not exceed the maximum application rate of 74.8 g of thiamethoxam per acre (Syngenta Crop Protection, n.d). To standardize non-pesticide impacts of agricultural production on the invertebrate communities, control wetlands were planted to similar proportions of corn using untreated seed. Following planting with treated or untreated corn, study wetlands remained mostly dry throughout the summer before being inundated mid-October and remaining inundated through the following spring sampling season to provide habitat for migrating waterfowl and waterbirds.

Field and laboratory methods

We sampled aquatic invertebrates by collecting nektonic net and benthic core samples from all study wetlands during three time periods. Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumn inundation 2016 (October), and approximately 1-year post-treatment spring 2017 (April). During each sampling period, we collected one benthic and one nektonic invertebrate sample at eight randomly selected locations within each wetland. After taking the first sample in the approximate center of the wetland, we took subsequent samples at a random bearing and distance until eight samples at adequate water depth (> 20 cm) were collected. Given 15% of wetlands were planted to corn, typically one or two samples would fall within the planted portion of the wetland with remaining samples located in adjacent habitats. Nektonic samples were collected by sweeping a 500-µm rectangular kick net 1.1 m along the wetland bottom twice at each sample location (Schepker et al., 2019). A 10-cm-diameter sediment core was also taken at each collection site to a depth of 5 cm resulting in 16 total samples (benthic and nektonic combined) per wetland per sampling period. Nektonic and benthic core invertebrate samples were preserved in 70% ethanol and transported to the University of Missouri for processing. Concurrent with invertebrate sample collection, and away from any sediment suspended by researchers, we also measured basic water quality parameters (e.g., temperature, pH, dissolved oxygen, and conductivity) at each sampling location using a handheld multiparameter instrument (YSI, Pro Plus 2030) and pH (Hanna instruments, pHep) meter. Additionally, we measured water depth and visually estimated percent vegetative cover on the sampling transect to account for environmental variables that may influence invertebrate communities (Schepker et al., 2020).

We processed invertebrate samples in the laboratory by first staining them with Rose-Bengal dye for 24 h before sieving into coarse (500 µm) and fine (250 µm) fractions (Tapp and Webb, 2015). Fine and coarse fractions of nektonic samples were then quartered by volume using a Folsom Plankton Sample Splitter (Aquatic Research Instruments) to expedite the processing of samples with greater number of invertebrates. All invertebrate individuals in our subsamples were enumerated and identified to family (insects) or order/class (non-insect taxa) (Hayasaka et al., 2012; Schepker et al., 2020). Nektonic samples were multiplied (4 ×) to account for their previous quartering in order to represent the original sample volume for all future analyses. Coarse and fine sample fractions were summed for each nektonic and core sample which resulted in 1009 total invertebrate samples. A completely sampled study design would have resulted in 1,056 samples; however, < 8 samples were collected at some wetlands due to inadequate water levels resulting in the inability to collect eight spatially independent samples in a given wetland.

Neonicotinoid sample collection and analysis

At each wetland, we collected water and sediment samples for neonicotinoid analysis during each sampling period. At three randomly selected invertebrate sampling locations, we collected water (approximately 330 mL) from 15 cm below the water surface taking care to avoid suspended sediments. Similarly, after all water samples were taken, we collected a sediment core to a depth of 5 cm at the same three random points. Individual samples were then combined and thoroughly mixed to form a composite sample which yielded 1 L of water and 250 mg of sediment for each wetland and sampling period. Water and sediment samples were immediately placed in coolers with ice for storage in the field and during transportation to the University of Missouri where they were stored at 4 °C and shielded from UV light until shipped at the end of each sample period.

To determine the levels of pesticides in water and sediment, all samples were analyzed for the presence of neonicotinoid and fungicide residues by the Water Sciences Laboratory at the University of Nebraska-Lincoln (Lincoln, Nebraska, USA). Pesticide concentrations of six neonicotinoid insecticides (imidacloprid, clothianidin, thiamethoxam, dinotefuran, thiacloprid, acetamiprid) and three fungicides (pyraclostrobin, azoxystrobin, and trifloxystrobin) were determined using microwave-assisted solvent extraction followed by liquid chromatography-tandem mass spectrometry (LC–MS/MS; Waters Model 2695 HPLC system combined with a Quattro-Micro triple quadrupole mass spectrometer; Satkowski et al., 2018). Specific instrument details and extraction methods followed in this study are provided elsewhere (Stamm et al., 2012). All quantification was performed using external calibration standards which were prepared from certified standard reference materials obtained from Sigma-Aldrich. Method detection limits (MDL) for all neonicotinoids and fungicides were 0.010 µg/L in water and 0.200 µg/kg in sediment, respectively. Quality assurance/quality control (QA/QC) included laboratory reagent and fortified blanks, as well as field duplicate samples for each sampling period. Despite being used in all planted seed treatments, we were unable to test for the fungicides fludioxonil, mefenoxam, and thiabendazole because of poor recoveries or lack of reference materials. Due to delays in method development, a complete dataset for aqueous fungicide concentrations was not available, and therefore fungicide concentration data were limited to sediment samples only.

Statistical analysis

Statistical certainty is lower for concentration values reported below the MDL; however, we opted to analyze all instrument-derived concentration values including those below the MDL (Antweiler et al. 2008; Helsel 2010). We recognize the uncertainty of using instrument-derived values below the MDL; however given a single MDL for all samples, we considered this method preferable to other substitutions for regression-type analyses (Antweiler et al., 2008; Helsel, 2010). Concentrations of the three most commonly detected neonicotinoids were used in calculating neonicotinoid toxic equivalency units (NI-EQs) in order to equate all concentrations to the toxicity of imidacloprid. Following the method for determining chronic toxicity to *Chironomus* sp. developed by Cavallaro et al. (2017b), we calculated NI-EQs using an additive model of toxic equivalency factors (TEFs) where NI-EQ = imidacloprid + 1.62*clothianidin + 0.11*thiamethoxam. We used chronic TEFs over measured concentrations because neonicotinoid active ingredients are variably potent to invertebrates (Raby et al., 2018; Taillebois et al., 2018). No similar toxic equivalency was available for fungicides, and concentrations were largely dominated by azoxystrobin. Therefore, we simply summed all sediment fungicides for statistical analysis. Further information on data pre-processing, data distribution testing, and model fit assessment can be found in the supporting information.

