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Movement of the Neonicotinoid Seed Treatment Clothianidin into Groundwater, Aquatic Plants, and Insect Herbivores

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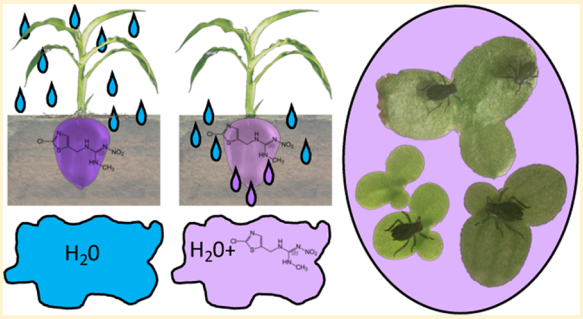
Supporting Information

ABSTRACT: Agricultural use of the neonicotinoid clothianidin (CLO) as a seed treatment of corn and soybeans has been linked to contamination of waterways and irrigation water. By analyzing samples collected from field lysimeters with liquid chromatography tandem mass spectrometry (LC-MS), this study reports the highest CLO concentrations within leachate following planting, with maximum concentrations occurring 4 weeks post-planting (3370 ng L^{-1}). This concentration is approximately 10× greater than previously reported CLO concentrations in streams/rivers and prairie wetlands, likely the result of reduced dilution and photolysis impacts. To document nontarget vegetation translocation dynamics, the macrophyte *Lemna gibba* was exposed to varying CLO concentrations for 12 h within a laboratory setting. Quantification of CLO uptake occurred every 4 h. Finally, trophic level impacts were investigated by exposing the water lily aphid *Rhopalosiphum nymphaeae* to *L. gibba* grown in CLO-contaminated water. Aphids lived and fed on contaminated duckweed for 48 h, after which an LC_{50} of 8.71 ng g of the plant tissue^{-1} was calculated. While uptake of CLO by duckweed was rapid, aphids are unlikely to suffer acute mortality at previously reported environmental CLO concentrations. Future research should expand on this work with other macrophytes/herbivores and longer-term experiments to more realistically mimic chronic field exposures.



The neonicotinoids have become the most widely used insecticide class in the world.[1,2] In many oilseed and grain crops, their main use is as a prophylactic neonicotinoid seed treatment (NST) approach to pest management, and by 2011, US adoption rates exceeded 80% in maize.[3] Both thiamethoxam (TMX) and its breakdown product clothianidin (CLO) are used solely as NSTs in US maize, where they are applied at rates of 0.25−1.25 mg of compound kernel^{-1} prior to being sold to the grower. Notably, there is a trend of increasing per kernel rates of AI, resulting in an overall increase of the active ingredient (AI) per hectare.[4]

Despite initial claims to the contrary, there are significant risks of nontarget exposure associated with planting seeds treated with NST. Planter dust, resultant from seed abrasion during planting, accounts for a loss of at least 2% of the active ingredient in maize, this is dispersed over a wide area by planter exhaust and prevailing winds.[4,5] Furthermore, the translocation efficiency (i.e., the amount that actually enters the target plant) is reported at <1.5% of the applied active ingredient in the field.[6] The remainder of NST applied to the seeds remains largely unaccounted for and provides the impetus for the work described here.

The inability to readily purchase NST-free seed[3,7] leads to a continual and repeated dose of neonicotinoid in the soil, which, in turn, raises concerns regarding the potential of NSTs to contribute to environmental loading and water contamination via leaching and field runoff.[8] The groundwater ubiquity score (GUS) reported by the Pesticide Properties Database,[9] while not the only metric used in leaching risk assessments, places CLO and TMX at a high leaching risk,[10] and initial concerns regarding environmental loading appear to have been justified with increasing reports of neonicotinoids in a range of surface and ground waters. Contamination has been suggested as a direct result of runoff and/or leaching.[11−14] Occasionally, concentrations have exceeded either acute or chronic freshwater invertebrate toxicity benchmarks.[15] However, the direct role NSTs applied to crops may play in contributing to environmental contamination has only been studied directly in potatoes[16] and sugar beets.[17] A study in maize, the largest application of NSTs, by area, has not been reported.[3]

Nontarget impacts of environmental neonicotinoids in aquatic systems have only been the subject of increased research effort relatively recently. Within aquatic systems, neonicotinoid translocation into nontarget macrophytes may serve as an unaccounted exposure route for nontarget phytophagous invertebrates. In one instance, maximum CLO and TMX concentrations of 2.01 and 8.44 ng g^{-1} plant tissue,

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respectively, were reported in aquatic vegetation.[18] Furthermore, several correlative studies have linked declines in aquatic macroinvertebrates and birds to environmental neonicotinoids.[19−21] In the latter example, the authors proposed this decrease as a result of a trophic cascade via food source reduction; many of the birds included in the study were obligate insectivores with most of their prey undergoing an aquatic larval stage.

