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Implications of Nanopesticides on Downstream Wetland Ecosystems

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IMPLICATIONS OF NANOPESTICIDES ON DOWNSTREAM WETLAND ECOSYSTEMS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food, and the Environment
at the University of Kentucky

By

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2024

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ABSTRACT OF THESIS

IMPLICATIONS OF NANOPESTICIDES ON DOWNSTREAM WETLAND ECOSYSTEMS

Nanopesticides are thought to be a promising course of action for reducing agricultural impacts on the environment, however, little is known regarding the fate and transport of nanopesticides, specifically their influence on downstream wetland ecosystems. The objective of this study was to assess the implications of a nano-Cu fungicide (Kocide 3000) and a neonicotinoid insecticide (imidacloprid) on downstream wetland habitats, particularly their effects on wetland nutrient cycling, using fifteen mesocosm wetlands. The complex interactions between nitrogen, Kocide 3000, and imidacloprid were found to increase nitrate removal rates, decrease phosphate removal rates, and inhibit nitrogen uptake in below-ground biomass. Each treatment, with the exception of the pure control, was found to remove 84 – 99% of nitrate over the sampling period, with removal rates ranging from 0.42 to 1.69 d^{-1}. Imidacloprid was observed to photodegrade but was not completely removed from the wetlands by the end of the sampling period. Large pre-existing copper concentrations in source water led to inconclusive results regarding Kocide 3000 removal. Findings from this study can provide insight on the fate of nanopesticides in downstream wetland habitats, as well as provide guidance for the design of best management practices for managing agroecosystem pesticide loads.

KEYWORDS: Nanopesticides, wetlands, neonicotinoids, nano-fungicides

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IMPLICATIONS OF NANOPESTICIDES ON DOWNSTREAM WETLAND HABITATS

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DEDICATION

To Dylan and Molly.

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CHAPTER 1. INTRODUCTION

1.1 Use of Pesticides in Agriculture

1.1.1 History of Pesticides

The use of additives to improve the biological performance of crops and increase yields dates to as early as the 18th century [1]. While these early additives consisted of simple ingredients such as flour, sugar, and molasses mixed with lime, sulfur, and copper, the practice prompted the creation of complex chemical pesticides that are still being refined today [1]. Late in the 20th century, the use of synthetic pesticides became widespread with organophosphates being introduced in the 1960s, followed by carbamates in the 1970s, and pyrethroids in the 1980s [2]. Over time, the use of pesticides skyrocketed. Their many benefits (i.e., improved productivity, protection against crop losses, control of vector diseases) prompted pesticide users to adopt the "if a little is good, a lot more will be better" mentality [2].

Today, pesticides are an integral part of agriculture production systems. In the United States alone, over 900 million kilograms of pesticides are applied to cropland annually [3]. A growing population, a lack of arable land, a preference towards meat-based diets, and a demand for bioenergy crops is driving the need for more efficient agriculture [4]. While the capacity of arable land has increased from 1.9 person/ha to 4.3 person/ha from 1908 to 2008, mainly due to the invention and refinement of synthetic pesticides, there is still concern regarding the exploitation of pesticides [4]. Previous studies have shown that excessive doses of synthetic pesticides can significantly alter the efficiency of nutrient uptake and use by plants [5]. Therefore, this increased dependency

on pesticides in recent decades has led to many issues, specifically regarding the environmental impacts and the hazards to human health [6].

1.1.2 Introduction of Nanopesticides

In the early 2000s, agricultural engineers and chemists turned to nanomaterials to improve traditional agriculture practices, including pesticide development. Due to their chemistry, size, and potentially non-biodegradable characteristics, nanomaterials have been an emerging field in agriculture for the use of fertilizers, pesticides, and sensors [6]. Kah et al. (2013) defined nanopesticides as "any pesticide formulation that intentionally includes entities in the nanometer size range, is designated with a 'nano' prefix, and/or is claimed to have novel properties associated with the small size" [7]. Nanopesticides have been shown to be more effective than their conventional counterparts. For instance, Gopal et al. (2012) determined nanohexaconazole was five times more effective as a fungicide than hexaconazole against *Rhizoctonia solani*, and nanosulfur was ten times more effective as a miticidal than sulfur when managing red spider mites [8]. Nanopesticides are also thought to be a promising course of action for reducing agricultural impacts on the environment and human health as nanopesticides are able to effectively target pests with smaller quantities and less frequent applications compared to conventional pesticides [5, 8, 9]. This is due to their targeted, prolonged release as well as their resistance to premature degradation [7, 9]. Guan et al. (2010) showed nanopesticides left the same amount of residue on crop leaves as their conventional counterparts but deposited less in the soil than conventional pesticides [10].

The impacts of nanomaterials in agriculture are predicted to surpass that of the green revolution and farm mechanization over the next two decades; however, a lack of

information regarding the effects of nanomaterials on environmental and human health is giving regulators pause before permitting their widespread use [8, 11]. The direct, repeated application of nanopesticides could lead to large masses of chemicals introduced into the environment; chemicals of which there is limited knowledge regarding their effects on plant microbiomes, bioavailability, and toxicity levels [9]. Previous studies regarding the impacts of nanopesticides have stressed concerns regarding plant and soil health, food quality and safety, soil fertility, and ecosystem health, as described in more detail in the following sections [9].

1.2 Fate and Transport of Nanopesticides

The goal of pesticides is the same, regardless of the formulation and application. Pesticides are designed to be harmful to target species and harmless to non-target species, including people and organisms that perform beneficial ecosystem services. While sound in theory, pesticides are often harmful to non-target species, leading to controversy of the use and abuse of pesticides [2]. Additionally, a very small percentage (2 - 20%) of pesticides actually make it to their intended biological targets; the remainder of the pesticides are released into the environment [12, 13, 14, 15]. Once released, pesticides face three outcomes: breakdown into harmless byproducts, interact with existing chemicals to form more toxic substances, or resist degradation and remain unchanged for extended periods [12]. Identifying the ecological effects of nanopesticides on ecosystems is vital for preventing negative consequences, including diminishing biodiversity, drinking water contamination, and loss of ecosystem services [16]. Many pesticides, especially those brought to the market in the past decade, have not been studied extensively in terms of their fate, transport, bioavailability, and toxicity limits; therefore,

there is insufficient knowledge regarding the safety of the pesticides commonly used around the US [6]. Two pesticides – imidacloprid and copper hydroxide ($CuOH_2$) – have been under scrutiny in the past few years due to several studies revealing their negative effects on human and ecosystem health.

1.2.1 Imidacloprid

Neonicotinoid insecticides were first developed in the 1990s, gained popularity in the mid to late 2000s, and are now the most widely used insecticides in the world due to their effective plant protection and low application inputs [14]. In the United States, over 3.3 million kg of neonicotinoids (including imidacloprid) were used in 2014. First introduced by Bayer AG in 1991, imidacloprid is categorized as a chloronicotinyl insecticide and is commonly used as a foliar, soil, and seed treatment [13]. Between 1992 and 2014, the use of imidacloprid grew from zero to one million kg per year [3]. By blocking the microtinergic neuronal pathway, imidacloprid is able to target and effectively eliminate many sucking pests, including aphids, thrips, whiteflies, termites, and turf insects [10, 12, 17].

Multiple studies have been conducted regarding the fate and transport of imidacloprid. Fossen (2006) completed a review and reported imidacloprid to have a high solubility in water and a low soil adsorption coefficient; therefore, imidacloprid is likely to be highly mobile in soils [18]. Thompson et al. (2020) completed a study in the United Kingdom and reported imidacloprid concentrations in soil increased from 6 – 8 ng/g one year after planting to 18 – 60 ng/g six years later [15]. The study also concluded neonicotinoids persist in soils for several years after repeated applications [15]. Both Sanabria (2014) and Khalaf (2013) studied the fate of imidacloprid in soils and

determined the half-life to be one to two years [19, 20]. Additionally, Fossen (2006) reported the half-life of imidacloprid to range from 27 days to 229 days, with factors such as crops, soil chemistry, and light affecting the rate of degradation [18]. Fossen (2006) concluded that the use of cover crops, a low pH, and the presence of light have been shown to correlate to more rapid degradation of imidacloprid in soils [18].

Even when only applied to cropland, imidacloprid can contaminate waterbodies through runoff or spray drift [13]. Imidacloprid has high water solubility; therefore, imidacloprid is prone to leaching and has been found to be prevalent in bodies of water in North America, Australia, Europe, and Asia [14, 20, 21]. Imidacloprid was detected near an ethanol production plant in Nebraska at concentrations exceeding 300 ppb, which greatly surpasses the EPA aquatic health benchmark of 0.385 ppb [22]. Satiroff et al. (2021) also detected imidacloprid in urban, agricultural, and herbaceous watershed surface waters at concentrations ranging from 1 to 1,000 ng/L [3]. According to Tisler et al. (2009), imidacloprid is persistent in the water column and does not easily biodegrade in aquatic habitats [13]. Previous studies have found the half-life of imidacloprid in water to be approximately 40 days depending on pH, temperature, and alkalinity [18, 20]. However, there is conflicting evidence regarding the occurrence of imidacloprid leaching into groundwater; laboratory studies have shown that imidacloprid can leach to a depth of 105 cm at concentrations up to 120 ppb; however, field sampling studies in California did not detect imidacloprid in any of the 33 wells sampled [18, 23]. On the other hand, the EPA detected imidacloprid in wells with water tables at depths of 18 ft during a groundwater monitoring program in New York [24]. Imidacloprid is not commonly found in surface waters as the combination of water and light results in rapid degradation and a

half-life of less than three hours [18]. However, turbid waters with reduced light penetration limit the rate of degradation of imidacloprid by photolysis [15].

1.2.2 Copper Hydroxide

Copper based pesticides are some of the oldest and most widely used pesticides due to their effective management of food spoilage microorganisms, fungi, and microbial pathogens [25]. Copper hydroxide ($Cu(OH)_2$), commercially known as Kocide 3000, is commonly used on forage crops, vegetables, fruits, and trees for its antibacterial and antifungal properties [9]. The formulation itself consists of nanoparticles of copper and nanosheets of $Cu(OH)_2$, which allows for the dissolution of $Cu(OH)_2$ particles followed by the sustained release of copper ions [9, 17]. Many metal nanoparticles have been successfully used as microbial agents including silver, copper, and zinc; however, copper is more cost effective and more readily available than other commonly used metals [26].

Copper is present at low concentrations in vegetables, meat, and fish as well as plants where it is a necessary micronutrient and contributes to chlorophyll synthesis, plant pigment synthesis, and protein metabolism [26]. In aquatic ecosystems, copper is typically identified with concentrations ranging from 0.2 to 30 µg/L [26]. The majority of copper in waterbodies is due to the mobilization of disturbed soils, while the remainder comes from industrial sources such as waste incineration, steel and iron production, metal mining, coal combustion, and fertilizer manufacturing [26]. Once introduced into aquatic habitats, copper accumulates within sediment beds. Recently, copper has been reported to become more stable and unreactive during this period; however, copper has low mobility and remains in sediments and soil for weeks to months after application (2 to 3 weeks as

determined by Willis and Bishop (2016); 6 months as observed by Simonin et al. (2018a)) [9, 25, 27].

