# Fate of Six Neonicotinoids During Full-scale Wastewater Treatment and Passage Through an Engineered Wetland

by

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ABSTRACT

Six high-production-volume neonicotinoids were traced through a municipal wastewater treatment plant (WWTP) and engineered wetland located downstream, in a study motivated by reports on these insecticides posing threats to non-target invertebrate species and potentially playing a role in the global honeybee colony collapse disorder. An array of automated samplers was deployed in a five-day monitoring campaign and resultant flow-weighted samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using the isotope dilution method. Concentrations in WWTP influent and effluent were 54.7 ± 2.9 and 48.6 ± 2.7 ng/L for imidacloprid, respectively, and 3.7 ± 0.3 and 1.8 ± 0.1 ng/L for acetamiprid, respectively. A mass balance over the WWTP showed no ($p$=0.09, $CI$ = 95%) removal of imidacloprid, and 56 ± 6% aqueous removal of acetamiprid. In the constructed wetland downstream, a lack of removal was noted for both imidacloprid (from 54.4 ± 3.4 ng/L to 49.9 ± 14.6 ng/L) and acetamiprid (from 2.00 ± 0.03 ng/L to 2.30 ± 0.21 ng/L). Clothianidin was detected only inconsistently in the WWTP and wetland (>2 to 288 ng/L; 60% detection frequency), whereas thiamethoxam (<10 ng/L), thiacloprid (<2 ng/L), and dinotefuran (<180 ng/L) were not detected at all. Thus, imidacloprid and acetamiprid were identified as recalcitrant sewage constituents (estimated U.S. WWTP discharge of 1920- 4780 kg/y) that persist during conventional wastewater treatment to enter U.S. surface waters at potentially harmful concentrations.

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## Chapter 1

## INTRODUCTION

Neonicotinoids are the world's most widely used insecticides, with global production valued at 2.5 billion dollars, and registration in more than 120 countries for commercial use on more than 140 crops. They are neurotoxic insecticides used for control of aphids, whiteflies, planthoppers, lepidoptera, and some coleopteran pests, among others. They can be applied as seed treatment, foliar treatment, soil injection, trunk application, and drench/drip application (Jeschke, Nauen et al. 2011). Neonicotinoids act on nicotinic acetylcholine receptors, disrupting synaptic transmission (Matsuda, Buckingham et al. 2001). The vertebrate nAChR is an agonist-gated ion channel responsible for rapid excitatory neurotransmission. The neonicotinoids have an electronegative tip consisting of a nitro or cyano pharmacophore, which binds to a unique cationic subsite of the insect receptor and disrupt excitatory cholinergic neurotransmission, imparting potency (Tomizawa and Casida 2005).

In December, 2013 the European Commission introduced a 2-year moratorium on clothianidin, imidacloprid, and thiamethoxam following reports by the European Food Safety Authority (EFSA) saying the substances pose an "acute risk" to honey bees essential to farming and natural ecosystem (EU Regulation No 485/2013). Adverse effects on many non-target organisms like phloem feeding insects (Bonmatin, Giorio et al. 2015), pollinators and bees (van der Sluijs, Simon-Delso et al. 2013), and aquatic invertebrate (Morrissey, Mineau et al. 2015) due to widespread use of neonicotinoids have been recently reported. Neonicotinoids cause excitation of the insect nerves, leading

to trembling and shaking, paralysis, and ultimately death. Median lethal dose values ($LD_{50}$) of neonicotinoids for bees varies from 5-70 ng/bee (Suchail, Guez et al. 2001). Sub-lethal doses cause ATP synthesis inhibition, resulting in impairment of foraging success, memory and learning, damage to the central nervous system, and increased susceptibility to diseases (van der Sluijs, Simon-Delso et al. 2013). According to a recent review, based on 214 toxicity tests of 48 species, average individual environmental concentration greater than 35 ng/L can severely affect sensitive aquatic invertebrate populations (Morrissey, Mineau et al. 2015). A recent study observed that aquatic macrofauna populations dropped sharply at concentrations between 13 and 67 ng/L (Van Dijk, Van Staalduinen et al. 2013). Insectivorous birds are also susceptible to exposure through the food chain(Goulson 2014). A study in the Netherlands observed a decline in bird population after the introduction of imidacloprid, the highest production volume insecticide in the world; imidacloprid concentrations of greater than 20 ng/L correlated with 3.5% average annual declines in bird populations (Hallmann, Foppen et al. 2014). Co-occurrence of multiple neonicotinoids is known to impart synergistic toxic effects (van der Sluijs, Amaral-Rogers et al. 2015).

During the past decades global contamination of neonicotinoids has been observed in surface water (Bonmatin, Giorio et al. 2015). In a 2013 study in Canada, neonicotinoids were detected in 91% of samples gathered from wetlands from the central-eastern region of Saskatchewan with a total average concentration of 52.7 ng/L ($n$=90) (Main, Headley et al. 2014). In several rivers around Sydney, Australia total average neonicotinoid concentrations of 118 ng/L were detected; imidacloprid was the most common neonicotinoid, detected in 93% of samples ($n$=15) (Sanchez-Bayo and Hyne

2014). In California imidacloprid was detected in 89% of surface water samples ($n$=75) in which 19% samples exceeded concentrations of 1.05 μg/L, the United States Environmental Protection Agency's chronic invertebrate Aquatic Life Benchmark value (Starner and Goh 2012). In Spain imidacloprid was detected in river water receiving wastewater treatment plant effluent at a maximum concentration of 19.2 ng/L, identifying wastewater treatment plants (WWTPs) as a potential but not well established source of neonicotinoids in the environment (Masiá, Campo et al. 2013).

The goal of the present study was to assess the presence of six neonicotinoids – imidacloprid, thiamethoxam, clothianidin, acetamiprid, thiacloprid and dinotefuran in a major metropolis in the southwestern U.S., and to trace their fate and transport through a conventional wastewater treatment train and engineered wetland located immediately downstream. We hypothesized that neonicotinoids in addition to being present in agricultural runoff, also may occur at detectable levels in urban wastewater, due to their use for control of insects on daily consumable products like rice, fruits, tea, and vegetables, for horticulture and grass management applications, as well as for domestic pet flea control (Jeschke, Nauen et al. 2011). Effluent of WWTPs is discharged into surface waters used by animals, plants, or other organisms, thereby posing a potential source of exposure to neonicotinoids. To determine the occurrence of neonicotinoids in various stages of wastewater treatment infrastructure commonly used in the U.S. and around the world, we developed a method for detection of six neonicotinoids and studied their behavior during passage through a conventional WWTP and engineered wetland downstream.

