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Neonicotinoid Pesticide and Nitrate Removal in Floating Treatment Wetlands

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NEONICOTINOID PESTICIDE AND NITRATE REMOVAL IN FLOATING TREATMENT WETLANDS

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Nutrient and pesticide concentrations in surface water are a growing concern in the Midwest. Floating treatment wetlands (FTWs) are often used to remove excess nutrients from surface water and should be considered for removal of emerging contaminants, such as neonicotinoids. Therefore, the objectives for this research project were: 1) Determine FTW neonicotinoid removal capacity, 2) Quantify neonicotinoid incorporation into floating macrophytes, and 3) Explore potential implications of neonicotinoids on microbial denitrification. A microcosm and mesocosm experiment was completed. The mesocosm experiment evaluated 3 treatments replicated 3 times (9 mesocosms) over a 21-day period. Treatments were: 1) FTW mesocosm with neonicotinoids, 2) FTW without neonicotinoids, and 3) mesocosm with neonicotinoids and without FTWs. All mesocosms were given a pulse enrichment of 10 ppm of nitrate-nitrogen while mesocosms with neonicotinoids also received a pulse enrichment of 100 ppb of the neonicotinoids imidacloprid and thiamethoxam. In contrast, microcosms evaluated the denitrification potential of roots and mesocosm water with and without neonicotinoids. FTW mesocosms exhibited significant removal of imidacloprid (38.3 ± 13.6%) when compared to mesocosms without FTWs. However, for thiamethoxam, mesocosms with and without FTWs had no significant difference in removal, with thiamethoxam removal of 38.0 ± 4.2% and 41.1 ± 2.0%, respectively. For imidacloprid

and thiamethoxam, 23.4% and 8.8%, respectively, was found in the above surface biomass and 6.9% of imidacloprid and 5.2% of thiamethoxam was stored in the below surface biomass. Neonicotinoid metabolites found in FTW biomass included imidacloprid desnitro, imidacloprid urea, and clothianidin. Significant differences were not observed in the nitrate-N removal potential of FTWs when neonicotinoids were present. Additionally, neonicotinoids did not have a significant effect on the abundance of nitrifying and denitrifying genes in the water surrounding FTW roots. The microcosm experiment indicated that denitrifying potential of FTW roots did not change with the addition of neonicotinoids. FTWs have potential to remove neonicotinoids from surface water through biomass incorporation. No evidence supports that the presence of neonicotinoids in the water column lowers the nitrate removing potential of FTWs.

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CHAPTER 1: Neonicotinoid Transport in Surface Water and Potential Removal within Wetland Systems: A Review

Introduction

Urban and agricultural fertilizer application has led to excess nitrogen and phosphorus in surface water, causing a rise of toxic and harmful algae blooms (HAB) in midwestern lakes (Carpenter, 2008). The death of algae and decomposition by microorganisms results in low dissolved oxygen in lakes and, in turn, fish kills (X. e. Yang, 2008). New innovations in best management practices (BMPs) have resulted in improved in-situ treatment practices. One example of in-situ treatment practices is floating treatment wetlands (FTWs). FTWs are used for nutrient removal in eutrophic lakes but may also uptake some contaminants of emerging concern (CECs) such as insecticides. Neonicotinoids, a common insecticide, have become increasingly popular in agricultural and urban systems and their impact on BMPs effectiveness is of interest. In this review, neonicotinoid insecticide properties and frequency of detection in surface water are explored, and their fate and transport in wetland plants as well as their effect on nutrient removing microbial communities is assessed.

Floating Treatment Wetlands as Nutrient Removing BMPs

Floating treatment wetlands (FTWs) are a new technology to the United States that is currently gaining popularity to treat urban (White and Cousins, 2013; Xu et al., 2017) and agricultural (Spangler et al., 2019) runoff. FTWs are vegetative mats with native wetland plants that are placed on eutrophic lakes to remove nutrients such as nitrogen and phosphorus, while also reducing total suspended solids (TSS) (Winston et al., 2013). Microorganisms around the plant rhizospheres result in nitrogen removal by denitrification and/or plant uptake of nitrogen and phosphorus into the plants roots and

shoots (Borne et al.2013). Keizer-Vlek et al. (2014) reported removal of total nitrogen (TN) and total phosphorus (TP) of 277 mg TN m^{-2} day^{-1} and 9.32 mg TP m^{-2} day^{-1} respectively in FTW mesocosms containing *Iris pseudacorus* with constant water concentrations of 4 mg N L^{-1} and 0.25 mg P L^{-1}. Luca et al, (2019) also reported phosphorus removal in FTWs planted with *Typha domingensi;,* however, significant removal of nitrate did not occur (Di Luca et al., 2019). NO3-N reduction has been observed with values ranging from 30.2 to above 90% (Samal et al., 2019).

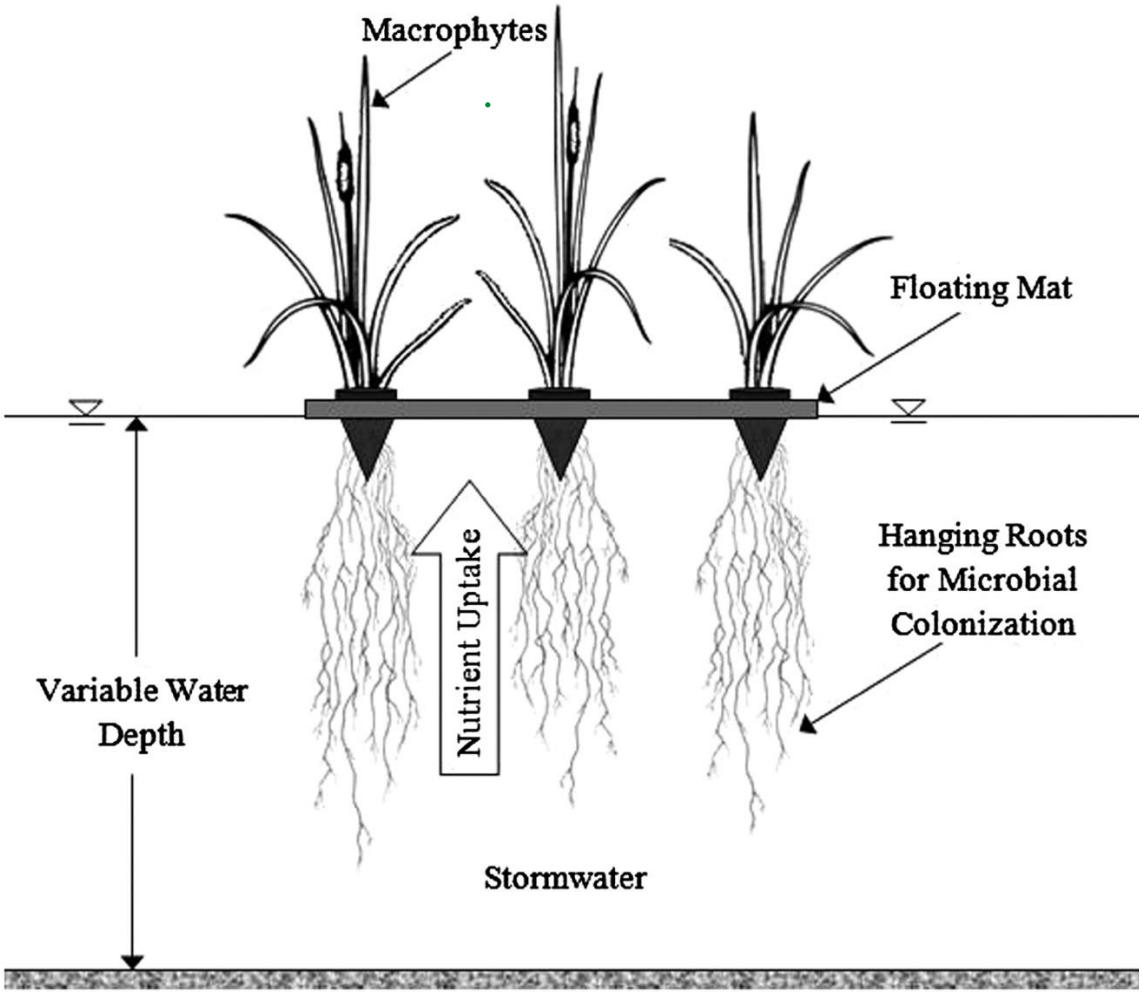

Figure 1. Floating Treatment Wetland (Taken from Hartshorn et al., 2016)

FTWs are a low-cost option for water treatment because mats can be placed directly on an impaired lake. Removal efficiency can vary depending on, plant species, hydraulic retention time, temperature, and carbon concentrations (Van De Moortel et al., 2010; Xu et al., 2017; Z. Yang et al., 2008). Plant species are important in nutrient removal. Xu et al. (2017) found *I. pseudacorus* outperformed *Thalia dealbata* having total nitrogen and total phosphorus removals of 3.95 ± 0.19 and 0.15 ± 0.01 g m^{-2} day^{-1}, respectively, for *I. pseudacorus* while removal was 3.07 ± 0.15 and 0.14 ± 0.01 g m^{-2} day^{-1} respectively for *T. dealbata* (Xu et al., 2017).

Nutrient Removal Pathways in FTW

Nitrogen and phosphorus accumulate in plant roots and shoots as the species grow. Estimates of nitrogen removal attributed to plant uptake vary by study. On the higher removal species, 74% and 60% of TN and TP are attributed to plant uptake in mesocosms with *I. pseudacorus*. (Keizer-Vlek et al., 2014). However, Gao et al, (2018) reported only 9.90% of nitrogen removal was credited to plant uptake with most nitrogen (4 times more) accumulating in the shoots rather than roots (Gao et al., 2018).

While plant uptake is a large factor in nutrient removal, microbial degradation is arguably more important to the success of FTWs due to the decomposition of plant material, and consequently, the recycling of nutrients into the system. Denitrification results in the complete removal of nitrogen to the atmosphere and is favored since nutrients are not recycled back into the system. Denitrification is an anaerobic process that requires a pH between 6-8, warm temperatures (18-24 °C), a nitrate source, and organic matter (Vymazal, 2007). The microbial community in the rhizosphere is responsible for denitrification, and the presence of denitrifying bacteria is necessary for

the process to occur. Denitrification rates decrease at colder temperatures, thus reduced rates of nitrogen removal will occur in the winter (Gao et al., 2018).

Nitrification is another important nitrogen transforming process in wetlands. Nitrification is a two step aerobic process that includes the conversion of ammonia to nitrite followed by nitrite conversion to nitrate. Reddy et al. (1989) suggests that nitrification occurs at the root surface, supported by oxygen from the plant. Microbes responsible for these processes are encoded with nitrifying and denitrifying genes (Figure 2). *AmoA* is a nitrifying gene that encodes the oxidation of ammonia to nitrite. *NirK*, *nir*S, and *nosZ* are denitrifying genes. Nitrite reductase genes include *nirK* and *nirS* and are involved in converting NO_2^- to NO. Finally, the reduction of N_2O to N_2 is encoded by *nosZ* and is the final step in denitrification (Chon et al., 2011).

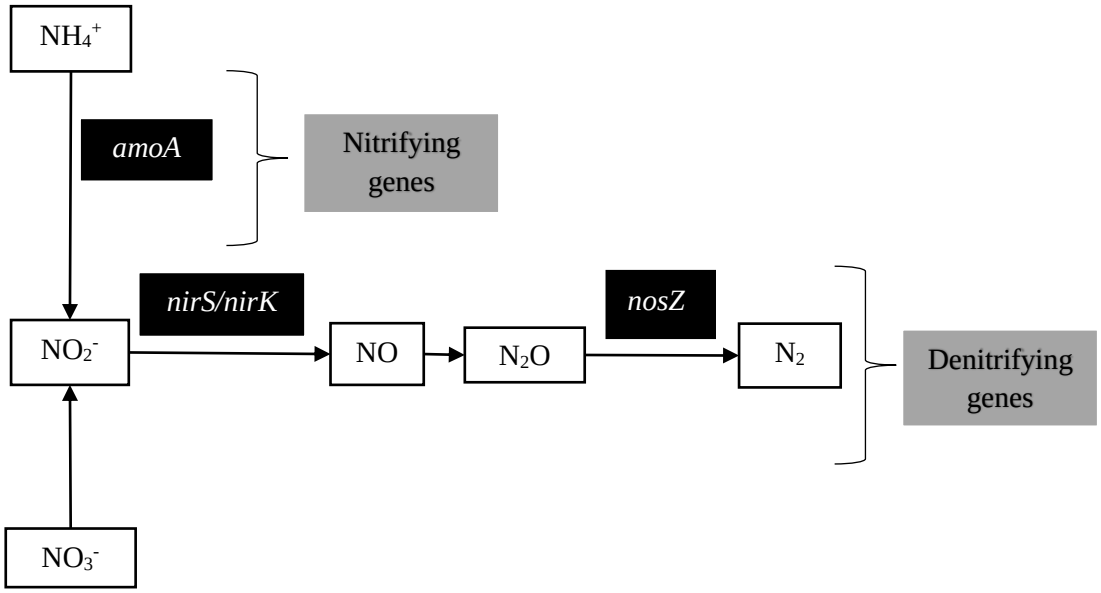

Figure 2. Nitrification and denitrification encoded genes

Neonicotinoid Development and Use

Neonicotinoids, a class of insecticides widely used in agricultural and urban settings, have been shown to bioaccumulate in aquatic environments and result in adverse effects on critical entomological species, such as honeybees. In 2014 an estimated 1.4 million pounds of thiamethoxam and 2.0 million pounds of imidacloprid, two common neonicotinoids, were used for agricultural purposes in the United States (USGS NAWQA). It is estimated that 1.6 to 20% of neonicotinoid active ingredient is absorbed into the applied crop, allowing for contamination of water through leeching (Goulson, 2013).

