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Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic Exposure for Bees

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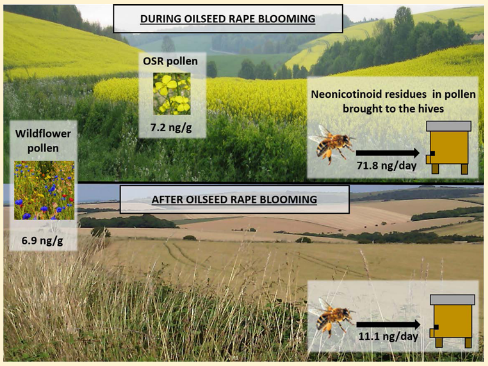
Supporting Information

ABSTRACT: In recent years, an intense debate about the environmental risks posed by neonicotinoids, a group of widely used, neurotoxic insecticides, has been joined. When these systemic compounds are applied to seeds, low concentrations are subsequently found in the nectar and pollen of the crop, which are then collected and consumed by bees. Here we demonstrate that the current focus on exposure to pesticides via the crop overlooks an important factor: throughout spring and summer, mixtures of neonicotinoids are also found in the pollen and nectar of wildflowers growing in arable field margins, at concentrations that are sometimes even higher than those found in the crop. Indeed, the large majority (97%) of neonicotinoids brought back in pollen to honey bee hives in arable landscapes was from wildflowers, not crops. Both previous and ongoing field studies have been based on the premise that exposure to neonicotinoids would occur only during the blooming period of flowering crops and that it may be diluted by bees also foraging on untreated wildflowers. Here, we show that exposure is likely to be higher and more prolonged than currently recognized because of widespread contamination of wild plants growing near treated crops.



INTRODUCTION

Bees currently face many interacting pressures, including loss of habitat and concomitant reductions in the availability of flowers and nest sites, impacts of parasites and pathogens (both native and introduced), and exposure to pesticides.[1] The contribution of pesticides, and in particular neonicotinoids, to pollinator declines has led to controversy across the United States and Europe.[2] Laboratory and semifield studies of honey bees and bumblebees suggest that exposure of colonies to concentrations approximating those found in pollen and nectar of flowering crops can impair pollen collection, increase worker mortality, weaken immune function, reduce nest growth, and reduce the production of new queens.[3−6] However, a key point of controversy is whether bees consume enough of these compounds during the flowering period of the crop to do them significant harm. It has thus been argued that the levels of exposure used in these studies may be higher than those most bee colonies are likely to experience in the field, based on the premise that exposure to neonicotinoids from flowering crops will be diluted by bees also foraging on untreated wildflowers.[7] Moreover, it has been shown that the concentrations of neonicotinoid residues present in food stores are extremely variable, going from no detectable level to >200 ng/g in bee-stored pollen.[8−10] Some field studies in which honey bee hives were exposed to plots of treated crops for the duration of their flowering period found no measurable impact on colony health.[11−14] A recent well-replicated and realistic field study found that exposure to a treated oilseed rape crop for one season was not enough to have measurable adverse effects on honey bee colonies but did have profound effects on bumblebee nests and on the reproduction of solitary bees, suggesting that honey bees may be more able to cope with exposure to neonicotinoids than wild bees.[14]

Here, we present data on environmental contamination with neonicotinoids from five predominantly arable farms in East Sussex, U.K. We sampled soil from fields under neonicotinoid-treated winter oilseed rape (OSR) in spring 2013 and also soil from beneath the herbaceous vegetation in the field margins of both OSR and winter wheat crops. We sampled by hand the pollen and nectar of the OSR crop and of the wildflowers growing in the margins of both winter wheat and OSR fields through the spring and summer. We also placed honey bee colonies on these farms and sampled the pollen returned to the hives, to estimate the level of exposure to neonicotinoids. Finally, we analyzed samples of neonicotinoid-dressed seeds and of crop seeds untreated with neonicotinoids for sowing during the EU moratorium. The objectives of this study were to evaluate the environmental contamination caused by the application of neonicotinoid seed treatments in conventional arable farms and to examine the role of nontarget vegetation as a source of exposure to neonicotinoid residues for bees.

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MATERIALS AND METHODS

Sample Collection Methods. *Sampling Locations.* Seven winter-sown oilseed rape (sown at the end of August 2012) and five winter-sown wheat (WW, sown at the end of September 2012) fields were selected at random from five conventional farms located in East Sussex, South-East England, U.K. The selected fields had varying cropping history following normal farming practices in the region (the predominant crops being WW and OSR). Previous crops had been treated with a range of pesticides, including neonicotinoids each year for at least the three previous years (Table S1a–g). The seeds from the OSR fields were all treated with Cruiser seed dressing in 2012 (active ingredients being 280 g/L thiamethoxam, 8 g/L fludioxonil, and 32.2 g/L metalaxyl-M), and the WW was treated with Redigo Deter (active ingredients being 50 g/L prothioconazole and 250 g/L clothianidin) following normal farming practice.

Analysis of Commercial Oilseed Rape, Wheat, and Barley Seeds. To determine relative concentrations of neonicotinoid insecticides in commercial seeds routinely used in U.K. farmland, we tested one sample of rape seeds treated at a purported rate of 4.2 g of active ingredient (a.i.) thiamethoxam/kg of seed (Cruiser OSR) and one wheat sample with 0.5 g of a.i. clothianidin/kg of seed (Redigo Deter). Additionally, seeds treated with fungicide only were analyzed, using oilseed rape seeds treated with Agrichem HY-PRO Duet (active ingredients being 150 g/L prochloraz and 333 g/L thiram), oilseed rape seeds treated with Beret Multi (active ingredients being 25 g/L fludioxonil and 25 g/L flutriafol), and barley seeds treated with Kinto (active ingredients being 20 g/L triticonazole and 60 g/L prochloraz).

Soil Sampling. Soil samples were collected from the seven OSR fields 10 months after sowing (June 2013). Three sites of 50 m^2 were sampled in each field, sites being at least 100 m apart. Within each site, 15 × 20 g subsamples were collected at depths of 0–10 cm and pooled to minimize variation caused by small-scale heterogeneity in pesticide concentrations.