Considering increasing documentation of neonicotinoid runoff and leaching, there is a clear need to describe more clearly: (1) the rate and chronology of when neonicotinoids leave crop seeds, when they enter the aquatic environment and (2) the potential effects of the resulting aqueous residues on aquatic ecosystems either through direct toxicity or via contamination of food and water sources. The objectives of the research described here were 2-fold: (1) to use a field study to quantify the leaching potential of NST throughout the growing season in Indiana maize and (2) using concentrations documented by objective 1 and from other published literature conduct manipulative laboratory experiments using an aquatic macrophyte, gibbous duckweed (*Lemna gibba,* Alismatales, Araceae) and the water lily aphid (*Rhopalosiphum nymphaeae,* L. Hemiptera, Aphididae) to document both nontarget macrophyte translocation dynamics and potential impacts upon higher trophic levels. Both species were chosen due to their history as model organisms and the fact that they are commonly found in our study area.[22,23] Duckweed represents an important connection between aquatic and terrestrial systems, and 39 insects have been reported to use duckweed mats as shelter, food, or both.[24] Furthermore, the eutrophic, nutrient-rich waters where duckweed is the most common are typified by ditches adjacent to the tile-drained fields found throughout our study area and provide a potential route for nontarget translocation in these plants. Finally, the dense mats duckweeds create dramatically reduced light penetration, potentially extending the half-life of neonicotinoids, which are highly photolytic compounds.[25]

MATERIALS AND METHODS

Experimental Site, Setup, and Sampling. Planting of maize hybrid 5337SX (Becks Hybrids, Atlanta IN, RM: 103 days) took place at the Purdue Water Quality Field Station (WQFS) (40.4903083°, −86.9952139°) on May 23/2016. The soil at this site is classified as a Drummer silty clay loam and received mean yearly precipitation of 880.74 ± 44.83 mm, from 2005 to 15.[26] Maize seed was pretreated with 1.25 mg of CLO (Bayer Crop Science, Monheim am Rhein, Germany) per kernel by the manufacturer and expressed the insecticidal Bt toxins *cry*2*Ab*2, *cry*1*A.105*, *cry*1*Fa*2 for lepidopteran (i.e., caterpillar pests) pest management and toxins *cry*3*Bb*1, *cry*34*Ab*1, and *cry*35*Ab*1 for corn rootworm pest management. Soybean variety P34T07 (DuPont Pioneer, Johnston, Iowa) was used for soy plots, NST-free soybean seed was used. Maize and soybeans were planted at 74,130 and 642 473 seeds hectare^{-1}, respectively.

The WQFS is comprised of 48 small plots and 8 large plots. Small plots measure 10 × 48.5 m and contain a clay box lysimeter (10 × 24 m) with walls that start ∼0.4 m below the soil surface and extend down to the glacial till layer (1.5 m). In contrast, large plots are 60 × 48.5 m and utilize guard tiles to maintain 10, 20, and 30 m tile spacing as they lack clay boxes. Each clay box lysimeter is tile line-drained (0.1 m diameter) and is placed 0.9 m below the soil surface. Each plot's tile drainage line is perforated only within its respective clay box to limit contamination between plots. Drainage lines run to collection huts, which protect samples from both rainfall and sunlight, and drain leachate directly into tile-specific tipping buckets, which deposit a water subsample (∼10 mL) into a 20 L collection bucket every other tip. A schematic of the sampling unit is provided in Figure S1. Samples were collected daily, when flow occurred, so that any pesticide present in the sample represented the average concentration over the previous ∼24 h period. Samples were stored at −20° C until further processing. Data were only collected from rainfall events when more than 35 tiles flowed irrespective of large/small plot designation. This decision was made to provide sufficient data for a given rainfall and tile flow event as both the period of time between precipitation and resultant tile flow as well as flow duration were variable between plots. This was likely due to a variety of factors including, but not limited to, crop history, tillage practices, and condition of the tile drainage system.

A complete description of treatments and relevant agronomic practices is presented in Table S1. Of the 48 small plots at the WQFS, 12 plots were planted with continuous maize, 8 plots were planted with maize rotated with soybean, 12 with soybean rotated with maize, and 4 plots each for the remaining treatments (sorghum, prairie grass, Miscanthus spp., switchgrass). The 6 large plots were planted with continuous maize. The prairie grass plots have been comprised of native Indiana grasses since 1996 and served as a negative control in which little or no CLO residues were expected in leachate samples. Plots received no supplemental irrigation beyond rainfall, which is provided in Figure S2.

Processing of Water Samples. Solid-phase extraction was used to prepare water samples for analysis with high-performance liquid chromatography (HPLC). First, Oasis HLB 12 cc filter cartridges (500 mg sorbent, 60 μm particle size, Waters Milford Massachusetts) were attached to a Preppy 12-port vacuum manifold (Sigma-Aldrich St. Louis Missouri) and conditioned with 5 mL of HPLC-grade acetonitrile (Sigma-Aldrich St. Louis Missouri) followed by water equilibration (5 mL). A water sample (60 mL) was then passed through the conditioned cartridge, eluted twice with 1.5 mL of HPLC-grade acetonitrile, and evaporated within a single tube in a Savant Automatic Environmental SpeedVac System AES2010 (Thermo). Samples were resuspended in 100 μL ACN and analyzed with an Agilent 1200 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6460 series QQQ mass spectrometer (MS) (Santa Clara, CA). Machine settings for the MS are reported in the Supporting Information. Calibration curves of 0.001−10 ng mL^{-1} were used to quantify CLO concentrations in WQFS samples and an internal standard was used in the quantification of CLO in all duckweed samples. The LOD of duckweed samples was 1−10 ppt.