Multiple studies have been conducted on the safety of $Cu(OH)_2$ and its effects on non-target species, including beneficial microbial communities and nitrogen-fixing bacteria. Since $Cu(OH)_2$ has antimicrobial properties, there is concern it negatively impacts microbial taxa in addition to target pathogens [27]. In terms of the fate of copper, Vencalek et al. (2016) reported copper pesticides in water had a half-life of approximately eight hours; however, Sharma et al. (2009) observed copper particles had a half-life ranging from 205 hours (at a depth of 23 cm) to 370 hrs (at a depth of 68 cm) [25, 26].

Two studies were conducted by Simonin et al. (2018a) and Carley et al. (2020) focused on testing the effects of $Cu(OH)_2$ at the mesocosm scale. Simonin et al. (2018a) found that while $Cu(OH)_2$ had no negative effects on the plant yields, only a small amount (< 8%) of the nanopesticide was recovered by the plants throughout the experiment with the remaining 92% accumulating in the soil [9]. Simonin et al. (2018a) also concluded copper nanopesticides have insignificant environmental implications to target terrestrial agroecosystems; however, downstream non-target ecosystems may be more vulnerable to contamination [9]. Similarly, Carley et al. (2020) tested the effects of $Cu(OH)_2$ on both terrestrial and wetland habitats [16]. The authors identified $Cu(OH)_2$ in the water column and found that it altered the activity of eukaryotic, fungal, and microbial communities in wetlands [16]. Carley et al. (2020) came to the same conclusion as Simonin et al. (2018a) – nontarget species in nontarget ecosystems were

more vulnerable to copper nanopesticides, while target species in target ecosystems suffered minimal effects [9, 16].

1.3 Negative Effects of Nanopesticide Use

Common agricultural practices, including pesticide applications, can negatively impact soil and water quality as well as harm ecosystem community composition and function [16]. With an increase in nanopesticide use, wetlands are becoming major sinks for these emerging contaminants due to agricultural runoff [28]. Pesticide usage has been shown to negatively impact human and environmental health, as well as harm organisms that perform beneficial ecological services, such as honeybees [29, 30].

1.3.1 Implications on Human Health

Extensive use of neonicotinoid pesticides results in pesticide residues in soils and waterbodies. Neonicotinoid contamination of water has been tested and studied in countries around the world, including in China, Canada, and the United States. In China, six neonicotinoids were found in 100% of samples from the Yangtze and Han rivers; in Canada, three neonicotinoids (including imidacloprid) were found in 90% of samples collected from freshwater streams [15]. In the United States, a study from 1999 to 2015 found imidacloprid at concentrations between 0.008 – 0.202 ng/L in both untreated and treated water samples; imidacloprid concentrations peaked in 2011 when imidacloprid was found in approximately 40% and 30% of untreated and treated water samples, respectively [15, 31, 32].

In addition to drinking water contamination, human exposure to pesticides can occur through consumption of foods with pesticides residues. Neonicotinoid pesticide

contamination of fruits and vegetables was found in almost all foods studied in Massachusetts, with foods testing positive for one or more neonicotinoids and imidacloprid being detected most frequently and at the highest concentrations [15]. Since copper-based nanopesticides are often used to prevent food spoiling, there is a risk of ingesting excessive amounts of $Cu(OH)_2$. The recommended intake guidelines for copper are 0.7 – 1 mg Cu/person-day; however, a diet heavy with fruits and vegetables protected with copper pesticides may exceed the recommended intake [27]. Additionally, copper exposure has the potential to affect the nutritional value of crops due to its effects on metabolites; however, this has not been studied extensively to date [27].

In terms of health risks, acute poisoning of imidacloprid has been shown to damage the respiratory, cardiovascular, and nervous systems as well as cause adverse birth outcomes for mothers exposed during pregnancy [15]. Signs of imidacloprid toxicity can include exhaustion, dizziness, vomiting, disorientation, sweating, and fever [20]. Excess copper exposure can result in neurological and gastrointestinal issues, including stomach pain, hematemesis, headaches, exhaustion, and concentration difficulties [30]. A study on copper nanoparticle exposure in rats found the colon eliminated the majority of unabsorbed particles; however, the remaining copper nanoparticles contributed to impaired liver and kidney function [33]. Due to the small size of nanoparticles, they have been shown to persist in the lungs following inhalation as well as permeate skin and cross cellular membranes to enter the blood stream [33].

1.3.2 Implications on Environmental Health

A study by the Unites States Geological Survey found that 61% of agricultural streams and 90% or urban streams were contaminated with one or more pesticides at

concentrations deemed unsafe for aquatic life [3]. Nanopesticides can enter non-target ecosystems through multiple pathways, including agricultural runoff, surface soil erosion, drift during application, subsurface drainage channels, and volatilization from the atmosphere [34]. The effects of nanopesticides on the environment have not been extensively studied. However, preliminary studies have determined nanopesticides negatively impact non-target aquatic ecosystems, particularly beneficial microbial populations. For example, microbial populations already stressed by a lack of available nutrients will have lower resilience and less energy to manage additional stress, including that caused by significant nanopesticide influxes [9, 16]. Wetland habitats and aquatic species have also been found to be more vulnerable to pesticide application compared to terrestrial or agricultural ecosystems [9].

The environmental effects of imidacloprid, particularly its effects on aquatic and terrestrial organisms, have been investigated due to imidacloprid's large-scale and widespread use. Tisler et al. (2009) tested the toxicity of imidacloprid on multiple aquatic organisms, including algae, bacteria, daphnids, and zebrafish and reported imidacloprid was not highly toxic to many species, but that combination of imidacloprid with solvents increased the toxicity [13]. Additionally, Tisler et al. (2009) observed water fleas were the most sensitive to imidacloprid, followed by bacteria, zebrafish, and algae [13]. In terms of ecotoxicity levels, Satiroff et al. (2021) reported imidacloprid concentrations at a recreational lake receiving urban runoff exceeded ecotoxicity levels for many aquatic invertebrates [3]. In a literature review by Khalaf (2013), imidacloprid was reported to potentially induce toxicity in aquatic invertebrates and fish at low levels [20]. Berheim et al. (2019) conducted a study regarding the effects of imidacloprid on white tailed deer

and reported fawns and adult deer with higher concentrations of imidacloprid in their spleen were smaller, less healthy, and less active than those with no or low levels of imidacloprid in their organs [14]. Other non-target vertebrates, such as rats, mice, rabbits, partridges, tilapia, and frogs, have also shown adverse effects after being exposed to neonicotinoid insecticides [14].

Similar to imidacloprid, $Cu(OH)_2$ has been found to have adverse environmental effects. While copper is a necessary element for many biological processes, excessive copper doses can be toxic to many species, primarily those that reside in aquatic habitats [27]. Carley et al. (2020) studied the effects of $Cu(OH)_2$ on multiple taxonomic groups, including proteobacteria, algae, fungi, amoeba, and cercozoans and concluded non-target wetland habitats were vulnerable to repeated pesticide exposure [16]. More specifically, eukaryotic taxa (microscopic worms, cercozoans, and amoeba) were the most sensitive to pesticide exposure and experienced the largest population decline throughout the experiment [16]. Keller et al. (2017) found copper exposure resulted in significant membrane damage and oxidative stress in two species of bacteria [27]. Similarly, soil microorganisms that degrade organic matter may be especially vulnerable to the application of antimicrobial products, as determined by Simonin et al. (2018a) [9]. However, Simonin et al. (2018a) also found that microbes resistant to metal contamination, including mycorrhizal, were not found to be impacted by copper exposure [9].

Even more concerning, the combination of $Cu(OH)_2$ and nutrient enrichment has led to algal blooms and reduction in dissolved oxygen in a second study conducted by Simonin et al. (2018b) [28]. While the addition of $Cu(OH)_2$ correlated to more frequent

and larger algal blooms, Simonin et al. (2018b) did not observe a decrease in ecosystem productivity typically observed in algal blooms caused by phosphorus and nitrogen [28]. In plants, copper-based pesticides have been shown to cause stunted growth, high stress, altered metabolite profiles, reduced photosynthesis rates, and increased transpiration rates [27]. Overall, $Cu(OH)_2$ has the potential to cause severe damage to vital ecosystem elements.

1.3.3 Implications on Honeybee Populations

The recent decline of honeybees has prompted further investigation into the effects of nanopesticides on honeybee (and other beneficial insect) populations, particularly neonicotinoid formulations. As bees are economically valuable pollinators – they are thought to support 9.5% of world food production – there is cause for concern over the effects of neonicotinoids on bees. Between 1947 and 2005, the North American honeybee population decreased by 50%, with similar trends occurring around the world [29]. Neonicotinoid pesticides are systemic, meaning they are absorbed by plants and spread to all tissues, including pollen and nectar; therefore, forager bees have the potential to encounter neonicotinoids through the pollen and nectar of treated plants, and transport the pesticide back to the hive [29, 30].

Multiple studies have been conducted regarding the effects of imidacloprid and its exposure and potential health implications to bees. When exposed at low, sublethal doses, bees express learning and homing behavior impairments as well as reduced immune capacities such as inhibited encapsulation, wound healing, and antimicrobial defense [29]. Following neonicotinoid exposure, queen production, reproductive outputs, and colony growth have also been observed to decline [30]. Woodcock et al. (2016) found

substantial evidence that neonicotinoid exposure resulted in negative impacts on wild bees, with forager bees being three times more negatively affected compared to non-forager bees [30]. Brandt et al. (2016) investigated the effects of neonicotinoid exposure on the immune system of honeybees and observed sublethal doses (5.7 μg/kg) to honeybees showed reduced immune defense and increased susceptibility to pathogens [29]. Gillam (2021) observed multiple honeybee colonies die off in addition to disoriented birds and butterflies in an area with high levels of imidacloprid detected in soils and water sources [22]. Based on the growing evidence of the implications of neonicotinoids to overall bee health, the European Commission banned the use of three neonicotinoid pesticides (including imidacloprid) in 2018 due to concern over honeybee colony collapse; however, neonicotinoid pesticides are still in use in most of the United States.

1.3.4 Implications of Nanoparticles on Wetlands – Silver Nanoparticles

Preliminary research has shown that nanopesticides can interact with nutrients and other emerging contaminants to alter organism uptake and act as environmental stressors [16]. While little research has been conducted regarding the environmental fate and transport of copper nanoparticles, many studies have investigated the effects of silver nanoparticles on wetland habitats. Silver nanoparticles are commonly used for textiles, plastic containers, medical devices, and other consumer products due to their antifungal, antibacterial, and antimicrobial agents [35, 36]. Similar to copper nanopesticides and neonicotinoid insecticides, silver nanoparticles have been shown to be toxic to aquatic and terrestrial organisms [37]. Silver nanoparticles enter water bodies primarily through

wastewater discharge and affect organisms through particle attachment and subsequent reactions, including membrane damage, genotoxicity, and oxidation of proteins [35].

Special focus must be placed on the influence of emerging contaminants on plants as they are a vital component of natural and constructed wetland ecosystems. In terms of ecosystem services, plants provide food, regulate flows, and drive wetland biogeochemistry cycles [37]. The health of aquatic plants is often difficult to assess; while plants may appear healthy, there is still the potential for plants to be experiencing phytotoxicity and physiological stress [35]. When nanoparticles are introduced to wetland habitats, they absorb onto the surface of plants or are taken up by plants [37]. Nanoparticles have the potential to cause stress to the plant due to their toxicity, which may result in reduced plant metabolic activity as well as kickstart reactive oxygen species production in plant cells [35].