We developed models for dependent variables in a hierarchical structure starting with the abundance and richness for all taxa, insect orders, and non-insect taxa. The abundances of the six most common insect orders were then analyzed to further investigate taxa-specific sensitivity. All models were fit as generalized linear mixed-effects models (GLMMs) in Program R (version, 3.6.0, R Development Core Team 2019) using the package glmmTMB (Brooks et al., 2017). For each dependent variable, a single global model was developed which allowed for investigation of variables of interest (i.e., chemical concentrations/treatment) while accounting for environmental variables known to influence aquatic invertebrate communities (e.g., dissolved oxygen, depth). Collinearity was determined not to be an issue for chemical or environmental variables ($|r| > 0.7$) after they were scaled and centered; therefore, each model contained an interaction of season and treatment, pesticide concentrations, and environmental covariates (Table 1). The interaction SEASON x TREATMENT was included to reflect the paired (treatment/control wetland) study design and allowed for multiple comparisons of invertebrate communities within seasons and among treatments. Data for this experiment were spatially (paired wetlands within CAs) and temporally (3 sampling periods) nested. We accounted for potential autocorrelation by designating season as a fixed effect and nested the random intercept of wetland within CA. A global model approach was chosen rather than model or variable selection as we desired to measure impacts of pesticide treatment on each dependent variable, rather than seek to find a single "best" model (Hobbs et al. 2012). Chemical covariates were considered significant if 95% confidence intervals did not overlap zero (Fowler et al., 2020). Finally, neonicotinoid and fungicide concentrations were determined to have a non-normal distribution and therefore were compared using the nonparametric Kruskall-Wallis one-way analysis of variance.

Table 1 Model variables included in the global generalized linear mixed-effects models (GLMM) for each dependent variable

Environmental	Pesticide	Random intercept
Water depth (cm)	Season*treatment	Conservation area/wetland
Vegetation cover (%)	Sediment NI-EQ	
Turbidity (ntu)	Water NI-EQ	
Dissolved oxygen (%)	Sediment fungicide	
Conductivity (µS/cm)		
pH^2		

Results

Chemical concentration in wetland soil and water samples

Water sampling across all sample periods resulted in the collection of 63 water samples for neonicotinoid analysis. As a result of two wetlands with inadequate water in spring 2016 and one water sample from spring 2017 that was compromised during shipping, there were three missing samples. Thiamethoxam, which was the active ingredient in our experimental treatment, was detected in 6% of water samples with a mean concentration below the MDL (<0.01 µg/L). In contrast, clothianidin, a major metabolite of thiamethoxam, still had a mean concentration below the MDL (0.01 µg/L); however, clothianidin was frequently (78%) detected in water samples. In addition to thiamethoxam and clothianidin, imidacloprid was detected in 30% of samples. Sediment sampling ($n = 64$) resulted in similar detection patterns as water for thiamethoxam (11% of samples), clothianidin (41%), and imidacloprid (19%). However, while the mean sediment concentration of thiamethoxam was again below the MDL (0.08 µg/kg), mean sediment clothianidin concentration was 0.30 µg/kg. Finally, dinotefuran, acetamiprid, and thiacloprid were never detected above the MDL and therefore were not included in any further analysis.

Concentrations of thiamethoxam, clothianidin, and imidacloprid in wetland sediments resulted in mean (± SD) neonicotinoid toxic equivalencies (NI-EQs) of 0.80 ± 1.64 µg/kg and 0.37 ± 0.80 µg/kg in treated and untreated wetlands, respectively. Water NI-EQs were below the US Environmental Protection Agency's acute imidacloprid threshold for freshwater aquatic invertebrates (0.385 µg/L) in treated (0.02 ± 0.02 µg/L) and untreated (0.02 ± 0.02 µg/L) wetlands. However, mean aqueous NI-EQs did exceed the chronic benchmark of 0.01 µg/L (U.S. Environmental Protection Agency, 2019) for both groups. Kruskall-Wallis tests of sample water determined concentrations of neonicotinoids and the respective NI-EQs within seasons did not differ by treatment as all comparisons resulted in p-values > 0.05. Post-treatment water NI-EQs remained similar in treated and control wetlands before having greater concentrations in treated wetlands spring 2017 (Fig. 2). Sediment NI-EQs were greater in treated wetlands post-treatment autumn 2016 with the trend continuing into spring 2017 (Fig. 3); however, these differences were not statistically significant due to substantial variation in concentrations ($P = 0.39$). Thiamethoxam in wetland sediments exhibited a post-treatment peak before returning to pre-treatment levels in spring 2017. However, post-treatment sediment NI-EQs appeared to be driven by the greater, and more potent, concentrations of clothianidin. Mean sediment fungicide concentrations were similar between treatments ($p > 0.05$); however, they exhibited different temporal patterns. Untreated wetlands experienced the greatest fungicide concentration pre-treatment while fungicides in treated wetlands peaked during spring 2017.

Aquatic invertebrates collected in wetlands

Over the three sampling periods, we collected 1,009 invertebrate samples that contained approximately 1.5 million individuals from at least 29 orders. However, we acknowledge uncertainty on the exact taxonomic total, as some taxa were only identified to class (e.g., Decapoda) or phylum (e.g., Nematoda) and likely consisted of multiple orders. The class Insecta contributed approximately 9% of individuals to the overall abundance with the remaining 91% represented by non-insect taxa. Among insect taxa, the majority of individuals were from the order Diptera (74%) followed by Hemiptera (16%) and Ephemeroptera (6%). Non-insect taxa were predominantly Cladocera (64%) followed by Copepoda (15%) and Ostracoda (9%).