Soil from the margins was also sampled from all four margins of five of the OSR fields and five of the WW fields. As described above, each sample comprised a pool of 15 subsamples collected along the length of the margin at depths of 0–10 cm. The average sample distance from the crop edge was 1.5 m (range of 1–2 m). Only soil samples from the margins where neonicotinoid pesticides were detected in wildflowers were analyzed (24 of 120 samples). Field margin soil samples were analyzed only if neonicotinoids were detected in wildflowers in that margin, because our goal was to examine whether soil was a plausible route for contamination of the flowers.

All soil samples were stored on ice in coolers in the field and then frozen immediately in the laboratory and kept at −80 °C.

Pollen and Nectar Samples Collected from Oilseed Rape Plants. Nectar and pollen samples were collected during the period of rape blooming (from May 19 to June 27, 2013) directly from rape flowers in the seven OSR fields using the same three sampling sites per field as for the soil samples. Additional details are provided in the Supporting Information.

Pollen and Nectar Samples Collected from Wild Plants in the Field Margins. Field boundaries in the region typically consist of a hedge of woody plants separated from the crop by a 0–2 m strip of herbaceous vegetation. Samples of pollen and nectar were collected from the wild flowers that were present in the field margins and hedge by choosing representatives of the main plant families of which honey bees and other bees feed, using the same methodology as for OSR plants (see the Supporting Information). A total of 57 nectar samples and 188 pollen samples from 54 different plant species were gathered from the same field margins where the soil samples were collected. The species of wildflowers collected varied considerably and depended upon which species were available. The average sample distance from the crop edge was 1.5 m (range of 1–2 m). When the weight of pollen samples or the volume of nectar samples was not sufficiently large to be analyzed separately, samples from different species growing in the same or neighboring margin were pooled and analyzed as a single sample. In total, 55 of 98 of the wildflower pollen samples (56.1%) and 21 of 32 of the wildflower nectar samples (67.7%) could be analyzed as single species, and the rest were all analyzed as pooled samples from different species (see Tables S2a–j and S3a,b).

Pollen Collected by Honey Bees. Five honey bee (*Apis mellifera*) colonies (one hive per farm) were placed in the vicinity of OSR fields at the beginning of the OSR flowering period (May 2013) and remained at the same sites until the end of August 2013. The hives were equipped with pollen traps during four consecutive days at the beginning of June 2013 and for four days in mid-August 2013 to collect pollen loads from the returning honey bee foragers during the OSR blooming period, and also when no OSR was in flower. After 4 days, the traps were removed and the honey bee-collected pollen loads were stored on ice and then at −80 °C in the laboratory until they were analyzed. Pollen loads within each sample were sorted by eye according to color, texture, size, and shape as indicators of different pollen types. All pollen types were separately weighed to calculate their relative abundance within the samples.[15,16] A representative sample of loads from each pollen type was mounted, and pollen grains were identified under a microscope following standard methods[17] and using reference specimens and published reference collections.[18−21]

Residue Analysis. Sample Preparation for Neonicotinoid Analyses. All samples were analyzed for concentrations of thiamethoxam (TMX), clothianidin (CLO), imidacloprid (IMC), and thiacloprid (THC). Additional details are provided in the Supporting Information.

Soil and Seed Samples. One hundred grams of each soil sample was homogenized and sieved (2 mm), and 100 g seed samples were ground to a fine powder with a mortar and pestle. An aliquot of soil or seed samples (0.5 ± 0.5 g for both matrices) was spiked with 1 ng of the deuterated pesticides in ACN and extracted using the QuEChERS method. First, 2 mL of water was added to form an emulsion, and samples were then extracted by adding 2.5 mL of ACN and 750 μL of hexane and mixing on a multiaxis rotator for 10 min. Then, 1.25 g of a magnesium sulfate/sodium acetate mixture (4:1) was added to each tube in turn with immediate shaking to disperse the salt and prevent clumping of the magnesium salt. After centrifugation [13000 relative centrifugal force (RCF) for 5 min], the supernatant was removed into a clean Eppendorf tube containing 625 mg of SupelQuE PSA/C18/ENVI-Carb and vortexed. The aqueous phase and salt pellet were extracted again using 1.75 mL of ACN and the supernatant combined with the previous ACN extract. The extract was mixed with PSA/C18/ENVI-Carb on a multiaxis rotator (10 min) and then centrifuged (10 min). The supernatant was transferred into a glass tube, evaporated to dryness under vacuum, reconstituted with 200 μL of an ACN/H_2O solvent (10:90), and spin filtered (0.22 μm). Seed samples were then further diluted to determine thiamethoxam and clothianidin concentrations. An aliquot of 1.5 g of each wet soil sample was dried for 24 h at 105 °C to determine the water content, and neonicotinoid concentrations were expressed as nanograms per gram of dry weight of soil.

Pollen. A 100 mg pollen sample was weighed into an Eppendorf tube; 150 pg of deuterated pesticides in ACN was added, and the samples were extracted using the QuEChERS method. The same ratio of solvents, salts, and PSA/C18/ENVI-Carb per gram of sample as for the soil extractions was used [i.e., 400 μL of water, 500 μL of ACN, 150 μL of hexane, 250 of a magnesium sulfate/sodium acetate mixture (4:1), and 125 mg of PSA/C18/ENVI-Carb]. After the first extraction, the aqueous phase and resuspended pellet were extracted again with 400 μL of ACN and the supernatants combined. Extracts were mixed with PSA/C18/ENVI-Carb (10 min) and centrifuged (10 min). The supernatant was evaporated to dryness under vacuum, reconstituted with 120 μL of an ACN/H_2O solvent (10:90), and filtered as described above.