A study by Stegemeier et al. (2017) tested the uptake and sorption of silver nanoparticles in duckweed, a common wetland plant; they found that 1-2% of the total silver applied was sorbed into the duckweed tissue [37]. Twenty-four hours after exposure, the silver nanoparticle residue was most prevalent in the apical meristem of the plant, with silver distributed throughout the entirety of the roots; because of this, the authors believed the primary uptake route to be attachment to the root surface followed by migration into the plant tissue [37]. Sixty hours after exposure to silver the plants began turning white, therefore extended exposure to silver nanoparticles can be determined to be toxic to duckweed [37]. Overall, Stegemeier et al. (2017) determined that silver nanoparticles are able to be rapidly accumulated by hydrophytic plants at

concentrations two times greater than commonly observed environmental silver concentrations [37].

Yuan et al. (2018) conducted a similar study in which the authors tested the phytotoxicities and physiological and enzymatic responses of aquatic plants exposed to silver nanoparticles [35]. The study showed that silver nanoparticles increased the prevalence of reactive oxygen species within the plant cells, resulting in oxidative stress; additionally, silver nanoparticle exposure led to reduced chlorophyll content and thus inhibited photosynthesis ability in duckweed [35]. Yuan et al. (2018) determined that silver nanoparticle exposure caused some stress to aquatic plants, however the plants also exhibited enzymatic defenses to tolerate low concentrations of silver [35].

In terms of environmental fate, Colman et al. (2018) conducted a study regarding the effects of chronic and pulse silver nanoparticle exposure in wetland mesocosms [38]. In the month following chronic silver nanoparticle treatment, silver was found to have accumulated in surficial sediment, mosquitofish, snails, and clams; after one year of chronic exposure, silver was observed in surficial sediment, snails, and mosquitofish at concentrations three times greater than those observed after one month of chronic exposure [38]. Colman et al. (2018) found that silver accumulated in primary producers (mainly periphyton), then spread to primary consumers (snails, clams, chironomids) and secondary consumers (dragonflies, mosquitofish, spiders) [38]. The authors also observed the accumulation of silver in Tetragnatha terrestrial spiders, which represented the transfer of silver nanoparticles across ecosystem boundaries from aquatic habitats to terrestrial habitats [38].

Silver nanoparticles have also been shown to affect aquatic microorganisms and microbes through altered community composition and reduced community diversity [44]. A study conducted by Ward et al. (2019) regarding the effects of pulse silver nanoparticle treatments on wetland mesocosm conditions found that the pulse treatments resulted in high silver concentrations, reduced dissolved oxygen concentrations, and declines in prokaryotic cell counts [44]. Specifically, the pulse treatments resulted in the death of sensitive bacterioplankton and membrane damage in plants; however, the microbial community returned to equilibrium one month after the pulse as silver was removed from the water column or transformed into less toxic forms, and silver resistant genes were passed through the community [44]. Ward et al. (2019) also studied long-term chronic silver nanoparticle exposure and observed altered community composition and decreased diversity after 100 days of exposure, with normal community functioning returning after 273 days [44].

1.4 Constructed Wetlands for Pesticide Management

Agricultural best management practices (BMPs) are robust, cost effective, environmentally friendly, and efficient means of mitigating agricultural non-point source pollution [39, 40]. BMPs can be structural or non-structural management practices, such as sediment ponds, vegetated buffers, vegetated ditches, and constructed wetlands that mitigate the effects of agrochemicals [39]. In addition to filtering pollutants, BMPs can perform many ecosystem services such as enhance wildlife habitat in agricultural areas, serve as natural recreation areas, and manage high influxes of water during floods [41, 42, 43].

This study focused on the use of free water surface constructed wetlands for agricultural runoff filtration. Natural wetlands act as ecosystem filters to improve water quality; constructed wetlands are pseudo-natural engineered systems that mimic the physical, chemical, and biological characteristics of natural wetlands in more controlled conditions [42, 44, 45]. They can be used to treat domestic and industrial wastewater, sewage, mine wastewater, stormwater pollution, agricultural runoff, and more [42, 44]. Constructed wetlands consist of plants, substrates, and native microorganisms that remove pollutants thorough physical, chemical, and biological degradation processes (i.e., sedimentation, plant uptake, microbial degradation, and photodegradation) [37, 44].

Microbial degradation and plant metabolization are key contributors in pesticide removal [40]. Typically, only a small percentage of pesticides are removed through plant uptake (approximately 13%) during the spring when the plants are in their vegetative stage [40, 43]. However, the presence of plants increases the degradation of pesticides in the rhizosphere [43, 44]. Nitrification in the aerobic water column and denitrification in the anaerobic litter layer, coupled with ammonia volatilization caused by algal photosynthesis, contribute to nitrogen removal [46].

BMPs have been shown to effectively remove chemicals and sediments from agricultural runoff [39]. Specifically, constructed wetlands are effective in managing high biochemical oxygen demand, chemical oxygen demand, and total suspended solids while exhibiting high total nitrogen and total phosphorous removal rates [44]. Vymazal (2007) determined total nitrogen removal rates for constructed wetlands to range from 40 – 50% with removed loads of 250 – 630 gN/m^2yr and total phosphorous removal rates to range from 40 – 60% with removed loads of 45 – 75 gN/m^2yr [45]. A later study by Vymazal

(2010) found the treatment efficiency for total nitrogen and total phosphorus to range from 40 – 60% and 35 – 50%, respectively [46]. According to Scholz and Lee (2005), constructed wetlands can reduce biochemical oxygen demand by 12%, suspended solids by 22%, overall metal concentrations, and ammonia and nitrate levels for polluted stormwater runoff [42].

Lv et al. (2016) studied the efficiency of constructed mesocosm wetlands on removing pesticides in agricultural runoff; the planted mesocosms successfully removed 60 – 95% of the pesticides during the summer and 30 – 60% in the winter [40]. When studying the removal of pyrethroids in agricultural runoff in California, Budd et al. (2009) found that the concentrations of the three tested pyrethroids decreased significantly from the wetland inlet to the outlet [41]. Overall, Budd et al. (2009) observed 50 – 60% reductions through the use of constructed wetlands [41]. Maillard and Imfeld (2014) studied the pesticide sink and source functions of wetlands and found the dissipation rate for the total pesticide loads to be 96.3% [43].

1.5 Research Objectives

One of the foremost issues facing the 21st century is the rising global population. There is a pressing need for increased food production; however, a balance between agriculture production and environmental health must be achieved [16]. The introduction of nanomaterials, specifically in terms of pesticide production, has the potential to meet rising food demands without sacrificing the health of downstream surface water and wetland ecosystems. However, little information is available regarding the fate and transport of nanopesticides when introduced into the agricultural environment.

Nanopesticides provide many benefits regarding agricultural production. However, they also pose risks to environmental and human health. Imidacloprid, a commonly used neonicotinoid insecticide, and Kocide 3000, a copper-based nano-fungicide, have both shown harmful effects to aquatic organisms and people. Particularly imidacloprid, which has been shown to contribute to the recent honeybee collapse [29, 30]. The development of effective BMPs for managing high nanopesticide loads is necessary to ensure the agricultural industry may continue using nanopesticides to meet rising food demands without harming vital wetland ecosystems.

Therefore, this study focused on the interactions between nanopesticides and wetland ecosystems to determine 1) how the introduction of nanopesticides affects nutrient cycling and 2) whether constructed wetlands are viable BMPs for managing high pesticide loads. The complex interactions between nitrogen, $Cu(OH)_2$, and imidacloprid were hypothesized to impact nutrient kinetic rates, while the chemical and biological processes observed in wetland ecosystems were predicted to alter nanopesticide transformation and degradation rates. Overall, the study aimed to provide insight on the fate and transport of nanopesticide and nutrients in downstream wetland habitats, as well as provide guidance for the design of BMPs for managing agroecosystem pesticide and nutrient loads.

CHAPTER 2. MATERIALS AND METHODS

2.1 Mesocosm Construction and Establishment

Fifteen wetland mesocosms were assembled in August of 2022 at the University of Kentucky's North Farm (Lexington, KY). Each mesocosm consisted of a Rubbermaid 100-gallon tank filled with local topsoil (Bluegrass-Maury silt loam) to a depth of 30 cm. Twelve of the mesocosm contained three different species of common wetland plants – cattail (*Typha latifolia*), soft stem bulrush (*Schoenoplectus tabernaemontani*), and pickerel weed (*Pontederia cordata*). Three mesocosms contained soil but no plants and acted as soil controls. Three 5-gallon buckets were used as pure controls and contained only water. The planting plan and images of a new and an established mesocosm are shown in Figure 1. The mesocosms were established during the Fall 2022 and Spring 2023, during which the mesocosms were watered weekly and slowly inundated to a water depth of 80 cm.

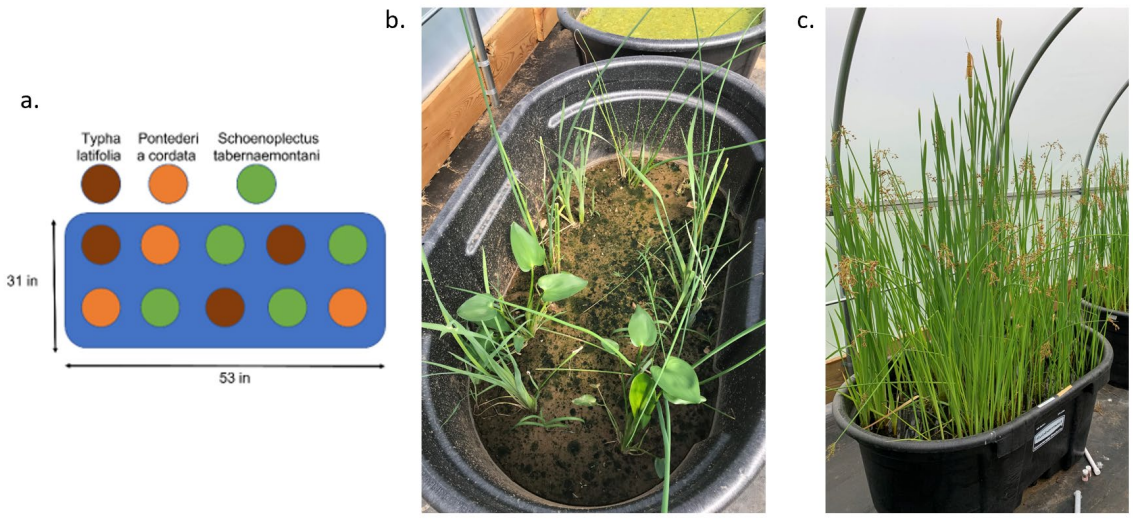

Figure 1. Mesocosm planting plan (a), a newly planted mesocosm in the Fall of 2022 (b), and a fully grown mesocosm in the Spring of 2023 (c).

2.2 Experimental Design

Five different treatments were evaluated in the mesocosms: soil control (C); planted control (CWP); imidacloprid (IMD); $CuOH_2$ (CU); imidacloprid and $CuOH_2$ (IMDCU). Starting in experiment 2, a pure control (PC) with just water was added to provide baseline nitrate, phosphate, and ammonium concentrations. Four experiments were conducted over the summer of 2023 with varying pesticide concentrations: no pesticide application, low pesticide application (50 ng/L), medium pesticide application (500 ng/L), and high pesticide application (1,000 ng/L). Concentrations of 50, 500, and 1,000 ng/L were chosen as they have been previously detected in surface waters and runoff in urban and agricultural watersheds [3]. Regardless of the treatment or experiment, each mesocosm received approximately 10 g of KNO_3 to achieve a target concentrations of 10 mg/L NO_3-N. The breakdown of mesocosm use, treatments, and experiments are shown in Tables 1 and 2 below.