Analysis of WQFS Data. Tiles were variable in initial flow and overall flow duration per precipitation event. For example, some tiles would flow 1−2 days, whereas others would flow for 3−5 days following the same precipitation event. These discrepancies are likely due to a variety of factors including differences in plot history, tillage practices, and condition of the drainage tiles themselves. To minimize the effect of differential flow for data comparison purposes, a weighted average was calculated for CLO. This approach utilized data generated from tip counters, which provided daily flow data for

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each tile, in combination with CLO concentrations as determined by LC-MS (previous section), to calculate an average CLO concentration per flow event.

To further account for variable tile flow and maximize data available for analysis, three groupings were created based upon initial seed treatment rate and plot history. These groupings were (1) maize plots, which were treated with 1.25 mg of CLO kernel^{-1}, (2) NST-free soybean plots, and (3) untreated plots consisting of a range of other crops planted without NST. As each field plot has undergone varying crop rotations and combinations of starter/fertilizer over the past decade, each prospective grouping was analyzed with a multivariate approach to repeated measures. Fixed main effects included treatment, sampling date, and a multivariate treatment × sampling date interaction effect as predictors of CLO concentration within leachate. The time × treatment interaction effect was assessed for each proposed grouping, with an insignificant result indicating the suitability of creating a new treatment group from each proposed grouping (Maize, Soy, NST-free control). The final category includes a range of different crops and, therefore, cannot be considered a "control" treatment in the strictest sense but includes a variety of annual crops where NST are not used in our study system and, thus, are grouped together with the expectation that this will provide a group of plots for comparison where NST have not been previously applied. Levene's test was used to assess homogeneity of variances in all multivariate models.

Because the time × treatment effect was insignificant for all three models (Table 1), each plot treatment was reclassified into a new Maize, Soy, or NST-free control treatment. These new treatment groupings were then analyzed with the same multivariate approach to repeated-measures model as described above, but results were followed by univariate analysis by sampling the event, to compare CLO concentrations in treated maize plots to untreated soy and control plots. All statistical models within this section were analyzed with Statistica (version 13.3).[27]

Table 1. F-statistics and Estimated Degrees of Freedom (df) for the Multivariate Repeated-Measures ANOVA Model Assessing the Suitability of Grouping Treatments by Initial Seed Treatment Rate for CLO[a]

group	factor	df	*F*-statistic
maize	time	5, 1	63.24
	time × treatment	20, 4.26	2.612
soy	time	5, 1	19.06
	time × treatment	10, 2	10.79
control	time	4, 1	78.99
	time × treatment	12, 2.94	2.95

[a]Significant results are denoted by an * with *P < 0.05, **P < 0.01, ***P < 0.001.

Finally, a mass balance was calculated for all small maize plots using an initial dose (# of kernels in a clay box lysimeter), flow per sampling date (recorded from tip counters), and CLO concentration per sample.

Maintenance of Duckweed and Aphid Cultures. The G3 strain of *L. gibba* was maintained in the axenic culture, as described by Brian and Solomon.[23] Axenic fronds of *L. gibba* were transferred within a laminar flow hood to autoclaved 2800 mL culture flasks, containing 1000 mL of fluid fortified with half-strength Hutner's growth media and stoppered with a cloth plug.[23] Initial *L. gibba* stocks were provided by Paul Fourounjian at Rutgers University. Newly established colonies were maintained at 25 °C, with constant light measured at 43 000 lx with a Lx-1010B digital luxmeter (HDE Manufacturing, Inc, Fort Worth TX). Colonies grew until they covered the surface of the 2800 mL flask. At that point, a portion of duckweed was transferred to a new culture flask as before, and the remaining duckweed was used for experimentation or as a food/substrate for aphid colonies.

As mentioned above, the water lily aphid, *R. nymphaeae,* was used as a model organism. While aquatic plants represent this insect's summer host, *R. nymphaeae* are heteroecious, using fruiting trees as overwintering and early spring hosts. While an occasional pest of spring plums, they are more commonly a pest of cultivated aquatic plants with a broad host range.[28,29]

Colonies of *R. nymphaeae* were maintained in 8 L aquaria (15 × 30 × 20 cm^3) at room temperature under constant light measured at 126 000 lx. Deionized water fortified with half-strength Hutner's media was used to propagate *L. gibba* from opened culture flasks. Aphids were initially collected from duckweed (*Lemna* spp.) located at the Purdue Wildlife Area (40.452293°, −87.054987°) in autumn 2016 and maintained on laboratory cultures of *L. gibba* after collection. Tanks were cleaned and water/nutrients replaced as needed when duckweed populations crashed due to aphid overfeeding or algal growth began outcompeting duckweed fronds for growing space. Aquaria were also supplemented with aeration pumps to provide gentle water movement. This reduced competition from various biofilm-creating microorganisms, which limited the lateral growth of duckweed colonies.