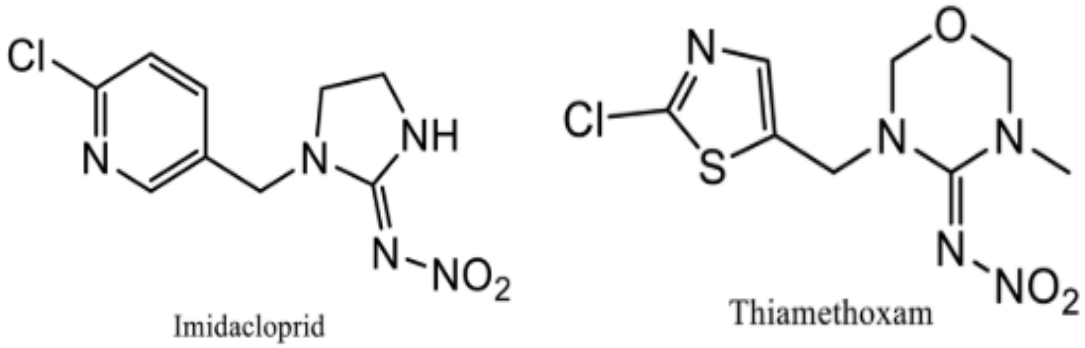

Figure 3. Imidacloprid and Thiamethoxam molecular structures

Neonicotinoids are a class of insecticides which operate similar to nicotine by binding to nicotinic acetylcholine receptors (nAChrs) in insects. This class of insecticides includes imidacloprid, cloprid, thiamethoxam, acetamiprid, thiacloprid, nithiazine, and nitenpyram (Figure 3). In 1991, imidacloprid was developed by Bayer CropScience. In subsequent years, many neonicotinoids, including thiamethoxam, were developed as a derivative of imidacloprid. These insecticides were immediately prevalent in the agricultural market. In recent years, neonicotinoids are predominantly applied via seed

application. Imidacloprid is also used as a pest repellant for domestic animals. The majority of imidacloprid agricultural use is for corn, while thiamethoxam is most often used for soybeans crop protection (USGS NAWQA).

Impact of Imidacloprid and Thiamethoxam use on ecosystems and human health

The widespread use of neonicotinoids has raised concerns about the impact of these insecticides on ecosystems (DiBartolomeis et al., 2019). Neonicotinoid exposure can occur with the consumption of exposed fruits, vegetables, and drinking water (Juraske et al., 2009). Neonicotinoids were detected in urine with a weighted average of 49.1% in an American sample population with age greater than 3. Neonicotinoid metabolites were also detected more often (35% N-desmethyl-acetamiprid and 19.7% 5-hydroxy-imidacloprid), with imidacloprid was detected with a weighted frequency of 4.3% (Ospina et al., 2019).

During recent years health concerns associated with neonicotinoid exposure have risen. Toxicity to humans of imidacloprid if ingested is quantified with an LD50 of 50-500 mg kg^{-1} for moderate toxicity (Kumar et al., 2013). Exposure to imidacloprid in pregnant women may cause negative birth outcomes such as anencephaly and tetralogy of Fallot (Han et al., 2018). Marfo, et al (2015) reported a correlation between N-des-methyl-acetamiprid concentrations found in urine and neurological problems (Marfo et al., 2015).

Finnegan et al, (2017) reported thiamethoxam toxicity for fish, molloscs, worms, and rotifers to be above concentrations that occur in the environment (LC50/EC50 $\geq$ 80 mg L^{-1} EC50 $\geq$ 100 mgL^{-1} for fish and molluscs, worms, rotifers respectively) but aquatic insects were more sensitive to thiamethoxam with acute EC < 1.0 mg L^{-1} (Finnegan et al.,

2017). Arguably the highest concern of neonicotinoid concentrations are the death of honey bees with oral LD50 of 0.0037 and 0.005 μg bee^{-1} for imidacloprid and thiamethoxam respectively (DiBartolomeis et al., 2019). Locomotion impairment was prevalent in a stingless bee *Tetragonisca angustula* when exposed to thiacloprid and imidacloprid while thiamethoxam caused hyperactivity (Jacob et al., 2019). Bees are crucial pollinators in agricultural systems and are necessary to grow food. Therefore, the uptake of neonicotinoids by wetland pollinator plants is of interest to quantify exposure risk to pollinator species.

Degradation pathways

Neonicotinoid insecticide chemical properties are important in characterizing their transport in environmental systems. Imidacloprid and thiamethoxam are both highly water soluble and adsorbent. Neonicotinoids degrade readily with UV light in aquatic environments, though high total suspended solids concentrations may limit this. Degradation pathways for most neonicotinoids leads to compounds that are more or equally toxic. Neonicotinoids degrade by two primary pathways: hydrolysis and photodegradation.

Various water characteristics have affected hydrolysis rates of neonicotinoids. Hydrolysis of imidacloprid occurs slowly in low and neutral pH but increases as water becomes more basic. Imidacloprid urea has been found as a common intermediate in hydrolysis and photolysis. Todey et al (2018) reported neonicotinoid hydrolysis, with pH levels ranging from 4 to 10, pseudo first order rate constants increased with increasing pH. Imidacloprid ranged from $4.3 \pm 1.6x10^{-4}$ (pH 4) to $1.8 \pm 0.1x10^{-2}$ (pH 10) while thiamethoxam ranged from $2.0 \pm 1.8x10^{-4}$ (pH 4) to $5.8 \pm 0.1x10^{-2}$ (pH 9). However, the

presence of minerals did not have a sign1ificant effect on hydrolysis rates (Todey et al., 2018) .

Surface water is rarely as pristine as evaluated in laboratory settings. For example, water from the Mississippi River was shown to have a smaller pseudo first order rate constant for imidacloprid while thiamethoxam reacted the same (Todey et al., 2018). Imidacloprid's absorption of UV light is highest between 211 and 268 nm, and is lower at frequencies greater than 300 nm, which is more indicative of PAR light (W. Liu et al., 2006). In a study of neonicotinoids in reservoir and wastewater treatment plant effluent, photodegradation occurred least in water with high natural organic matter (NOM). This was to be expected, as the presence of NOM and TSS interferes with the ability of light to penetrate below the surface. However, thiamethoxam degraded faster than imidacloprid with respective pseudo-first order rate constants of 1.11 min^{-1} and 0.43 min^{-1} in ultrapure water and 0.85 min^{-1} and 0.33 min^{-1} in reservoir water (Acero et al., 2019).

Environmental Fate and Transport

Neonicotinoids pollute surface water and groundwater, with the primary source believed to be from agricultural usage (Goulson, 2013). Neonicotinoids are often applied as seed coatings in an effort to minimize surface runoff (Jeschke et al., 2011). Coated seeds allow the crop to absorb the chemical while growing. This application method is more accurate than spray application. Regardless of application technique neonicotinoids can still transport off crop fields (Chrétien et al., 2017; Van Cuyk et al., 2004). For example, thiamethoxam coated seeds have been shown to result in adjacent ecosystem contamination via surface runoff, shallow lateral drainage, and deep drainage (Radolinski

et al., 2019). Additionally, neonicotinoid dust residues are present during seed sowing in amounts toxic to honey bees (Krupke et al., 2017).

Urban pesticide use is another contributor to neonicotinoid water contamination. Masoner et al., (2019) found a maximum imidacloprid concentration of 331 ng L^{-1} and detection frequency of 86% in a study of 50 stormwater samples from 21 industrial, commercial, and residential sites throughout the United States. Furthermore, imidacloprid concentrations in San Francisco Bay wastewater treatment plants were as high as 306 ng L^{-1} with domestic animal pest controls a potential source of contamination (Sadaria et al., 2017).

Neonicotinoid Detection

While neonicotinoids have been detected in surface water throughout the world, there are limited studies on the severity of contamination in the United States. Seventy four percent of monthly grab samples taken from ten major tributaries to the Great Lakes (Bad River, WI, Cuyahoga River, OH, Genesee River NY, Grand River, MI, Indiana Harbor Canal, IN, Manitowoc River, WI, Maumee River, OH, River Rouge, MI, Saginaw River, MI, St. Joseph River, MI) had at least one detectable neonicotinoid (maximum individual concentration: 230 ng L^{-1}), with 53% containing imidacloprid and 22% containing thiamethoxam (Hladik et al., 2018). A study of 91 irrigation wells in Wisconsin revealed groundwater contamination with detectable levels of thiamethoxam in 67% of samples with a mean concentration of 0.28 µg L^{-1} (Bradford et al., 2018). In a Maryland study of surface water contamination near honey bee hives neonicotinoid concentrations were measured between 7 and 131 ppb in 8% of the samples (Johnson and Pettis, 2014).

Neonicotinoids contamination of surface water in turn contaminates drinking water. Clothianidin, imidacloprid, and thiamethoxam have been found in the tap water at the University of Iowa in concentrations ranging 3.89 - 57.3 ng L^{-1}, 1.22 - 39.5 ng L^{-1}, and 0.24 - 4.15 ng L^{-1} respectively, with higher concentrations corresponding to peak flow rates in source surface water. Concentrations were shown to be higher in water from traditional treatment systems in comparison to systems with granular activated carbon, which removed clothianidin, imidacloprid, and thiamethoxam neonicotinoids with removal efficiencies > 80% (Klarich et al., 2017).

Wetlands as Potential Removers of Neonicotinoids

Wetlands are known to remove nutrients and agrochemicals from surface water and are often used to treat runoff containing pesticides with inconsistent success (Vymazal and Březinová, 2015). Plant uptake is an important aspect of wetland treatment for pesticides. A field study of neonicotinoids in Canadian prairie wetlands susceptible to clothianidin runoff demonstrated lower neonicotinoid concentrations and detection frequencies in sites with heavier wetland vegetation. In fact, 43% of wetland plant species had detectable neonicotinoids concentrations in plant tissue. The wetland species *Equiestum arvense*, *Alisma triviale*, and *Typha latifolia* contained the highest neonicotinoid concentrations (Main et al., 2016). However, a study of neonicotinoid removal in traditional treatment plants vs treatment wetlands found no removal of imidacloprid and acetamiprid in the treatment wetland (Sadaria et al., 2016).

Neonicotinoid Plant Uptake

Neonicotinoids may accumulate in plants because they are highly water soluble. This is a concern for pollinator wetland plant species, such as milkweed, as well as

harvestable wetland plants, such as rice. Many studies have shown neonicotinoids accumulate in harvested plants such as maize (Sun et al., 2017), cabbage (Y. Li et al., 2018), and rice (Ge et al., 2017). Imidacloprid may also accumulate in leafy vegetables depending on the growth stage of the plant (Y. Li et al., 2018). Wetland species have also shown to accumulate neonicotinoids. Clothianidin, imidacloprid, and thiamethoxam had concentrations up to 2.01, 2.61, and 8.44 μg kg^{-1} respectively in an established wetland next to clothianidin treated canola fields (Main et al., 2017). Additionally, wetland mesocosms containing South American wetland plants removed 86%-100% of imidacloprid with low concentration (60 μg L^{-1}) water (Mahabali and Spanoghe, 2014). While neonicotinoid plant uptake from soil has been explored, no research has been done on uptake in hydroponic systems and more research is needed in floating treatment wetland systems.

FTW Microbial Communities

Denitrification, the complete removal of nitrogen through reduction of nitrate-nitrogen (NO_3-N) to nitrogen gas (N_2), is driven by microbes surrounding the root systems of the FTW plants. Urakawa et al., (2017) evaluated the microbial community of a FTW implemented in a Naples, Florida urban stormwater pond using 16S rRNA gene amplification sequencing. Root, water, and mat/pot samples were taken to determine key factors of bacterial communities in various mediums. It was also of interest to determine how the microbiomes are formed, i.e., how strongly the microbiome of the roots affects the microbiome of the surrounding water. In the study, the biofilm from the mats and plant pots showed less diversity than that of the roots and rhizosphere. The roots and surrounding water contained similar microbiomes. It is suggested that the microbiome of

the roots is influenced by the surrounding water and the roots. The study did not identify any denitrifying bacterium in samples from any sampled locations. Instead, a large quantity of sulfur cycle microbes was found in root and rhizosphere samples.

Microbial communities are critical to nitrogen removal processes. Therefore studies need to be conducted to determine negative effects pesticides may have on richness, diversity, and activity of microbial communities as well as effects on specific genes involved in nitrogen conversion. Pesticide toxicity in FTW microbial communities has not been investigated and research on aquatic microbial communities is limited. Additionally, pesticide toxicity is highly variable across pesticide type. For example, (Milenkovski et al., 2010) showed a reduction in denitrification potential in wetland water for two fungicides (thiram and captam) but no effect for six other fungicides. Other studies show negative effect on aquatic microbial community richness, diversity, and production for insecticides (malathion, carbaryl, and permethrin) and herbicides (glyphosate) but have not explored their effects on denitrification (Muturi et al., 2017; Sura et al., 2012).

Research Objectives

Neonicotinoid insecticides are an emerging contaminant of increasing concern for ecosystem and human health (DiBartolomeis et al., 2019; Finnegan et al., 2017; Han et al., 2018; Jacob et al., 2019). Further research on the fate and transport of neonicotinoids in FTW systems needs to occur to determine any effect the insecticide may have on this BMP. Therefore, my research objectives are to:

1. Determine neonicotinoid removal capacity of FTWs
2. Quantify incorporation of neonicotinoids into FTW biomass

3. Explore potential implications of neonicotinoids on microbial nitrogen removing processes

I hypothesize that FTWs will significantly reduce imidacloprid, thiamethoxam, and metabolite concentrations in the water column. Concentrations will be detectable in roots and shoots. Neonicotinoids will reduce diversity in the FTW microbial community and therefore reduce rates of nitrate removal.

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CHAPTER 2: Neonicotinoid Floating Treatment Wetland Experiment

Introduction

The Midwest is vulnerable to nutrient contamination in surface and groundwater. High concentrations of nitrogen and phosphorus can lead to harmful algae blooms (HABs) and fish kills (X. e. Yang, 2008). Best management practices such as buffer strips and treatment wetlands are often recommended to reduce nitrogen and phosphorus loading into water bodies (Y. Liu et al., 2017; O'Geen et al., 2010). While these practices are effective, implementation in the Midwest is often minimal due to cost and land requirement. Floating treatment wetlands (FTWs) are an innovative, low cost water treatment option currently used in lakes, stormwater ponds, and lagoons to reduce nutrients as well as total suspended solids (Di Luca et al., 2019; Keizer-Vlek et al., 2014; Tanner and Headley, 2011; Winston et al., 2013).