## Chapter 2

## MATERIALS AND METHODS

### Chemicals and Reagents.

Organic solvent of high performance liquid chromatography (HPLC) grade and formic acid of American Chemical Society (ACS) grade (98%) were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. Ultrapure LC-MS grade water was purchased from Thermo Fisher Scientific, Waltham, MA, USA. Analytical standards for six neonicotinoids and deuterated labeled standards for imidacloprid (imidacloprid-$d_4$, Chemical Abstracts Service (CAS) number 1015855-75-0) and acetamiprid (acetamiprid-$d_3$, Molecular Design Limited (*MDL*) number MFCD17019132) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Stock solutions of analytical standards and their mixtures were prepared in acetonitrile and stored at -20$^{o}$C.

### Sample Collection.

Sampling was conducted in early December 2014 for a period of five consecutive days (Thursday through Monday) at a large activated sludge sewage treatment plant and an engineered wetland downstream, located in the southwestern region of USA. The plant is designed to serve a population of over 2.57 million with design capacity of 870 million liters per day, received sewage being comprised of 94% domestic wastewater and 6% industrial wastewater, and producing Class B+ reclaimed water discharged into a river and Class B sludge used for land application. The treatment plant consists of 5 parallel but similar treatment trains, merging before discharge into the constructed wetland. Unit processes performed at the WWTP include: screening, grit removal, primary

sedimentation, activated sludge biological treatment, secondary clarification, chlorine disinfection, centrifuge thickening of primary sludge and waste activated sludge, anaerobic sludge digestion, and centrifuge dewatering of digested sludge. Primary sludge and waste activated sludge are digested at 35$^{o}$C, with an average solid retention time of 21 days. Average values of carbonaceous biochemical oxygen demand (cBOD) for plant influent and wetland effluent were 292.4 ± 18.4 mg/L and 6.5 ± 1.0 mg/L respectively, demonstrating cBOD removal of 97.8 ± 0.4%. Average values of total suspended solid particles for plant influent and wetland effluent were 442.8 ± 122.2 mg/L and 13.4 ± 2.3 mg/L, respectively, demonstrating total suspended solids (TSS) removal of 96.8 ± 1.0 %.

The treatment train on which sampling was conducted received wastewater at a flow rate averaging 230 ML/D. Seven portable automated samplers (6712 Full-Size Portable Sampler, Teledyne Isco, Lincoln, NE, USA) were programmed based on three-week average hourly–daily flow rate data to collect 2.5 liters of flow weighted composite samples of primary influent, primary effluent, secondary effluent, waste activated sludge, tertiary effluent, wetland influent and wetland effluent over a period of 24 hours for 5 consecutive days. Samples were collected into pre-cleaned 2.5 liter amber wide- glass mouth bottles. Grab samples of primary sludge and dewatered sludge were collected into pre-cleaned 1 liter amber glass bottles and 40 ml amber VOA glass vials, respectively.

After collection, samples were placed into coolers and shipped to the laboratory, where 600 mg/L Kathon preservative(Groot and Weyland 1988) and 80-100 mg/L sodium thiosulfate (MacCrehan, Bedner et al. 2005) were added to disinfect and dechlorinate the samples, respectively, preventing biological and chemical degradation of

analytes during storage. Then, 500 mL of sample aliquots were fortified with 200 ng of the deuterated surrogate standards imidacloprid-$d_4$, acetamiprid-$d_3$ to account for losses during storage, extraction and analysis. Solid samples were fortified with 400 ng per gram of the surrogate standards. All samples were stored at 4$^{o}$C prior to processing.

**Sample Extraction and Cleanup.**

**Extraction of Water Samples.** An automatic solid-phase extraction instrument (Dionex AutoTrace 280, Thermo Scientific, Waltham, MA, USA) was used to concentrate and elute analytes from water samples from the sorbent bed for analysis. Following screening of extraction efficiency of a combination of sorbents and sample volumes, reverse-phase, functionalized polymeric styrene divinylbenzene sorbent (Strata X & XL, 500 mg/3 mL, Phenomenex, Torrance, CA, USA) was selected and loaded with 500 mL of sample. Before loading, cartridges were conditioned with 3 mL methanol, followed by 3 mL water. Then, water samples were loaded onto the cartridges at a flow rate of 2 mL/min, washed with water, and dried with nitrogen gas for 5 minutes. Two consecutive elutions were performed, each with 4 mL of methanol and formic acid mixture (95:5, v/v). Equal volumes of serial eluates were combined, evaporated, and reconstituted to half the volume of water and methanol solution (80:20, v/v) in 0.1% formic acid for LC-MS analysis. Waste activated sludge and primary sludge samples featuring approximately 2 and 6% TSS content, respectively, were centrifugated at 7500 *g* for 10 minutes. Resultant supernatants and solids were extracted separately.

**Extraction of Solid Samples.** Solid samples were dried under nitrogen using an evaporator (Reacti-Therm TS-18821, Thermo Scientific, Waltham, MA, USA). Later,

1 gram of solids was transferred into 40 mL VOA vials and extracted into 10 mL acetone for 24 hours followed by 1 hour of sonication. Resultant solutions were centrifugated and supernatants transferred into another vial. To maximize analyte recovery, 10 mL acetone was added again to the extracted solids, vortexed for a minute, centrifugated, and the resultant supernatant combined with the first extract. After exchanging solvents from acetone to 6 mL of hexane, extract cleanup was performed by solid phase extraction (EPA Method 1698, USA) with a sorbent bed featuring a blend of magnesium oxide and silica gel (Sep-Pak Vac Florisil Cartridge 6 cc containing 1 g of sorbent, Waters Corporation, Milford, MA, USA). Before loading, the sorbent was conditioned successively with 6 mL methylene chloride (DCM), 6 mL acetone and 6 mL hexane. Extracts in hexane were loaded, the resin bed washed with 6 mL of hexane and analytes eluted subsequently with 4 mL DCM and 4 mL acetone. Lastly, from resultant eluates 1 mL extracts were transferred into separate 2 mL vials, dried under a gentle stream of nitrogen, and reconstituted with 1 mL of a solution of water, methanol and formic acid (80/20/0.1, v/v/v) for analysis.

**Liquid Chromatography Separation.**

Separation was carried out using a Shimadzu Ultra Performance Liquid Chromatography (UPLC) system, equipped with the SIL-20AC autosampler and 20-AD solvent delivery system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Simultaneous chromatographic separation for all six neonicotinoids was performed by reverse phase liquid chromatography using a 4.6 x 150 mm $C_8$ column (XBridge, Waters Corporation Milford, MA, USA) with 3.5 µm bridged ethylene hybrid (BEH) particles. A

binary gradient with acidified water and methanol (100:0.1, v/v) at a total flow rate of 0.5 mL/min was applied. The mobile phase consisted of 20% organic with an initial 1-min ramp of 10% solvent content increase $min^{-1}$, followed by a 6-minute ramp of 10.8% $min^{-1}$ to 95% organic, where it was held for 3.5 min, for a total run time of 14 min.