Translocation Dynamics of Duckweed. The uptake dynamics of aqueous CLO was investigated with the use of *L. gibba*. Selected test concentrations were well below the no-observed-effect concentration of 59 000 ng mL^{-1}.[30] As the environmental chamber dehumidifier was nonoperational, addition of water-filled tanks to the environmental chamber was used to increase relative humidity. To determine if this change in humidity would impact the rate of CLO uptake and ultimate in-plant CLO concentration, two test humidities were used (60 and 80% relative humidity). These humidities correspond to both the minimum and maximum humidity observed during the aphid LC_{50} trials discussed below. Each experimental tank was composed of ∼95 cm^2 of duckweed exposed to three CLO concentrations (0, 2, or 10 ng mL^{-1}) in a 4 L aquarium (20.5 × 11.5 × 18.5 cm^3), at 25 °C, under constant light (95 000 lx). CLO concentrations within experimental tanks were made from dilutions of a stock solution of HPLC-grade CLO, which was created at the start of each experimental replication (≥98% purity, Sigma-Aldrich St. Louis Missouri). The 2 and 10 ng of CLO mL^{-1} treatments were both replicated three times, whereas the control tank was only replicated once. Each humidity trial was conducted a single time with full replication of the 2 and 10 ng of CLO mL^{-1} test concentrations. Preliminary trials found that CLO concentrations reached equilibrium within plant tissues within 8 h under these conditions, and all subsequent translocation trials were carried out over a 12 h time span. Immediately preceding the introduction of duckweed and every 2 h following duckweed introduction, the humidity was recorded from the environmental chamber's internal hygrometer. Every 4 h post-exposure, ∼0.5−1 g of duckweed was removed from each experimental unit, quickly rinsed under tap water, and then dried between two paper towels to remove excess water,

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Table 2. Mean concentration (ng mL^{-1}) ± SE of CLO concentrations following selected rainfall events and number (n) of tiles contributing to average[a]

date	maize	soy	control
5/11/16	0.143 ± 0.031 n = 19	0.155 ± 0.027 n = 9	0.024 ± 0.008 n = 14
6/23/16	3.371 ± 0.214 n = 24 **a**	0.585 ± 0.085 n = 10 **b**	0.081 ± 0.015 n = 3 **b**
7/18/16	1.371 ± 0.221 n = 21 **a**	0.447 ± 0.108 n = 10 **b**	0.041 ± 0.011 n = 14 **b**
12/26/16	0.072 ± 0.009 n = 21	0.071 ± 0.012 n = 9	0.002 ± 0.001 n = 10
1/17/17	0.070 ± 0.009 n = 21	0.042 ± 0.008 n = 9	0.008 ± 0.002 n = 14
3/31/17	0.058 ± 0.014 n = 18	0.069 ± 0.011 n = 10	0.005 ± 0.002 n = 15

[a]Sampling events coincided with precipitation events that produced flow in >35 tiles. Groupings were made by initial NST application rate irrespective of tile history. This decision was made after an insignificant time × treatment effect in a multivariate approach to repeated-measures model was reported for each grouping. Time points denoted by different letters within a single date indicate significant differences (P = 0.05) at that sampling event as determined by univariate Tukey comparisons.

fresh weight recorded, and briefly stored in a 7 mL homogenization tube. After duckweed was sampled from each experimental unit, plants were homogenized and processed with a modified QuECHeRs protocol, as described in Alford and Krupke.[6] A 50 mL water sample was also collected prior to duckweed introduction to confirm clothianidin concentrations. Quantification was performed using liquid chromatography tandem mass Spectrometry (LC-MS) with a LOD of 0.1 ng g^{-1}. Following quantification, the fresh-weight bioconcentration factor (BCF) was calculated, as described by Carvalho et al.,[31] as follows:

$$\mathrm{BCF} = \frac{\text{concentration of CLO in plant } (\mu\text{g mL}^{-1})}{\text{concentration of CLO in water } (\mu\text{g mL}^{-1})}$$

A multivariate approach to repeated measures followed by univariate results by sampling event was used to compare BCF as a function of relative humidity in Statistica (version 13.3).[27] Separate models were run for each concentration (2 and 10 ng of CLO mL^{-1}).

Aphid LC_{50} Trials with Contaminated Duckweed. Aphids were exposed to contaminated duckweed in a series of experiments to assess whether feeding upon CLO-contaminated plant tissue resulted in mortality. Each trial was conducted at the same tank size, temperature, light duration, and intensity as in the translocation dynamics experiment. Due to a nonoperational dehumidifier in the environmental chamber, relative humidity ranged from 61 to 81%. Each experimental replication consisted of one control tank, in which no CLO was added to the water (0 ng mL^{-1}), and 4−6 experimental tanks with varying concentrations of CLO. The number of experimental tanks per replication was influenced by the number of suitable aphids and duckweed fronds available. A variety of target concentrations were tested, ranging from 0 to 34 ng mL^{-1} (0, 2, 3, 4, 5, 7.5, 9, 10, 12, 15, 18, 22, 25, 28, 31, and 34 ng mL^{-1}). At the start of each trial, ∼32 cm^2 of duckweed was placed in a 4 L tank. Duckweed was allowed to equilibrate and grow for the first 48 h to both expand the surface area upon which aphids could walk and provide sufficient plant material for future samples. After 48 h, 15 apterous aphids were added to a floating plastic Petri dish (60 mm diameter × 15 mm in height) and left to disperse and feed. Water samples (50 mL) were collected immediately prior to addition of duckweed (0 h), after addition of aphids (48 h), and at the end of the trial (96 h) to both ensure and monitor how target concentrations changed over time. Similarly, ∼0.33−0.5 g of duckweed was collected prior to aphid addition (48 h) and removal (96 h) and processed as in the translocation experiment. Duckweed samples ensured that equilibration had been reached and determination of initial CLO concentrations. Aphids were collected at 96 h, and mortality of apterous adults recorded. This species of aphid is parthenogenic,[32] and data recording nymphs resulting from live birth were not collected during the 96 h experimental runs.