FTWs consist of buoyant hydroponic vegetated mats, with wetland plant shoots growing above the surface water and roots residing in the water column. Vegetation takes up excess nitrogen and phosphorus from the water column to incorporate into the plant biomass while facultative microbes in the rhizosphere convert inorganic nitrogen forms, specifically nitrate-N, into N_2 permanently removing nitrogen from the pond or lake ecosystem through the process of denitrification. Water quality benefits vary based on factors such as plant species (Xu et al., 2017), hydraulic retention time (Z. Yang et al., 2008), temperature (Van De Moortel et al., 2010), and carbon availability (Keilhauer et al., 2019) ; however, FTWs have been shown to effectively reduce total nitrogen (TN) (30.6%-90.9%) and total phosphorus (TP) (23.4%-87.5%) (Samal et al., 2019). One mesocosm experiment exhibited complete NO_3-N removal after 2 weeks (West et al., 2017).

With current agricultural and urban practices, water quality concerns extend beyond nutrient contamination and treatment. There is a growing concern for a variety of emergent contaminants that affect nutrient removal processes. Pesticides can reduce the abundance and expression of genes responsible for nitrogen fixation, nitrification, and denitrification in soil (Singh et al., 2015). While many studies have explored the negative effects pesticides have on biogeochemical processes in soil (Wołejko et al., 2020; Yeomans and Bremner, 1985; Zhang et al., 2018), fewer studies focus on aquatic microbial communities. Researchers that have explored these communities report a decrease in community richness, diversity, and production, but have not quantified specific gene abundance (Muturi et al., 2017; Sura et al., 2012). However, a decrease in denitrification potential with the addition of certain fungicides to wetland water has been reported (Milenkovski et al., 2010). Nevertheless, little is known about pesticide toxicity for denitrifying microbes in hydroponic rhizosphere communities.

Among those contaminants of concern are neonicotinoids, a class of insecticides used widely in agriculture for soybean production, tree treatment, as well as urban settings as pest repellents. Named for their structural qualities similar to nicotine (Jeschke et al., 2011), neonicotinoids target the central nervous system in insects by binding to the nicotinic acetylcholine receptors (nAChrs) (Jacob et al., 2019). Neonicotinoids are the fastest growing class of insecticides, accounting for over 25% of the global market in 2014 (Bass et al., 2015). Additionally, only an estimated 1.6 to 20 % of active ingredient on neonicotinoid seed treatments, which makes up the majority of its agricultural use, is absorbed into the treated crop, creating potential for water contamination via leeching and runoff (Goulson, 2013). While degradation of these insecticides occurs with photolysis,

suspended solids in the contaminated water column inhibit light penetration and metabolites can be as toxic or more toxic than the parent compound (Acero et al., 2019; Klarich et al., 2017). Negative impacts have been shown in nontarget organisms such as pollinators and aquatic invertebrates (DiBartolomeis et al., 2019; Finnegan et al., 2017; Jacob et al., 2019). There is also concern for association with birth defects in exposed pregnant women and negative neurologic symptoms in humans (Han et al., 2018).

Imidacloprid and thiamethoxam specifically are two neonicotinoids widely used in the United States. Neonicotinoids have been found in midwestern groundwater, surface water, and even in tap water (Bradford et al., 2018; Hladik et al., 2018; Klarich et al., 2017). Therefore, new pesticide removal practices are needed in response to the increasing concentrations and frequency of detection. While FTWs are commonly used for nutrient removal, other treatment benefits are being explored. Studies have shown that the presence of wetland vegetation can reduce pesticides, including neonicotinoid, concentrations and detection frequency (Mahabali and Spanoghe, 2014; Main et al., 2017; Vymazal and Březinová, 2015). Therefore, we hypothesized pesticide removal would be an additional benefit of FTWs. Neonicotinoid water solubility allows the insecticide to be taken up into the treated crops; however, few studies have explored the uptake of neonicotinoids by wetland species and no studies have been completed to investigate hydroponic uptake. Therefore, the objectives of this study were to: 1. determine the FTW neonicotinoid removal capacity, 2. quantify neonicotinoid incorporation into biomass, and 3. explore potential implications of neonicotinoids on microbial nitrogen removing processes.

Materials and Methods

FTW Mesocosm Setup

A mesocosm experiment was conducted during the summer of 2019 in the Messer Ecological Systems Observation Laboratory (*meso*LAB), a climate controlled greenhouse at the University of Nebraska-Lincoln (UNL). FTW mesocosms consisted of 380 L black Rubbermaid feeding troughs filled with simulated greenhouse water (Figure 1). Due to limited greenhouse space, control mesocosms (no FTWs) were smaller 56 L buckets, which have been used successfully in similar mesocosm experiments (Keilhauer et al., 2019; Messer, Burchell, Birgand, et al., 2017). 60 cm X 60 cm FTW mats were purchased from Beemats and contained ten established native Nebraska wetland plants that were planted in spring 2017. Plant species consisted of longhair sedge (*Carex comosa*), fox sedge (*Carex vulpinoidea*), swamp milkweed (*Asclepias incarnate*), common rush (*Juncus effuses*), and torrey's rush (*Juncus torreyi*). Plants were established prior to the experiment. A HOBO light and temperature sensor was situated underneath each FTW mat to monitor temperature and light conditions in the mesocosms throughout the experiment. A total of 9 mesocosms were used, consisting of three FTW mesocosms enriched with neonicotinoids and nitrate-N (NO_3-N), three control mesocosms (no plants) with neonicotinoids and NO_3-N, and three FTW mesocosms enriched with NO_3-N only.

Figure 1: Climate controlled greenhouse with FTW and control mesocosms. Tin foil was placed on mesocosms to limit neonicotinoid degradation via photolysis.

FTW Neonicotinoid Experiment

All mesocosms were cleaned and refilled with greenhouse tap water four days before the start of the experiment. Mesocosms were filled using a flow meter (P3 International Corporation; New York, NY) to approximately 285 L for those with FTWs and 50 L for controls. On the first day of the experiment (day 0), foil was placed over the mesocosms to limit UV light on water surface, which is known to contribute to substantial photodegradation of the insecticides (Todey et al., 2018). Biomass samples were collected from the six FTWs before NO_3-N and neonicotinoid enrichment occurred.

All mesocosms were amended with KNO_3 (Fisher Scientific International, Inc; Pittsburgh, PA) to reach initial NO_3-N concentrations of approximately 10 mg L^{-1}. However, due to NO_3-N concentrations in tap water actual NO_3-N concentrations were slightly above 10 mg L^{-1}. Imidacloprid and thiamethoxam are two commonly used

neonicotinoids in the Midwest (Hladik et al., 2018) and were chosen due to their ubiquitous nature in Nebraska water systems (Satiroff et al., In Review). Stock solutions of the insecticides were used to enrich three of the six FTW mesocosms and the three control mesocosms (no FTWs) to 100 ppb for both imidacloprid and thiamethoxam based on recently observed concentrations in rivers and lakes around eastern Nebraska (Satiroff et al., In Review).

Sample collection occurred on days 0, 1, 2, 3, 5, 7, 10, 15, and 21 of the experiment. On each sampling day, water characteristics (temperature, pH, dissolved oxygen, conductivity, ORP) were measured using a YSI EXO2 Sonde (Xylem, Yellow Spring, OH). Water depth measurements were taken and water recirculated in each of the mesocosms for three minutes prior to sample collection to ensure the water was well mixed. Grab samples were collected 15 cm below the surface placed a cooler with ice and taken immediately back to the Messer Lab. Samples were filtered with GF/F filters and stored in refrigerator to be analyzed for NO_3-N, ammonium, and phosphorus concentrations on every sampling day and dissolved organic carbon (DOC) on days 0 and 21. On days 0, 7, 10, 15, and 21, 20 mL of unfiltered samples were stored in a freezer for microbial assays using quantitative polymerase chain reaction (qPCR). Insecticide samples were collected on days 1 and 21 by pipetting 2 mL of water directly from each mesocosm into amber glass bottles, placed in a cooler with ice, and stored in a freezer until analyzed for thiamethoxam, imidacloprid and byproducts. Neonicotinoid byproducts that were analyzed for in water included imidacloprid desnitro, imidacloprid olefin, imidacloprid urea, clothianidin, 6-chloronicotinic acid, 6-cloronicotinic aldehyde, 6-chloro-N-methylnicotinamide, and 6-hydroxynicotinic acid.

Above and below surface biomass samples were collected before neonicotinoid enrichment and on the last day of the experiment for neonicotinoid and neonicotinoid byproduct analysis. Composite shoot samples from mesocosms with identical treatments were made for the three plant types: sedge, rush, and milkweed. Milkweed above surface biomass samples consisted of leaves while sedge/rush samples were cut from the top 10 inches of the plant. Root samples were cut from the bottom six inches of the rhizome but were not categorized by plant type. All plant samples were placed in ziplock bags and stored in a freezer until they were freeze dried and analyzed. Additionally, a destructive harvest was carried out at the end of the experiment to measure nitrogen and carbon content in the FTW biomass. One plant was taken from each of the six FTWs, the roots were separated from the shoots, and plants were dried in a heated room until the dry mass could be measured. Dry biomass was sent to Ward Lab and analyzed for percent nitrogen and total carbon.

In order to characterize the microbial community in the FTW mesocosms and determine if a change occurs following the addition of neonicotinoids, microbial assays were conducted throughout the mesocosm experiment. Microbial assays consisted of analyzation of mesocosm water on days 0, 7, 10, 15, 21 for six genes using qPCR: *16*S, ammonia oxidizing archaea (*AOA*), ammonia oxidizing bacteria (*AOB*), two nitrite reductase genes (*nirS* and *nirK*), and nitrous oxide reductase (*nosZ*). While the 16S gene quantifies the total microbial community, *nirS*, *nirK*, and *nosZ* are denitrifying genes.

Denitrification Potential Incubations

In addition to the mesocosm experiment, a denitrification incubation experiment was conducted using mesocosm water and FTW roots to measure the effect of imidacloprid

and thiamethoxam on potential denitrification. In order for denitrification to occur, five conditions must be met: NO_3-N source, carbon source, anaerobic environment, suitable temperature/pH, and denitrifying microbes. The purpose of these incubation experiments was to ensure the first four criteria are in excess, therefore, the denitrifying potential of the microbes could be assessed. These experiments have been used in previous studies to measure the denitrifying potential of a microbial community (Daum and Schenk, 1997; Dodla et al., 2008; Holmes et al., 1996; Teissier and Torre, 2002). Four treatments in triplicate were evaluated: mesocosm water, mesocosm water with neonicotinoids, buffer water with root matter, buffer water with root matter with neonicotinoids (figure 2). Mesocosm water only vials contained 90 mL of water collected from previously used mesocosms while root matter vials contained approximately 10 g of FTW roots. Potassium phosphate buffer (pH 7.4) and glucose was amended to achieve 1 mM and 5 mM final concentration respectively. Chloramphenicol (0.5 g L^{-1} final concentration) was added prior to incubation to inhibit new microbial growth, and potassium nitrate was added to achieve a 10 mg L^{-1} final concentration. Neonicotinoid

stock solutions were used to bring neonicotinoid treatments to 100 ppb of both thiamethoxam and imidacloprid, the same level as in the mesocosm experiment.

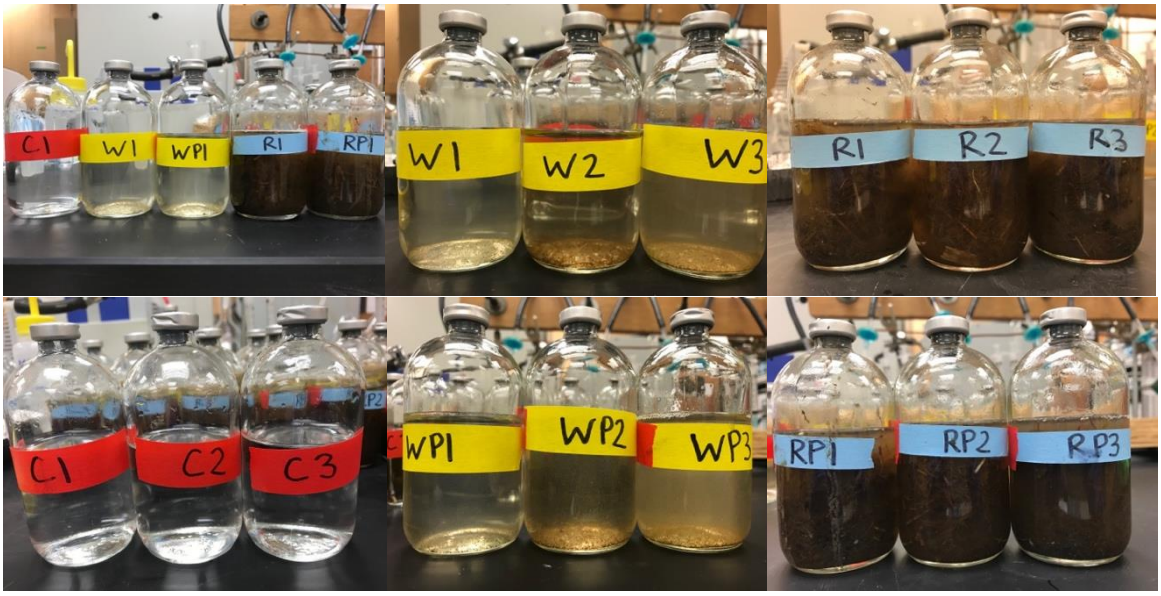


Figure 2: Vials from microcosm experiment. Every mesocosm water and FTW root vial has a corresponding vial with water/roots from the same mesocosm sample, with the addition of 100 ppb of imidacloprid and thiamethoxam. Therefore, each mesocosm has 4 vials: mesocosm water (W), mesocosm water with neonicotinoids *(WP), FTW roots (R), and FTW roots with* neonicotinoids *(RP). Control vials (C) contain sterilized buffer.*

On the morning of the incubation experiment, three previously used mesocosms (D2, D3, D3P) were chosen randomly for water and root sampling (Figure 3a). Mesocosms were mixed for three minutes before sampling 250 mL of mesocosm water, approximately 15 cm below the surface, from each mesocosm. FTW roots were cut from multiple locations on each FTW mat to total approximately 40 g per mesocosm. Mesocosm water and FTW roots were kept in a cooler to be transported to the lab and processed immediately. Root biomass was cut into approximately 2 cm pieces and was mixed to give a homogenous root sample for each mesocosm (Figure 3b). Root samples were divided so that two 10 g samples came from each mesocosm (Figure 3c). During root processing, excess liquid in bags were saved. An additional 50 mL of water and 10 g of FTW roots

from each mesocosm were placed in a freezer for qPCR microbial assays. 10 g of FTW were sent to Ward lab (Kearney, NE) for nutrient content analysis.