**Tandem Mass Spectrometry.**

Identification and quantitation were performed using an API 4000 tandem mass spectrometer (ABSciex, Framingham, MA, USA) in positive electrospray (ESI+) mode by monitoring the first and second most abundant ion transitions for quantification and confirmation, respectively. Mass spectrometry was performed at a source heating temperature of 700°C, ion spray voltage of 4500 V, curtain gas (nitrogen) pressure of 50 psi, nebulizer gas pressure of 90 psi, heater gas pressure of 75 psi, and dwell time of 70 ms. Analyst software, version 1.5 (ABSciex, Framingham, MA, USA) was used for LC-MS/MS system control and data analysis.

**Quantification, Isotope Dilution, Method Validation and Quality Assurance.**

Quantification was performed using 8-point, linear calibration curves for each analyte in the specific concentration range of interest. Calibration curve with a coefficient of determination $R^2 > 0.99$ was considered satisfactory. When background signal was detected in field blank and instrumental blank, detected concentrations in samples were corrected by background subtractions. For imidacloprid and acetamiprid isotope dilution technique was utilized to determine losses during extraction and to compensate for ion suppression during the LC-MS/MS detection. Water samples were spiked with 200 ng of each deuterated isotopes (imidacloprid-$d_4$, and acetamiprid-$d_3$). Calibration samples were

spiked with 50 ng of labeled standards for relative recovery determination of isotopes. Calibration curves for imidacloprid and acetamiprid were built by plotting the area ratio of analyte with the internal standard (IS) to the concentration of each analyte. Similarly, solid samples were spiked with 400 ng of isotope. For the other four analytes not having labled standards, the method of standard addition was performed to compensate for ion suppression during analysis.

Concentrations below detection limits were considered to be half of the method detection limit for calculation purposes.

Relative percentage difference (RPD) was determined with the following equation to determine precision between samples and duplicates.

$$\text{RPD, \%} = \frac{C_{sample} - C_{duplicate}}{\frac{C_{sample} + C_{duplicate}}{2}} \times 100 \quad (1)$$

where $C_{sample}$ and $C_{duplicate}$ are the detected concentrations in the original sample and its duplicate, respectively.

**Mass Balance Calculations.**

Analyte mass balances were performed for the full-scale wastewater treatment train, combining primary, secondary and tertiary treatment, using the following equation:

$$\dot{m}_{transformed} = Q_{1'inf} \times C_{1'inf} - Q_{3'eff} \times C_{3'eff} - \dot{m}_{DWS} \quad (2)$$

where,

$\dot{m}_{transformed}$ = mass input of neonicotinoids lost to transformation (g/day)

$Q_{1'inf}$ = flowrate of influent to primary clarifier (L/day)

$C_{1'inf}$ = concentration of neonicotinoids in influent entering primary clarifier (g/L)

$Q_{3'eff}$ = flowrate of tertiary effluent after chlorine disinfection (L/day)

$C_{3'eff}$ = concentration of neonicotinoids in tertiary effluent leaving treatment plant (g/L)

$\dot{m}_{DWS}$ = mass of neonicotinoids accumulated in digested dewatered sludge (g/day)

= $M_{DWS}$ x $C_{DWS}$

$M_{DWS}$ = mass of dewatered sludge produced (kg/day)

$C_{DWS}$ = concentration of neonicotinoids in dewatered sludge (g/kg)

Mass balance for wetland was calculated by following equation.

$$\dot{m}_{lost} = Q_{WL,inf} \text{ x } C_{WL,inf} - Q_{WL,eff} \text{ x } C_{WL,eff} \quad (3)$$

where,

$\dot{m}_{lost}$ = mass input of neonicotinoids lost to transformation and accumulation during passage through wetland (g/day)

$Q_{WL,inf}$ = flowrate of influent entering wetland (L/day)

$C_{WL,inf}$ = concentration of neonicotinoids in influent entering wetland (g/L)

$Q_{WL,eff}$ = flowrate of effluent leaving wetland (L/day)

$C_{WL,eff}$ = concentration of neonicotinoids in effluent leaving wetland (g/L)

Mass balances for primary and secondary treatment were calculated using the equations 3 and 4, respectively:

$$\dot{m}_{PT,transformed} = Q_{1'inf} \text{ x } C_{1'inf} - Q_{1'eff} \text{ x } C_{1'eff} - Q_{PS} \text{ x } C_{PS} \quad (4)$$

where,

$\dot{m}_{PT,transformed}$ = mass input of neonicotinoids lost to transformation during primary treatment (g/day)

$Q_{1'eff}$ = flowrate of primary effluent leaving primary clarifier (L/day)

$C_{1'eff}$ = concentration of neonicotinoids in effluent leaving primary clarifier (g/L)

$Q_{PS}$ = flowrate of sludge leaving primary clarifier (L/day)

$C_{PS}$ = concentration of neonicotinoids in primary sludge (g/L)

$= C_{PS,aq} + (C_{PS,particulates} \times TSS_{PS})$

$C_{PS,aq}$ = concentration of neonicotinoids in aqueous phase of primary sludge (g/L)

$C_{PS,particulates}$ = concentration of neonicotinoids in sorbed phase of primary sludge (g/g-solids)

$TSS_{PS}$ = concentration of total suspended particles in primary sludge (g-solids/L)

$$\dot{m}_{ST,transformed} = Q_{1'eff} \times C_{1'eff} - Q_{2'eff} \times C_{2'eff} - Q_{WAS} \times C_{WAS} \quad (5)$$

where,

$\dot{m}_{ST,transformed}$= mass input of neonicotinoids lost to transformation during secondary treatment (g/day)

$Q_{2'eff}$ = flowrate of secondary effluent leaving secondary clarifier (L/day)

$C_{2'eff}$ = concentration of neonicotinoids in secondary effluent leaving secondary clarifier (g/L)

$Q_{WAS}$ = flowrate of waste activated sludge (L/day)

$C_{WAS}$ = concentration of neonicotinoids in waste activated sludge (g/L)

$= C_{WAS,aq} + (C_{WAS,particulates} \times TSS_{WAS})$

$C_{WAS,aq}$ = concentration of neonicotinoids in aqueous phase of waste activated sludge (g/L)

$C_{WAS,particulates}$ = concentration of neonicotinoids in sorbed phase of waste activated sludge (g/g-solids)

$TSS_{WAS}$ = concentration of total suspended particles in waste activated sludge (g-solids/L)

**Sludge Water Partitioning Coefficient (or Distribution Coefficient), $K_D$.**

To determine the sorption affinity of analytes onto sludge particulates, a laboratory study was conducted (EPA 1991). Ten mL aliquots of water having 1 ppm, 10 ppm and 100 ppm of all six neonicotinoids was added to 1 gram of dewatered sludge and after 10 days of shaking in absence of light at 22$^{o}$C, water and solids were analyzed to establish the partitioning behavior. To determine $K_D$ values, equation 5 was used, for all six neonicotinoids,

$$K_D = C_S / C_D \quad (6)$$

Where,

$K_D$ = distribution coefficient, L/kg

$C_S$ = sorbed concentration on the solid particulates, mg/kg dry weight of dewatered solids

$C_D$ = bulk concentration remaining after sorption, mg/L

**Statistical Data Analysis.**

To determine standard error (SE) of the population (daily average parameter data), following formula (Altman and Bland 2005) was used, where $\bar{x}$ is sample mean average and n is sample size.