Nectar. Nectar in the capillary tube was expelled into an Eppendorf tube, and the capillary was then flushed in 100 μL of a H_2O/ACN solvent (90:10) and the sample combined with the nectar sample. The nectar samples were centrifuged at 13000 RCF for 10 min to remove pollen and plant debris, and the supernatant (between 10 and 110 μL depending on collection volume) was transferred into a clean Eppendorf tube and the volume increased to 200 μL using a H_2O/ACN solvent (90:10). Fifty picograms of deuterated pesticide standard mixture was added to 200 μL of diluted nectar, and the samples were extracted using the first step of the QuEChERS method. For this, 250 μL of ACN was added, and samples were extracted on a multiaxis rotator for 10 min. Then 125 mg of a magnesium sulfate/sodium acetate mixture (4:1) was added and the sample shaken (3 min) and centrifuged (13000 RCF for 5 min). The supernatant was removed and the aqueous phase extracted again with 250 μL of ACN, and the supernatants were combined. Samples were reconstituted in 50 μL of a H_2O/ACN solvent (90:10) and centrifuged (13000 RCF for 10 min) prior to UHPLC−MS/MS analysis.

UHPLC−MS/MS Analyses. Ultra-high-performance liquid chromatography and tandem mass spectrometry (UHPLC−MS/MS) analyses were conducted using a Waters Acquity UHPLC system coupled to a Quattro Premier triple-quadrupole mass spectrometer from Micromass (Waters, Manchester, U.K.). Samples were separated using a reverse phase Acquity UHPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, Manchester, U.K.) fitted with an ACQUITY UHPLC BEH C18 VanGuard precolumn (130 Å, 1.7 μm, 2.1 mm × 5 mm, Waters) maintained at 22 °C. The injection volume was 20 μL, and mobile phase solvents consisted of 95% water, 5% ACN, 5 mM ammonium formate, and 0.1% formic acid (A) and 95% ACN, 5% water, 5 mM ammonium formate, and 0.1% formic acid (B). The initial ratio (A:B) was 90:10, and separation was achieved using a flow rate of 0.2 mL/min with the following gradient: 90:10 to 70:30 over 10 min, from 70:30 to 0:100 over 2 min and held for 7 min, and return to initial condition and equilibration for 7 min.

MS/MS was performed in multiple-reaction mode (MRM) using ESI in the positive mode, and two characteristic fragmentations of deprotonated molecular ion $[M + H]^+$ were monitored; the most abundant one was used for quantitation and the second as a qualifier. Retention times, ionization, and fragmentation settings are reported in Table S4. Other parameters were optimized as follows: capillary voltage, −3.3 kV; extractor voltage, 8 V; multiplier voltage, 650 V; source temperature, 100 °C; desolvation temperature, 300 °C. Argon was used as the collision gas (collision cell P, 3×10^{-3} mbar), while nitrogen was used as the desolvation gas (600 L/h). Mass calibration of the spectrometer was performed with sodium iodide. Samples were analyzed in a random order, and QC samples (i.e., standards) were injected during runs every 10 samples to check the sensitivity of the machine. Data were acquired using MassLynx version 4.1, and the quantification was conducted by calculating the response factor of neonicotinoid compounds to their respective internal standards. Concentrations were determined using a least-squares linear regression analysis of the peak area ratio versus the concentration ratio (native to deuterated). At least five-point calibration curves ($R^2 > 0.99$) were used to cover the range of concentrations observed in the different matrices for all compounds, within the linear range of the instrument. Method detection and quantification limits (MDL and MQL, respectively) were determined from spiked samples that had been extracted using the QuEChERS method. Nonspiked samples were also prepared. MDLs were determined as the minimal amounts of analyte detected with a signal:noise ratio of 3 and MQLs as the minimal amounts of analyte detected with a signal:noise ratio of 10, after accounting for any levels of analyte present in nonspiked samples (Table S5a).

Quality Control. One blank workup sample (i.e., solvent without matrix) per batch of 11 samples was included and injected onto the UHPLC−MS/MS instrument to ensure that no contamination occurred during sample preparation. Solvent samples were also injected between sample batches to ensure that there was no carryover in the UHPLC system that might affect adjacent results in analytical runs. Several replicates per site were analyzed, and all samples in which pesticides were detected were extracted and analyzed at least in duplicate for confirmation. Identities of detected neonicotinoids were confirmed by comparing the ratio of MRM transitions in samples and pure standards. The QuEChERS method is used routinely for neonicotinoid analyses (e.g., 24), and recovery experiments performed on spiked [1 ng/g of dry weight (dw); $n = 4$], pollen (1.2 ng/g of dw; $n = 4$), and soil (10 ng/g of dw; $n = 4$) samples gave absolute recovery values ranging from 85 ± 8 to 111 ± 5% for the four pesticides, in agreement with other published studies[22,23] (Table S5b). The concentration of any pesticides detected in unspiked samples was also determined and subtracted from the spiked concentration to estimate the true recovery of the test chemical. Finally, gas chromatography with tandem mass spectrometry was also used to confirm the high thiamethoxam concentrations observed in some wildflower pollen samples (see the Supporting Information).

Statistical Analysis. All statistical analyses were conducted using SPSS 21 software. To test for differences in the concentrations of the neonicotinoids in soil from OSR fields and field margins, a two-way analysis of variance (ANOVA) procedure was used (OSR fields 1−5, where samples from both cropland and margins were collected) with the origin of samples (cropland or field margins) as fixed factors and the concentrations for the different neonicotinoids (TMX, CLO, IMC, THC, and total neonicotinoid residues) as response variables. When no statistically significant interaction was found, this term was removed from the model and the analysis was rerun to test for the main effects of the fixed factors, using a Tukey post hoc test for multiple comparisons.

Table 1. Numbers of Samples Analyzed, Percentages with Detectable Levels of Neonicotinoid Insecticides, and Ranges, Means (±standard deviation), and Medians of the Levels Found in Soil Samples Collected from Oilseed Rape (OSR) Cropland and Field Margins (where the seeds were treated with thiamethoxam at an application rate of 4.2 g of a.i. thiamethoxam/kg of seed) and from the Field Margins of Winter Wheat Crops (WW, where the wheat seeds were treated with clothianidin at an application rate of 0.5 g a.i. clothianidin/kg of seed)[a]