Table 1. Mesocosm treatments.

Treatment	Number of Mesocosms
Pure control (PC)	3
Soil control (C)	3
Control with plants (CWP)	3
Imidacloprid (IMD)	3
$CuOH_2$ (CU)	3
Imidacloprid and $CuOH_2$ (IMDCU)	3

Table 2. Experiments and respective pesticide concentrations.

Experiment	Pesticide Concentration
No pesticide application	0 ng/L
Low pesticide application	50 ng/L
Medium pesticide application	500 ng/L
High pesticide application	1,000 ng/L

A randomization scheme was used to determine the layout of the mesocosms within the greenhouse to limit bias. Figure 2 below shows the final layout of mesocosms and their respective treatments. Three extra mesocosms, shown in black, were established as backups.

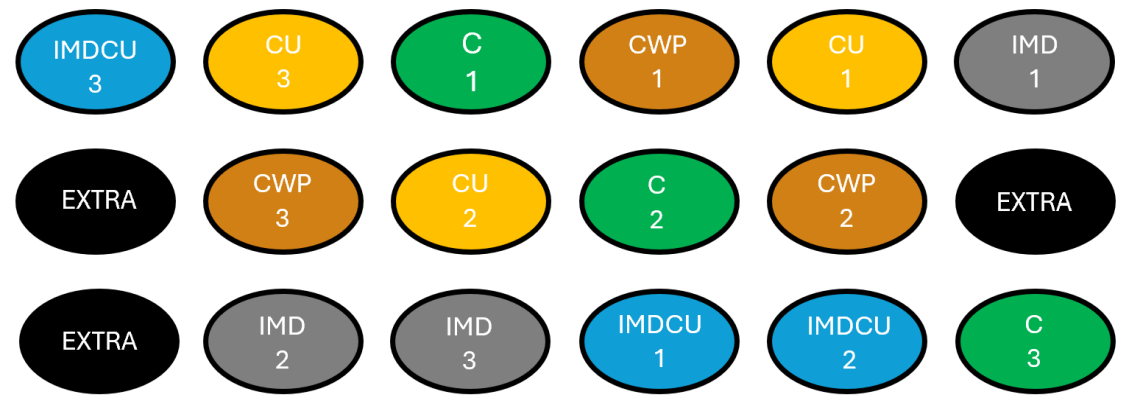

Figure 2. Randomized layout of mesocosms in the greenhouse.

2.3 Sampling Procedure

Sampling was conducted in a greenhouse at the University of Kentucky's North Farm over eight weeks during the summer of 2023. Prior to pesticide application, HOBO pendant temperature and light dataloggers (Bourne, MA) were deployed in each mesocosm to measure temperature and light exposure. All mesocosms were drained before the start of each experiment and filled to a depth of 80 cm with water before day 0. Potassium nitrate (KNO_3) and pesticide concentrations were measured out in the *meso*Lab at the University of Kentucky (Lexington, KY), then added to the designated mesocosm on day 0 of each experiment. The mesocosms were stirred for approximately 30 seconds using a PVC pipe stir rod to ensure adequate mixing before water samples were collected.

Water samples were collected on days 0, 1, 2, 3, 5, 7, and 10 following pesticide application. Samples analyzed for nitrate, phosphate, and ammonium were collected each day; these samples were filtered with 0.7 µm filters to remove soil, algae, seeds, and other large particles and collected in 20 mL glass scintillation vials. The ammonium samples were pre-acidified with a drop of hydrochloric acid for preservation. Samples were stored at 4°C until analyzed; nitrate and phosphate samples had a 48-hour holding period, while ammonium samples had a 28-day holding period. On days 1, 5, and 10, water samples were collected to measure dissolved organic carbon and nanopesticide concentrations. Samples collected for dissolved organic carbon analysis were acidified with hydrochloric acid and filtered with 0.45 µm filters into 40 mL glass vials. Imidacloprid samples were unfiltered and collected in 20 mL amber glass vials to limit light exposure; these samples were later filtered with a 0.22 µm PVDF filter prior to analysis at Western Washington University (Bellingham, WA). Three different samples were collected to be analyzed for copper concentrations: 30 mL unacidified and unfiltered; 60 mL filtered and acidified with nitric acid; and 60 mL unfiltered and acidified with nitric acid. The latter two copper samples were digested for metals prior to analysis for copper concentrations. All samples were stored at 4°C until analyzed.

Soil samples were collected prior to experiment 1 and following the completion of experiment 4. Initial soil samples were collected during the construction of the mesocosms in August of 2022 to provide baseline soil characteristics. Post-experiment soil samples were collected in September of 2023 to show changes in soil characteristics before and after pesticide application. Above and below ground biomass samples were collected prior to experiment 1 and after experiment 4. Above ground plant samples

consisted of the stem and leaves of both cattail and soft stem bulrush plants. The plants were cut where the stem meets the roots and the below ground samples were dug out of the soil and rinsed to remove the soil. All biomass samples were frozen until later analyzed. A more in-depth sampling standard operating procedure (SOP) is included in Appendix A.

2.4 Nutrient Analysis

Water samples were collected and analyzed for nitrate-N (NO_3-N), phosphate-P (PO_4-P), ammonium-N (NH_4-N), dissolved organic carbon (DOC), and nanopesticide concentrations. NO_3-N, PO_4-P, and NH_4-N samples were analyzed on an AQ400 Discrete Analyzer (Mequon, WI) using methods EPA-126-C Rev. 2, EPA-145-C Rev. 1, and EPA-129-C Rev. 3, respectively. DOC samples were analyzed using a Shimadzu TOC-L combustion analyzer (Columbia, MD) following EPA Method 9060A. NO_3-N, PO_4-P, NH_4-N, and DOC samples were analyzed at the *meso*Lab, while water samples to be analyzed for nanopesticide concentrations were shipped to Western Washington University. Imidacloprid samples were analyzed with an Agilent 6545XT AdvanceBio LC/Q-ToF mass spectrometer (Santa Clara, CA); 880 µL of sample was filtered into 2 mL amber glass vials and 200 µL of IMI D4 internal standard was added prior to analysis. Kocide samples were analyzed using an Agilent 7900 ICP-MS (Santa Clara, CS); 1 mL of sample was filtered with a 0.45 µm filter into a 15 mL centrifuge tube with 9 mL of 2% nitric acid prior to analysis. A portion of the samples to be analyzed for copper concentrations were digested for metals at the *meso*Lab before being shipped to Western Washington University for analysis; the metals digestion was conducted based on guidance from EPA 3005A.

Percent removal and first-order removal rates were calculated using equations (1) and (2) below, respectively, as used by (Keilhauer et al., 2019) [47].

$$Percent\ removal = \frac{C_o - C_T}{C_o} \times 100 \quad (1)$$

$$C_T = C_o e^{-kt} \quad (2)$$

in which

C_T = final concentration (mg/L)

C_o = initial concentration (mg/L)

t = time (days)

k = removal rate (d^{-1})

With regards to NO_3-N concentrations, C_O refers to the concentration on day 1 and C_T refers to the concentration on the last day before the level of detection is reached. PO_4-P concentrations, however, use day 1 concentrations and day 10 concentrations for C_O and C_T, respectively, due to the fluctuations of PO_4-P levels over time.

Both above ground and below ground biomass samples were shipped to Ward Laboratories, Inc. (Kearney, NE) to be analyzed for total carbon (%C), nitrogen (%N), phosphorus (%P), potassium (%K), calcium (%Ca), magnesium (%Mg), sulfur (%S), zinc (ppm Zn), iron (ppm Fe), manganese (ppm Mn), copper (ppm Cu), boron (ppm B), and molybdenum (ppm Mo) concentrations using Total Nitrogen and Total Carbon in Plants (Combustion Method) (Code 1096, Rev. 7) and Minerals (Ca, Mg, K, Zn, Fe, Mn, Cu, P, S, Na, Mo, B) via ICP-OES (Code 1045, Rev. 6), respectively. Pre- and post-experiment plant data is included in Appendix B. Above ground and below ground

biomass samples were collected to be later analyzed for imidacloprid concentrations following the neonicotinoid residue analysis outlined by Lindgren et al. (2022) [48].

Soil samples were sent to the University of Kentucky's Regulatory Service's Soil Testing Lab (Lexington, KY) for analysis. The soil samples collected prior to experiment 1 were analyzed following the tests listed in Table 3. The soils samples collected after experiment 4 were tested following the tests listed in Table 3, in addition to a metals analysis that provides information regarding manganese, copper, iron, and aluminum concentrations. The routine soil analysis and cation exchange capacity analysis were conducted following procedures in the Soil Analysis Handbook of Reference Methods [49]. Soil texture was determined based on a micropipette method outlined by Miller and Miller (1987) [50]. Organic matter was measured following guidance from Methods of Soil analysis, Part 2: Chemical and microbiological properties [51]. Pre- and post-experiment soil data is included in Appendix B. Additional pre- and post-experiment soil samples were collected to be later analyzed for imidacloprid concentrations following Lindgren et al.'s (2022) neonicotinoid residue analysis [48].

Table 3. Soil analysis tests ran at the University of Kentucky's Regulatory Services' Soil Testing Lab.

Test Name	**Analytes Reported**
Routine Soil Test	P, K, Ca, Mg, Zn, I M KCl-pH, calculated H_2O pH, buffer pH
Soil Texture	Sand, Silt, Clay, Textural class
Cation Exchange Capacity	CEC, Base saturation, Exchangeable Ca, Mn, K, and Na
Organic Matter and Nitrogen	OM = C*1.72, TN
Water Holding Potential	Field capacity H_2O, Wilting point H_2O, Plant available H_2O
Micronutrients	B, Mn, Cu, Fe

2.5 Statistical Analysis

All statistical analyses were conducted in SAS Studio (SAS Institute Inc., Cary, NC). NO_3-N, PO_4-P, NH_4-N, DOC, and nanopesticide concentrations were adjusted to account for a decrease in water depth over the 10-day sampling period prior to the natural log being applied. Day 0 concentrations were not analyzed due to insufficient mixing. One-way ANOVA and Tukey's Honest Significant Difference tests were run for each experiment with consideration to treatment, sampling day, and treatment by sampling day at a significance level of $\alpha = 0.05$. General linear modeling (GLM) and a linear mixed effects model (GLIMMIX) were also used for the aforementioned analytes to better understand the relationship between sampling day and treatment. Analytes from the biomass and soil samples collected post-experiment were normalized with the natural log and compared for significant differences between treatments using ANOVA. A printout of the SAS code for nutrient, nanopesticide, biomass, and soil analysis is included in Appendix C.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Physical Characteristics of Mesocosms

A handheld YSI probe (Yellow Springs, OH) was used to measure temperature, dissolved oxygen (DO), specific conductivity (SPC), total dissolved solids (TDS), pH, and oxidation-reduction potential (ORP) each sampling day. Minimum, maximum, and mean values for each sampling period are shown in Table 4. Sampling occurred in central Kentucky during the months of June through August, therefore temperatures remained within the 17 to 28°C range (63 to 83°F). Large changes occurred in the C and PC treatments in all four experiments, most noticeably between sampling days 0 through 5 for DO and pH. SPC and TDS were observed to increase between days 0 and 2 before stabilizing; DO displayed the opposite trend and decreased between days 0 and 2 before stabilizing. pH remained stable throughout the sampling period for the majority of the mesocosms, with the exception of the C and PC treatments, which fluctuated between 7 and 8.5. ORP fluctuated throughout the sampling period for all mesocosms, most often increasing between days 0 and 1, decreasing between days 1 and 2, increasing between days 2 and 3, then decreasing and stabilizing after day 3. Between days 0 and 3 during the second sampling period, recalibration of the YSI resulted in a noticeable drop in pH and ORP. After day 3 the pH and ORP levels returned to expected levels.