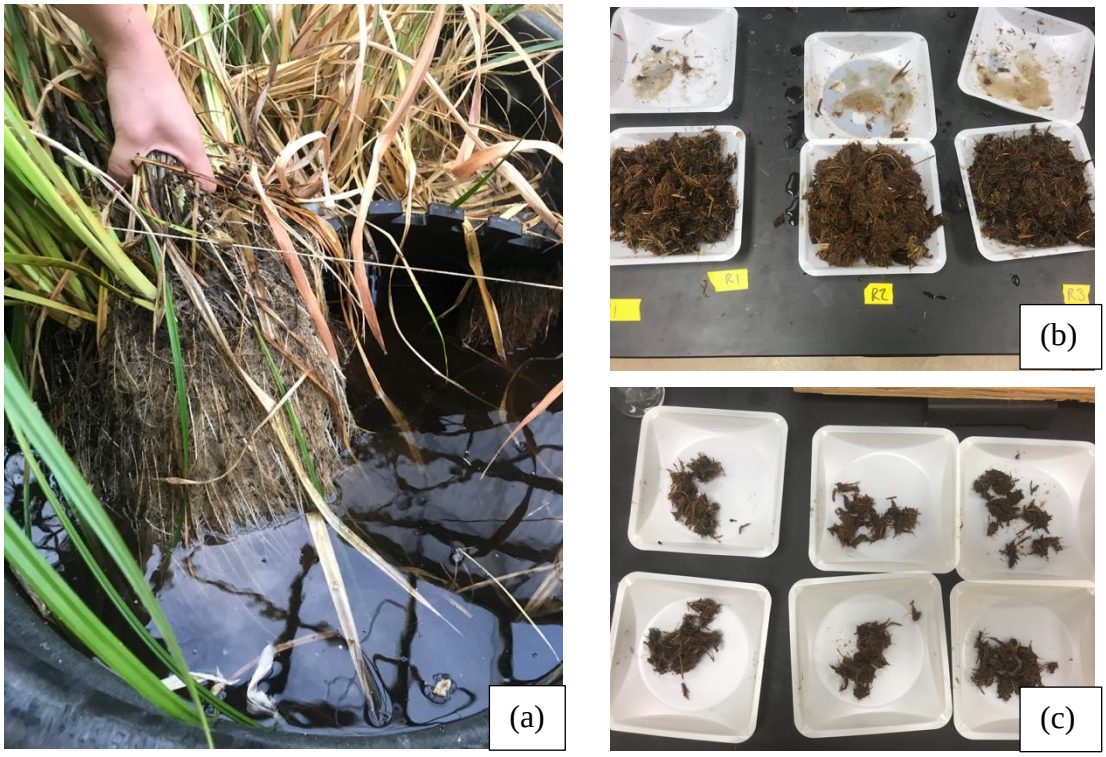


Figure 3: FTW roots in mesocosm prior to sampling (a), samples were cut up and two 10 g amounts were allocated from each mesocosm for corresponding vials (b) and (c).

Vials were sealed and were made anaerobic by replacing the headspace with nitrogen gas during three cycles of evacuation and nitrogen flushing. The vials were shaken vigorously and a new syringe was used immediately to collect the time zero 1.8 mL sample. Sampling occurred on days 0, 1, 2, 3, 4, 5, 7, and 10 at the same time each morning. At each sampling event, vials were shaken vigorously and 1.8 mL of water was extracted. After sampling, approximately 10 mL of nitrogen gas was amended into the vials to ensure the vials remained anaerobic. Water samples were stored immediately in a freezer to be analyzed

for NO_3-N concentrations. After ten days, root mass was recovered from the six FTW root vials for volume, dry mass, and nutrient concentration measurements.

Analytical Methods

Nutrient Analysis

After collection, water samples were stored at 4°C for up to 24 hours prior analysis at the USDA ARS Agroecosystem Management Research Unit laboratories (Lincoln, NE). Ammonium and NO_3-N in water samples were measured by automated spectrophotometry using a Seal Analytical AQ300 autoanalyzer according to EPA method 351.2 and 353.2, respectively. Dissolved phosphorous was also measured spectrophotometrically according to EPA method 365.3 using a Beckman DU-800 spectrophotometer. Water samples were analyzed for dissolved organic carbon (DOC) at the UNL Water Sciences Laboratory (Lincoln, NE) using a 1010 TOC Analyzer (Oceanography International Corporation; College Station, TX) with the Standard Method 5301D. Above and below surface biomass samples were analyzed for total nitrogen (TN) content at Ward Laboratories, Inc. (Kearney, NE) using the Dumas Combustion Method (Plank, 1991; Sweeney, 1989).

Plant and Water Neonicotinoid Analysis

Solvent and reagents used were high purity and reagent grade or better. Standards for target pesticides, including clothianidin, thiamethoxam, imidacloprid, acetamiprid, thiacloprid, dinotefuran, metalaxyl, dimethoate, pyraclostrobin, trifloxystrobin, azoxystrobin, picoxystrobin, imidacloprid urea, imidacloprid olefin, imidacloprid desnitro HCL, thiamethoxam urea, 6-hydroxynicotinic acid, 6-chloronicotinic acid, 6-chloronicotinic aldehyde, 6-chloro-N-methylnicotinamide, sulfoxaflor, and indoxacarb were purchased either from Sigma-Aldrich (St. Louis, MO USA), PlusCHEM (San Diego, CA USA) or (ChemService West Chester, PA USA). Stable isotope labelled

internal standards were purchased from Sigma-Aldrich or Cambridge Isotope Laboratories (Andover, MA USA). Stock solutions (1.0 μg μL^{-1}) of each analyte and standard were prepared in methanol (Optima, Fisher Scientific) and stored at -20°C. Diluted mixes of target and standard compounds were prepared in methanol for spiking and preparation of calibration solutions.

Plant tissue samples were stored frozen (-20°C) in polyethylene zipper bags. Prior to analysis plat tissue samples were freeze-dried using a Labconco 4.5L Freezone system and then individually ground using a mortar and pestle. Extraction and subsequent analysis of freeze-dried plant tissue for pesticide residues generally followed procedures outlined in (Botías et al. 2015). Briefly, 0.2 grams of freeze-dried tissue was weighed out in a 50 milliliter polypropylene centrifuge tube, mixed with 2 mL of reagent water to rehydrate, followed by 2.5 mL acetonitrile and 0.75 mL hexane. The mixture was spiked with 10 μL of a surrogate mix (0.10 ng μL^{-1} nitenpyram and terbuthylazine) to measure recovery, capped and shaken for 10 minutes on a wrist action shaker. A salting out reagent (1.25 grams 4:1 magnesium sulfate:sodium acetate) was then added and the mixture hand shaken to disperse the reagent. The mixture was then centrifuged at 2500 rpm for 5 minutes. Liquid supernatant was pipetted off into a clean centrifuge tube containing 625 mg SupelQue cleanup sorbent (PSA/C18/ENVI-Carb, Sigma-Aldrich, St. Louis, MO USA) and vortexed. The tissue sample was extracted a second time using an additional 1.75 mL acetonitrile, shaken by hand, centrifuged at 2500 rpm for 5 minutes, and the supernatant combined with the first portion. The purified extract was evaporated to approximately 1 mL and filtered using 25 mm 0.45 μm pore size glass microfiber into

a glass culture tube. Solvent was evaporated to near dryness, spiked with 50 µL deuterium labelled internal standards (0.2 ng μL^{-1} d3-clothianidin, d3-thiamethoxam, d4-imidacloprid, d6-metalaxyl, pyraclostrobin-(N-methoxy-d3) and mixed with 200 µL of purified reagent water to a solvent ratio 20:80 methanol:water.

Compounds were separated and analyzed on a Aquity^{TM} UPLC interfaced with a Xevo TQS triple quadrupole mass spectrometer using a UniSpray^{TM} source (Waters Corporation, Manchester, UK). Chromatographic separation used an Aquity BEG C_{18} 50mm x 2.1mm x 1.7µm reverse phase column. Mobile phase solvents A) 0.1% (v/v) formic acid in water and B) 0.1% (v/v) formic acid in methanol at a flow rate of 0.6 mL min^{-1} began with 95:5 A/B, increasing to 5:95 A/B until 3 min, hold for 0.5 min before switching back to original conditions 95:5 A/B at 3.60 min for a total run time of 5 min per injection.

Multiple reaction monitoring was used for each compound and five deuterium-labeled internal standards were used for quantitation (Table 1).

Table 1. Multiple reaction monitoring and five deuterium-labeled internal standards

Compound	Parent-Daughter m/z	Cone (V)	Collision (V)	Retention Time (min)
Clothianidin-d3 (IS)	252.968>171.922	34	12	1.70
	252.968>131.914	34	14	
Imidacloprid-d4 (IS)	260.032>179.059	40	16	1.70
	260.032>213.138	40	12	
Metalaxyl-d6 (IS)	286.16>226.141	36	12	2.67
	286.16>44.94	36	32	
Pyraclostrobin-d3 (IS)	391.096>197.069	44	10	3.25
	391.096>162.99	44	24	
Thiamethoxam-d3 (IS)	294.968>213.938	28	10	1.47
	294.968>183.951	28	22	
Nitenpyram (Sur)	271.032>55.997	28	28	1.29
	271.032>98.93	28	14	
Terbuthylazine (Sur)	230.096>173.948	38	14	2.89
	230.096>95.946	38	26	
Dimoxystrobin (Sur)	327.096>205.056	2	8	3.15
	327.096>115.96	2	22	
Acetamiprid	223.032>125.92	62	18	1.85

	223.032>55.996	62	14	
Clothianidin	249.968>168.86	34	10	1.70
	249.968>131.908	34	12	
Dimethoate	229.968>198.864	26	8	1.81
	229.968>124.896	26	20	
Dinotefuran	203.096>129.052	20	10	1.13
	203.096>86.965	20	14	
Imidacloprid	256.032>174.982	32	18	1.70
	256.032>209.066	32	14	
Metalaxyl	280.096>220.08	32	12	2.68
	280.096>44.94	32	28	
Thiacloprid	253.032>125.929	54	22	1.99
	253.032>90.02	54	34	
Thiamethoxam	291.968>210.941	28	10	1.47
	291.968>180.954	28	22	
Azoxystrobin	404.032>372.014	18	12	2.83
	404.032>328.962	18	30	
Picoxystrobin	368.032>144.981	18	20	3.13
	368.032>205.013	18	6	
Pyraclostrobin	388.032>194.01	28	10	3.24
	388.032>163.114	28	24	
Trifloxystrobin	409.096>185.947	38	14	3.36
	409.096>144.972	38	46	
Sulfoxaflor	278.047>173.989	36	6	1.91*
	278.047>154.021	36	26	
Indoxacarb	528.042>149.972	38	22	3.35
	528.042>292.978	38	12	
Imidacloprid urea	213.065>127.956	78	20	1.02
	213.065>90.024	78	34	
Imidacloprid olefin	253.97>205.187	28	14	1.55
	253.97>125.978	28	26	
Imidacloprid desnitro	211.064>125.947	64	24	1.02
	211.064>90.01	64	34	
Thiamethoxam urea	248.03>174.913	54	18	1.86
	248.03>44.026	54	14	
6-Hydroxynicotinic acid	140.004>50.994	40	28	0.54
	140.004>77.937	40	24	
6-Chloronicotinic acid	157.957>121.955	56	16	1.77
	157.957>125.922	56	22	
6-Chloronicotinic aldehyde	141.944>105.916	82	14	1.58**
	141.944>77.935	82	22	
6-Chloro-N-methylnicotinamide	170.994>77.991	32	26	1.47
	170.994>141.947	32	14	

Quality controls analyzed at a frequency of 5% or better included laboratory method blanks, laboratory fortified blanks, laboratory fortified matrix and laboratory duplicates. Method detection limits, determined from 8-10 replicates of a low-level fortified blank matrix (USEPA 1986), averaged 0.030 (±0.030) ng/g

DNA Extraction and Quantitative Polymerase Chain Reaction Analysis

Twenty mL water samples were stored frozen until DNA extraction occurred. Samples were centrifuged at 23-25°C for 5 minutes with RPM of 16000 and RCF 37000 before decanting and transferring to 2 mL vials. Vials were vortexed to re-suspend biomass, boiled to lyse microbial cells releasing DNA into the liquid, and then centrifuged for 10 minutes at 2°C before transferring the supernatant to a clean tube and discarding the remaining cell pellet.

Microbial assays consisted of quantification of six genes (16S ribosomal RNA, archaeal *amoA,* bacterial *amoA, nirS, nirK, nosZ*) using quantitative polymerase chain reaction (qPCR). 16S quantification was done using primers and procedures described by White et al. (1993). Archaeal *amoA* and bacterial *amoA* genes were quantified using procedures found in Tourna et al. (2008) and Liesack et al. (1997) respectfully. Nitrite reductase genes (*nirS* and *nirK*) were quantified using procedures from Braker et al. (1998). Finally, *nosZ* genes were quantified with primers and procedures from Scala and Kerkhof (1998). Primer descriptions and procedures can be found in Table 2.

qPCR reactions were carried out using QuantiTect Syber Green master mix (QIAGEN, Hilden, Germany). StepOnePlus real-time PCR system (Applied Biosystems Inc., Foster City, California) was used for gene quantification. Each sample analysis was carried out in triplicate and averaged before being converted to concentration in the original 20 mL sample (copies/mL) using a dilution factor of 100. Before statistical analysis, 16S ribosomal RNA concentrations were normalized on a logarithmic scale. Archaeal amoA, bacterial *amoA, nirS, nirK* and *nosZ* were normalized using 16S concentrations.