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{n\,(n-1)}} \quad (7)$$

To determine the deviation value ($s_p$) for the percentage removal of masses, pooled variance was determined by following formula (Bucchianico 2014),

$$S_p^2 = \frac{\sum_{i=1}^{k}(n_i-1)s_i^2}{\sum_{i=1}^{k}(n_i-1)} \quad (8)$$

A paired, two-tailed *t*-test (alpha = 0.05) was performed to test the null hypothesis that differences in the means between paired observations of the daily mass of analyte in two different streams were distinct.

## Chapter 3

## RESULTS AND DISCUSSION

### Analytical Method Performance.

The tandem mass spectrometry method developed for this study targeted six neonicotinoids at part-per-trillion levels simultaneously with monitoring of two ion transitions by multiple reaction monitoring (MRM). Mass spectrometry parameters optimized for detection are shown in Table 1.

Table 1

*Mass Spectrometric Parameters for Detection of Six Neonicotinoids and Two Isotope-Labeled Surrogate Standards*

| analyte | $Q$ *(m/z)* | $Q_1$ *(m/z)* | $Q_2$ *(m/z)* | $t_R$ (min) | DP (V) | CE (V) | EP (V) | CXP (V) |
|---|---|---|---|---|---|---|---|---|
| acetamiprid | 223.1 | 126.0 | 99.0 | 7.95 | 56 | 31 | 15 | 6 |
| clothianidin | 250.0 | 169.0 | 132.0 | 7.70 | 50 | 30 | 8 | 8 |
| dinotefuran | 203.0 | 129.3 | 113.1 | 6.06 | 50 | 30 | 15 | 8 |
| imidacloprid | 256.0 | 175.1 | 209.2 | 7.50 | 50 | 30 | 10 | 8 |
| thiacloprid | 253.0 | 126.0 | 73.1 | 8.27 | 50 | 30 | 15 | 12 |
| thiamethoxam | 292.0 | 211.1 | 181.0 | 7.01 | 50 | 30 | 8 | 8 |
| internal standards | | | | | | | | |
| imidacloprid-$d_4$ | 261.0 | 214.0 | 180.0 | 7.50 | 76 | $25^F$, $33^S$ | 6 | $4^F$, $8^S$ |
| acetamiprid-$d_3$ | 226.0 | 125.9 | 99.0 | 7.95 | 61 | $31^F$, $55^S$ | 15 | $10^F$, $8^S$ |

*Q* mass-to-charge ratio *(m/z)* of precursor ion; $Q_1$ *m/z* of most abundant fragment ion; $Q_2$ *m/z* of second most abundant fragment ion; $t_R$ retention time; DP declustering potential; CE collision energy; EP entrance potential; CXP collision cell exit potential; $^{F}$ quantifier ions; and $^{S}$ qualification ions.

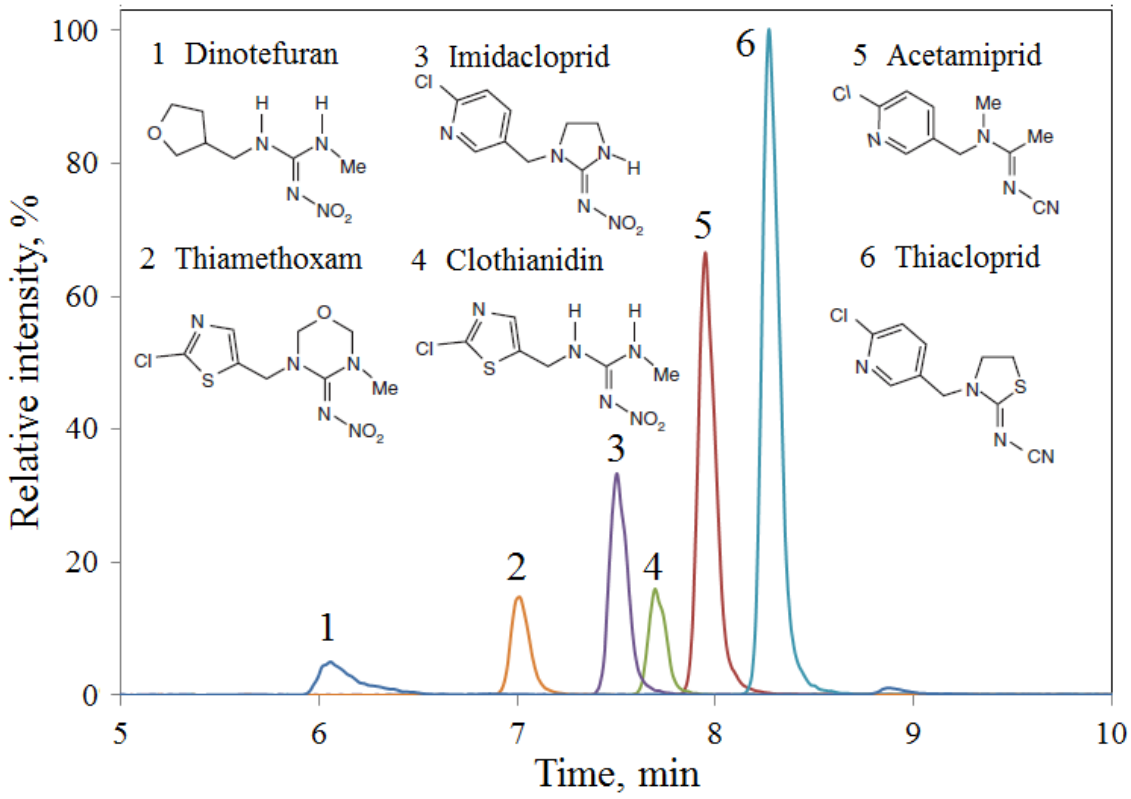


*Figure 1.* Chromatograms of all six neonicotinoids representing distinguishable separation; relative response for 5 ppb of each analyte in 1 mL solution of water, methanol and formic acid (80/20/0.1, v/v/v). The relative intensity has been scaled to the highest response for thiacloprid for better representation.

Table 2

*Estimated Method Detection Limits in Different Matrices*

| analyte | wastewater (ng/L) | sludge (μg/kg dry weight) |
|---|---|---|
| acetamiprid | 2 | 5 |
| clothianidin | 2 | 5 |
| dinotefuran | 180 | 200 |
| imidacloprid | 5 | 15 |
| thiacloprid | 2 | 8 |
| thiamethoxam | 10 | 15 |

Estimated limit of detection of analytes in different matrices are shown in Table 2. To assure the quality and validity of results, each analysis batch of environmental samples contained a field blank, instrument blank, and method blank. No false positives suggesting contamination were detected during the analysis of all samples. Check samples were analyzed between runs and calibration set was also repeated after each run to verify response fluctuations, if any. For imidacloprid and acetamiprid, RPD values were 25.3% and 38.9%, respectively.