Both water and duckweed samples were analyzed as in the translocation dynamics experiment, and a BCF was calculated as well. The BCF is used to infer a plant's ability to remove a contaminant from the environment. Species with a BCF > 1 are typically considered candidates for phytoremediation of a given contaminant as they can accumulate contaminants at a concentration greater than that of the surrounding environment.[33] Aphid results were analyzed with PROC probit in SAS to determine the LC_{50}, and Abbott's formula was used to correct treatment mortality.[34,35] As aphids in our oral LC_{50} experiments were also potentially exposed to CLO through contact with contaminated water, this experiment is admittedly confounded by this variable; however, the identical scenario would arise in the aphids' natural setting as well. The oral LC_{50} is typically lower than the contact LC_{50} for insects exposed to neonicotinoid insecticides. Therefore, while contact with contaminated water may contribute to aphid mortality, we hypothesize that feeding on contaminated plant tissue is likely to be a more important driver. Finally, while *R. nymphaeae* can break the surface tension to feed on submerged portions of plants, this behavior was not observed in any experimental tank or aphid colony tank.[24] Aphids were observed walking on the water surface when not feeding.

RESULTS

WQFS Leachate Concentrations. The varying combinations of fertilizer and tile history were determined not to be significant predictors of CLO concentration in leachate, within a given crop group (Table 1). As such, all CLO-treated maize plots, all soybean plots, and untreated control plots were grouped together for the CLO analysis. A significant multivariate treatment × time was recorded in the CLO model indicating that CLO concentrations were different across the sampling period (Time: Wilks, $F_{5,13}$ = 11.84, P < 0.001; time × treatment: Wilks, $F_{10,26}$ = 6.82, P < 0.001). Univariate treatment effects by sampling date interactions in the CLO model were highly significant on the first (6/23/16) and second (7/18/16) sampling events following planting (P < 0.01).

The weighted average approach provided 251 tile flow/date combination data points for analysis. In 20 instances of multiday flow, tip counter malfunction resulted in an inability to produce a weighted average. In this scenario, an equal

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weighting was assigned to each day of flow and a final weighted CLO concentration produced and used for analysis. The highest average CLO concentration in maize leachate across the season (3.37 ± 0.21 ng mL^{-1}; n = 24), corresponded with the first rainfall event (6/23/16), after planting (5/23/16), that resulted in >35 tiles flowing (Table 2). Portions of the Midwest experienced drought conditions during the 2016 growing season, including parts of Indiana. While our study location, Tippecanoe county, was not in official "drought" status, abnormally dry weeks were reported several times throughout the season (Figure S2). Notably, 100% of Tippecanoe county was considered abnormally dry from 6/14/16 to 6/21/16, which limited the flow into tiles, and correspondingly, the resolution with which CLO could be quantified in the first few weeks following plantingoccurred on 5/23/16.[36] The next precipitation event resulting in flow from >35 tiles occurred on 7/18/16 with an average CLO concentration of 1.37 ± 0.22 ng mL^{-1} (n = 21) in maize plots. No further samples were collected for the remainder of the growing season due to an insufficient number of tiles flowing per rainfall event. The next three rainfall events resulting in flow of >35 tiles were 12/26/17, 1/17/17, and 3/31/17, with respective CLO concentrations of 0.07 ± 0.01 (n = 21), 0.07 ± 0.008 (n = 21), and 0.06 ± 0.01 ng mL^{-1} (n = 18). CLO concentrations in the control plots never exceeded 0.1 ng mL^{-1} at any point indicating limited CLO contamination between tiles.

A maximum of 0.2399% ± 0.0663% (n = 18) CLO recovery was reported for the first precipitation event (6/23/16) resulting in >35 tiles flowing for small tile maize plots. Approximately, 4 weeks later (7/18/16), CLO recovery was ∼50% of the 6/23/16 date at 0.1206% ± 0.0274% (n = 16) of the initial CLO dose. Finally, the cumulative CLO % recovery for each tile produced an average of 0.3333% ± 0.0738% (n = 21) and represents the total CLO recovered throughout the sampling period (6/23/16−3/31/17).