Table 2. Primer descriptions and procedure references for assayed genes.

Gene	Primer	Primer Sequence	Cycling Conditions	Reference
16S rRNA	FW RV	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	95°C -15 min (1 cycle) 95°C - 15 sec, 55°C - 20 sec, 72°C – 10 sec (35 cycles)	(David C. White, Cory Lytle, Aaron Peacock and Jonas S. Almeida, 1993)
Archaeal *amoA*	FW RV	ATGGTCTGGCTWAGACG GCCATCCATCTGTATGT CCA	95°C – 5 min (1 cycle) 95°C – 30 sec, 55°C – 30 sec, 72°C 30 sec (35 cycles)	(Tourna et al., 2008)
Bacterial *amoA*	FW RV	GGGG ATTCTACTGGTGGT CCCGGATAGAACAGCAG ACC	95°C – 5 min (1 cycle) 95°C – 30 sec, 60°C – 30 sec, 72°C 30 sec (35 cycles)	(Liesack, Werner; Jan-Henrich, 1997)
nirK	FW RV	ATCATGGTSCTGCCGCG CCTCGATCAGRTTGTGG TT	94°C – 4 min (1 cycle) 94°C – 30 sec, 55°C – 30 sec (30 cycles) 60°C – 6 min (1 cycle)	(Braker et al., 1998)
nirS	FW RV	TACCACCC(C/G)GA(A/G) CCGCGCGT GCCGCCGTC(A/G)TG(A/C /G)AGGAA	94°C – 4 min (1 cycle) 94°C – 30 sec, 55°C – 30 sec (30 cycles) 60°C – 6 min (1 cycle)	(Braker et al., 1998)
nosZ	FW RV	CGGCTGGGGGCTGACCA A ATRTCGATCARCTGBTC GTT	94°C – 5 min (1 cycle) 95°C – 30 sec, 56°C – 90 sec, 72°C – 2 min (35 cycles) 72°C – 10 min (1 cycle)	(Scala and Kerkhof, 1998)

NO_3-N Removal

First order NO_3-N removal rates were calculated for all mesocosms following both experiments (Benjamin, 2010; Brezonik and Arnold, 2011; Keilhauer et al., 2019; Messer et al., 2017):

$$C_T = C_0 * e^{-kt} \qquad (1)$$

Where C_T was the final NO_3-N concentration (mgL^{-1}), C_0 was the initial NO_3-N concentration (mgL^{-1}), t was time from the beginning experiment to when NO_3-N concentrations were below detectable limits (days), and k was the removal rate ($days^{-1}$).

Neonicotinoid, NO_3-N Percent Removal, and NO_3-N Removal Rates

NO_3-N, thiamethoxam, and imidacloprid percent removals were calculated for each mesocosm using concentrations from day 1 the last day concentrations were above the minimum detection limit of 0.05 mgL^{-1} for NO_3-N and xxx for imidacloprid and thiamethoxam (Benjamin, 2010; Brezonik and Arnold, 2011; Keilhauer et al., 2019):

$$\% \, Removal = \frac{C_0 - C_T}{C_0} * 100\% \qquad (2)$$

Daily NO_3-N removal rates and overall neonicotinoid removal rates were calculated for all mesocosms after experiments:

$$J_{XX} = \frac{(X_{i-1} - X_i)}{A*t} \qquad (3)$$

Where J_{xx} was the analyte removal rate ($mg\ m^{-2}\ day^{-1}$), X_{i-1} was analyte loading from the previous sampling day (mg), X_i was analyte loading from given sampling day (mg), A is the area of the FTW mat, and t is the time since nutrient enrichment (days).

Statistics

Statistical analysis was performed on NO_3-N concentrations, DO, conductivity, ORP, pH, and temperature to determine if statistically significant differences were observed between the three treatments through time during the first 10 days of the experiment. All data was normalized, where appropriate, outliers removed, and ANOVA regression analysis was performed on each treatment using Minitab 17 (Champaign, IL, 2020). Significant differences between treatments were assessed using Tukey pairwise comparison test at a significance test of $\alpha = 0.05$.

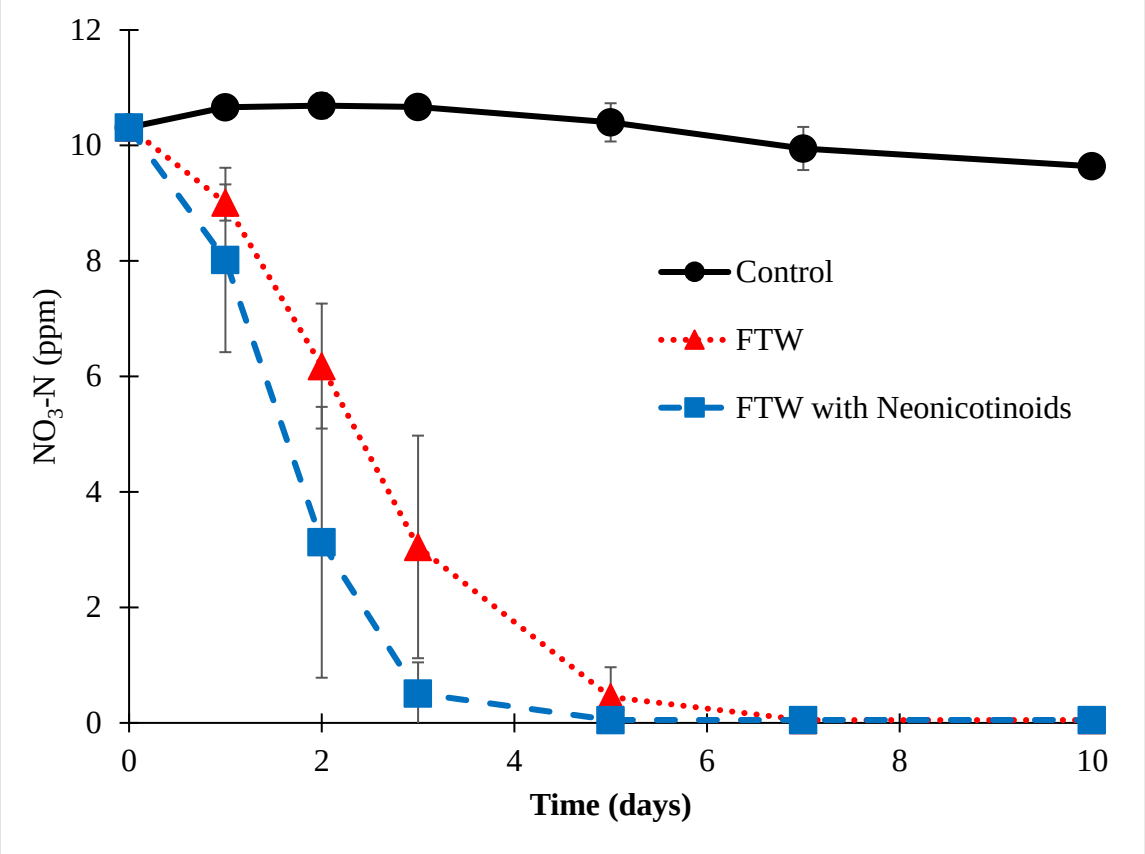
Results/Discussion

Mesocosm NO_3-N Removal

After the NO_3-N enrichment, NO_3-N concentrations were monitored on days 1, 2, 3, 5, 7, 10, 15, and 21. However, by day 10, all 6 FTW mesocosms had NO_3-N concentrations below the analytical detection limit (0.05 mg/L) while control mesocosm NO_3-N concentrations remained around the initial 10 mg NO_3-N mg/L^{-1}. Since FTW and control mesocosms lost 0.6 – 4 inches of water due to evapotranspiration during the experiment, statistical comparisons were carried out after adjusting NO_3-N concentrations with daily water depth measurements. Average NO_3-N removal rates for neonicotinoids and non neonicotinoids FTWs were 3.33 ± 1.17 and 1.47 ± 0.17 g m^{-2} day^{-1}. This is larger than the removal rate observed by Saeed et al., (2016) (0.21 g m^{-2} day^{-1}) for a horizontal flow pilot FTW. First order removal rates were 1.16 ± 0.43 and 0.61 ± 0.14 day^{-1} for mesocosm FTWs with and without neonicotinoids. Nutrient concentrations for all mesocosms can be found in Table A.3 and Table A.4.

A one way ANOVA test performed within the first 10 days of the mesocosm experiment identified significant differences ($\alpha = 0.05$) for NO_3-N concentrations between control mesocosms and FTW mesocosms with and without neonicotinoids, but no statistical significance between the two FTW treatments. Days 1-2 show no significant differences between the three treatments. However, on day three, NO_3-N concentrations in FTWs with neonicotinoids were significantly different from the control mesocosms while FTW mesocosms without neonicotinoids did not significantly differ from the control or FTW neonicotinoid treatments. While neonicotinoid and non-neonicotinoid treatments were very similar, there was a 1 to 2 day lag between significant treatment effects compared

to control mesocosms that could be attributed to differences in mesocosm biomass. No significant differences between the two FTW treatments were observed. The experiment



did exhibit FTW nitrogen removal was not negatively impacted by the neonicotinoids, contrary to the original hypothesis. After day 3 all sampling days exhibited no differences between the FTW treatments and both FTW treatments were significantly different than control mesocosms (Figure 4).

Figure 4: NO_3-N concentrations, adjusted for evapotranspiration, in control, FTW without neonicotinoids, and FTW with neonicotinoids for the first 10 days of the mesocosm experiment. NO_3-N was completely removed in mesocosms with FTWs by day 7.

By examining water characteristics such oxidation reduction potential (ORP), conductivity, and dissolved oxygen (DO) throughout the experiment, it can be deduced that

denitrification was occurring (Table 2). NO_3-N reduction occurs with ORP below 250 mV (Li and Irvin, 2007) and with low dissolved oxygen. When there is low dissolved oxygen, NO_3-N becomes the preferred electron acceptor in the system. All FTW mesocosms had a DO below 1 mg DO L^{-1} by day 2, although FTWs with neonicotinoids showed anaerobic conditions slightly sooner (Table A.1 and Table A.2). Additionally, ORP began below 250 mV and was negative in all FTW mesocosms by day 7. This indicated sufficient conditions for NO_3-N removal through denitrification. Control mesocosms had aerobic conditions and higher ORP readings throughout the experiment. While ORP dropped below 250 after day 5 in control mesocosms, the presence of dissolved oxygen and the lack of carbon source may be credited to the limited NO_3-N removal.

Table 2. DO, Conductivity, ORP, pH, temperature, and DOC ranges for FTW mesocosms with neonicotinoids (FTW + N), FTW mesocosms (FTW), and control mesocosms throughout the first 10 days of the mesocosm experiment.

	DO mg L^{-1}	**Conductivity µS cm^{-1}**	**ORP mV**	**pH**	**Temperature °C**	**DOC mg L^{-1}**
FTW + N	0.06 to 4.41	676 to 802	-352.7 to 289.1	6.29 to 6.63	23.2 to 26.9	**10.84 to 19.35**
FTW	0.1 to 4.4	685 to 822	-342.6 to 233	6.36 to 7.14	23 to 26.9	**4.97 to 16.68**
Control	0.15 to 7.94	685 to 717	23.6 to 396.6	6.45 to 7.45	23.9 to 27.4	**3.57 to 8.88**

Plant Uptake of Nitrogen

Above and below surface biomass were analyzed for nitrogen content to determine the amount of nitrogen that was incorporated into the plants. A mass balance of nitrogen added to mesocosms during the summer of 2019 exhibited only 3.9% of applied NO_3-N was incorporated into the biomass. Previous wetland mesocosm experiments have used ^{15}N

enrichments to determine the amount of nitrogen leaving the system as gas and observed higher plant uptake rates at lower NO_3-N concentrations and high denitrification rates at higher NO_3-N concentrations (Messer, Burchell, and Bírgand, 2017). While this analysis was not conducted for our study, all amended NO_3-N was removed from the mesocosm water by the FTWs; with 96.1% presumably removed through the process of denitrification. Water characteristics, as mentioned previously, indicated denitrification conditions were present, consistent with the majority of NO_3-N leaving the system in gaseous N_2 form.

Neonicotinoid Removal

Neonicotinoid removal occurred in FTW mesocosms for both imidacloprid and thiamethoxam. However, when compared to mesocosms without FTWs, imidacloprid exhibited a significant reduction ($\alpha = 0.05$), while the decrease observed in thiamethoxam concentrations were insignificant between the FTW and control mesocosms (Figure 5). Further, neonicotinoid byproduct concentrations were minimal compared to the parent insecticides after the 21-day period. Neonicotinoid concentrations on days one and 21 can be found in Table A.5.