origin of soil samples	N		TMX 0.04 ng/g (MDL) 0.12 ng/g (MQL)	CLO 0.07 ng/g (MDL) 0.20 ng/g (MQL)	IMC 0.07 ng/g (MDL) 0.20 ng/g (MQL)	THC 0.01 ng/g (MDL) 0.04 ng/g (MQL)
OSR cropland	21	frequency of detection (%)	100	100	100	42.86
		range (ng/g)	0.49–9.75	5.10–28.6	0.74–7.90	≤0.01–0.22
		mean ± SD (ng/g)	3.46 ± 2.98	13.28 ± 5.73	3.03 ± 2.05	0.04 ± 0.07
		median (ng/g)	2.43	13.05	2.10	≤0.01
OSR field margins	16	frequency of detection (%)	100	100	93.75	25
		range (ng/g)	0.28–1.76	2.25–13.33	≤0.07–7.17	≤0.01–0.13
		mean ± SD (ng/g)	0.72 ± 0.44	6.57 ± 3.12	1.92 ± 2.06	≤0.01
		median (ng/g)	0.59	5.61	0.70	≤0.01
WW field margins	8	frequency of detection (%)	50	100	75	25
		range (ng/g)	≤0.04–0.45	0.41–19.12	≤0.07–6.30	≤0.01–0.13
		mean ± SD (ng/g)	0.18 ± 0.21	7.71 ± 6.9	1.36 ± 2.19	≤0.01
		median (ng/g)	≤0.12	7.36	0.48	≤0.01

[a]All fields were sowed with harrow power drill combination.

A one-way ANOVA procedure was used to test for possible differences in concentrations of neonicotinoid residues among the seven fields where OSR pollen samples were collected (OSR fields 1–7), followed by Tukey or Tamhane post hoc tests for multiple comparisons depending on the homogeneity of variance in each case (determined using Levene's test). Levels in nectar were also compared among the seven OSR fields using the Kruskal–Wallis test (K–W) due to non-normality in the distribution of the data.

Nonparametric Mann–Whitney (M–W) U tests were used to compare the concentrations of neonicotinoids present in pollen and nectar collected from OSR flowers, to compare pollen and nectar collected from OSR flowers versus pollen and nectar from wildflowers growing in the OSR field margins, for pollen collected from wildflowers growing in OSR field margins versus wildflowers from WW field margins, for pollen collected from wildflowers growing in the OSR and WW margins versus honey bee-collected pollen of wildflower origin, and for pollen collected by the honey bees in June versus that collected in August. To perform the statistical analyses, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the value considered as the MDL in each case (Table S5a). Concentrations below the MDL were considered to be zero.

Pearson's coefficient of correlation (for normally distributed data) and Spearman's rank correlation (for data not normally distributed) were used to assess the relationship among levels of neonicotinoids in nectar, pollen, and soil collected in the OSR fields. When the relationship between levels in nectar and pollen or soil was evaluated, as the number of samples for nectar was reduced from 21 to 13 due to small volumes for some samples, the number of data for pollen ($N = 21$) and soil ($N = 21$) was reduced accordingly by calculating means where necessary. The number of samples was not reduced when the relationship in the levels of neonicotinoids was evaluated between pollen and soil.

The coefficient of variation (C_V) in the concentrations of neonicotinoids found in OSR pollen and OSR nectar, and in wildflower pollen, was used to analyze the consistency in the levels found in these sets of samples, using t tests to compare between the variability found in OSR pollen and OSR nectar, and in OSR pollen and wildflower pollen.

The diversity of plant taxa represented in pollen collected by honey bees per site and sampling period was calculated using Simpson's index of Diversity (1-D).[24]

RESULTS AND DISCUSSION

Soil Samples from OSR Cropland and Margins and WW Field Margins. All soil samples taken under OSR ($N = 21$) tested positive for thiamethoxam, which was the dressing applied to the seeds of the current crop, and for clothianidin, a breakdown product of thiamethoxam (Table 1). However, samples also all tested positive for imidacloprid, and 42.9% tested positive for thiacloprid, though these two compounds had not been applied in the previous three years (Table S1a–g). The field margin soils adjacent to OSR ($N = 16$) also all contained thiamethoxam and clothianidin, but the concentrations of these two compounds were significantly lower than those found in soil from OSR cropland [via two-way ANOVA, $F(1,25) = 12.78$, $P = 0.001$, and $\eta_p^2 = 0.338$ for thiamethoxam and $F(1,25) = 14.51$, $P = 0.001$, and $\eta_p^2 = 0.367$ for clothianidin]. Imidacloprid was detected in all but one (93.8%) of the OSR margins, and thiacloprid, with lower levels in margins than in cropland as well [via two-way ANOVA, $F(1,25) = 1.326$, $P = 0.260$, and partial $\eta_p^2 = 0.05$ for imidacloprid and $F(1,25) = 7.18$, $P = 0.013$, and partial $\eta_p^2 = 0.223$], was present in 25% of the samples. The insecticide applied as seed dressing in the WW fields was also found in all the soil samples from the WW margins [clothianidin; $N = 8$ (Table 1)] together with imidacloprid in 75% of the samples, thiamethoxam in 50% of them, and thiacloprid in 25% of them. This widespread prevalence both in cropland and in field margins is to be expected given the high persistence of these compounds in soils[25,26] and their high potential for lateral movement and leaching.[27−29] The persistence of neonicotinoids increases under cool conditions, and in soils with higher pH, organic matter content, and mineral clay content,[26] but as

Table 2. Numbers of Samples Analyzed, Frequencies of Detection, and Ranges, Means (±standard deviation), and Medians of Levels Found in Pollen and Nectar Samples Collected from Oilseed Rape (OSR) Flowers (seven fields) and from Wildflowers Collected from the Margins of Five OSR and Five Winter Wheat (WW) Fields, and from Pollen Collected by Honey Bees