Translocation Dynamics of Duckweed. At the 2 ng mL^{-1} of the concentration, only time was significant (Time: Wilks, $F_{2,3}$ = 16.46, P = 0.024; time × humidity: Wilks, $F_{2,3}$ = 2.64, P = 0.218). At the 10 ng mL^{-1} of the concentration, neither variable was significant (Time: Wilks, $F_{2,3}$ = 7.61, P = 0.067; time × humidity: Wilks, $F_{2,3}$ = 9.03, P = 0.04). The univariate results found significant differences at all sampling points at the 2 ng mL^{-1} of the concentration, but only at the 4 and 8 h time point, at the 10 ng mL^{-1} of concentration, indicating similar concentrations at the 12 h time point (Figure 1). The average humidities for the high- and low-humidity trials were 80.71 ± 0.51% (n = 14) and 60.5 ± 0.27% (n = 7). Measured CLO concentrations within experimental aquaria were 158.74 ± 31.31% (n = 12) of target concentrations of 2 and 10 ng of CLO mL^{-1} across all trials and humidity levels.

Aphid LC_{50} Trials with Contaminated Duckweed. Aphid LC_{50} was assessed over the course of 7 experimental replications and used 27 experimental tanks of varying CLO concentration. Measured CLO concentrations within water were 89.49 ± 1% (n = 27) of target test concentrations, with a corresponding BCF of 58.93 ± 2.86% (n = 27) for all experimental tanks. The relative humidity ranged from 61 to 81% over all 7 experimental trials, and 3 experimental tanks were not included in the analysis due to CLO contamination during the homogenization step. A total of 397 aphids were exposed to CLO-contaminated duckweed and found to have a probit-estimated (95% fiducial limits) LC_{50} and LC_{99} of 8.61 (6.23−9.76) and 20.46 (15.25−70.88) ng of CLO g plant tissue^{-1}, respectively (Figure 2). Due to a significant result following a goodness-of-fit test (χ^2 = 58.88, df =25, P < 0.0001), a t-value of 2.06 was used in the calculation of 95% fiducial limits. Despite this, the lowest concentration at which 100% mortality was observed was at 16.78 ng of CLO g plant tissue^{-1}. The average control mortality across all trials was 3.8%.

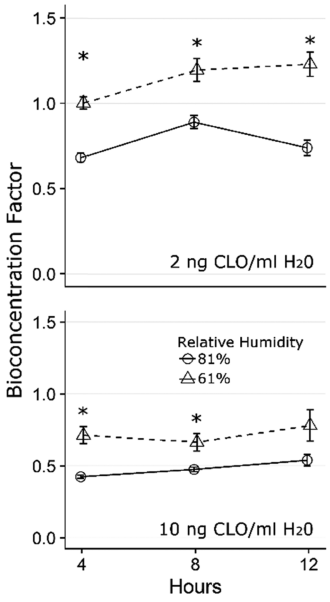

Figure 1. Mean BCF and SE of *L. gibba* grown in half-strength Hutner's media fortified with 2 and 10 ng mL^{-1} of CLO solutions at 61 and 81% relative humidity. Time points denoted by * indicate significant differences (P = 0.05) at that sampling event as determined by univariate Tukey comparisons.

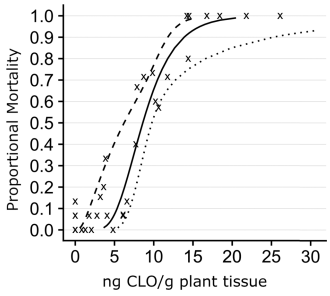

Figure 2. Probit-estimated dose response curve for *R. nymphaeae* (solid line) with lower (dashed line) and upper bounds (dotted line) of the 95% fiducial limits. "X" represents uncorrected mortality observations.

DISCUSSION

The work reported here provides documentation of the timing and magnitude of the potential contribution of NSTs to waterway contamination. The maximum CLO concentrations we report here of 3.37 ng mL^{-1} are approximately an order of magnitude greater than CLO concentrations previously reported in streams/rivers (Sánchez-Bayo and Hyne:[13] 0.42 ng mL^{-1}; Hladik et al.:[14] 0.257 ng mL^{-1}; Hladik et al.:[37] 0.226 ng mL^{-1}) and prairie wetlands (Main et al.:[38] 0.142 and 0.059 ng mL^{-1} for 2012 and 2013, respectively). This is not surprising, as our sampling approach differs from the studies

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cited above in offering more direct, undiluted measurements of neonicotinoid leachate from crop seeds in the field. In addition, water collected directly from belowground tiles is not subject to photodegradation. These are likely to be the two principal factors explaining the higher concentrations found in our samples. Furthermore, this work demonstrates that CLO concentrations within leachate are greatest at precipitation events that follow planting and largely conform to a first-order decay pattern (i.e., initially high concentrations with a rapid concentration decrease as the growing season progresses). A similar first-order decay pattern was also reported by Wettstein et al.[17] This result is expected as rainfall increases surface water contamination potential, and as cumulative rainfall increases, less active ingredient is likely left in the soil due to a combination of plant uptake, leaching, and breakdown.[39] A similar first-order decay pattern was also reported in Indiana maize with initially high in-plant CLO concentrations being followed by a rapid decrease of in-plant CLO concentrations.[6] Our findings, in combination with our work cited above and Wettstein et al.,[17] directly connect field-applied neonicotinoids with aquatic systems, and not the target crop, as a key environmental sink for these compounds.