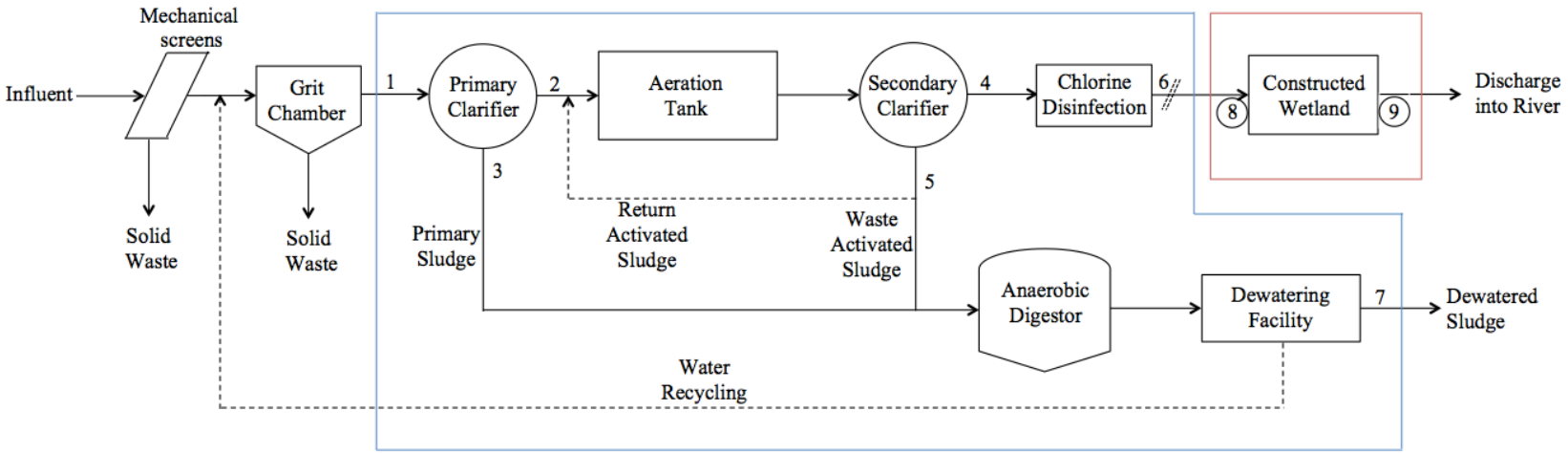




*Figure 2.* Flow diagram showing treatment processes for wastewater and sludge in the investigated activated sludge treatment plant. Numbers indicate the sampling locations used. At locations 1, 2, 4, 5, 6, 8, and 9 flow-weighted, 24-hour composite samples were collected using automated samplers. At locations 3 and 7 grab samples were collected. The blue and brown boxes represent, respectively, the control volumes used to conduct mass balances on the wastewater treatment train and an engineered wetland located immediately downstream.

**Fate of Neonicotinoids Across Wastewater Treatment Process.**

Three out of six targeted neonicotinoids, thiacloprid, thiamethoxam and dinotefuran, were absent from samples or present at levels below their respective method detection limits (Table 2) in all WWTP process streams shown in Figure 2. Consistent loading with imidacloprid and acetamiprid into the treatment facility was observed over the 5-day sampling period as shown by the data compiled in Table 3.

**Fate of Imidacloprid Across WWTP.**

During the 5-day period of sampling, concentrations of imidacloprid in plant influent fluctuated moderately between 43 and 65 ng/L. Based on the daily average flow received by the treatment train, these concentrations corresponded to 13.3 ± 0.8 grams/day of loading in the aqueous phase over the 5-day period. This mass entered the primary clarifier in which settling occurred, diverting 1% of total flow away as sludge showing a 17 times higher level of suspended solids relative to clarifier effluent. Analyte loading in primary effluent was 14.1 ± 0.8 grams/day, implying insignificant sorption on sludge and persistence during primary treatment. Secondary treatment was an activated sludge unit operation, a biological process aimed at breaking down organic compounds by microbial degradation. However, the mass of imidacloprid in secondary effluent was 11.7 ± 0.6 grams/day, implying insignificant oxidation, hydrolysis and microbial degradation in the aeration basin. A prior study also showed imidacloprid to undergo insignificant transformation in both acidic and neutral conditions. According to a laboratory study conducted at pH 7, after 3 months only 1.5% of mass was lost (Zheng and Liu 1999). To meet microbial removal criteria, the here examined facility uses a chlorine dosage of 2.5 mg/L. Although chlorine has the potential to oxidize organic

compounds, no change in imidacloprid concentration and mass was observed during the chlorination process unit, indicating resistance to oxidation under the conditions studied. Thus, during the 5-day period the average mass entering in raw sewage experienced little removal from 13.3 ± 0.9 grams to 11.7 ± 0.7 grams detected in the effluent. To determine statistical significance of the difference between change in mass of imidacloprid during treatment, paired t-test was performed, and with $p=0.09$ and $CI = 95\%$, it showed that difference was not statistically significant, implying no discernible aqueous removal of imidacloprid.

The average concentration in the aqueous phase of primary sludge was 30.7 ± 1.3 ng/L and the mass of this pesticide sorbed to sludge particles was below the method detection limit (<15 µg/kg). The aqueous phase of waste activated sludge featured imidacloprid concentrations of 22.3 ± 1.8 ng/L, with levels on the solids (particulate) fraction registering below the detection limit (<15 µg/kg), similar to findings for primary sludge. Based on the computed partitioning coefficient, the estimated concentration of imidacloprid sorbed onto solid particulates of primary sludge and waste activated sludge was 0.30 ± 0.01 and 0.22 ± 0.02 µg/kg, respectively. Therefore average daily mass of imidacloprid leaving in primary sludge and waste activated sludge was 91.1 ± 3.3 and 43.7 ± 4.5 mg/days, respectively. Primary sludge and waste activated sludge were subjected to anaerobic digestion at 35$^{o}$C for 21 days followed by dewatering. Similarly concentrations in dewatered sludge were below the detection limit (<15 µg/kg) and are estimated to be in the range of 0-0.5 µg/kg. As primary sludge and waste activated sludge were only 2% of total flow and due to less sorption of imidacloprid onto particles and high water solubility, the mass accumulated onto particles had no effect on the mass

balance of the wastewater treatment train, thus aqueous removal and total removal were similar.