Effects of pesticides and fungicides on aquatic invertebrate abundance

Dependent variables were initially evaluated at the broadest community scale where we quantified total invertebrate, non-insect, and insect abundance. We found no significant effects related to chemical stressors on total invertebrate abundance in autumn 2016. However, there was a significant positive effect of treatment on total invertebrate abundance in spring 2017 ($\beta = 0.59 \pm 0.28$, $P = 0.04$; Fig. 4). Non-insect abundance was similarly unimpacted by neonicotinoid or fungicide concentrations and showed only a weak relationship to treatment in spring 2017 ($\beta = 0.48 \pm 0.29$, $P = 0.10$). In contrast, across all seasons insect abundance was negatively affected by sediment fungicide concentration ($\beta = -0.33 \pm 0.07$, $P < 0.01$) and marginally negatively impacted by sediment NI-EQ ($\beta = -0.11 \pm 0.06$, $P = 0.08$) and water NI-EQ ($\beta = -0.16 \pm 0.09$, $P = 0.07$). Additionally, treated wetlands had an overall lower insect abundance compared with control wetlands during post-treatment autumn 2016 ($\beta = -0.83 \pm 0.31$, $P = 0.01$) before recovering during spring 2017 ($\beta = 0.85 \pm 0.33$, $P = 0.01$).

Effects of pesticides and fungicides on aquatic invertebrate taxonomic richness

Similar to our methods for modeling invertebrate abundance, we also modeled taxa richness separately for total invertebrates, non-insect taxa, and insect families. Richness for non-insect taxa was not reliably fit with a negative-binomial model

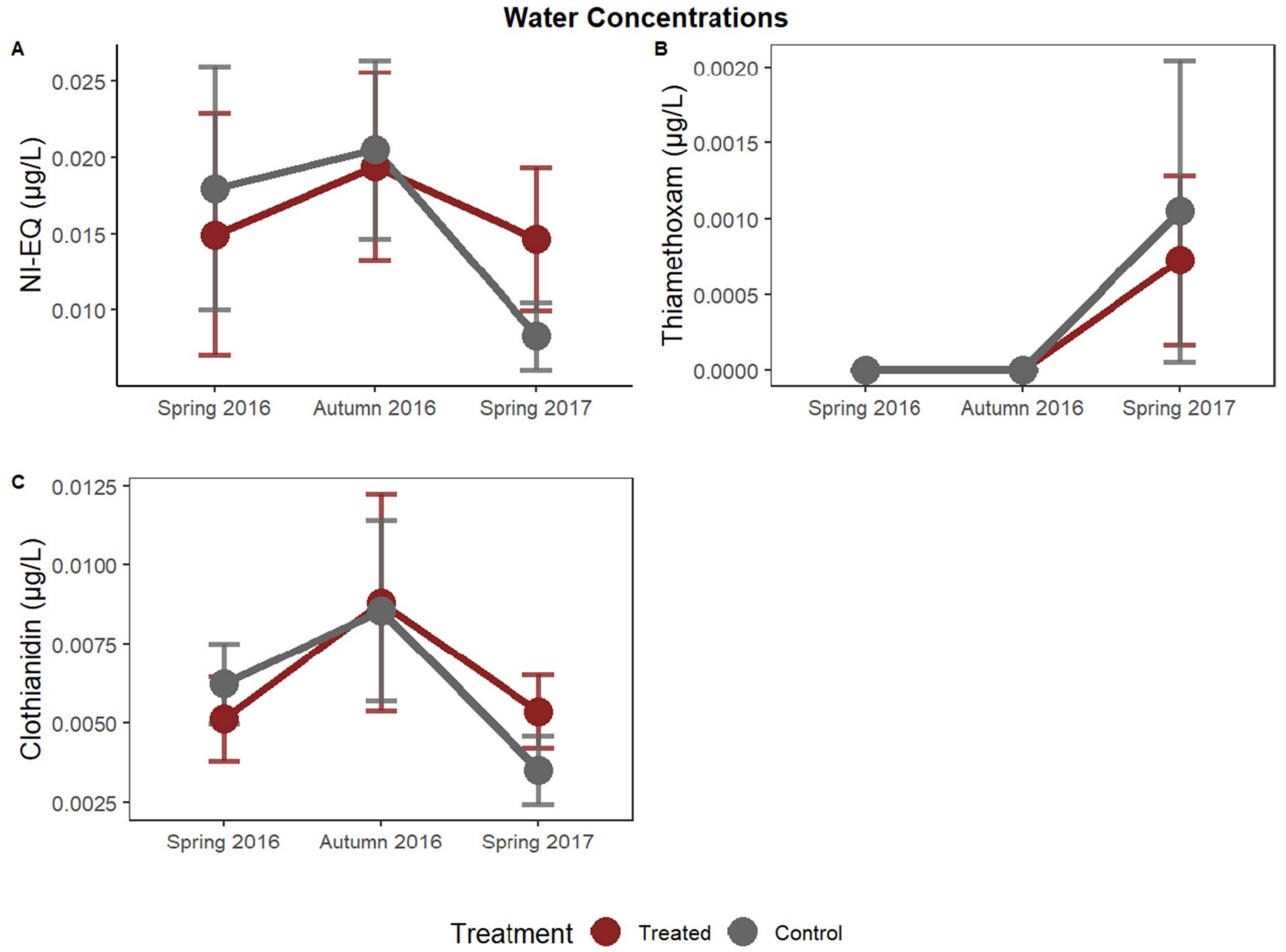


Fig. 2 Mean water concentrations ($n = 63$) and 95% confidence interval for (A) neonicotinoid toxic equivalency (NI-EQ), (B) thiamethoxam, and (C) clothianidin across two treatment categories and three sampling periods. Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumn inundation 2016 (October), and approximately 1-year post-treatment in spring 2017 (April)