Table 4. Minimum, maximum, and mean values for each parameter measured with the YSI.

		Experiment 1	Experiment 2	Experiment 3	Experiment 4
Temperature (C)	Min	16.9	17.5	22.3	19.3
	Mean	19.8	21.4	25.0	23.6
	Max	24.5	24.2	28.4	27.3
DO (%)	Min	4.7	13.1	1.2	2.8
	Mean	33.5	43.0	38.5	46.9
	Max	99.2	121.8	141.7	156.0
DO (mg/L)	Min	0.4	1.1	0.1	0.22
	Mean	3.0	3.8	3.2	4.0
	Max	8.8	10.7	11.7	13.5
SPC (µs/cm)	Min	385.5	525.0	431.5	427.9
	Mean	558.6	714.9	661.4	584.2
	Max	698.0	898.0	870.0	807.0
TDS (mg/L)	Min	250.8	341.4	280.4	60.8
	Mean	363.1	593.6	432.3	375.3
	Max	453.5	465.9	802	525.0
pH	Min	6.7	7.0	6.9	6.9
	Mean	7.5	7.4	7.6	7.6
	Max	8.6	9.5	9.9	10.7
ORP (mV)	Min	40.3	20.5	-183.2	-17.8
	Mean	195.7	146.2	37.9	66.1
	Max	491.7	324.0	160.2	187.4

3.2 Nutrient Assessment

3.2.1 Nitrogen Cycling

Potassium nitrate (KNO_3) was added to each mesocosm on day 0 to achieve a concentration of 10 mg/L nitrate-N (NO_3-N). As shown in Figure 3 each treatment was reduced to less than 0.2 mg/L of NO_3-N by day 7 of the sampling period. The PC treatment was the only exception and remained at a concentration of approximately 10 mg/L for the duration of the sampling periods. The PC treatment was emptied and refilled prior to each sampling period; however, algae continued to grow within the PC over the summer and likely resulted in the small decline in NO_3-N levels at the end of experiment

3 and during experiment 4. Sampling day and treatment were found to be significant for NO_3-N in all four experiments (p-value < 0.0001) with sampling days 0, 1, 2, and 3 being significantly different than sampling days 5, 7, and 10. C was found to have significantly higher NO_3-N concentrations than all other treatments in experiment 1 and PC had significantly higher NO_3-N concentrations than all other treatments in experiments 2, 3, and 4. While Simonin et al. (2018b) found total nitrogen (TN) concentrations in mesocosms treated with copper nanoparticles to be 30% higher than mesocosms without copper nanoparticle application, no similar trend in NO_3-N concentrations was observed in this study [28].

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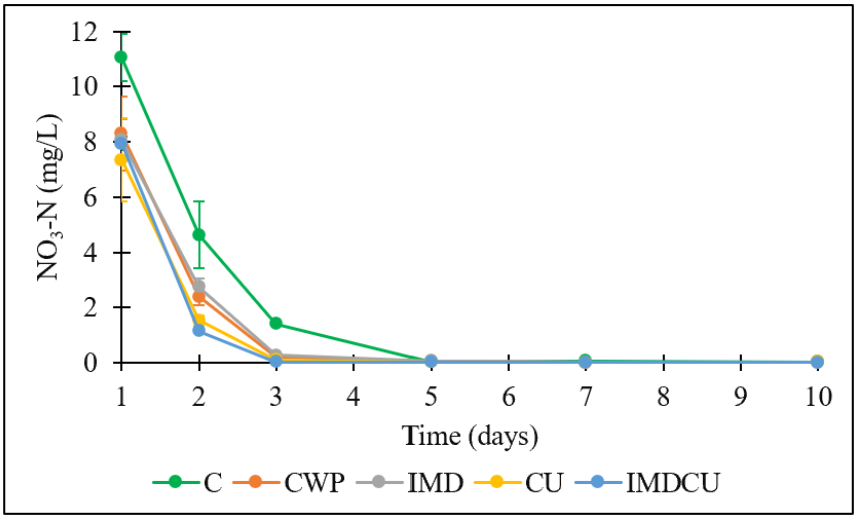

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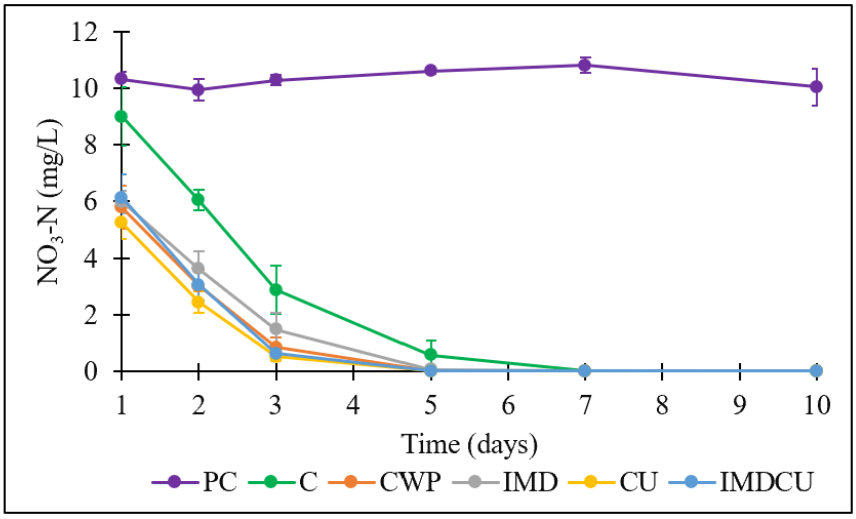

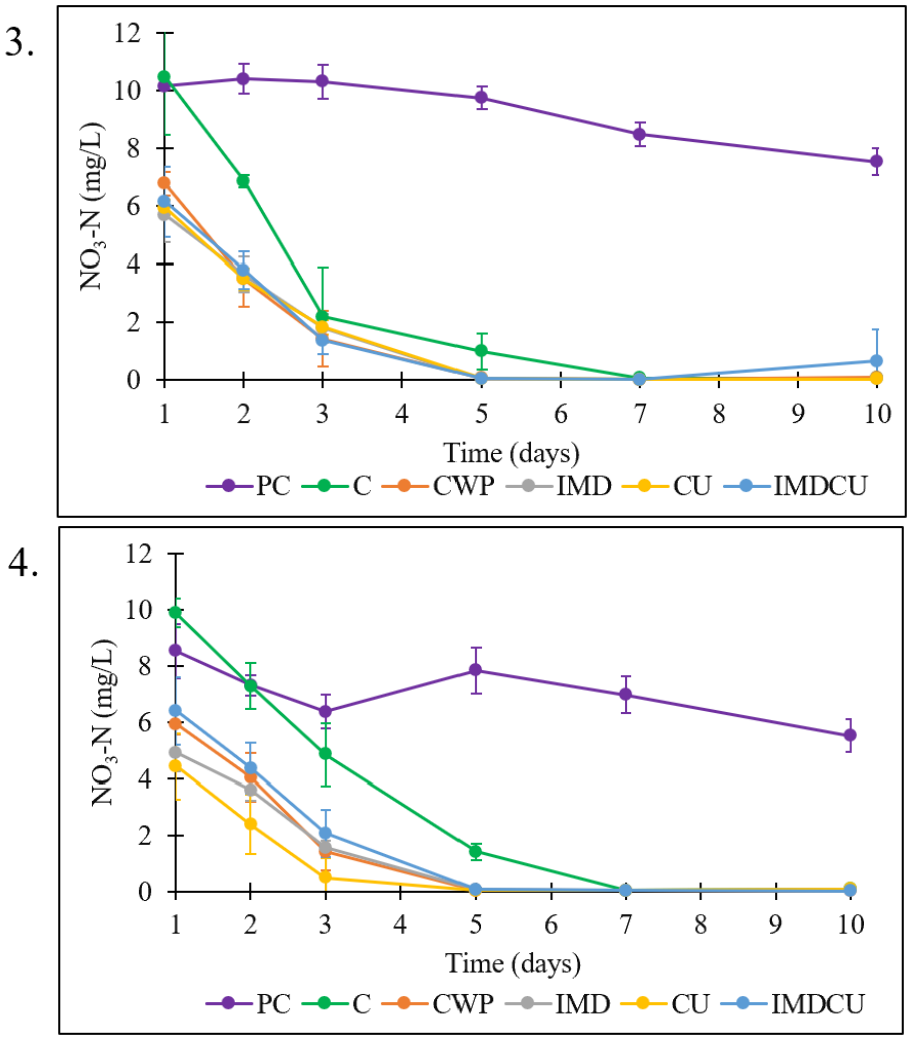

Figure 3. NO_3-N loss in mesocosm wetlands over the 10-day sampling period during (a) experiment 1 at 0 ng/L, (b) experiment 2 at 50 ng/L, (c) experiment 3 at 500 ng/L, and (d) experiment 4 at 1,000 ng/L.

Significant differences in NO_3-N concentrations were observed in treatments by days 2 or 3, with the exception of the IMD and CU treatments in experiment 3 and the IMD and IMDCU treatments in experiment 4, which became significantly different from initial NO_3-N concentrations after 5 days. When comparing treatments, it was observed that the C treatment had significantly higher NO_3-N levels than the planted treatments (CWP, IMD, CU, and IMDCU) by either day 2 or day 3 for all experiments. During

experiment 2, the IMD treatment was significantly different compared to the CU treatment on day 3 (p-value = 0.0146) and the IMDCU treatment on day 3 (p-value = 0.0176). However, during experiment 3 significant differences between the PC and all other treatments were only observed; this was hypothesized to likely be due to the higher temperatures and accelerated NO_3-N removal rates that took place during the experiment 3 sampling period, which has been observed in other wetland studies [45, 47, 52, 53]. During experiment 4, NO_3-N in the CU treatment was found to be significantly lower than the CWP treatment (p-value = 0.0242), the IMD treatment (p-value = 0.0063), and the IMDCU treatment on day 3 (p-value = 0.00071).