			TMX	CLO	IMC	THC
			0.12 ng/g (MDL)	0.12 ng/g (MDL)	0.16 ng/g (MDL)	0.04 ng/g (MDL)
origin of pollen samples	N		0.36 ng/g (MQL)	0.36 ng/g (MQL)	0.48 ng/g (MQL)	0.12 ng/g (MQL)
OSR flowers	21	frequency of detection (%)	100	90.5	0	85.7
		range (ng/g)	1.02–11.10	≤0.12–14.50	≤0.16	≤0.04–7.25
		mean ± SD (ng/g)	3.26 ± 2.16	2.27 ± 3.52		1.68 ± 1.84
		median (ng/g)	3.16	1.40		1.19
wildflowers from OSR margins	43	frequency of detection (%)	58.1	14	11.6	4.7
		range (ng/g)	≤0.12–86.02	≤0.12 to ≤0.36	≤0.16–12.29	≤0.04–0.46
		mean ± SD (ng/g)	14.81 ± 25.17		0.56 ± 2.10	≤0.04
		median (ng/g)	≤0.36		≤0.16	≤0.04
wildflowers from WW margins	55	frequency of detection (%)	1.8	0	3.6	3.6
		range (ng/g)	≤0.12–7.47[a]	≤0.12	≤0.16–0.58	≤0.04–0.64
		mean ± SD (ng/g)	0.14 ± 1.01		≤0.16	≤0.04
		median (ng/g)	≤0.12		≤0.16	≤0.04
collected by honey bees during OSR bloom (June)	34	frequency of detection (%)	50	23.5	20.6	58.8
		range (ng/g)	≤0.12–1.81	≤0.12–1.12	≤0.16–25.55	≤0.04–2.77
		mean ± SD (ng/g)	0.20 ± 0.44	≤ 0.12	2.51 ± 6.28	0.30 ± 0.65
		median (ng/g)	≤0.12	≤0.12	≤0.16	≤0.12
collected by honey bees after OSR bloom (August)	46	frequency of detection (%)	43.5	4.3	15.2	19.6
		range (ng/g)	≤0.12–0.31	≤0.12–0.28	≤0.16–2.52	≤0.04
		mean ± SD (ng/g)	≤0.12	≤0.12	≤0.16	
		median (ng/g)	≤0.12	≤0.12	≤0.16	

			TMX	CLO	IMC	THC
			0.10 ng/g (MDL)	0.17 ng/g (MDL)	0.17 ng/g (MDL)	0.03 ng/g (MDL)
origin of nectar samples	N		0.30 ng/g (MQL)	0.50 ng/g (MQL)	0.50 ng/g (MQL)	0.08 ng/g (MQL)
OSR flowers	13	frequency of detection (%)	53.9	30.8	0	53.9
		range (ng/g)	≤0.10–13.30	≤0.17–13.24	≤ 0.17	≤0.03–1.23
		mean ± SD (ng/g)	3.20 ± 4.61	2.18 ± 3.99		0.26 ± 0.36
		median (ng/g)	≤0.10	≤0.17		0.11
wildflowers from OSR margins	24	frequency of detection (%)	20.8	20.8	0	25
		range (ng/g)	≤0.10–1.80	≤0.17 to ≤0.50	≤ 0.17	≤0.03 to ≤0.08
		mean ± SD (ng/g)	0.10 ± 0.37			
		median (ng/g)	≤0.10			
wildflowers from WW margins	8	frequency of detection (%)	0	0	0	0
		range (ng/g)	≤0.10	≤0.17	≤0.17	≤0.03
		mean ± SD (ng/g)				
		median (ng/g)				

[a] Only one sample with detectable levels of this compound.

these features were not evaluated in our samples, their role in the persistence and concentrations found cannot be elucidated.

Pollen and Nectar Samples Collected from OSR Plants. Thiamethoxam used in the seed dressing was present in all pollen samples (21 of 21) and a majority of nectar samples (7 of 13) collected from the OSR crops, at concentrations similar to those found in previous studies,[26,30] with no differences in the values for both matrices [mean ± SD, 3.26 ± 2.16 ng/g in pollen, 3.20 ± 4.61 ng/g in nectar; M–W test, $U(32) = 90$, $P > 0.05$, $Z = -1.65$ (Table 2)]. Maximal concentrations were 11.1 and 13.3 ng/g for pollen and nectar, respectively. In addition to thiamethoxam, 90.5% of the pollen samples contained clothianidin and 85.7% contained thiacloprid. With regard to OSR nectar, 53.9% of the samples presented thiacloprid, with lower levels than in pollen [M–W test, $U(32) = 50.0$, $P = 0.002$, $Z = -3.09$] and 30.8% contained clothianidin. The concentrations of the neonicotinoids detected in the different samples were similarly highly variable for pollen and nectar [$C_{\mathrm{V\_OSR\ pollen}} = 82.75 \pm 66.04\%$; $C_{\mathrm{V\_OSR\ nectar}} = 118.45 \pm 81.14\%$; t test, $t(6) = -0.681$, $P = 0.521$] and did not show differences among the seven fields where they were collected [e.g., for TMX in pollen samples, via ANOVA, $F(6) = 2.46$ and $P = 0.078$; for TMX in nectar samples, via the K–W test, $H(6) = 10.12$, $P = 0.120$]. Furthermore, the concentrations for thiamethoxam in pollen were positively correlated with the concentrations in the soil samples collected from the same sites