Table 3

*Average Flow Rate and Average Aqueous Concentration of Imidacloprid and Acetamiprid in Wastewater Treatment and Wetland Streams (n=10). The Error Values Given Represents Standard Errors (SE).*

| process stream | flow rate (MLD)** | 5-day average aqueous concentration (ng/L) | |
|---|---|---|---|
| | | imidacloprid | acetamiprid |
| wastewater treatment plant | | | |
| influent | 243.8 ± 1.8 | 54.7 ± 2.9 | 3.7 ± 0.3 |
| primary effluent | 241.9 ± 1.8 | 58.4 ± 3.3* | 3.7 ± 0.2 |
| secondary effluent | 240.2 ± 1.7 | 48.6 ± 2.5 | 1.8 ± 0.1 |
| disinfection effluent | 240.2 ± 1.7 | 48.6 ± 2.7 | 1.7 ± 0.1 |
| engineered wetland | | | |
| influent | 283.6 ± 3.4 | 48.2 ± 1.5 | 2.1 ± 0.2 |
| effluent | 247.2 ± 6.5 | 41.5 ± 3.6 | 2.0 ± 0.1 |

*$n$=15, **$n$=5

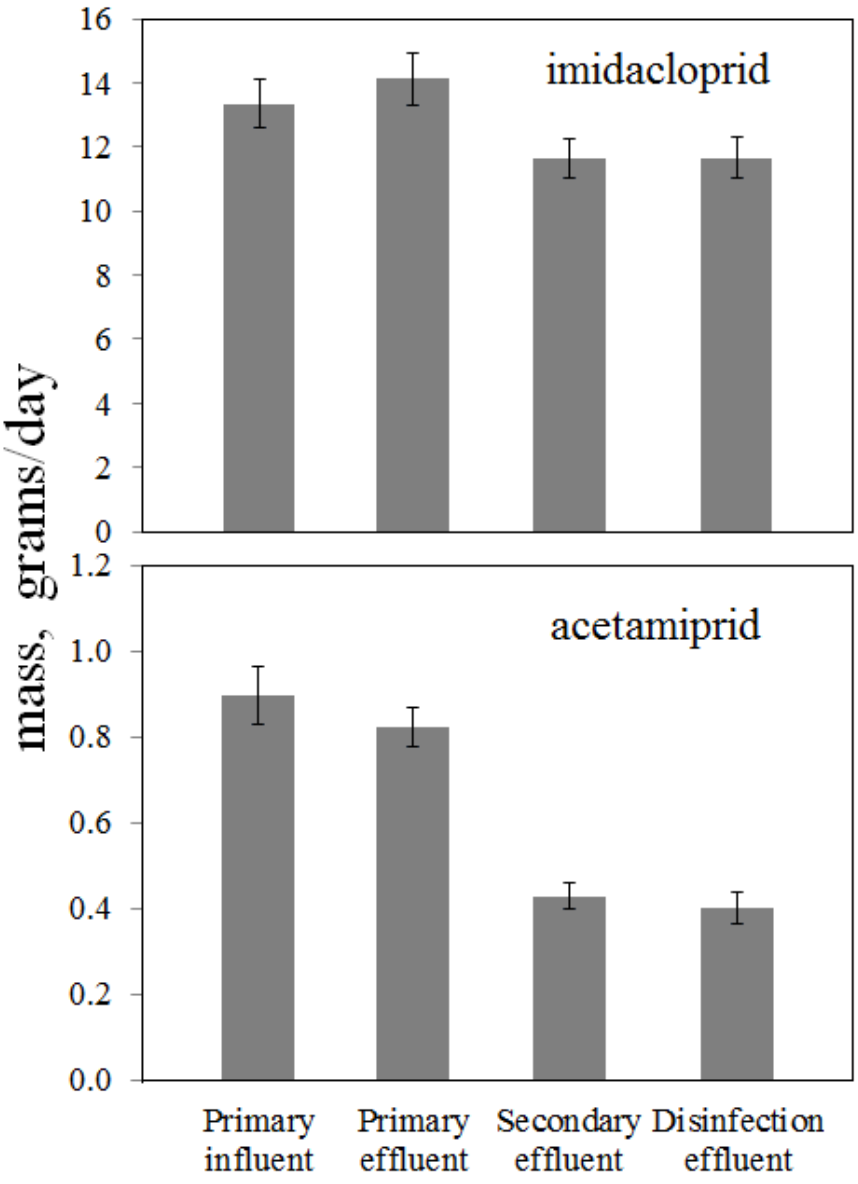


*Figure 3*. Average mass of imidacloprid and acetamiprid in wastewater streams over a 5-day period. Aqueous removal of imidacloprid and acetamiprid was less than 10% ($p$ = 0.09) and 56 ± 6% ($p < 0.01$), respectively, during tertiary wastewater treatment.

Table 4

*Partitioning and Persistence Properties of Neonicotinoids*

| analyte | Log $K_{OW}$[a] | log $K_D$ for dws | soil persistence half life, days[a] | water photolysis half life, days[a] | water dissipation half life, days[a] | GUS leaching potential index[b] |
|---|---|---|---|---|---|---|
| acetamiprid | 0.80 | 0.93 | 2-20 | 34 | 4.7 | 0.94 (very low) |
| clothianidin | 0.91 | 1.92 | 13-1386 | <1 | 40.3 | 4.91 (very high) |
| dinotefuran | -0.55 | - | 50-100 | <2 | - | 4.95 (very high) |
| imidacloprid | 0.57 | 0.99 | 104-228 | <1 | 30 | 3.76 (high) |
| thiacloprid | 1.26 | - | 9-27 | 10-63 | 8.5 | 1.44 (low) |
| thiamethoxam | -0.13 | 1.85 | 7-72 | 2.7-39.5 | 30.6 | 3.82 (high) |

dws, dewatered sludge - dry.

[a] Morrissey, Mineau et al. 2015; [b] Bonmatin, Giorio et al. 2015

**Fate of Acetamiprid Across WWTP.**

During sampling period, average concentration of acetamiprid detected in plant influent was 3.7 ± 0.3 ng/L, corresponding to an average loading of 0.90 ± 0.07 grams/day. After primary treatment, the average mass leaving the primary clarifier was 0.82 ± 0.05 grams/day. To determine the significance of difference between masses, t-test was performed and with $p$=0.06 it concluded that the difference is not statistically significant, implying no removal due to primary treatment. Prior studies have shown that acetamiprid undergoes relatively fast dissipation in neutral environment having an aqueous dissipation half-life of 4.7 days (Table 4). Similar results were observed during the secondary treatment with effluent concentration being half of the influent, confirming microbial and chemical degradation of acetamiprid in the aeration basin with a resulting mass leaving in the secondary clarifier effluent of 0.43 ± 0.03 grams/day. No change in acetamiprid concentration was observed after disinfection, causing the average mass leaving in disinfected effluent of 0.40 ± 0.04 grams/day with $p$=0.33 indicating no chemical oxidation by chlorine. The average massload of acetamiprid lost during full-scale treatment was 0.50 ± 0.08 grams/day, which was transformed or experienced dissipation and/or accumulated onto the sludge particulates. The average mass of acetamiprid for 5 day period in each treatment stream is shown in Figure 3 and resultant mass balance on the wastewater treatment train showed 56 ± 6 % aqueous removal of acetamiprid.