Above and below ground biomass were analyzed for nitrogen (N) content following pesticide application; the mean N concentrations are shown in Tables 5 and 6. No significant differences were observed in regard to N concentrations between treatments in both the above ground biomass (p-value = 0.4270) and below ground biomass (p-value = 0.2984). While not significant, the CWP treatment did show much higher N concentrations in the below ground biomass compared to the three treatments that received pesticide application. The difference in N uptake between the CWP treatment and the other treatments demonstrates a potential for inhibition of N uptake in below ground biomass following nanopesticide exposure. Multiple other studies have reported similar findings. For example, Fox et al. (2007) conducted a study regarding the effects of five pesticides (chrysin, methyl parathion, DDT, bispenol A, and pentaclorophenol) on nitrogen-fixing rhizobia and concluded that pesticide application leads to disruptions in signaling between plants and N-fixing rhizobia bacteria [54]. Sun et al. (2015) also studied the effects of two synthesized herbicides with phosphorus

containing heterocyclic rings on *Arabidopsis thaliana* seedlings and found that adsorption of N by the seedlings was significantly inhibited at herbicide applications of 40 and 320 µg/L [55].

Table 5. N concentrations in above ground biomass post-experiment.

	N content (gN/m^2)
CWP	1,048 ± 116.60
IMD	861.97 ± 206.68
CU	1,058.80 ± 166.80
IMDCU	1,062.24 ± 158.27

Table 6. N concentrations in below ground biomass post-experiment.

	N content (gN/m^2)
CWP	8,198.92 ± 814.77
IMD	5,213.90 ± 4,351.02
CU	4,859.63 ± 1,196.52
IMDCU	4,795.26 ± 982.84

Soil samples collected pre- and post-experiment were analyzed for N content (Table 7). N concentrations were provided as percentages; to convert from %N to gN/m^2, it was assumed one acre contains 2,000,000 lb of soils, an assumption used by Kentucky Regulatory Services when analyzing soil samples. Pre-experiment samples showed nitrogen concentrations of 526.80 gN/m^2; post-experiment samples showed similar concentrations ranging from 480 to 560 gN/m^2. N content in soils before and after pesticide application were not significantly different for any treatment. This finding aligns with that of Vyzamal (2007) who found that soil adsorption had one of the lowest magnitudes for nitrogen transformation in FWS wetlands [45].

Table 7. N concentrations in soil samples pre- and post-experiment.

	Pre-experiment (gN/m^2)	Post-experiment (gN/m^2)	T-test p-value
C	526.80 ± 28.94	560.42 ± 54.40	0.2747
CWP	526.80 ± 28.94	514.10 ± 23.55	0.7374
IMD	526.80 ± 28.94	524.56 ± 37.71	0.9588
CU	526.80 ± 28.94	486.45 ± 42.77	0.4119
IMDCU	526.80 ± 28.94	511.11 ± 6.73	0.4708

The percent removal of NO_3-N from the water column for each treatment, with the exception of the PC, ranged from 84% and 99% (Table 8). These values are comparable to those observed by Keilhauer et al. (2019) in FTW mesocosms (82% to 96%) and Messer et al. (2017) in FWS mesocosms (up to 98%) [47, 52]. The removal rates ranged from 0.42/day to 1.69/day for all treatments except the PC. The removal rates increased between experiments 1, 2 and 3, but decreased between experiments 3 and 4; this correlated with an increase in temperature between experiments 1, 2, and 3 and a decrease in temperature between experiments 3 and 4. The PC and C had consistent removal rates of approximately 0.02/day and 0.6/day for all experiments, respectively. The PC was the only treatment that was found to have significantly lower removal rates (p-values < 0.0001). Though not significant, treatments with nanopesticides experienced higher NO_3-N removal rates. More specifically, at 50 ng/L the IMDCU treatment had a higher removal rate than the CWP; at 500 ng/L and 1,000 ng/L all three pesticide treatments (IMD, CU, IMDCU) had higher removal rates. In all experiments average NO_3-N removal rates were determined to be highest for the CU treatment, followed by the IMDCU, IMD, CWP, and C treatments.

The NO_3-N removal rates observed in this study are similar to those observed by Keilhauer et al. (2019) (0.15 - 0.54/day in planted treatments, 0 - 0.01/day in control

treatments) and slightly lower than those observed by Lindgren et al. (2022) (0.61 – 1.16/day) in their FTW studies [47, 48]. Lindgren et al. (2022) also studied the effects of neonicotinoid insecticides on NO_3-N removal and found no significant differences in removal rates in FTW mesocosms with and without pesticides [48]. In this study, the removal rates of the planted mesocosms did increase slightly with pesticide application, though they were also not significantly different as Lindgren et al. (2022) observed.

In terms of removal mechanisms, the observed removal rates indicate denitrification and plant uptake were the two main NO_3-N removal mechanisms. The C treatment did not have plants; therefore, it is likely denitrification was the main NO_3-N removal mechanism in this treatment. Based on the calculated removal rates, denitrification was responsible for 0.4 to 0.6/day removal of NO_3-N. The CWP, IMD, CU, and IMDCU treatments did have plants, however, and it is likely that plant uptake coupled with denitrification were responsible for NO_3-N loss. If denitrification was responsible for 0.4 to 0.6/day of NO_3-N removal, it can be assumed that plant uptake or stimulation of microbes by the nanopesticides was responsible for the remaining 0.14 to 1.17/day observed removal of NO_3-N (calculated by subtracting the C removal rates from the planted treatment removal rates).

Table 8. Percent removal and removal rates for NO_3-N in different mesocosm treatments following retention time of 10 days.

	Removal (%)				Removal rate (d^{-1})			
Treatment	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
PC	NA	-6.21	15.77	25.50	NA	-0.01	0.02	0.03
C	99.64	84.11	99.09	97.73	0.63	0.61	0.52	0.42
CWP	99.47	99.24	98.94	99.44	0.58	0.98	1.14	0.58
IMD	99.63	89.69	99.48	98.56	0.62	0.76	1.32	1.06
CU	99.55	89.39	99.88	99.80	0.60	0.75	1.69	1.55
IMDCU	99.64	99.79	99.44	99.68	0.63	1.24	1.30	0.64

Ammonium-N (NH_4-N) levels were significantly different in terms of sampling day ($p\text{-value}_{exp1} < 0.0001$, $p\text{-value}_{exp2} = 0.0462$, $p\text{-value}_{exp3} = 0.0029$, $p\text{-value}_{exp4} = 0.0103$) and treatment ($p\text{-value}_{exp1} < 0.0001$, $p\text{-value}_{exp2} < 0.0001$, $p\text{-value}_{exp4} = 0.006$) for all experiments except experiment 3, in which case treatment did not significantly influence NH_4-N concentrations (Figure 4). In experiment 1 the NH_4-N concentrations remained consistent before increasing on day 10. NH_4-N concentrations fluctuated in experiments 2 and 3 between days 1 and 5 and then stabilized for the remainder of the sampling period, with the exception of the PC treatment in experiment 2 that increased sharply on day 2 and decreased over the next 5 days. Fluctuating NH_4-N concentrations were observed in experiment 4 over the entirety of the sampling period. Sampling day and treatment were also observed to significantly affect NH_4-N concentrations; specifically, it was observed that day 10 was significantly different than all other sampling days and planted mesocosms were significantly different than the C and PC treatments.

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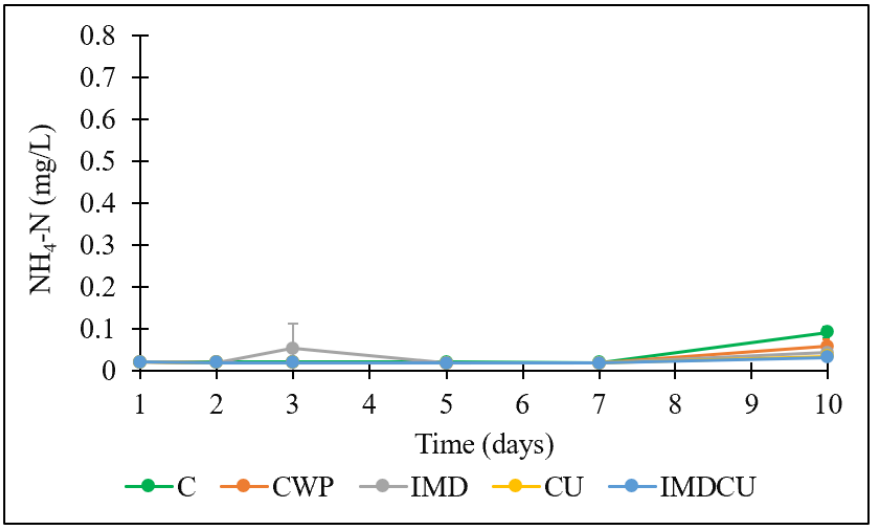


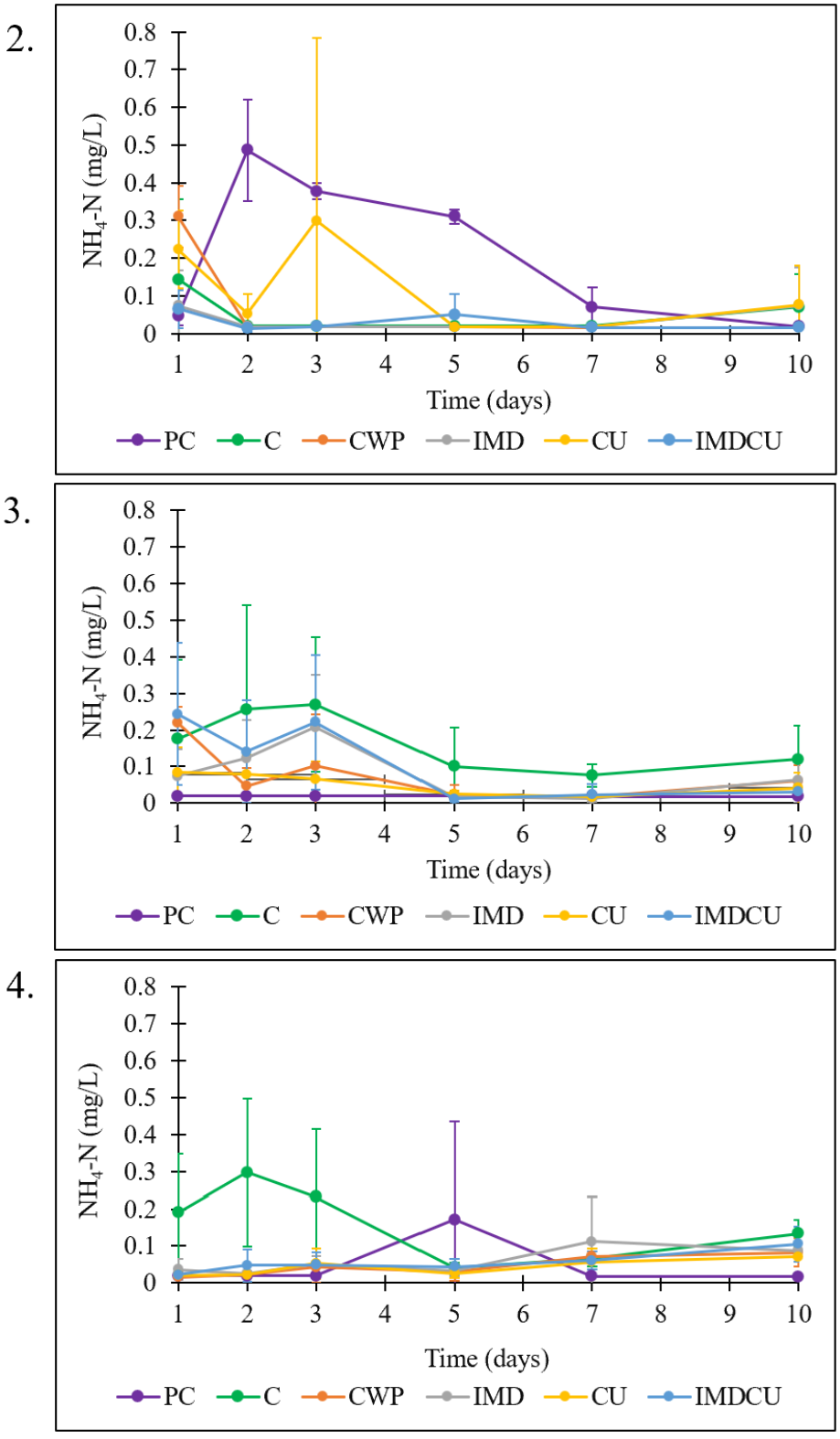


Figure 4. NH_4-N loss in mesocosm wetlands over the 10-day sampling period during (a) experiment 1 at 0 ng/L, (b) experiment 2 at 50 ng/L, (c) experiment 3 at 500 ng/L, and (d) experiment 4 at 1,000 ng/L.