(i.e., convergence errors) likely due to small variance explained in model random effects, and therefore results should be interpreted with caution. Non-insect richness was positively impacted by sediment fungicide concentration ($\beta = 0.04 \pm 0.02$, $P = 0.02$) with no other chemical variables being significant (Fig. 5). Insect richness was negatively affected by sediment NI-EQs ($\beta = -0.11 \pm 0.04$, $P < 0.01$) as well as treatment in autumn 2016 ($\beta = -0.29 \pm 0.16$, $P = 0.04$). Similarly, overall invertebrate richness was negatively influenced by sediment NI-EQs ($\beta = -0.07 \pm 0.02$, $P < 0.01$); however, the effect was smaller compared to insect richness response.

Effects of pesticides and fungicides on aquatic insect abundance

Insect abundance of the five most prevalent orders was evaluated using the global model including chemical and environmental predictors. Fly larvae (Dipterans) were negatively impacted by sediment NI-EQs ($\beta = -0.28 \pm 0.07$, $P < 0.01$) and sediment fungicides ($\beta = -0.32 \pm 0.08$, $P < 0.01$) and experienced a moderately negative impact from water NI-EQs ($\beta = -0.17 \pm 0.10$, $P = 0.08$; Fig. 6). Dipterans also displayed a negative relationship to treated seed in autumn 2016 ($\beta = -0.80 \pm 0.31$, $P = 0.01$) followed by a positive response in treated wetlands during spring 2017 ($\beta = 1.29 \pm 0.33$, $P < 0.01$). Aquatic true bugs (Hemiptera) were negatively affected by treatment during autumn 2016 and spring 2017; however, this relationship was only significant in autumn 2016 ($\beta = -1.54 \pm 0.74$, $P = 0.04$). Mayflies (Ephemeroptera) were not impacted by the use of treated seed, but were sensitive to sediment fungicide concentrations ($\beta = -0.56 \pm 0.22$, $P = 0.01$) and moderately impacted by water NI-EQs ($\beta = -0.39 \pm 0.23$, $P = 0.09$). Aquatic beetles (Coleoptera) were negatively

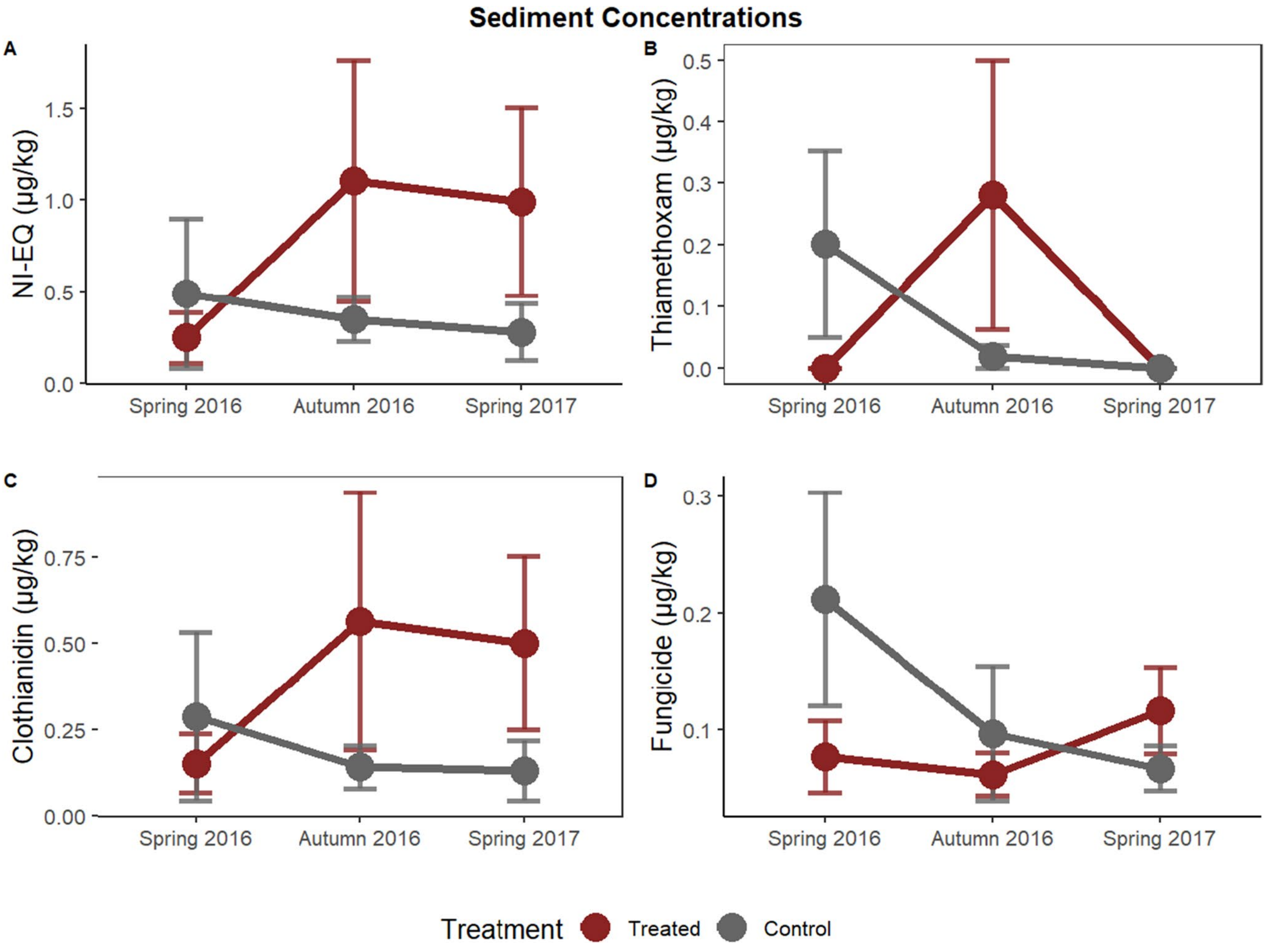


Fig. 3 Mean sediment concentrations ($n=64$) and 95% confidence interval for (A) neonicotinoid toxic equivalency (NI-EQ), (B) thiamethoxam, (C) clothianidin, and (D) strobularin fungicides across two treatment categories and three sampling periods. Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumn inundation 2016 (October), and approximately 1-year post-treatment in spring 2017 (April)