Using a multivariate approach to repeated-measures, post-planting concentrations of CLO in leachate samples were statistically similar to untreated plots by the 12/26/16 rainfall event. Concentrations of CLO peaked at the first rainfall event and decreased throughout the year (Table 2) along with percent CLO recovery in small maize plots (Table 3). Limited

Table 3. Percent of CLO Recovered from Initial Dose ± SE for Selected Rainfall Events and Number (n) of Small Plot Maize Tiles Contributing to Average[a]

date	average % recovery	SE	n
6/23/16	0.2399	0.0663	18
6/27/16	0.0437	0.0174	5
7/18/16	0.1206	0.0274	16
11/29/16	0.0101	0.0046	4
12/26/16	0.0090	0.0020	17
1/17/17	0.0110	0.0031	11
2/7/17	0.0008	0.0002	10
3/31/17	0.0109	0.0031	19
total over sampling period	0.3333	0.0738	21

[a]The total average constitutes each small plot maize tile which produced measurable flow since planting.

tile flow after the 7/18/16 rainfall event limited the resolution for detecting further changes in concentrations throughout the latter part of the season. Additionally, while the concentrations in the leachate of the untreated plots (switchgrass, prairie, *Miscanthus* spp., and sorghum) were extremely low for CLO (<0.1 ng mL^{-1}), they were measurable. Subsurface lateral flow has been reported as a contamination pathway in the transport of neonicotinoids to the nontarget soil.[40,41] While the clay box lysimeters likely prevented the majority of intertile subsurface flow, maize seed is planted in the first few centimeters of the soil profile and the clay box lysimeters do not extend to the soil surface; walls start at approximately 40 cm below the soil surface. Given the high water solubility of NST, this is a likely explanation for CLO presence in nonmaize plots. Another, complementary, explanation of intertile contamination is the occasional flooding that occurs throughout the year at the WQFS. This can result in floodwater transferring soil-bound NST from one tile and depositing it elsewhere during percolation. These factors, in combination with the high soil DT_{50} of CLO, may explain why CLO is present in soybean plots. However, the reported concentrations cannot wholly be attributed to intertile contamination or carryover from the previous year's NST maize planting. Soybean plots had an overall higher CLO concentration within leachate in comparison to untreated plots alone. If carryover from the previous years' planting was the predominant factor, pre-plant CLO concentrations would be similar to post-plant concentrations. If intertile contamination was the predominant factor, control plots would have similar CLO concentrations within leachate to soybean plots. This latter explanation, however, assumes intertile contamination to be equal across plots. While the ultimate cause of higher than expected CLO concentrations within soy plots was beyond the scope of this experiment, additional studies looking at how NST interact with the surrounding soil may provide an explanation.

Our subsequent experiments, investigating the uptake dynamics of CLO into duckweed found in-plant concentrations rapidly increased within 4 h of exposure to CLO-contaminated water. This rapid uptake and consistent BCF corroborate the findings of Carvalho et al., who reported equilibration occurred by 24 h in *Lemna minor* when exposed to a variety of pesticides (NSTs not tested) with varied physiochemical properties.[31] Despite the humidity-mediated uptake differences at most sampling points within the uptake trials, any humidity-mediated impacts to in-plant CLO concentrations appear to be minor within the scope of our aphid LC_{50} trials as the average BCF across all trials was 58.93 ± 2.86% (n = 27).

While the protocols described here were designed to limit the impacts of photolysis and dilution, this may be analogous to the situation in the field, where the dense mats of plant tissue that duckweeds and other floating macrophytes create in agricultural ponds and lakes are likely to reduce light penetration, and, therefore, limit the impact of photolysis. This plant-associated reduction, in light penetration in combination with the overall decrease in photodegradation rate resultant from a compound's position within the water column, provide a mechanism for these otherwise photolytic compounds to persist within the environment.[42] The nontarget translocation of aquatic neonicotinoids has only been reported in submerged and rooted macrophytes,[18] and future environmental monitoring studies would benefit from the targeted sampling of floating macrophytes such as duckweed as well as sampling the water and sediment in areas with and without duckweed present.

While these results demonstrate the rapidity with which aqueous CLO is translocated into *L. gibba*, the translocation mechanism is relatively unknown. The uptake of nutrients remains poorly understood within the Lemnaceae.[43] Organic chemicals with an octanol−water partition coefficient ($\log K_{ow}$) between 0.5 and 3 are considered hydrophobic compounds that are capable of moving through the lipid bilayer of membranes but still waters-oluble enough to travel into cell fluids.[44] The log K_{ow} represents the 1-octanol/water partition coefficient and is used as a measure of lipophilicity. A combination of lipid bilayer penetration and uptake by the plant is likely to have occurred in this study as CLO has a log K_{ow} of 0.905. Independent of the mechanism(s) at play, this research and previous work demonstrate that translocation of CLO into nontarget vegetation within the environment is

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likely to occur quickly and provides a route of nontarget exposure to organisms that utilize or feed on contaminated vegetation.