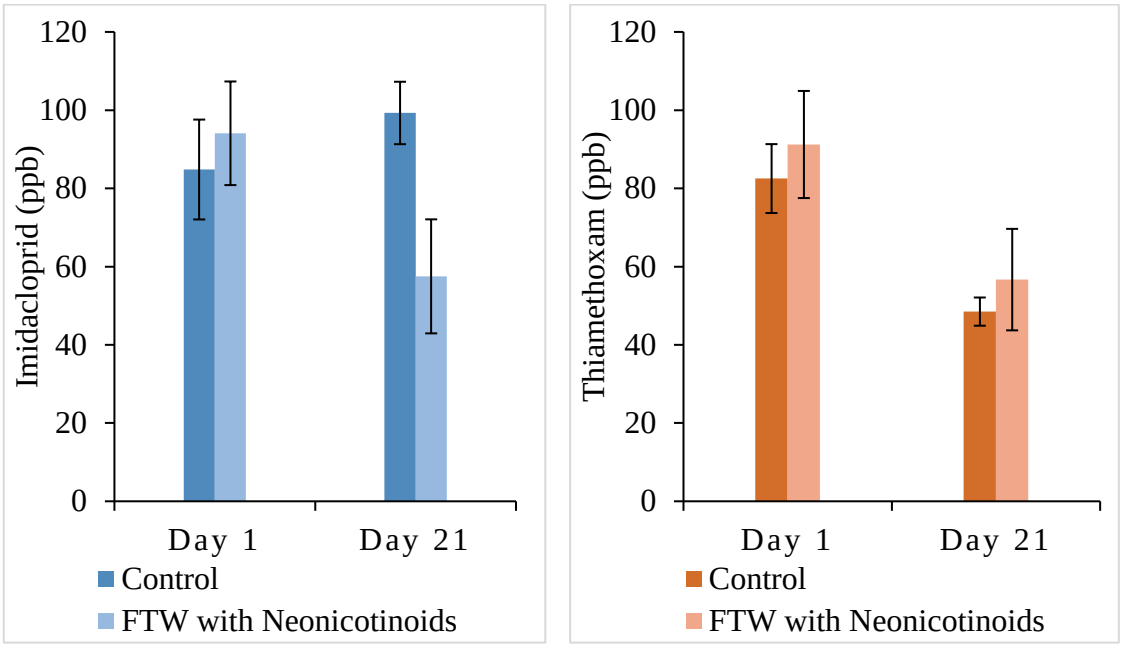


Figure 5: Average imidacloprid and thiamethoxam water concentrations from the beginning and end of experiment for mesocosms with no FTWs (control) and mesocosms with FTW and pesticides (FTW w/ Pesticides). Concentrations were adjusted for evapotranspiration and error bars show standard deviation for the three mesocosms on the corresponding day

Neonicotinoids accumulated in the above and below surface biomass for thiamethoxam, imidacloprid, and their byproducts including imidacloprid desnitro and imidacloprid urea (Figure 6). Above surface concentration ranges for clothianidin, imidacloprid, imidacloprid desnitro, imidacloprid urea, and thiamethoxam were 352.98 to 556.76 ng g^{-1}, 617.34 to 832.21 ng g^{-1}, 40.73 to 97.21 ng g^{-1}, 123.40 to 288.87 ng g^{-1}, and 170.01 to 2,274.94 ng g^{-1}, respectively. Biomass concentrations are recorded in Table A.6. Clothianidin concentrations fell within the range of a past insecticide study where milkweed leaves contained 10.8 to 2,193 ng g^{-1} of clothianidin after soil was dosed with 0.6 to 1.5 g of clothianidin per pot. The same study found LC_{50} for monarch butterflies to be 47 to 205 ng clothianidin g^{-1} with effect on larval growth at 277 and 1,154 ng

clothianidin g^{-1} (Bargar et al., 2020). Monarch larvae often feed on milkweed leaves; therefore, the milkweed leaf concentrations observed in this study would be concerning for full scale implementation.

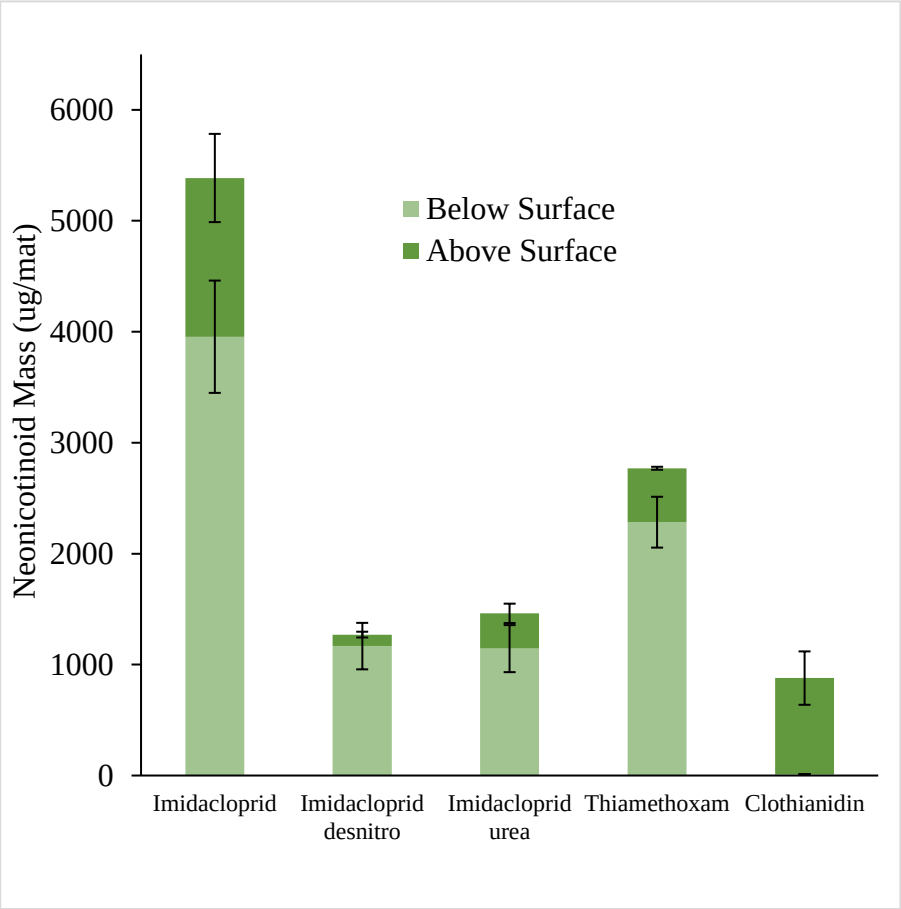


Figure 6. Neonicotinoid and neonicotinoid byproduct mass in above and below surface biomass.

Parent compounds and most byproducts were stored in the roots of the biomass with less neonicotinoid stored in the above surface biomass (Table A.7). This is similar to a glyphosate study where glyphosate was metabolized to aminomethyl phosphonic acid in the roots of tea plants and both compounds were translocated to the leaves (Tong et al.,

2017). Clothianidin, a byproduct of thiamethoxam, was found exclusively in the above surface biomass. Similar metabolism occurred in thiamethoxam treated rice plants where concentrations of clothianidin were found in plants 6-10 days before any clothianidin was detected in the surrounding soil (Ge et al., 2017). A mass balance of the neonicotinoids in the FTW systems accounted for ~80 to 90% of added imidacloprid and thiamethoxam either in the water or plant biomass (Figure 7). The 10 to 20% unaccounted insecticides could have been lost through adsorption onto mesocosm walls and FTW mat, conversion into an unknown/undetected byproduct, or decomposition via photolysis and/or hydrolysis (Todey et al., 2018).

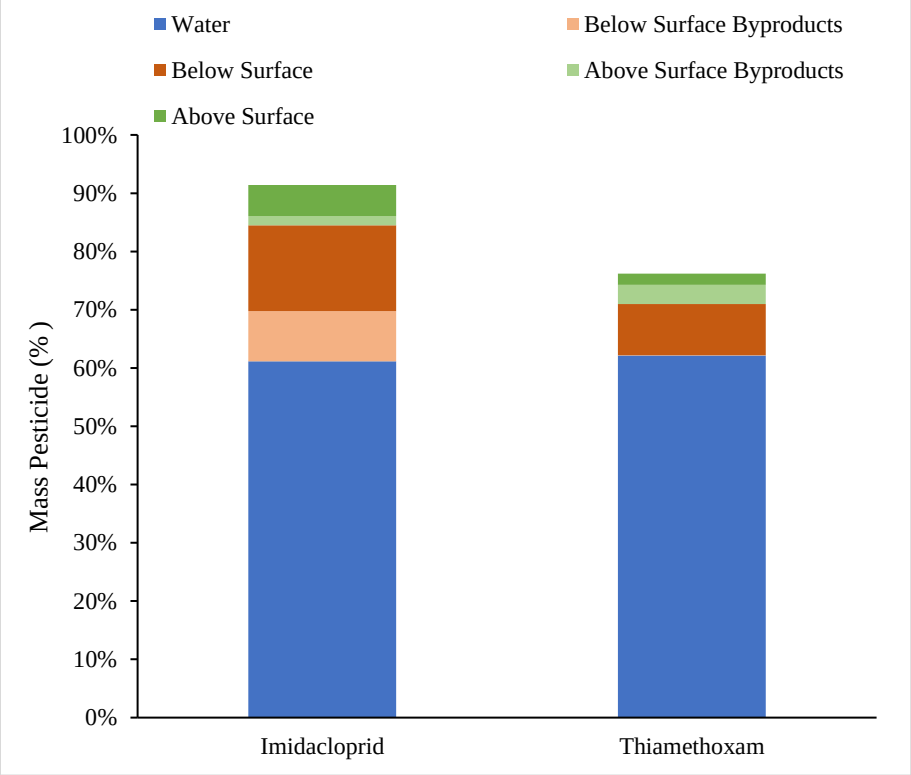


Figure 7. Mass balance of imidacloprid and thiamethoxam in FTW mesocosms on experiment day 21. All mass values are averages of the three FTW with pesticides mesocosms.

Pesticide Persistence in Wetland Plants

The persistence of neonicotinoids in plants have been reported to be minimal. A study investigating imidacloprid and thiamethoxam uptake following seed treatments in cotton found leaf concentrations were nearly 10 times lower 30 days after planting than 14 days following planting, suggesting neonicotinoids degrade relatively quickly in plant material (Kohl et al., 2019). However, other factors may be at play since sampled cotton plants were in the growing stage. One year after the mesocosm experiment, above and below surface biomass samples were analyzed for neonicotinoids and byproducts (table 3). Most imidacloprid and imidacloprid byproducts (desnitro, urea, 6-chloronicotinic acid) resided in the below surface biomass, with 2.98% of the original imidacloprid mass found in the roots. Conversely, thiamethoxam and thiamethoxam byproducts (clothianidin, urea) resided in the above surface biomass. Only 0.1% of the original thiamethoxam added was detected in the biomass. Biomass neonicotinoid and neonicotinoid byproduct concentrations approximately one year after the mesocosm experiment are listed in table A.8)

Table 3. Imidacloprid, thiamethoxam, and byproduct concentration in above and below surface biomass approximately one year after original mesocosm experiment.

	6-Chloronicotinic acid	Clothianidin	Imidacloprid	Imidacloprid desnitro	Imidacloprid urea	Thiamethoxam	Thiamethoxam urea
Above Surface ($ng\ g^{-1}$)	1.34 ± 2.33	1.53 ± 2.65	2.65 ± 3.14	2.96 ± 2.84	3.13 ± 2.80	10.99 ± 19.04	5.19 ± 8.99
Below Surface ($ng\ g^{-1}$)	0.36 ± 0.62	0.00 ± 0.00	4.73 ± 2.18	250.88 ± 91.37	252.16 ± 88.85	0.98 ± 0.41	0.00 ± 0.00

Microbial Assays

When nutrients are removed through plant uptake, biomass decay recycles nutrients back into the water column unless biomass is harvested. FTW roots may provide an attachment site for microbes, allowing a biofilm to form and denitrification, the anaerobic conversion of NO_3-N to nitrogen gas, to occur (Samal et al., 2019). Microbial assays were conducted on mesocosm water samples to determine if neonicotinoids affected the abundance of microorganisms involved in nitrification and denitrification. Ammonia oxidizing archaea and bacteria (*amoA*), nitrite reductase gene (*nirS, nirK*), nitrous oxide reductase gene (*nosZ*) and community microbial abundance (16S ribosomal RNA gene) were analyzed using qPCR (Table A.9). Community abundance was significantly larger in the six mesocosms with FTWs when compared to control mesocosms but there was no significant differences in microbial abundance between the two FTW treatments. There was no difference in the three mesocosm treatments for archaeal *amoA*, *nirS*, and *nosZ* after quantities were normalized based on 16S values. Differences in mesocosms with FTWs and control mesocosms for bacterial *amoA* and *nirK* were significantly different with control mesocosms having larger percentages of total abundance. *NirS* and *nirK*, although different genes, function the same and indicate that the denitrification process is where nitrite (NO_2^-) is transformed to nitric oxide (NO). Conversely, *nosZ* indicates the production of nitrogen gas (N_2), which is the preferred method of nitrogen removal via denitrification.

Mesocosm water contained very low microbial abundance. In retrospect, microbes were likely predominantly located on the FTW roots causing biological activity to occur closer to the rhizosphere. However, rhizosphere samples were not analyzed by qPCR for

this report. While there was no difference between mesocosms with neonicotinoids and without neonicotinoids, FTWs had a significant impact increasing the microbial abundance in mesocosm water. These results led to a potential denitrification experiment to further explore neonicotinoid effects on denitrification.

Denitrification Potential

Incubation experiments confirmed microbial activity associated with FTW roots compared to the water column, which is consistent with low abundance of microbes observed in mesocosm water during the FTW mesocosm experiment. NO_3-N concentrations during the incubations varied between the two microbe sources: mesocosm water and FTW roots (Table A.10). NO_3-N concentrations in the root vials fell below the detection limit (<0.05 mg L^{-1}) after day 3 of the experiment while vials with only mesocosm water remained unchanged through the course of the incubation (Figure 8). This suggests microbes attached to FTW roots were primarily responsible for denitrification in the system and very little NO_3-N was removed in the surrounding water. This is most likely because more microbes are present around FTW roots (Urakawa et al., 2017). The presence of organic matter in FTW roots would help to create anoxic conditions quickly, but in mesocosm water vials, trace oxygen could have inhibited denitrifying conditions and could have limited NO_3-N removal rather than a lack of denitrifying microbes. NO_3-N first order removal rates in root vials with and without neonicotinoids were 1.10 ± 0.27 and 0.85 ± 0.1 day^{-1} respectively, which is comparable to values in FTW mesocosms. While, root vials with neonicotinoids seemed to slow denitrification, NO_3-N concentrations between the two root treatments were not significantly different throughout the 5-day experiment.

Therefore, there is little evidence that presence of neonicotinoids slowed denitrification rates in FTW root systems.