Upon analysis aqueous phase of the primary sludge and waste activated sludge showed concentration of 1.0 ± 0.2 ng/L and 1.5 ± 0.4 ng/L, respectively. Though corresponding sorbed concentrations onto the particulates were below the limit of

detection (< 5 µg/kg), based on experiential distribution coefficient (Table 4), predicted sorbed concentration value from Equation 5 was 8.4 ± 2.1 ng/kg for primary sludge and 12.7 ± 3.7 ng/kg for waste activated sludge. Hence, the average mass in primary sludge and waste activated sludge was 2.8 ± 0.7 mg/day and 3.0 ± 0.9 mg/day, respectively, negligible in comparison to mass in aqueous phase. The concentration of acetamiprid in dewatered sludge was below the detection limit but based on primary sludge and waste activated sludge concentration it was estimated to be in the range of 0-10 ng/kg.

**Detection of Clothianidin Across WWTP.**

Clothianidin was detected, but not consistently in all wastewater treatment streams with detection frequency ranging between 40-60% making fate determination by mass balance indeterminate. Influent concentrations of clothianidin entering treatment facility was 64.6 ± 56.2 ng/L (40% detection frequency) and corresponding effluent concentrations leaving facility was 70.4 ± 48.3 ng/L (60% detection frequency). Detected concentrations of clothianidin in all wastewater and wetland streams with corresponding detection frequency are shown in Table 5 and respective average masses over 5 day period are shown in Figure 4.

Aqueous phase of the primary sludge and waste activated sludge showed clothianidin concentration of 4.9 ± 3.9 ng/L and 3.7 ± 1.5 ng/L, respectively. Though corresponding sorbed concentrations onto the particulates were below the limit of detection (< 5 µg/kg), based on experiential distribution coefficient, predicted sorbed concentration value from Equation 5 was 41.8 ± 33.0 ng/kg for primary sludge and 27.2 ± 6.5 ng/kg for waste activated sludge. Hence, the average mass in primary sludge and

waste activated sludge was 14.5 ± 11.6 mg/day and 6.9 ± 2.3 mg/day, respectively, negligible in comparison to mass in aqueous phase. The concentration of clothianidin in dewatered sludge was below the detection limit but based on primary sludge and waste activated sludge concentration it was estimated to be in the range of 0-0.05 µg/kg.

Table 5

*Aqueous Concentration of Clothianidin in Wastewater Treatment and Wetland Streams with Respective Detection Frequency*

| process stream | range of concentrations (ng/L) | detection frequency (%) |
|---|---|---|
| wastewater treatment plant | | |
| influent | 32-288 | 40 |
| primary effluent | 178-191 | 40 |
| secondary effluent | 21-260 | 60 |
| disinfection effluent | 19-256 | 60 |
| engineered wetland | | |
| influent | 25-208 | 60 |
| effluent | 24-80 | 60 |

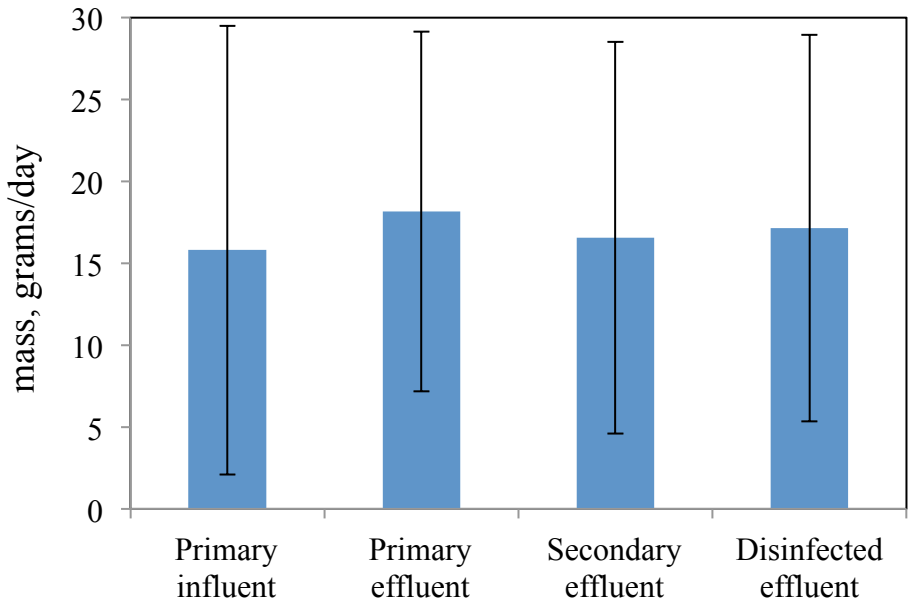


*Figure 4*. Average mass of clothianidin in wastewater streams over a 5-day period.

**Fate of Neonicotinoids Across Wetland Treatment System.**

Effluent water from all five parallel treatment trains was combined and a portion of it was directed into the engineered wetland located downstream studied having hydraulic retention time (HRT) of 4.7 days. The difference in concentration of imidacloprid between the effluent of the studied treatment train and the wetland influent was statistically insignificant ($p$=0.9, $CI$=95%), implying similar removal of imidacloprid in all treatment trains. However, for acetamiprid this difference was significant ($p$< 0.01, $CI$=95%) suggesting discrepancy in the removal between treatment trains, with overall treatment plant acetamiprid removal efficiency being 43 ± 13% vs 56 ± 9% of the treatment train analyzed. Average wastewater received and discharged by the wetland for the sampling period was around 280 MLD and 250 MLD, respectively. Considered possible mechanism triggering change in the concentration of these compounds during

passage through the constructed wetland are photodegradation, chemical transformation, biological degradation, accumulation into sediments, plant uptake, leaching into groundwater because of infiltration, and possible biotic uptake if any.

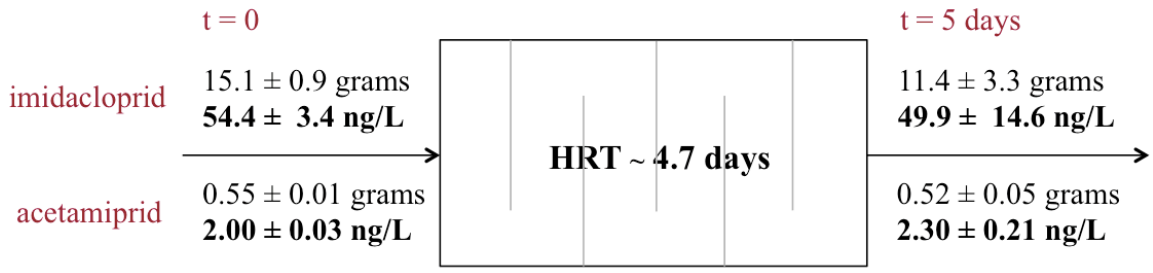


*Figure 5.* Mass and concentrations of imidacloprid and acetamiprid in engineered wetland streams, implying persistence to treatment. Wastewater treatment train and wetland were analyzed as different control volumes as the wetland received effluent from multiple parallel but similar treatment trains.