impacted post-treatment autumn 2016 ($\beta=-1.14\pm0.60$, $P=0.06$) with the relationship becoming more pronounced spring 2017 ($\beta=-1.28\pm0.62$, $P=0.04$). Dragonflies/damselflies (Odonata) showed no relationship to the use of treated seed, but were negatively affected by sediment NI-EQs ($\beta=-0.78\pm0.32$, $P=0.01$).

Discussion

Globally, neonicotinoids have been identified as a potential driver of insect declines (Morrissey et al., 2015; Sánchez-Bayo and Wyckhuys, 2019). Although acute toxicity values are useful in setting regulatory and water quality thresholds, they may not fully represent risk to aquatic invertebrates in field settings. The impact of neonicotinoids on insects has, until recently, been primarily assumed based on toxicity thresholds developed in laboratory studies and concurrent surface water monitoring (Hladik and Kolpin, 2015; Raby et al., 2018). However, recent field and semi-field studies have demonstrated negative effects of neonicotinoids at levels below acute LC_{50} values (Cavallaro et al., 2019; Schepker et al., 2020). Here, we expanded on previous research by experimentally manipulating agricultural seed treatment (including neonicotinoids and fungicides) exposure in a field setting at the wetland scale and measuring the response of existing invertebrate communities. We found aquatic insect abundance and taxa richness were negatively affected by the use of agricultural seed treatments, even when measured neonicotinoid and fungicide concentrations were below effect thresholds.

Our results indicate that the impacts of agricultural seed treatments and the associated neonicotinoid and fungicide concentrations to aquatic invertebrates varied by taxonomic class, with class Insecta being more sensitive to chemical

Fig. 4 Standardized parameter estimates and 95% CI for the generalized linear mixed-effect model that evaluated the relative importance of chemical and environmental variables on invertebrate, non-insect, and insect abundance. Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumn inundation 2016 (October), and approximately 1-year post-treatment in spring 2017 (April). Parameters are statistically significant if 95% confidence intervals do not overlap zero and are denoted with an asterisk. NI-EQ, neonicotinoid toxic equivalency

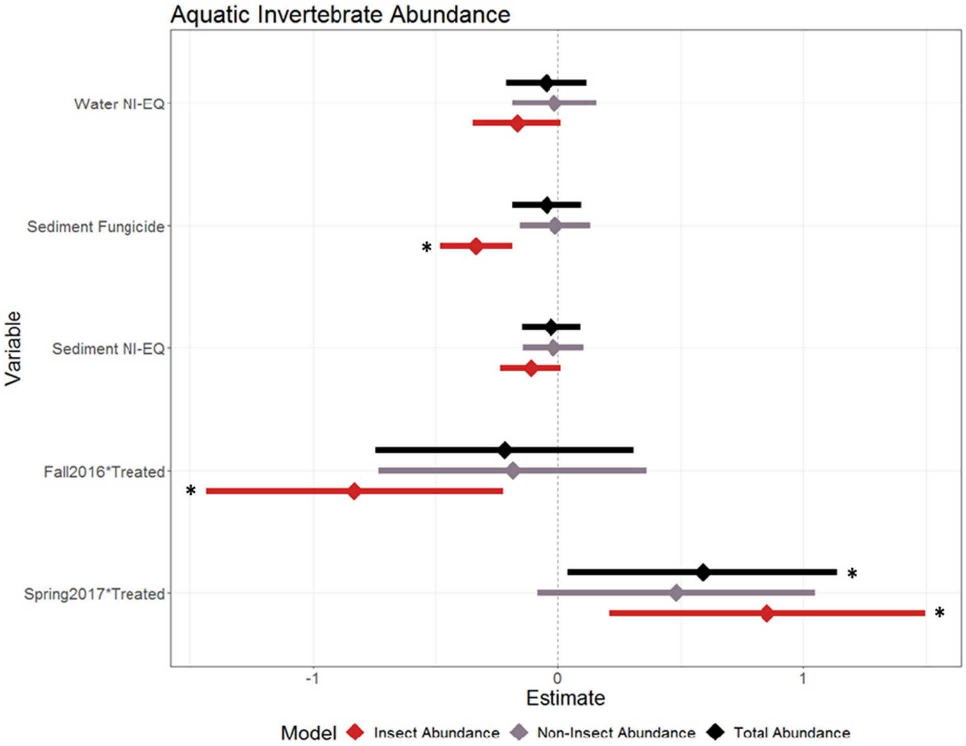
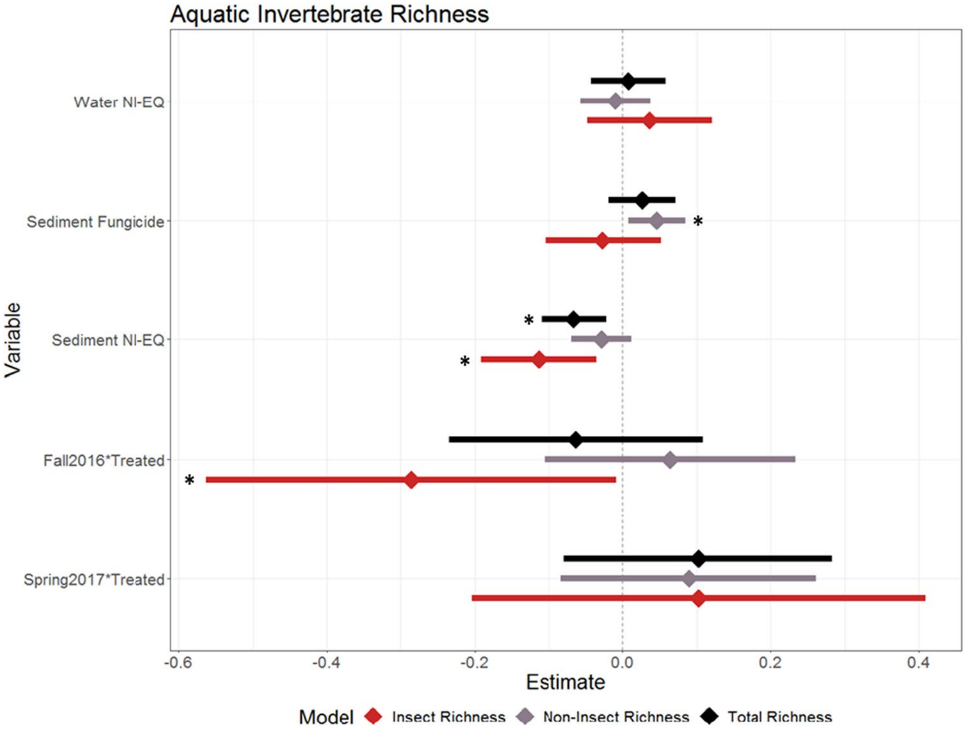