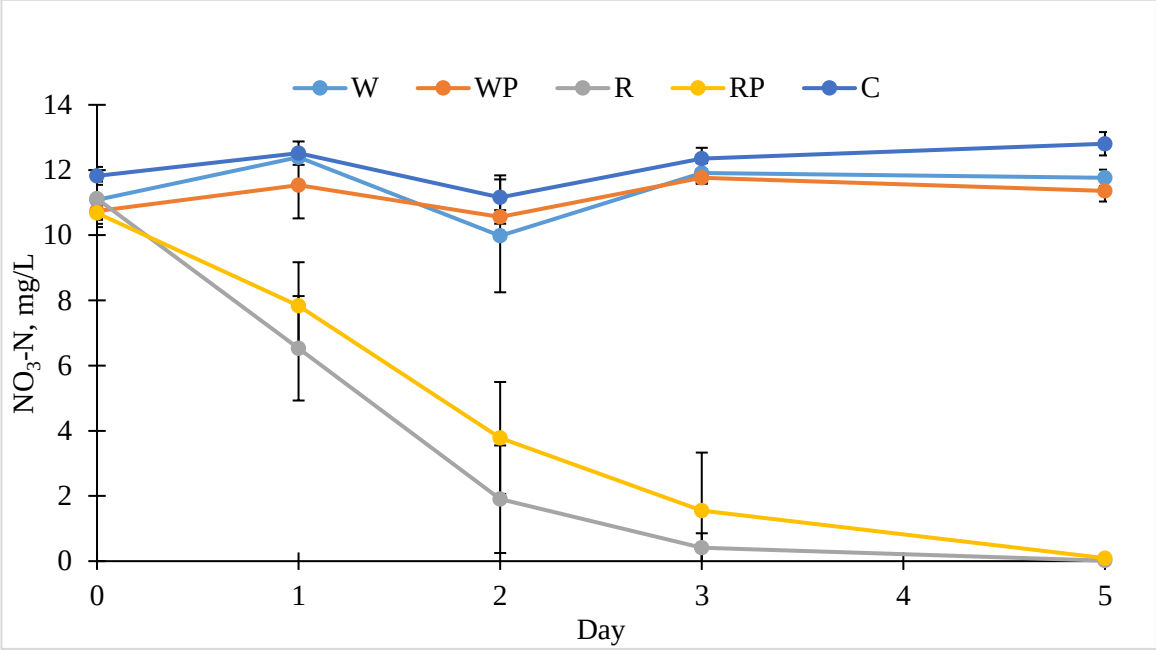


Figure 8. Average NO_3-N concentrations (n=3) in vials with mesocosm water (W), mesocosm water with neonicotinoids (WP), roots (R), roots with neonicotinoids (RP), and sterilized buffer (C). Error bars indicate concentration standard deviation for each treatment.

In order to confirm that NO_3-N removal in FTW roots were not due to biomass incorporation, total nitrogen in roots were analyzed before and after the experiment (Table A.11). Analysis showed a total nitrogen mass decrease of 38.1 ± 4.4% and 40.9 ± 3.8% in roots from no neonicotinoid and neonicotinoid vials respectively from day 0 to day 5. Presumably, nitrogen was released and used by microbes as roots began to break down.

CHAPTER 3: Conclusions

FTWs have been used previously to remove nutrients currently being considered for removal of other contaminants. In this study, neonicotinoid removal by FTWs was explored. FTW mesocosms were enriched with NO_3-N and neonicotinoids thiamethoxam and imidacloprid and compared with FTW mesocosms excluding neonicotinoids to determine neonicotinoid removal potential and nitrogen removing effects. Furthermore, a microcosm incubation experiment compared denitrification potential of FTW microbial communities with and without neonicotinoids.

In the mesocosm experiment, FTWs showed potential to be used for neonicotinoid removal in contaminated surface waters. Imidacloprid and thiamethoxam concentrations decreased in the water column when FTWs were present. This may be contributed to plant uptake with the majority of neonicotinoids stored in the below surface biomass. Neonicotinoid byproducts were not present in the water column, but imidacloprid byproducts were stored in the roots while thiamethoxam byproduct, clothianidin, was found in the above surface biomass.

Neonicotinoids did not significantly alter FTW NO_3-N removal potential in both the greenhouse mesocosm experiment and the potential denitrification microcosm experiment. Furthermore, the microbial community in mesocosm water was not affected by neonicotinoids. During the microcosm experiment, microbes attached to FTW roots were responsible for denitrification while mesocosm water produced little nitrogen removal during the potential denitrification experiments, suggesting denitrifying genes reside in the rhizosphere, not the surrounding water. Microbial analysis of FTW roots

provided insights to specific community changes after neonicotinoids were added. Although, qPCR only quantifies DNA present in the microbial community (Yi et al., 2014), specific nitrifier and denitrifier gene detection indicates these communities are present. Furthermore, the community's capability to denitrify was assessed and found to be most active in the roots of the FTW.

Future studies should analyze mRNA to determine if quantity of expressed genes changes when pesticides are added (Wallenstein et al., 2006). Further research is also needed to investigate the fate and transport of emerging contaminants including pesticides, antibiotics, and microplastics in native wetland plants and potential contaminant recycling as biomass decays. Lastly, FTWs exhibit potential for neonicotinoid removal and should be considered for other emerging contaminants.

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Appendix A: Chapter 2 Supporting Materials

Table A.1: Dissolved oxygen (DO), conductivity, oxidation reduction potential (ORP), pH, temperature, and dissolved organic carbon (DOC) in mesocosms for days 0, 1, 2, 3, and 5 of the mesocosm experiment

Date	Mesocosm Treatment	Rep	DO mg/L	Conductivity µS/cm	ORP mV	pH	Temperature °C	DOC mg/L
8/6/2019	Control	1	7.94	685	305.7	6.45	25.4	8.88
		2	6.85	688	337.9	6.56	25.2	8.27
		3	6.95	688	353.1	6.69	24.6	8.59
	FTW	1	4.4	697	145.4	6.55	23.3	4.97
		2	3.37	735	132.1	6.37	23.4	5.43
		3	2.06	685	131.3	6.38	23.7	6.38
	FTW + N*	1	2.05	715	220	6.32	23.9	15.82
		2	3.67	686	196.5	6.42	24	12.24
		3	4.41	676	187.6	6.48	23.2	14.38
8/7/2019	Control	1	5.44	688	358.2	6.89	25.1	-
		2	4.63	689	379.3	7.03	25.5	-
		3	4.51	687	396.6	6.97	25	-
	FTW	1	1.73	721	128.6	7.14	23.8	-
		2	0.99	709	205.7	6.69	24	-
		3	0.45	734	233	6.57	24.8	-
	FTW + N	1	0.18	727	146	6.52	24.37	-
		2	0.27	708	230.6	6.63	24.7	-
		3	0.5	708	289.1	6.42	24	-
8/8/2019	Control	1	6	688	330	6.99	25.9	-
		2	5.05	691	352.5	7.14	26.4	-
		3	5.22	687	376.2	7.03	25.5	-
	FTW	1	0.6	724	179	6.51	25	-
		2	0.72	728	139.7	6.54	25	-
		3	0.3	742	179.2	6.44	25.8	-
	FTW + N	1	0.08	727	122.4	6.49	25.8	-
		2	0.19	712	162.9	6.52	25.7	-
		3	0.18	710	180	6.44	25	-
8/9/2019	Control	1	0.1	689	239.9	7.11	26	-
		2	0.24	691	271.9	7.28	26.5	-
		3	0.26	688	305.1	7.2	25.6	-
	FTW	1	0.7	729	182	6.58	25	-
		2	0.33	735	130	6.7	25.2	-
		3	0.26	762	141	6.47	26	-
	FTW + N	1	0.1	734	-296.2	6.41	26	-
		2	0.24	713	2.8	6.49	25.8	-
		3	0.26	707	29.3	6.46	25.2	-
8/11/2019	Control	1	4.02	688	192.5	7.37	26.7	-
		2	4.4	717	218.4	7.45	27	-
		3	5.12	705	244.3	7.38	26.4	-
	FTW	1	0.31	752	80.1	6.54	26.3	-
		2	0.38	760	36	6.46	26.4	-
		3	0.18	797	-252.8	6.36	26.9	-
	FTW + N	1	0.1	786	-345	6.29	26.7	-
		2	0.13	727	-232.1	6.43	26.8	-
		3	0.18	735	-242	6.43	26.3	-

"-" indicates that no DOC water sample was collected on the given day

*FTW + N: FTW mesocosms with neonicotinoids

Table A.2: Dissolved oxygen (DO), conductivity, oxidation reduction potential (ORP), pH, temperature, and dissolved organic carbon (DOC) in mesocosms for days 7, 10, 15, and 21 of the mesocosm experiment

Date	Mesocosm Treatment	Rep	DO mg/L	Conductivity µS/cm	ORP mV	pH	Temperature °C	DOC mg/L
8/13/2019	Control	1	4.56	689	98.9	7.18	27.1	-
		2	0.15	696	23.6	7.23	27.4	-
		3	3.69	685	89	7.2	26.2	-
	FTW	1	0.27	766	-116.4	6.63	25.9	-
		2	0.22	754	-185.7	6.49	26.2	-
		3	0.1	794	-317.5	6.36	26.9	-
	FTW + N*	1	0.14	786	-352.7	6.31	26.9	-
		2	0.13	742	-298.1	6.43	26.8	-
		3	0.06	729	-303.6	6.39	26.1	-
8/16/2019	Control	1	0.19	698	-64.5	6.93	24.1	-
		2	2.87	700	28.2	7.19	24.6	-
		3	1.55	697	55.2	7.14	23.9	-
	FTW	1	0.53	777	-220.2	6.98	23	-
		2	0.16	775	-222.1	6.65	23.7	-
		3	0.12	822	-342.6	6.52	24.1	-
	FTW + N	1	0.17	802	-316.2	6.49	23.9	-
		2	0.12	763	-283.6	6.53	23.9	-
		3	0.11	747	-267	6.5	23.6	-
8/21/2019	Control	1	4.3	706	6.9	7.19	26.8	-
		2	4.01	703	30.2	7.46	27.6	-
		3	3.7	724	50.8	7.37	26.7	-
	FTW	1	0.24	821	-331.2	6.52	26.8	-
		2	0.16	815	-305.6	6.45	26.9	-
		3	0.11	866	-279.1	6.46	27.9	-
	FTW + N	1	0.09	844	-337.3	6.4	27.4	-
		2	0.13	812	-344.6	6.4	27.6	-
		3	0.14	780	-347.9	6.35	26.8	-
8/27/2019	Control	1	5.32	709	-35.9	6.51	25.8	3.62
		2	5.44	706	28.8	6.44	25.9	3.57
		3	5.42	705	79.1	6.39	24.5	3.64
	FTW	1	0.6	871	-276.6	6.76	23.8	11.29
		2	0.28	851	-254.9	6.49	24.2	10.11
		3	0.18	898	-325.2	6.5	24.8	16.68
	FTW + N	1	0.09	871	-373.9	7.1	25	19.35
		2	0.14	852	-330.7	7.36	24.3	10.88
		3	0.22	813	-335.5	7.27	23.9	10.84

"-" indicates that no DOC water sample was collected on the given day

*FTW + N: FTW mesocosms with neonicotinoids

Table A.3: Nutrient concentrations in mesocosm water and water depth measurements for days 0, 1, 2, 3, and 5 of the mesocosm experiment

Date	Mesocosm Treatment	Rep	Depth in	NO_3-N mg/L	Phosphorus mg/L	NH_4-N mg/L
8/6/2019	Control	1	13.25	10.47	0.21	0.22
		2	12.75	10.58	0.23	0.26
		3	13.25	10.88	0.22	0.26
	FTW	1	19.00	9.82	0.33	0.10
		2	19.00	10.81	0.29	0.13
		3	19.25	6.06	0.33	0.05
	FTW + N*	1	19.00	12.27	0.38	0.21
		2	19.00	10.43	0.28	0.08
		3	18.50	11.16	0.26	0.08
8/7/2019	Control	1	13.25	10.71	0.23	0.19
		2	12.50	10.47	0.23	0.19
		3	13.00	10.60	0.23	0.21
	FTW	1	18.75	9.16	0.38	-
		2	18.75	9.22	0.33	-
		3	19.00	8.65	0.39	-
	FTW + N	1	19.00	6.20	0.54	0.23
		2	18.75	8.68	0.29	-
		3	18.25	9.13	0.31	-
8/8/2019	Control	1	13.50	10.77	0.22	0.14
		2	12.75	10.65	0.25	0.14
		3	13.00	10.44	0.22	0.16
	FTW	1	18.50	7.04	0.40	-
		2	18.50	6.54	0.32	-
		3	18.80	4.96	0.48	-
	FTW + N	1	19.00	0.49	0.57	-
		2	18.75	3.93	0.25	-
		3	18.25	4.94	0.23	-
8/9/2019	Control	1	13.25	10.50	0.22	0.13
		2	12.75	10.60	0.22	0.11
		3	13.00	10.71	0.25	0.13
	FTW	1	18.25	4.67	0.39	-
		2	18.25	3.55	0.31	-
		3	18.50	0.92	0.54	-
	FTW + N	1	18.75	-	0.72	0.04
		2	18.25	1.10	0.24	-
		3	18.00	0.38	0.19	-
8/11/2019	Control	1	13.25	10.20	0.31	0.20
		2	13.00	10.58	0.22	0.20
		3	13.00	10.22	0.24	0.23
	FTW	1	17.00	1.03	0.45	-
		2	17.80	0.27	0.33	-
		3	18.25	-	0.68	0.04
	FTW + N	1	17.60	-	1.11	0.44
		2	18.00	-	0.36	-
		3	18.60	-	0.23	-

"-" indicates a nutrient concentration below the detection limit

*FTW + N: FTW mesocosms with neonicotinoids

Table A.4: Nutrient concentrations in mesocosm water and water depth measurements for days 7, 10, 15, and 21 of the mesocosm experiment