As shown in Figure 4, little to no NH_4-N was detected in experiment 1. In experiments 2, 3, and 4, all treatments fluctuated between 0 and 1.8 mg/L NH_4-N between days 0 and 5, but by day 7 all treatments had NH_4-N concentrations less than 0.3 mg/L. However, between days 7 and 10 all treatments exhibited an increase in NH_4-N levels. The NH_4-N values observed here are comparable to those measured by Messer et al. (2022) in their FWS mesocosm study (<0.05 to 0.48 mg/L); however, the authors reported a decrease in NH_4-N values to below the detectable level after 48 hours [56]. Lindgren et al. (2022) also observed NH_4-N values between 0.20 and 0.84 mg/L, but also identified a drop in NH4-N levels to below 0.05 mg/L after 24 hours [48]. Han et al. (2020) observed rapid decreases in NH_4-N levels over time, which they attributed to the reduction of NH_4^+ to NO_3^- [57].

Within the water column, three main methods of NH_4 removal are possible – volatilization to NH_3 gas, mineralization to inorganic N, and nitrification to NO_3 [58]. It is unlikely that volatilization was a primary NH_4-N removal mechanism in this experiment as volatilization predominantly occurs in water bodies with high pH levels (pH > 9.0) [58]. Both nitrification – the oxidation of NH_4 to NO_3 – and mineralization – the decomposition of organic N into plant available inorganic N – most likely explain the decrease in NH_4-N concentrations between days 0 and 5 [58]. It is believed that the increase in NH_4-N between days 7 and 10 is caused by the oxidation reduction of the soils; as organic matter breaks down and dissolved oxygen levels drop, both NH_4 and PO_4 are released (Figure 5).

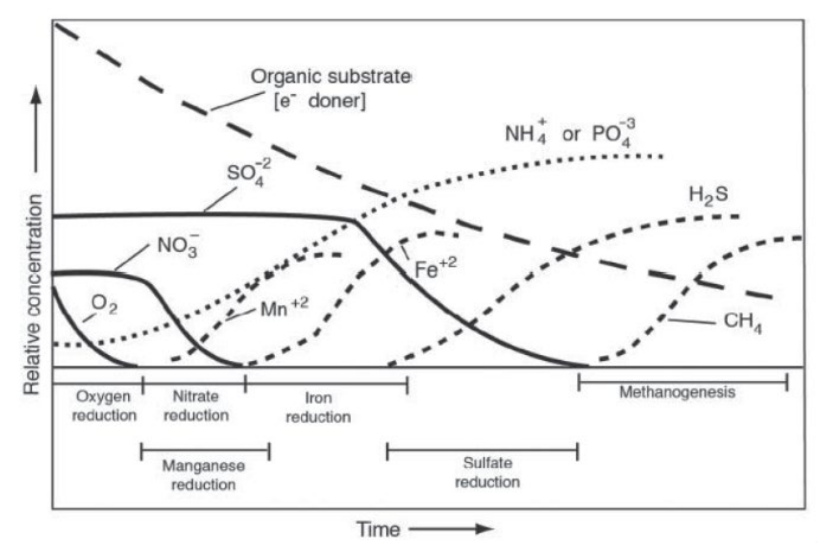


Figure 5. Oxidation reduction of newly flooded wetland soils over time [Taken from Reddy and Delaune, 2008].

The biogeochemical environment observed within the mesocosm wetlands coupled with a consistent loss of NO_3-N supported conditions for denitrification of NO_3-N to N_2. Previous studies found a favorable environment for denitrification includes warm temperatures, a moderate pH, low dissolved oxygen, and low oxidation-reduction potential [48, 53, 60]. Lai et al. (2019) studied the effects of temperature on denitrification and found a rise in temperature correlated to a rise in denitrification rates, with ideal temperatures ranging from 35 to 40ºC [53]. Temperatures for the mesocosms ranged from 16.9 to 27.4ºC; while they do not equate the peak denitrification temperatures observed in Lai et al.'s study, temperatures were warm enough to support active denitrification. A moderate pH (ideally 7.5 to 9.5, as determined by Albina et al. (2019)) supports denitrification; pH for the mesocosms in this study ranged from 6.66 to 9.02 [60]. Lindgren et al. (2022) and Messer et al. (2022) reported low ORP and DO levels were conducive for denitrification [48, 56]. In this study, ORP and DO levels

ranged from -183.2 to 255.7 mV and 0.09 to 10.72 mg/L, respectively, which were similar to the ORP values observed by Messer et al. and the ORP and DO values observed by Lindgren et al. (2022) [48, 56].

While denitrification was not specifically measured in this study, NO_3-N loss was observed in all treatments with the exception of the PC, most likely due to the lack of plant biomass and soils in the PC treatment. Vymazal (2005) describes the process of nitrogen cycling in wetland ecosystems over time; during cold periods, organic nitrogen accumulates as dormant plants are unable to uptake nitrogen [42]. In the warmer months, nitrogen is available through plant decay, and as vegetation matures it is able to uptake nitrogen more readily; at the same time, anaerobic conditions in the soils encourage denitrification [42]. Several similar studies have identified the benefits of planted wetlands in NO_3-N removal; both Messer et al. (2022) and Keilhauer at al. (2019) observed significant NO_3-N reductions in FTW compared to control treatments [47, 56]. Han et al. (2020) observed planted mesocosms with high water levels dramatically reduced NO_3-N concentrations compared to planted low water level mesocosms and control mesocosms [57]. Similarly, Messer et al. (2017) observed NO_3-N concentrations to drop to less than 2 mg/L in planted mesocosms during the summer [52]. Lindgren et al. (2022) found that FTW mesocosms, including those enriched to 1 mg/L of imidacloprid, had NO_3-N levels below the analytical detection limit by day 10, while control mesocosms remained at 10 mg/L on day 10 [48]. Scholz and Lee (2005) studied the influence of planted vertical-flow treatment wetlands on NO_3-N removal and found that the NO_3-N concentrations in the planted treatment wetlands were much lower than the NO_3-N concentrations in the unplanted treatment wetlands, which was most likely due to

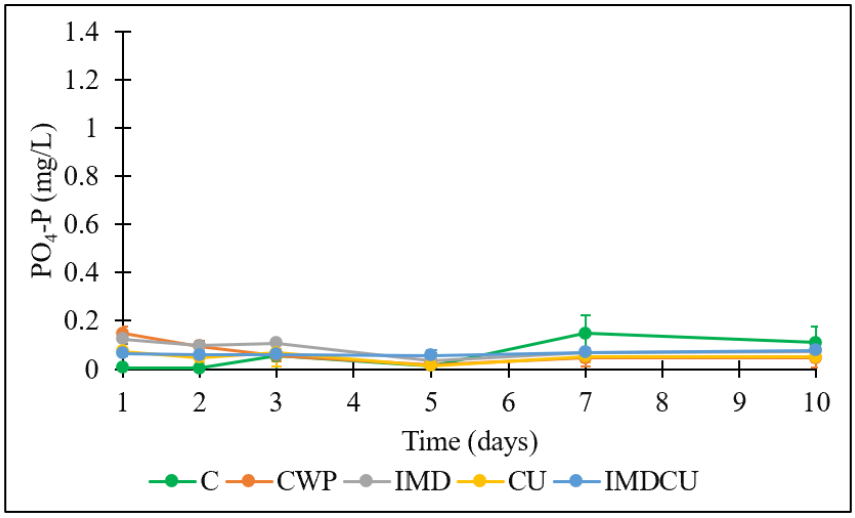
plant uptake [42]. The biogeochemical environment displayed in each of the aforementioned studies, coupled with the presence of soils and plants, contribute greatly to the decrease in NO_3-N through denitrification and plant uptake.

In summary, pesticide application was not found to significantly affect NO_3-N removal in wetland habitats and all treatments with the exception of the PC were reduced to less than 0.2 mg/L of NO_3-N by the end of each 10-day sampling period. This finding supports the importance of plants and soils in NO_3-N loss in constructed wetlands, which is also emphasized by Keilhauer et al. (2019), Lindgren et al. (2022), and Messer et al. (2022) in FTWs and Vymazal (2007), Messer et al. (2017), and Han et al. (2020) in FWS wetlands [45, 47, 48, 52, 56, 57]. Each treatment, with the exception of the PC, was found to remove 84 – 99% of NO_3-N over the sampling period, with removal rates ranging from 0.42 to 1.69 d^{-1}. It is believed that denitrification and plant uptake were the two main methods are NO_3-N removal in the mesocosm wetlands based on nitrogen concentrations in plants and the biogeochemical environment observed in the mesocosms.

3.2.2 Phosphorus Cycling

Phosphate-P (PO4-P) concentrations for each experiment are shown in Figure 6. Sampling day ($p\text{-value}_{exp1} = 0.0286$, $p\text{-value}_{exp2} = 0.0004$, $p\text{-value}_{exp3} = 0.0026$, $p\text{-value}_{exp4} = 0.0005$) and treatment (p-values < 0.0001) were found to be significantly different regarding PO_4-P concentrations for all four experiments.

1.


1.4
1.2
1
0.8
0.6
0.4
0.2
0
PO4-P (mg/L)
1
2
3
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7
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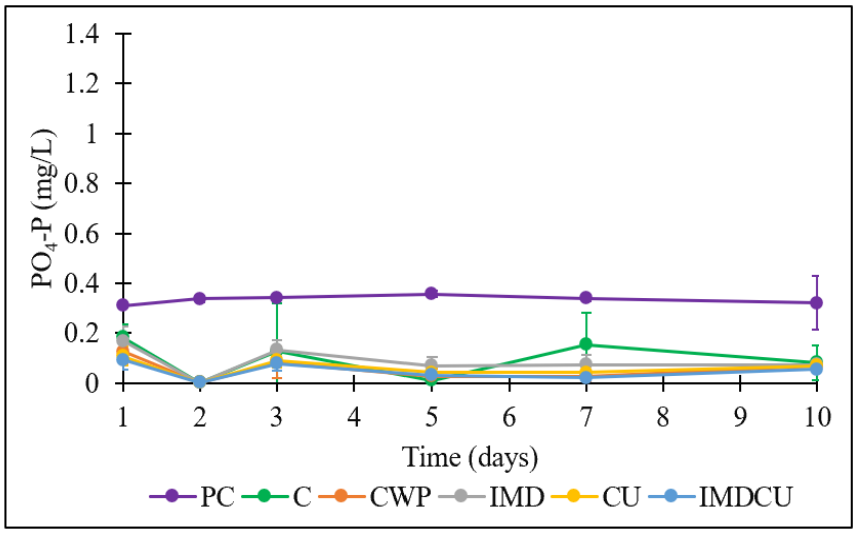
9
10
Time (days)
C
CWP
IMD
CU
IMDCU

2.