**Fate of Imidacloprid Across Wetland Treatment.**

Imidacloprid concentrations entering and leaving the engineered wetland after 5 days was 54.4 ± 3.4 ng/L and 49.9 ± 14.6 ng/L, respectively; consequent average daily mass loading and output was 15.1 ± 0.9 grams/day and 11.4 ± 3.3 grams/day as shown in Figure 5. Though studies have shown that water photolysis half-life is less than 1 day (Wamhoff and Schneider 1999), no significant removal of imidacloprid was observed after wetland treatment suggesting possible persistence in surface water bodies, too.

During the sampling period (5 days) average concentration of imidacloprid entering and leaving the engineered wetland was 48.2 ± 1.5 ng/L and 41.5 ± 3.6 ng/L, respectively; consequent average daily mass loading and output was 13.6 ± 0.4 grams/day

and 10.2 ± 0.8 grams/day. Though comparison of these results don't consider hydraulic retention time (HRT) of wetland, data consistently aligns with the HRT considered value as steady mass loading was received by the wetland, also implying no significant removal of imidacloprid.

**Fate of Acetamiprid Across Wetland Treatment.**

As shown in figure 5, the concentration of acetamiprid in influent and effluent of the wetland was 2.00 ± 0.03 and 2.30 ± 0.21 ng/L respectively, showing no decline in the concentration during the passage. Corresponding daily mass entering and leaving the wetland was 0.55 ± 0.01 and 0.52 ± 0.05 grams/day, indicating no aqueous removal by the wetland treatment. Similar consistency was observed during the five-day sampling period as the average concentration of acetamiprid in influent and effluent of the wetland was 2.1 ± 0.4 and 2.0 ± 0.2 ng/L, respectively.

**Detection of Clothianidin in Wetland.**

Clothianidin was detected in 60% composite samples during the sampling days, with concentrations ranging from 25 – 208 ng/L and 24 – 80 ng/L in the influent and effluent streams, respectively. Based on the HRT of the wetland, though concentration of clothianidin on day 1 was below the method limit of detection (< 2 ng/L) corresponding detected clothianidin concentration in effluent was 80 ng/L; making fate determination inconclusive.

**Environmental Emission of Neonicotinoids Through WWTPs.**

Based on detected concentration of neonicotinoids in treatment plant influent and population served by the studied treatment facility, the total neonicotinoid annual loading in sewage will range between 5.6-16.0 mg/person. This will correspond to national level surface water contamination by approximately 1.9-4.8 metric tons of neonicotinoids – acetamiprid, clothianidin, and imidacloprid by discharged effluent of WWTPs. Obtaining state specific information on annual trends in relative quantities used and mode of agricultural application from peer review literature was tough thus calculated emission might be under or over predicted. Crude estimates for mass loading in influent and effluent of imidacloprid, acetamiprid and clothianidin are shown in Table 6.

Table 6

*Estimated Emissions of Neonicotinoids by WWTP Effluent*

| mass estimates | value | | | total neonicotinoids |
|---|---|---|---|---|
| | imidacloprid | acetamiprid | clothianidin | |
| estimated annual mass entering the plant (kg/y) | 9.8-11.0 | 0.6-0.8 | 1.6-23.0 | 12.1-34.8 |
| estimated annual mass discharged from the plant (kg/y) | 8.6-9.6 | 0.3-0.4 | 4.2-22.6 | 13.0-32.5 |
| calculated average per-capita loading (mg/y) | 4.5-5.1 | 0.3-0.4 | 0.7-10.6 | 5.6-16.0 |
| estimated national mass loading to the WWTPs (kg/y) | 1440-1620 | 100-110 | 240-3390 | 1780-5110 |
| estimated national annual mass discharged into surface water (kg/y) | 1260-1410 | 40-50 | 610-3320 | 1920-4780 |

US population is considered 318.9 million (2014) (Source. United States Census Bureau).

**Study Limitations.**

Since portable automated samplers could not be deployed at the sludge outlet of the primary clarifier, grab samples were collected once daily. As flow and composition of wastewater may change with time, grab samples yield limited information about the daily composition of primary sludge, though acetamiprid and imidacloprid were detected consistently. But as primary sludge represented only 1% of the total flow, and for all compounds most of the mass was detected in the clarifier effluent, ambivalence in primary sludge mass would not affect the mass balance and be negligible. The mass sorbed onto the sludge particulates was calculated from theory yielding conservative estimates. Sorption coefficients were determined for dewatered sludge particles – comprised of both primary and waste activated sludge. Differences in the composition of the two sludge may result in minor differences in sorption of neonicotinoids, a phenomenon that was not further investigated here. However, regardless of the individual value, the respective $K_D$ values and volumes were not high enough to influence the mass balance significantly. Influent to primary clarifier was considered the treatment plant influent, and it can under-predict the calculated per capita loading per person, as mass lost during pretreatment, for example, grit removal, will be unaccounted for. Emission extrapolation is a function of per capita pesticides usage, state wide annual trends of relative usage, and characteristics of treatment; thus, having inherent unpredictability. No wetland sediments were collected during the study thus making determination of accumulation into sediments inconclusive.

## Chapter 4

## CONCLUSION

Imidacloprid, thiamethoxam, clothianidin, and acetamiprid are frequently detected in global surface waters. This study detected three neonicotinoids, imidacloprid, acetamiprid and clothianidin in raw wastewater and in WWTP effluent. According to a recent study, 74% of global surface waters exhibit individual neonicotinoids concentrations exceeding 35 ng/L ($n$=17) (Morrissey, Mineau et al. 2015). Treated waste discharge, according to the results of this study, could contribute to the reported global surface water contamination. Though the clothianidin loading was not consistent enough to enable performing a mass balance, it was detected during the sampling period in all treatment streams. The results of this study demonstrate the occurrence of neonicotinoids at considerable concentrations in wastewater streams at all locations within a treatment train, whereas mass balances conducted over primary, secondary, tertiary, and quaternary treatment showed these compounds to resist aerobic digestion, chlorine disinfection, and wetland attenuation. Imidacloprid migrated through the WWTP without undergoing any significant partitioning and transformation, whereas acetamiprid experienced limited aqueous removal of 56 ± 9%. Additionally, imidacloprid and acetamiprid experienced no significant mass reduction during passage through the wetland. The fates of these compounds in the wetland are illustrative of their slow natural attenuation in the environment.

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