Fig. 5 Standardized parameter estimates and 95% CI for the generalized linear mixed-effect model that evaluated the relative importance of chemical and environmental variables on invertebrate, non-insect, and insect richness. Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumn inundation 2016 (October), and approximately 1-year post-treatment in spring 2017 (April). Parameters are statistically significant if 95% confidence intervals do not overlap zero and are denoted with an asterisk. NI-EQ, neonicotinoid toxic equivalency



concentrations. Overall, non-insect taxa were not measurably affected by agricultural seed treatment chemicals and therefore we conducted no further examination. Despite the lack of impacts to non-insects in study wetlands, neonicotinoids have been proposed as a control agent for some non-insect taxa (e.g., burrowing shrimp, *Callianassa* sp.; *Upogebia* sp.) (Felsot and Ruppert, 2002). The absence of a detected effect to non-insects in Missouri floodplain wetlands may be attributed to fewer potentially sensitive organisms (e.g., Decapoda) (Morrissey et al., 2015), as samples

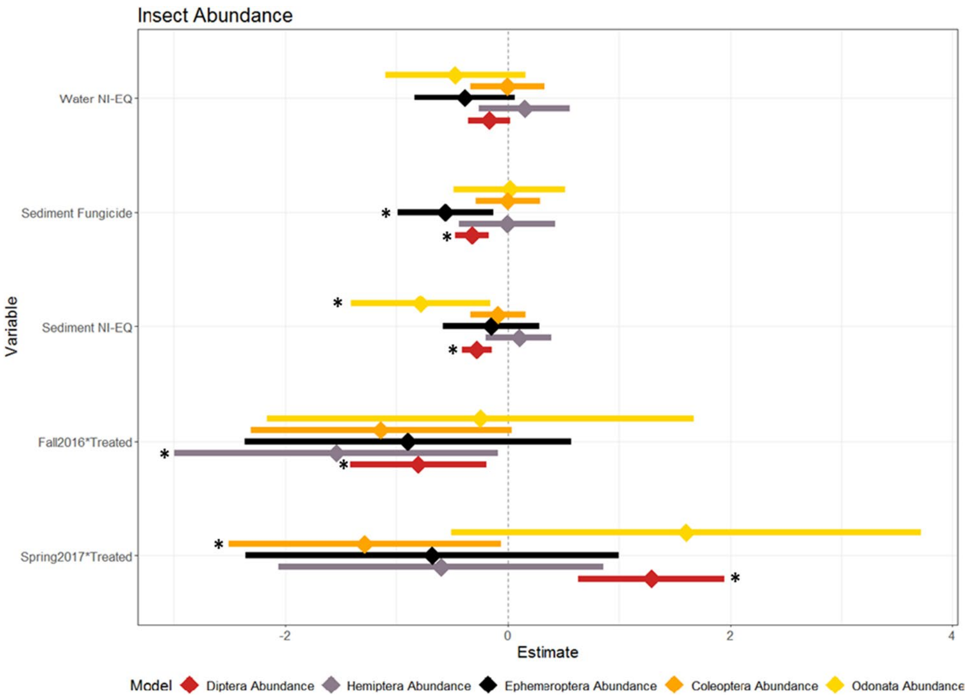


Fig. 6 Standardized parameter estimates and 95% CI for the generalized linear mixed-effect model that evaluated the relative importance of chemical and environmental variables on the abundance of five common insect orders (Diptera, Hemiptera, Ephemeroptera, Coleoptera, Odonata). Samples were first collected prior to any experimental manipulations in spring 2016 (March) to establish a baseline for both treatment and control wetlands. After the experimental treatment occurred (May/June 2016), wetlands were sampled following autumninundation 2016 (October), and approximately 1-year post-treatment in spring 2017 (April). Parameters are statistically significant if 95% confidence intervals do not overlap zero and are denoted with an asterisk. NI-EQ, neonicotinoid toxic equivalency

were dominated by cladocerans and copepods. Regardless, insects exhibiting a greater degree of toxicity in field settings are consistent with previous laboratory studies of aquatic invertebrates, and represent targeting specificity of neonicotinoids for insect nicotinic acetylcholine receptors (Jeschke et al., 2011; Raby et al., 2018).