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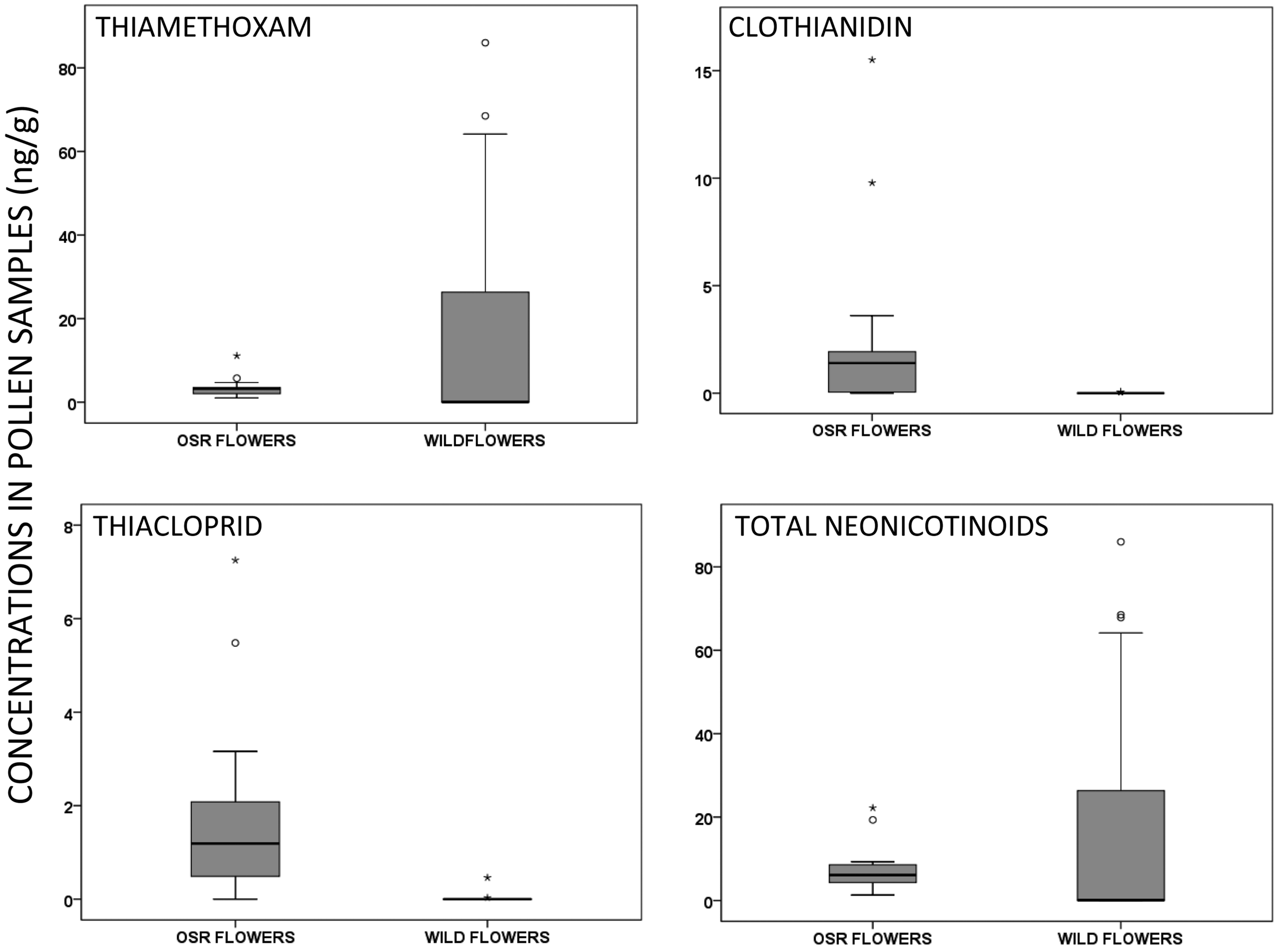

Figure 1. Levels of thiamethoxam, clothianidin, thiacloprid, and total neonicotinoids (TMX, CLO, IMD, and THC) in pollen collected from OSR flowers and wildflowers from OSR field margins. Black horizontal bars inside boxplots show median values. Upper and lower whiskers represent scores outside the middle 50%. Empty circles represent mild outliers and asterisks extreme outliers.

[Pearson correlation coefficient $r(19) = 0.52$, and $P = 0.017$ (Figure S1)], but the same correlation was not found for nectar [Spearman's rank correlation $\rho(11) = -0.12$, and $P = 0.70$].

Pollen and Nectar Samples from Wild Plants in the Field Margins. Pollen collected by hand from wildflowers in OSR field margins frequently contained thiamethoxam (58% of 43 samples), sometimes at high concentrations, as in the case of a pollen sample from *Heracleum sphondylium* (86 ng/g) collected in margin M2 of OSR field 4 and one from *Papaver rhoeas* (64 ng/g) collected in margin M2 of OSR field 1 (Table S2a,d). However, neonicotinoid residues were not always detected in pollen samples of the same species collected from different field margins (Table S2a–j). The possible heterogeneity in soil properties and environmental factors along the field margins (e.g., organic matter content, microbial communities, humidity, degree of slope, and sunlight exposure) may have influenced the persistence of neonicotinoids and their sorption onto soil particles in specific sites,[26] thus resulting in differential exposure and uptake of these active ingredients by field margin plants growing in different field locations.

Overall, the total concentrations of neonicotinoids present in the pollen from wildflowers in the OSR field margins were higher than in pollen from the treated OSR plants [via the M–W test, $U(62) = 287.0$, $P = 0.018$, and $Z = -2.37$ (Figure 1)], though as might be expected when testing a range of different plant species, levels were more variable in wildflower samples [$C_{V\_\text{wildflower pollen}} = 350.35 \pm 189.31\%$; $C_{V\_\text{OSR pollen}} = 82.75 \pm 66.04\%$; t test, $t(6) = -2.669$ and $P = 0.037$]. The higher residue levels detected in wildflower pollen were mainly due to the significantly greater concentrations of thiamethoxam when compared to that in OSR pollen [via the M–W test, $U(62) = 302.0$, $P = 0.03$, and $Z = -2.165$]. In contrast, clothianidin and thiacloprid were typically found at lower concentrations than in the crop [via the M–W test, $U(62) = 61.0$, $P < 0.001$, and $Z = -6.36$ for clothianidin and $U(62) = 70.0$, $P < 0.001$, and $Z = -6.64$ for thiacloprid (Figure 1)]. Imidacloprid, absent in OSR pollen, was detected in 11.6% of the wildflower pollen samples.

Residues of thiamethoxam, imidacloprid, and thiacloprid were detected in pollen collected from wildflowers adjacent to winter wheat fields, but the levels were lower (total neonicotinoid residues, 0.17 ± 1.01 ng/g) than in wildflowers growing in OSR field margins [total neonicotinoid residues, 15.40 ± 25.45 ng/g; via the M–W test, $U(96) = 507.0$, $Z = -5.75$, and $P < 0.001$]. The seed treatment in the winter wheat fields, clothianidin, was not detected in any of the pollen or nectar samples gathered from wildflowers growing in the WW field margins (Table 2) despite being present in the soil beneath this margin vegetation (Table 1). Thiamethoxam is