As mentioned above, the maximum CLO concentrations we report here are an approximate order of magnitude higher than the other published literature. Despite this, the highest leachate concentration recorded (3.37 ng mL^{-1}) was less than half of the LC_{50} (8.71 ng g plant $tissue^{-1}$) generated here for *R. nymphaeae* and corresponds to a probit-estimated mortality probability of <1. Using mortality as our sole metric, no toxic effects were observed in *R. nymphaeae* at field-relevant CLO concentrations. However, toxic effects have been elicited on both organismal and community scales with other neonicotinoids within the same order of magnitude as our data. Sublethal effects (immobilization) have been elicited in the mayflies *Cloeon dipterum* and *Caenis horaria* following 96 h of imidacloprid exposure for an EC_{50} of 1 and 1.8 ng of imidacloprid mL^{-1} respectively.[45] Sublethal effects were recorded in a predatory water bug (*Belostoma flumineum*) at 0.1 ng/mL with a 62% reduction in prey consumption. Another mesocosm experiment investigated the colonization of water bodies as a result of neonicotinoid contamination.[46] Mesocosms were created from 400 g of the loamy soil and 10 L of water, contaminated at several concentrations (CLO and TMX at 0.1, 1, 3, 7, 10, 15 ng mL^{-1}) and left open to the environment. The three most prominent colonizing groups were chironomids (midges), ostracods (crustaceans), and Culex mosquitoes. Chironomids and ostracods were most negatively impacted by increasing neonicotinoid concentrations, whereas Culex larvae were relatively unaffected. These experiments, in combination with the correlative field studies mentioned earlier,[19,21] have allowed researchers to better describe and predict how neonicotinoid contamination of water bodies can lead to nontarget impacts in both aquatic and terrestrial environments on a community scale.

Previous research has shown that a small fraction of NST applied to seeds (<1.5%) is actually translocated into the maize plant tissue, with the remaining ∼98% presumably entering the environment via multiple pathways.[6] Perhaps due, in part, to this inefficient translocation into target plants, the documentation of plant protection from pests has been shown to be variable in NSTs, ranging from approximately equivalent to other approaches, to studies showing no, or even negative, effects upon plant yields.[5,7,47,48] Notably, NSTs are often largely redundant in the systems where they are used most, maize and soybeans. In maize, this occurs both as a consequence of >90% of maize hybrids expressing Bt toxins targeting key pests and a relative scarcity of the entire suite of secondary, below-ground, corn pests.[7,49] In soybeans, recent work has documented similar rapid declines of the active ingredient in soybean foliage, coupled with a poor synchrony between peak concentrations and populations of the soybean aphid, the key pest of soybean in our study area.[5]

Although our goal was a full assessment of concentrations for a calendar year following planting, several abnormally dry periods[36] (Figure S2), and the consequent low tile flow, limited data collection between 2 and 7 months after planting. During the initial 2 months of the study, CLO concentrations entered groundwater consistently. Based on our laboratory studies, we would expect nontarget macrophytic translocation to be both rapid and to reach equilibrium within plant tissues by 12−24 h upon entering aquatic systems. While these laboratory studies only used *L. gibba*, these results are likely relevant for other duckweeds and free-floating/submerged macrophytes. Measurement of neonicotinoid residues in aquatic plants in the field is a logical next step as well as quantification of any subsequent effects upon the multitude of organisms that feed upon them. Results from these experiments will allow a greater understanding of neonicotinoid persistence in the nontarget plant tissue and may expand environmental monitoring opportunities.

This work adds to a growing body of research, which demonstrates that the overwhelming majority of NST-active ingredient intended for management of crop pests can be expected to reliably enter waterways where their effects remain largely unquantified. Specifically, our findings highlight the rapid nature of the movement of the neonicotinoids applied to crop seeds into waterways, where they can be readily translocated into aquatic vegetation. In contrast to the off-site movement of pesticides via drift, for example, that can be addressed using application technology improvements, the highly water-soluble nature of the neonicotinoids used in NST applications makes contamination of aquatic systems a hazard that is difficult to address without reducing the rates of the active ingredient applied to the landscape. This could be readily achieved by reintroducing elements of integrated pest management to maize, soybeans, and other cropping systems where NST use is the predominant form of insect management.[3,50,51]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b05025.

List of treatments and relevant agronomic practices for the WQFS in 2016 (Table S1); Schematic of a Purdue Water Quality Field Station sampling unit. Individual tile lines would drain (a) into a tipping bucket (b), which at every other tip would deposit ∼10 mL of drainage water through a 1 cm slit (c) into the 20 L collection bucket's (d) stainless steel protective (Figure S1); graph of percent of Tippecanoe County Indiana considered "Abnormally Dry" by the National Drought Mitigation Center by week (Top) as well as daily rainfall as determined by onsite precipitation gauge (Bottom) (Figure S2); box and whisker plot of total flow per selected precipitation event (Figure S3); machine settings for LC/MS/MS analysis of water and duckweed samples (Text S1)(PDF)

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Notes

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■ ABBREVIATIONS:

CLO Clothianidin
NST Neonicotinoid seed treatment
TMX Thiamethoxam

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