Within class Insecta, neonicotinoids are known to show variation in toxicity as different taxa of insects can be affected at concentrations separated by more than an order of magnitude; Ephemeroptera, Diptera, and Trichoptera are among the most sensitive taxa (Morrissey et al., 2015). In Missouri floodplain wetlands, we found Dipterans to be the most abundant order among class Insecta (74% of insects) and also had a significant negative response to treatment, sediment NI-EQ, and fungicide concentrations. Conversely, ephemeropterans were the third most abundant insect order (6%), but only exhibited a negative response to sediment fungicide concentrations. The disparity in impacts to dipterans and ephemeropterans may be attributed to the route of chemical exposure. The majority of aquatic invertebrate toxicity tests and monitoring studies focus on aqueous neonicotinoid concentrations; however, our study systems showed NI-EQ concentrations were greatest in wetland sediments. Therefore, it may be that the benthic dwelling dipterans (e.g., chironomids) are more susceptible to the presence of neonicotinoids in wetland sediments as chironomids more frequently interact in the benthic zone. The dipteran response in our study system may also be the result of their relative abundance which allowed for a more precise estimation of effect size. For example, although the effect of treatment was negative for all models of tested insect orders (Diptera, Hemiptera, Ephemeroptera, Coleoptera, Odonata), only Diptera and Hemiptera (the two most abundant orders) experienced significant reductions in abundance in response to treatment. Furthermore, Trichoptera, an order often considered sensitive to chemical stressors, did not have sufficient abundances to reliably fit a model.

Insects are known to respond to chemical stressors differently depending on functional group and life histories (Main et al., 2018). Specifically, mesocosm studies have demonstrated that although multivoltine taxa, such as the dipteran family Chironomidae, experience initial declines when exposed to neonicotinoids, they are able to recover more quickly than univoltine taxa (Beketov et al., 2008). Our results indicate dipterans, which primarily consisted of the multivoltine family Chironomidae, were initially negatively impacted by neonicotinoid seed treatment in autumn before recovering the following spring. Conversely, there was a greater decline in abundance of longer-lived taxa such as coleopterans 1-year post-treatment than immediately following treatment. Aquatic coleopterans generally only produce one generation per year, and therefore may recover more slowly, if at all, to chemical disturbances (Voshell, 2002). The longer-term impacts of neonicotinoids to univoltine invertebrate predators such as the common coleopterans Dytiscidae and Hydrophilidae also have potential to alter community trophic structure. Reduced predator abundances may cause top down trophic interactions which could have ultimately led to the ecological release of their prey such as dipterans (Atwood et al., 2018). Consequently, the increased abundance of dipterans 1-year post-treatment in our study wetlands might have been a combined result of population recovery due to life history characteristics (e.g., multiple generations per year, dispersal from nearby wetlands) as well as population release due to reduced predation (Miles et al., 2017). A semi-field study of agricultural contaminants, including the neonicotinoid thiacloprid, found that invertebrate community effects were present beyond the time frame that the chemicals were detectable in the system, indicating broader impacts to the ecological community beyond those realized through direct toxicity (Barmentlo et al., 2019).

Greater toxicity of sediment fungicides to insects versus non-insects is not as readily explained by chemical mode of action. Azoxystrobin, the most frequently detected fungicide in study sediment, is an electron transport inhibitor designed to prevent certain fungal disease, and unlike neonicotinoids, is not intended to cause insecticidal activity (EPA, 1997). Midges and cladocerans were the most common insect and non-insect taxa to our study system, and likely had disproportionate weight when assessing the effects of azoxystrobin on overall abundances. A study of chronic toxicity showed azoxystrobin to have approximately equal toxicity at the most sensitive endpoints to a midge (*Chironomus dilutus*) and a cladoceran (*Ceriodaphnia dubia*), with lowest-observed-effect-concentrations (LOECs) of 13 and 10 μg/L, respectively (Kunz et al., 2017). However, these LOECs were developed in a laboratory setting for aqueous test conditions and did not specifically test toxicity in sediments. Because our fungicide measurements were only taken in the sediment, they may display greater toxicity to the benthic associated midges than the nektonic cladoceran. It is also possible that greater fungicide toxicity to insects was the result of an interaction with thiamethoxam (or other neonicotinoids) in wetland sediments. A study of azoxystrobin in the presence of lindane (an organochlorine insecticide) found that leaf decomposition in a model stream system was more impacted by azoxystrobin than lindane, a result the authors attributed to reduced fungal resources and subsequent detritivore mortality (Dawoud et al., 2017). It is likely that both direct chronic toxicity and complex trophic interactions played a role in impacts of fungicides in our study wetlands.

In this study, it is unlikely that we evaluated a naïve wetland invertebrate community, but rather one adapted and evolved in the presence of neonicotinoids since neonicotinoid uses have gained in popularity since the early 2000s. Resistance to neonicotinoids among certain target pests (e.g., *Bemisia tabaci* and *Leptinotarsa decemlineata*) was identified as early as 2005 (Nauen and Denholm, 2005). We might expect similar patterns of invertebrate resistance and species persistence developed among non-target organisms with a close proximity to agriculture if exposed to frequent doses of sub-lethal neonicotinoid concentrations (Bendis and Relyea, 2016). Studying an insect community previously exposed to neonicotinoids, in some cases for over a decade, also raises the possibility that more sensitive taxa may have been greatly reduced or eliminated prior to our experimental manipulation of neonicotinoids. The post-treatment decrease in insect richness measured in our study could result in landscape-level losses to diversity if impacted taxa are dispersal limited, or if relatively few unimpacted communities remain as a source of dispersing individuals. Rapid decline of the Odonate *Sympetrum frequens* in Japan has been linked to the introduction of systemic insecticides, including imidacloprid, into common rice farming practices (Nakanishi et al., 2018). Therefore, it is possible a similar impact may have occurred in Missouri wetlands prior to this research; however, since we lack species-level invertebrate data, it is impossible to document such a decline. If neonicotinoids are introduced into a previously unexposed ecosystem, it is likely initial negative effects on the invertebrate community will be greater than reported in our study.