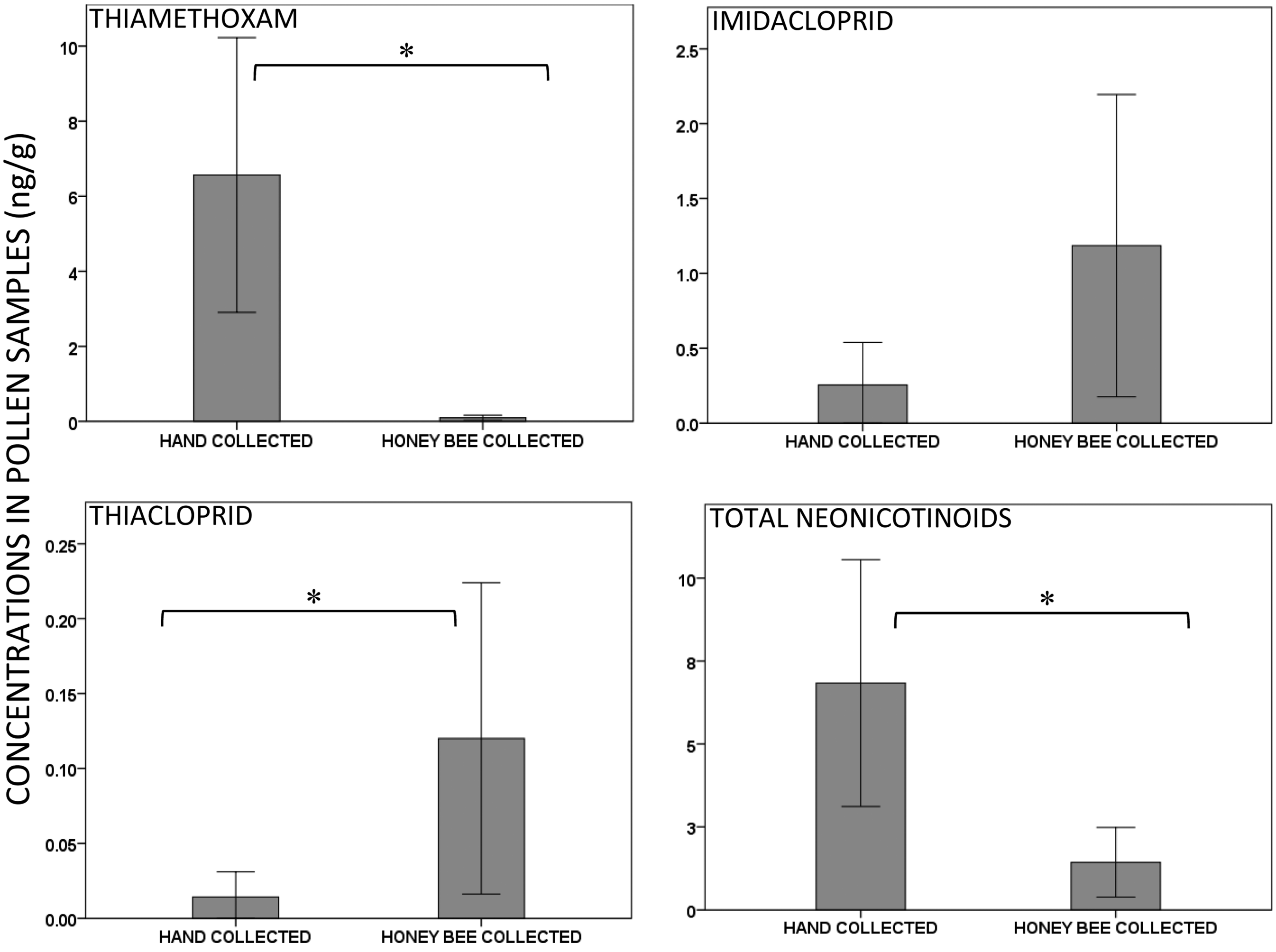

Figure 2. Mean levels of thiamethoxam, imidacloprid, thiacloprid, and total neonicotinoid residues detected in hand-collected pollen from the wildflowers present in the margins of OSR and WW fields and the mean levels in corbicular pollen of wildflower origin trapped in honey bee hives located in the vicinity of the same fields. Standard error bars are represented in the graphs, and statistically significant differences ($P < 0.05$) are marked with an asterisk.

more soluble in water (4.1 g/L) than is clothianidin (0.30−0.34 g/L),[31] and thus, it may have better systemic properties, increasing the probability of the uptake of this compound by plants compared to that of clothianidin.

Only 20.8% (5 of 24 samples) of the nectar samples obtained from wildflowers adjacent to OSR crops contained thiamethoxam, and the concentrations for this compound [0.10 ± 0.37 ng/g (Table 2)] were significantly lower than for OSR nectar [3.20 ± 4.61 ng/g; via the M−W test, $U(35) = 94.5$, $P = 0.049$, and $Z = -2.3$ (Table S3a,b)]. We also found clothianidin in 20.8% of the nectar samples and thiacloprid in 25%, the latter presenting lower levels (all detected levels were below the MQL) than in OSR nectar [0.24 ± 0.36 ng/g; via the M−W test, $U(35) = 90.0$, $P = 0.036$, and $Z = -2.47$]. The number of nectar samples obtained from wildflowers adjacent to WW was low ($N = 8$), and none of them contained neonicotinoid residues. The lower prevalence of neonicotinoid residues in nectar samples in comparison with pollen both in OSR flowers and in wildflowers growing in the field margins may be due to the shorter half-life of these compounds in aqueous matrices caused by higher rates of hydrolysis, photolysis, and microbial degradation.[32]

Given that field margin soils were found to be consistently contaminated with all of the commonly used neonicotinoids, this is the mostly likely source of wildflower contamination. Three previous studies have demonstrated neonicotinoid contamination of wild plants growing in field margins or surrounding areas of seed-treated crops, but in these studies, the whole flower was analyzed[33] or the information about the part of the plant analyzed was not provided;[14,34] therefore, the concentrations found in the nectar or pollen and the subsequent exposure to bees were not clear. Our study marks a significant step toward understanding the prevalence and concentrations of neonicotinoid residues present in pollen and nectar from nontarget plants, which are essential foraging sources for bees.[35]