Date	Mesocosm Treatment	Rep	Depth inch	NO_3-N mg/L	Phosphorus mg/L	NH_4-N mg/L
8/13/2019	Control[A]	1	13.00	10.36	0.22	0.21
		2	12.30	9.44	0.08	-
		3	12.75	9.87	0.18	0.03
	FTW[B]	1	17.75	0.05	0.45	-
		2	17.30	-	0.29	-
		3	17.90	-	0.85	0.15
	FTW + N*[B]	1	18.25	-	1.36	0.84
		2	17.75	-	0.30	-
		3	17.40	-	0.21	-
8/16/2019	Control[A]	1	12.75	9.78	0.13	0.08
		2	12.30	9.48	0.16	0.30
		3	12.60	9.48	0.14	0.25
	FTW[B]	1	17.00	-	0.42	-
		2	16.75	-	0.20	-
		3	17.00	-	0.97	0.07
	FTW + N[B]	1	17.25	-	1.58	0.79
		2	17.00	-	0.32	-
		3	16.80	-	0.18	-
8/21/2019	Control[A]	1	13.00	10.52	0.20	0.01
		2	12.25	10.26	0.23	0.02
		3	12.50	10.32	0.22	0.01
	FTW[B]	1	16.25	-	0.40	0.06
		2	15.75	-	0.17	0.01
		3	16.25	-	1.13	0.14
	FTW + N[B]	1	17.50	-	1.97	2.13
		2	16.25	-	0.30	0.10
		3	16.25	-	0.20	0.07
8/21/2019	Control[A]	1	12.60	9.97	0.22	0.02
		2	12.00	9.78	0.22	0.02
		3	12.60	10.17	0.23	0.02
	FTW[B]	1	15.00	-	0.38	0.04
		2	15.50	-	0.17	0.02
		3	15.00	-	1.23	0.13
	FTW + N[B]	1	16.75	-	2.23	2.73
		2	15.25	-	0.40	0.09
		3	15.00	-	0.05	0.07

"-" indicates a nutrient concentration below the detection limit (<0.05 mg L^{-1})
*FTW + N: FTW mesocosms with neonicotinoids

Table A.5: Neonicotinoid and byproduct concentrations (µg L^{-1}) in mesocosms at the beginning and end of mesocosm experiment

Compound	Date	Control			FTW with Neonicotinoids		
		1	2	3	1	2	3
Imidacloprid	8/7/19	74.38	81.05	99.06	99.25	79.03	103.97
	8/27/19	103.65	91.68	106.24	48.73	55.53	69.35
Imidacloprid desnitro	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	10.16	4.10	2.73
Imidacloprid olefin	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	-	-	-
Imidacloprid urea	8/7/19	-	-	-	-	-	-
	8/27/19	3.15	2.77	3.60	-	-	-
Thiamethoxam	8/7/19	78.70	76.25	92.58	97.14	75.56	100.96
	8/27/19	47.26	46.54	53.55	60.50	44.87	66.66
Clothianidin	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	-	-	-
6-Chloronicotinic acid	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	-	-	-
6-Chloronicotinic aldehyde	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	-	-	-
6-Chloro-N-methylnicotinamide	8/7/19	-	-	-	-	-	-
	8/27/19	-	-	-	-	-	-
6-Hydroxynicotinic acid	8/7/19	9.56	-	-	-	-	-
	8/27/19	-	-	-	2.11	-	-

"-" indicates neonicotinoid concentrations were below detection limit (<2.00 µg L^{-1})

Table A.6: Neonicotinoid and byproduct concentrations (ng g^{-1}) in above and below surface biomass at the end of mesocosm experiment

Compound	Above Surface Biomass			Below Surface Biomass		
	Sedge	*Milkweed*	*Rush*	*Roots 1*	*Roots 2*	*Roots 3*
Acetamiprid	0.32	-	0.37	0.30	0.14	0.35
Azoxystrobin	1.62	8.39	0.55	-	-	-
Clothianidin	556.76	352.98	512.25	3.53	2.40	4.49
Dimethoate	-	-	-	-	-	-
Dinotefuran	-	-	-	-	-	-
Imidacloprid	925.97	617.34	832.21	1020.79	838.72	1703.79
Imidacloprid desnitro	40.73	97.21	89.98	366.27	154.20	530.52
Imidacloprid urea	123.40	207.24	288.87	345.29	151.56	533.66
Indoxacarb	-	-	-	-	-	-
Metalaxyl	-	-	-	-	-	-
Picoxystrobin	-	-	-	-	-	-
Pyraclostrobin	-	-	-	-	-	-
Sulfoxaflor	-	-	-	-	-	-
Thiacloprid	-	-	-	-	-	-
Thiamethoxam	170.01	2274.94	235.81	749.70	454.89	852.63
Thiamethoxam urea	0.81	18.95	2.94	8.50	6.13	11.50
Trifloxystrobin	-	-	-	-	-	-

"-" indicates neonicotinoid concentrations were below detection limit (<0.05 ng g^{-1})

Table A.7: Biomass dry mass from destructive harvest and estimated neonicotinoid/byproduct mass in biomass

	Dry Mass (g)	Number of plants per mat	Clothianidin (µg)	Imidacloprid (µg)	Imidacloprid desnitro (µg)	Imidacloprid urea (µg)	Thiamethoxam (µg)
Sedge	225.0	4	501	833	37	111	153
Milkweed	39.0	2	28	48	8	16	177
Rush	165.0	4	338	549	59	191	156
Roots 1	111.0	10	4	1133	407	383	832
Roots 2	111.0	10	3	931	171	168	505
Roots 3	111.0	10	5	1891	589	592	946

Table A.8: Neonicotinoid and byproduct concentrations (ng g^{-1}) in above and below surface biomass one year after mesocosm experiment was completed

Compound	Above Surface Biomass			Below Surface Biomass		
	FTW1	*FTWA2*	*FTWA3*	*FTW1*	*FTWA2*	*FTWA3*
6-Chloronicotinic acid	4.03	-	-	1.07	-	-
6-Chloronicotinic aldehyde	-	-	-	-	-	-
6-Chloro-N-methylnicotinamide	-	-	-	-	-	-
6-Hydroxynicotinic acid	-	-	-	-	-	-
Acetamiprid	-	-	-	-	-	-
Azoxystrobin	-	0.46	-	-	1.71	-
Clothianidin	4.60	-	-	-	-	-
Dimethoate	-	-	-	-	-	-
Dinotefuran	-	-	-	-	-	-
Imidacloprid	6.25	1.21	0.49	2.37	5.12	6.69
Imidacloprid desnitro	6.16	1.99	0.73	271.04	330.48	151.12
Imidacloprid olefin	-	-	-	-	-	-
Imidacloprid urea	6.27	2.22	0.90	273.49	328.39	154.58
Indoxacarb	-	-	-	-	-	-
Metalaxyl	-	-	-	-	-	-
Picoxystrobin	-	-	-	-	-	-
Pyraclostrobin	-	-	-	-	-	-
Sulfoxaflor	-	-	-	-	-	-
Thiacloprid	-	-	-	-	-	-
Thiamethoxam	32.97	-	-	0.82	0.67	1.45
Thiamethoxam urea	15.57	-	-	-	-	-
Trifloxystrobin	-	-	-	-	-	-

"-" indicates neonicotinoid concentrations were below detection limit (<0.05 ng g^{-1})

Table A.9: Microbial concentration in mesocosm water during the mesocosm experiment for 16S ribosomal RNA (log scale) and archaeal amoA, bacterial amoA, nirK, nirS, and nosZ (normalized by 16S)

Date	Mesocosm Treatment	Rep	log 16S[1]	archaeal *amoA*[2]	bacterial *amoA*	*nirK*	*nirS*	*nosZ*
8/6/2019	Control	1	5.60	-[4]	3.48E-03	7.03E-03	2.31E-02	4.88E-03
		2	5.29	-	7.86E-03	3.43E-02	1.07E-02	-
		3	5.37	-	8.00E-03	-	9.41E-04	5.23E-03
	FTW	1	6.52	4.96E-03	4.59E-03	3.61E-03	1.19E-02	3.87E-04
		2	6.77	2.67E-03	2.57E-03	2.47E-03	1.46E-02	3.25E-04
		3	6.91	4.87E-03	1.62E-03	5.15E-03	2.28E-02	4.01E-04
	FTW + N[3]	1	7.13	4.25E-03	9.46E-04	5.54E-03	3.41E-02	2.12E-04
		2	6.34	6.14E-03	7.81E-03	5.77E-03	2.41E-02	1.51E-03
		3	6.53	4.92E-03	3.89E-03	3.13E-03	2.94E-02	1.51E-03
8/13/2019	Control	1	5.30	-	7.14E-03	1.64E-02	5.15E-04	-
		2	6.86	4.18E-03	6.02E-04	7.23E-04	1.04E-04	-
		3	5.22	-	9.31E-03	-	1.77E-03	-
	FTW	1	7.00	2.03E-03	6.79E-04	7.93E-04	1.00E-02	5.05E-04
		2	6.23	-	7.36E-03	2.08E-03	1.41E-02	-
		3	6.67	6.02E-03	2.24E-03	1.56E-03	9.39E-03	-
	FTW + N	1	6.81	4.80E-03	1.68E-03	3.27E-03	9.55E-03	4.29E-04
		2	6.60	2.21E-03	2.95E-03	2.18E-03	1.66E-02	-
		3	6.62	3.81E-03	3.24E-03	1.17E-03	1.15E-02	2.76E-04
8/16/2019	Control	1	6.59	1.40E-03	1.48E-03	1.65E-03	5.49E-04	1.37E-03
		2	5.91	1.12E-02	7.27E-03	1.39E-02	2.95E-03	-
		3	7.14	2.48E-04	1.11E-04	9.97E-04	6.23E-04	1.98E-04
	FTW	1	6.57	1.70E-03	2.17E-03	1.15E-03	2.54E-03	-
		2	6.79	1.18E-03	1.65E-03	7.02E-03	2.71E-03	-
		3	6.16	3.98E-03	6.38E-03	-	3.93E-03	-
	FTW + N	1	6.94	1.33E-04	1.01E-03	1.72E-03	4.41E-03	1.89E-04
		2	6.40	1.42E-03	3.34E-03	7.14E-03	3.37E-02	7.71E-04
		3	7.21	4.19E-05	4.77E-04	6.89E-04	1.12E-02	6.65E-05
8/21/2019	Control	1	5.37	5.28E-03	3.95E-02	9.41E-02	3.92E-02	4.37E-03
		2	5.16	1.50E-02	6.15E-02	1.79E-01	1.39E-02	-
		3	5.37	1.66E-02	3.40E-02	2.52E-01	1.85E-03	-
	FTW	1	7.55	1.75E-03	2.22E-04	2.61E-03	1.28E-02	-
		2	7.46	5.53E-04	3.73E-04	8.43E-04	1.73E-03	7.85E-05
		3	6.79	2.38E-03	1.39E-03	3.54E-03	1.65E-02	2.84E-04
	FTW + N	1	6.94	1.31E-04	8.13E-04	1.59E-03	1.20E-02	1.59E-04
		2	7.05	5.87E-03	7.62E-04	1.28E-03	2.03E-03	-
		3	6.14	4.21E-04	6.00E-03	7.70E-03	3.10E-02	1.91E-03
8/27/2019	Control	1	5.00	1.60E-02	5.76E-02	1.74E-01	9.42E-04	1.04E-02
		2	4.95	5.24E-02	5.42E-02	1.10E-01	8.20E-03	-
		3	5.48	1.31E-02	2.35E-02	4.43E-02	9.63E-04	3.38E-03
	FTW	1	7.05	2.72E-03	6.73E-04	2.65E-03	5.60E-03	1.00E-04
		2	6.79	1.36E-03	1.23E-03	2.88E-03	1.04E-02	3.05E-04
		3	6.99	2.04E-03	9.27E-04	1.29E-03	4.81E-03	1.45E-04
	FTW + N	1	6.19	1.60E-01	6.28E-03	1.24E-02	1.32E-03	1.31E-03
		2	6.51	4.49E-03	3.17E-03	5.29E-03	1.08E-03	5.86E-04
		3	7.48	6.54E-04	2.33E-04	1.02E-03	2.83E-03	8.78E-05

1. 16S ribosomal RNA measured in log (copies mL^{-1})
2. Archaeal *amoA*, bacterial *aomA*, *nirS*, *nirK*, and *nosZ* are normalized by dividing gene concentration (copies mL^{-1}) by 16S ribosomal RNA concentration (copies mL^{-1})
3. FTW + N: FTW mesocosms with neonicotinoids
4. "-" indicates concentration below level of detection

Table A.10: Nitrate-nitrogen (NO_3-N) concentrations in microcosm vials on days 1-5 of potential denitrification experiment

Vial Treatment	Rep	Day 1	Day 2	Day 3	Day 4	Day 5
Roots	1	10.56	4.77	0.24	0.39	0.06
	2	10.80	6.91	1.92	-	-
	3	12.00	7.91	3.54	0.87	-
Roots + Neonicotinoids	1	10.80	8.51	3.96	1.01	-
	2	10.80	6.30	1.98	0.11	0.09
	3	10.44	8.70	5.40	3.54	0.19
Mesocosm Water	1	11.28	12.51	7.98	11.99	11.79
	2	11.52	12.28	10.98	11.57	11.50
	3	10.44	12.39	10.98	12.16	12.00
Mesocosm Water + Neonicotinoids	1	10.86	12.05	10.68	11.97	11.56
	2	11.16	12.19	10.68	11.63	10.98
	3	10.20	10.36	10.32	11.68	11.52
Control	1	11.88	12.16	10.74	12.61	12.78
	2	11.52	12.52	11.94	11.99	13.17
	3	12.06	12.88	10.80	12.47	12.46

"-" indicates NO_3-N concentrations were below detection limit (<0.05 mg L^{-1})

Table A.11: Mass, volume, and nitrogen content for root biomass in microcosm vials

	Rep	Dry Weight (g)	Volume (mL)	% Nitrogen		Nitrogen Mass (g)	
				Day 0	*Day 5*	*Day 0*	*Day 5*
Root Vials	1	0.5	14.2	1.64	1.09	8.19	5.47
	2	0.51	16.3	1.98	1.20	10.12	6.14
	3	0.42	14.6	2.06	1.20	8.65	5.04
Root + Neonicotinoid Vials	1	0.47	13.8	1.64	1.02	7.70	4.78
	2	0.43	16.1	1.98	1.20	8.53	5.16
	3	0.37	12.0	2.06	1.13	7.62	4.18