Concentrations of neonicotinoids in study wetlands, regardless of treatment, rarely met or exceeded acute toxicity thresholds for aquatic invertebrates (Raby et al., 2018; Sappington et al., 2017). Individual neonicotinoid concentrations below effect thresholds may have been due to a low percentage of the overall wetland area being planted to treated seed (~ 15%) or could be due to peak concentrations being missed with low concentration sampling frequency (three times during study duration) (Wolfram et al., 2019). For these reasons, we chose to use chronic toxic equivalency factors to calculate NI-EQs such that water NI-EQs can be compared to toxicity thresholds for imidacloprid (Cavallaro et al., 2017b). Water NI-EQs met or exceeded the United States Environmental Protection Agency's imidacloprid chronic threshold for aquatic invertebrates (0.01 μg/L) in all treatments and seasons ("U.S. Environmental Protection Agency," 2019). However, the threshold of 0.01 μg/L is at the analytical MDL meaning our values at or near the threshold should be interpreted with caution. Furthermore, no similar thresholds are available for sediment neonicotinoid concentrations, which may ultimately underrepresent risk to aquatic ecosystems if monitoring studies measure only aqueous concentrations.

Despite the direct application of thiamethoxam via treated corn seed to wetlands, mean neonicotinoid concentrations did not significantly vary between treated and control wetlands. Given that many of the study wetlands were likely planted with neonicotinoid-treated seed in previous years, there may have been a carryover in concentrations from that previous use. Or, as neonicotinoids are moderately persistent and environmentally mobile (Bonmatin et al., 2015), study wetlands may have been contaminated by outside or off-site sources. This aligns with results from our study which found the neonicotinoid compounds clothianidin and thiamethoxam in sediments of our control sites and imidacloprid, which was not applied as part of a seed treatment in sediments of study wetlands. A study of neonicotinoid contamination in Missouri wetland sediments found that the use of treated seed in wetlands was not an important predictor of neonicotinoid concentrations and that neonicotinoids were detected in sediments of wetlands that had not been planted with treated seed for at least 5 years, indicating either long-term persistence in wetland sediments or contamination from external sources (Kuechle et al., 2019). Kuechle et al. (2019) also reported 60% samples of source water used to inundate floodplain wetlands in autumn contained imidacloprid and 80% contained clothianidin. In our study, clothianidin was detected with greater frequency and in greater concentrations than the applied active ingredient thiamethoxam. Greater clothianidin concentrations during autumn 2016 and spring 2017 may have resulted from external sources, as imidacloprid was also detected, but also likely was the result of thiamethoxam from seed treatments degrading into clothianidin (Žabar et al., 2012). Thiamethoxam degrading into clothianidin is concerning for aquatic systems, as clothianidin has the potential to be more toxic to aquatic invertebrates than the parent compound thiamethoxam (Cavallaro et al., 2017b).

Missouri floodplain wetlands and the associated aquatic invertebrates represent an important habitat type and food resource to migrating avifauna (e.g., waterfowl and shorebirds); consequently, any reduction in invertebrates may have implications for foraging waterbirds (Fredrickson and Taylor, 1982). The use of treated seed resulted in approximately 40% fewer dipteran larvae than in untreated wetlands during autumn migration periods. Although dipterans (e.g., family Chironomidae) are an important food source for migrating waterfowl and shorebirds (Davis and Smith, 1998; Tidwell et al., 2013), it is unclear whether a 40% decrease in Diptera abundance is meaningful to avian predators and additional research is needed on this topic. Furthermore, insect larvae emerging as adults represent an important transfer of energy to terrestrial food webs (Nakano and Murakami, 2001). Aerial insectivorous birds, a group that is particularly dependent on emergent aquatic insects as a food source, have experienced widespread population declines; a pattern that has been correlated with imidacloprid concentrations in surface waters (Hallmann et al., 2014). Experimental data linking the occurrence of neonicotinoids in the environment and adverse population impacts to wildlife are currently lacking and future studies could help elucidate these relationships.

Broader implications for wetland ecosystems

Agricultural insecticides, specifically neonicotinoids, use a mode of action that does not discriminate between harmful pests and beneficial insects (Pisa et al., 2014). Thus, when selecting pesticide-treated versus untreated seed, it becomes important to weigh the benefits of potential pest suppression with the risk to non-target insects. This risk assessment becomes especially relevant when crops are planted on lands managed for wildlife; it is important for managers to simultaneously consider the benefits crops provide to vertebrate wildlife and the risk any associated chemicals may pose to non-target invertebrates. Aquatic invertebrate populations are foundational in many food webs, but currently face multiple environmental stressors including climate change, habitat loss, and chemical inputs (Collier et al., 2016). Agrochemical-related impacts to aquatic invertebrates have the potential to affect the broader ecosystem beyond sheer abundance of these organisms and those animals which rely on them as a food source. Healthy wetlands provide important ecosystem services, which among other functions, include plant material decomposition and nutrient cycling, processes which are supported by a diverse community of aquatic invertebrate taxa (Gleason et al., 2008). Benthic dipterans play a crucial role in nutrient cycling and a disruption in the abundance or taxa richness of dipterans may reduce their influence on wetland function and thereby alter a wetland's role in landscape nutrient dynamics (Covich et al., 1999; Wolfram et al., 2019). With fewer wetlands remaining on the landscape, it is vital that wetlands function at the highest level possible to perform the ecosystem services that serve an ever-growing human population and their accompanying demands.

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Author contribution E.B.W and D.M conceived the idea for and designed the study. K.J.K collected data, performed the data analysis, and wrote the first draft of the manuscript. E.B.W, D.M, and A.R.M provided guidance throughout data analysis and writing of earlier manuscript drafts.

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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