Pollen Collected by Honey Bees. Pollen traps were used to collect pollen brought back to honey bee hives placed on the five farms, both during the OSR blooming period (beginning of June 2013) and later in the summer (mid-August 2013). Identification of pollen types revealed that the majority of pollen collected by honey bees in June was *Crataegus monogyna* (62.5%), with just 9.9% of pollen coming from OSR (Table S6a,b). Previous studies have indicated that honey bees may not use OSR flowers as a major source of pollen,[36] but their frequent presence as pollinator visitors in OSR crops[37−39] could indicate that they may forage in OSR flowers mainly to collect nectar.[40] In August, the pollen loads were more diverse (Simpson's index of Diversity, 1-D = 0.85) than in June (1-D = 0.54), comprising a range of wildflowers, with *Epilobium hirsutum* (23.1%) and *Rubus fruticosus* (13.5%) being the most visited plants. Honey bee-collected wildflower pollen commonly contained thiamethoxam, clothianidin, imidacloprid, and thiacloprid, but mean concentrations of total neonicotinoid residues were generally lower [mean ± standard deviation (SD), 1.48 ± 4.56 ng/g] compared to those in pollen collected by hand from field margin wildflowers [6.85 ± 18.40 ng/g; via the M−W test, $U(171) = 2635.0$, $P = 0.001$, and $Z = -3.389$] or from the crop [7.20 ± 5.08 ng/g; via the M−W test, $U(94) = 110.5$, $P < 0.001$, and $Z = -6.037$ (Figure 2)]. This is to be expected because bees will have been foraging over a large area, visiting patches of wildflowers that were not adjacent to crops, resulting in a dilution effect. It is notable that a significant drop in the concentrations of neonicotinoids detected in wildflower pollen was observed between June (3.09 ± 6.45 ng/g) and August [0.20 ± 0.43 ng/g; via the M−W test, $U(78) = 339.0$, $P < 0.001$, and $Z = -4.358$], perhaps suggesting a reduction in plant tissue concentrations through summer because of photolysis[41] and increasing temperatures.[26]

Of the total neonicotinoid residues present in the pollen collected by honey bees in June (287 ng of residues in 514 g of pollen; 0.56 ng of residues/g of pollen), only 3% had its origin in the OSR pollen, the remaining 97% coming from wildflowers. In August, all identified pollen taxa were wild plants (Tables S6a,b), residue levels were lower than in June, and the amount of pollen collected was smaller (44.28 ng of residues in 224.84 g of pollen; 0.20 ng of residues/g of pollen). If one considers these values in terms of the quantity of neonicotinoid residues entering hives per day, honey bee foragers brought back an amount of 71.8 ng of residues per day in June, and 11.1 ng per day in August. According to our current understanding, these concentrations are lower than those likely to cause significant harm to honey bee colonies in the short term,[30,10] as for instance the oral LD_{50} values (dose required to kill 50% of a population of test animals in 48 h) for thiamethoxam and clothianidin in honey bees are 5 and 3.7 ng/bee, respectively.[42] Considering the mean values for neonicotinoid content in corbicular pollen collected during oilseed rape bloom in this study (0.56 ng/g), a honey bee would need to eat ∼10 g of pollen to obtain an LD_{50} dose, which is unlikely because honey bees consume <10 mg of pollen per day.[43,44] However, it should be noted that these figures do not include the residues brought back to the hive in nectar, and that a long-term chronic exposure to field realistic sublethal levels of thiamethoxam (5.31 ng/g) and clothianidin (2.05 ng/g) has been shown to cause an impact on honey bee colony performance and queen supersedure.[45] It is also worth mentioning that the number of colonies we used to evaluate levels and origin of exposure to neonicotinoids on honey bee colonies was limited, and because the overall foraging pattern may differ among colonies placed on the same landscapes due to varying factors,[46,47] a different outcome with another experimental design cannot be ruled out. Likewise, exposure of other bee species in this landscape will depend on their foraging range and floral preferences and may be quite different.

Commercial Oilseed Rape, Wheat, and Barley Seeds. Analysis of thiamethoxam-dressed OSR seeds revealed contamination with clothianidin (a breakdown product of thiamethoxam) but also imidacloprid and thiacloprid (Table S7). Most surprisingly, samples of OSR, winter wheat, and barley seeds that had not been treated with neonicotinoids and had been dressed only with fungicides also contained residues of various mixtures of neonicotinoids, albeit at concentrations much lower than those found in dressed seeds. This may result from contamination via the machinery used to dress or count the seeds, as suggested in a previous study in which a similar contamination was detected in cotton seeds,[34] or perhaps residues remaining from treatments of the crop from which the seeds were harvested. The role of these additional neonicotinoid residues present in coated seeds as a potential source of environmental contamination warrants further research.

Previous field studies of the impacts of neonicotinoids on bee colonies have often suffered from contamination of control colonies.[12,48] Our study provides a potential explanation for this widespread presence of residues in bee colony food stores; much of the exposure of free-flying bees is likely to be caused by residues in wildflowers, which cannot readily be manipulated. Under these circumstances, we would not expect any differences in the performance of colonies placed next to experimental plots of treated versus untreated crops, unless the experiment is performed in a landscape where minimal neonicotinoids have been used previously.[14]

Farmers are often encouraged to sow wildflower strips in arable field margins as a means of boosting pollinator populations and to attract and conserve natural enemies of arthropod pests.[49,50] Our data suggest that such wildflowers are likely to be contaminated with neonicotinoids; whether the benefits accrued from providing more food and suitable habitat would exceed the cost via impacts of the pesticide is unclear. However, when possible, it would seem best to promote the creation of wildflower patches that are not adjacent to treated crops or on soil in which treated crops have previously been grown to avoid exposure to neonicotinoid residues via this route.

Overall, our results demonstrate that the application of neonicotinoid seed dressings to autumn-sown arable crops results in contamination of pollen and nectar of nearby wildflowers throughout the following spring and summer, and that wildflowers were the major route of exposure for bees in this study. It has been suggested that chronic intake of neonicotinoid pesticides may lead to weakening and failure in bee colonies,[45,51] but the consequences of prolonged exposure to mixtures of these compounds in wildflower pollen and nectar have not been examined in any field study conducted to date. Furthermore, widespread contamination of wild plants and soil is also likely to lead to chronic exposure of a broad range of nontarget invertebrates in farmland.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03459.

Additional details for materials and methods, and tables and figures as noted in the text (PDF)

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Notes

The authors declare no competing financial interest.

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