1.4
1.2
1
0.8
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0.4
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PO4-P (mg/L)
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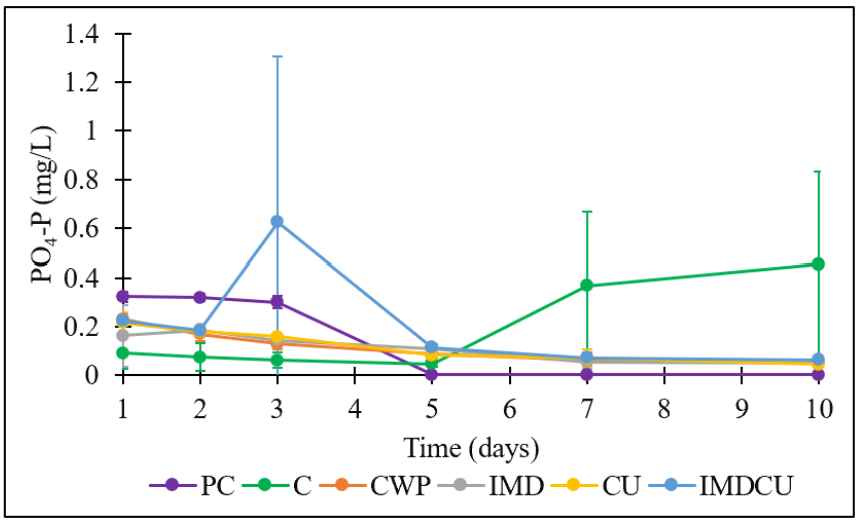
Time (days)
PC
C
CWP
IMD
CU
IMDCU

3.


1.4
1.2
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0.8
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PO4-P (mg/L)
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Time (days)
PC
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CWP
IMD
CU
IMDCU

4.

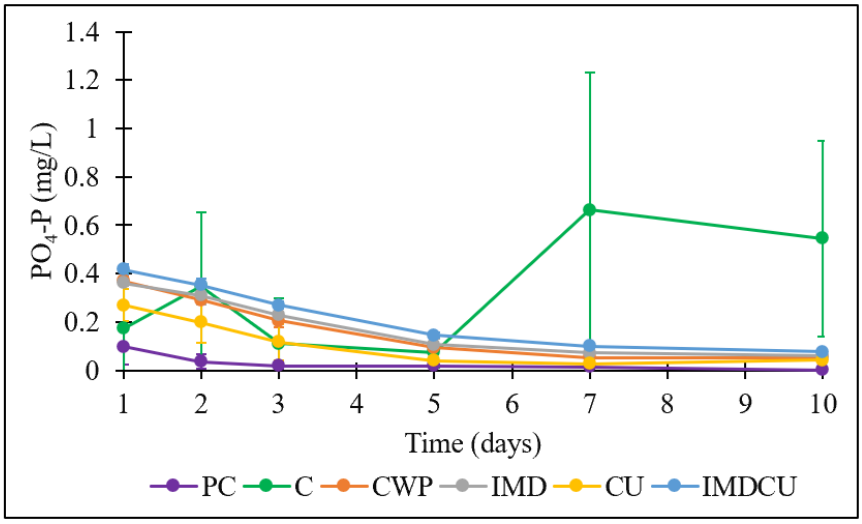


Figure 6. PO_4-P concentrations during the 10-day sampling period during (a) experiment 1 at 0 ng/L, (b) experiment 2 at 50 ng/L, (c) experiment 3 at 500 ng/L, and (d) experiment 4 at 1,000 ng/L.

PO_4-P concentrations fluctuated in all treatments over the 10-day sampling period; however, PO_4-P concentrations ultimately decreased in all planted treatments. The C treatment was an exception, as PO_4-P concentrations were observed to increase between days 1 and 10. The oxidation-reduction of inundated soils explains the increase in PO_4-P concentrations in the C mesocosms (as shown in Figure 5 in section 3.2.1). O_2 is quickly depleted, followed by NO_3, Mn, and Fe; as these compounds are reduced, PO_4-P and NH_4-N levels rise. Since the C mesocosms did not contain plants, there was a lack of dissolved oxygen which led to a release in PO_4-P as Fe was used as an electron donor. The planted mesocosms had higher dissolved oxygen concentrations, therefore they did not progress to Fe reduction or experience a release of PO_4-P.

The PC treatment remained consistent through experiment 2 but decreased to below detection by day 5 in experiment 3 and day 3 in experiment 4. As stated previously, it is likely that algae growth throughout the summer affected nutrient levels

within the PC treatment. The changes in PO_4-P levels observed in these four experiments are different than the trends reported by Messer et al. (2022), who observed initial PO_4-P concentrations between 0.82 and 1.91 mg/L in their FTW mesocosms with little to no change in the concentrations throughout the study [56]. The Messer et al. (2022) study did not use soils in their mesocosms, however, which most likely explains the difference in PO_4-P concentrations [56].

During experiment 1 C was significantly different than all other treatments, while during experiments 2, 3, and 4 both the C and PC were significantly different than all other treatments. Further, significant differences were observed between the earlier sampling days (i.e., days 1, 2, and 3) and the later sampling days (i.e., days 5, 7, and 10). Experiment 3 stood out among the other experiments as no sampling days were found to be significantly different. Experiment 3 took place during the warmest time of the summer; therefore, it was hypothesized temperature influenced the dissolved oxygen levels within the mesocosms and PO_4-P levels during this period. When comparing PO_4-P levels by treatment, the C treatment had significantly higher PO_4-P concentrations compared the planted treatments for all experiments.

Percent removal and removal rates for PO_4-P in each treatment and experiment are shown in Table 9. Treatment type significantly affected PO_4-P removal rates (p-value < 0.0001), especially between the planted treatments and soil and PC treatments. While not significantly different, the IMD, CU, and IMDCU treatments had lower average PO_4-P removal rates compared to the CWP across each experiment. The IMDCU treatment had consistently lower PO_4-P removal rates; therefore, it is likely that the combination of both imidacloprid and $Cu(OH)_2$ affects PO_4-P removal rates.

Table 9. Percent removal and removal rates for PO4-P in different mesocosm treatments following retention time of 10 days.

	Removal (%)				Removal rate (d^{-1})			
Treatment	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
PC	NA	-12.90	99.22	97.44	NA	-0.01	0.54	0.41
C	-1850.00	32.05	72.70	-231.79	-0.33	-0.56	-0.19	-0.13
CWP	61.07	46.27	63.23	81.28	0.10	0.04	0.14	0.19
IMD	28.08	21.80	74.62	78.53	0.04	0.07	0.11	0.17
CU	18.94	27.03	65.94	78.11	0.02	0.03	0.15	0.17
IMDCU	-43.78	-15126.67	-474.01	75.57	-0.04	0.04	0.12	0.16

Above and below ground biomass were analyzed for phosphorus (P) content following pesticide application (Tables 10 and 11). No significant differences were observed in terms of P concentrations in both above ground biomass (p-value = 0.3427) and below ground biomass (p-value = 0.5968) between treatments. Soil samples were also analyzed for P content pre- and post-experiment (Table 12). P content was observed to decrease in all treatments, however only the C treatment was found to decrease significantly (p-value = 0.0248), which is most likely due to the PO_4-P release described earlier.

Table 10. P concentrations in above ground biomass post-experiment.

	P content (gP/m^2)
CWP	259.74 ± 10.22
IMD	192.85 ± 58.27
CU	272.94 ± 85.60
IMDCU	235.38 ± 18.47

Table 11. P concentrations in below ground biomass post-experiment.

	P content (gP/m^2)
CWP	5,765.35 ± 2,547.83
IMD	4,159.00 ± 1,419.86
CU	4,667.83 ± 392.42
IMDCU	6,217.34 ± 2,740.05

Table 12. P concentrations in soil samples pre- and post-experiment.

	Pre-experiment (gP/m^2)	Post-experiment (gP/m^2)	T-test p-value
C	56.61 ± 2.98	35.05 ± 1.88	0.0248
CWP	56.61 ± 2.98	51.93 ± 4.84	0.4690
IMD	56.61 ± 2.98	54.96 ± 4.06	0.6505
CU	56.61 ± 2.98	48.50 ± 3.19	0.0715
IMDCU	56.61 ± 2.98	50.40 ± 1.66	0.0657

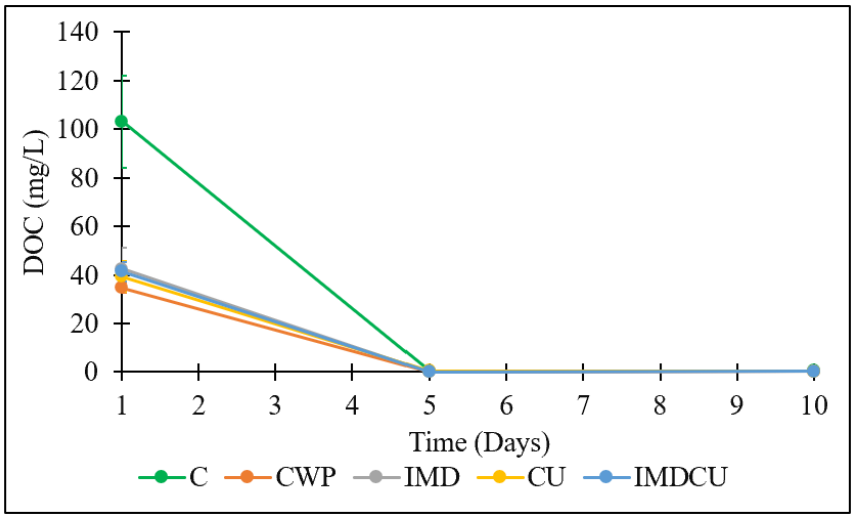
This study was novel in that it is one of the only studies to analyze the relationship between nanopesticides and neonicotinoid insecticides on PO_4-P removal in constructed wetlands. Findings from these experiments exhibit a combination of nanopesticide application influence on PO_4-P removal rates in FWS mesocosm wetlands. Similar to NO_3-N, it was observed that plants are vital to nutrient cycling and PO_4-P removal.

3.2.3 Dissolved Organic Carbon

Unlike NO_3-N, PO_4-P, and NH_4-N, dissolved organic carbon (DOC) was only sampled on days 1, 5, and 10 (Figure 7). Based on ANOVA results, sampling day significantly impacted DOC concentrations ($p\text{-value}_{exp1} < 0.0001$, $p\text{-value}_{exp2} < 0.0001$) along with treatment ($p\text{-value}_{exp1} = 0.0002$, $p\text{-value}_{exp2} < 0.0001$) in experiments 1 and 2 only. Experiments 3 and 4 had no significant differences between treatments for DOC concentrations. Experiment 2 only had a significant difference in DOC concentrations between the C treatment and the planted treatments. However, significant differences

were observed between the C mesocosms; the IMD and CWP mesocosms; and the CU and IMDCU mesocosms in experiment 4.

1.



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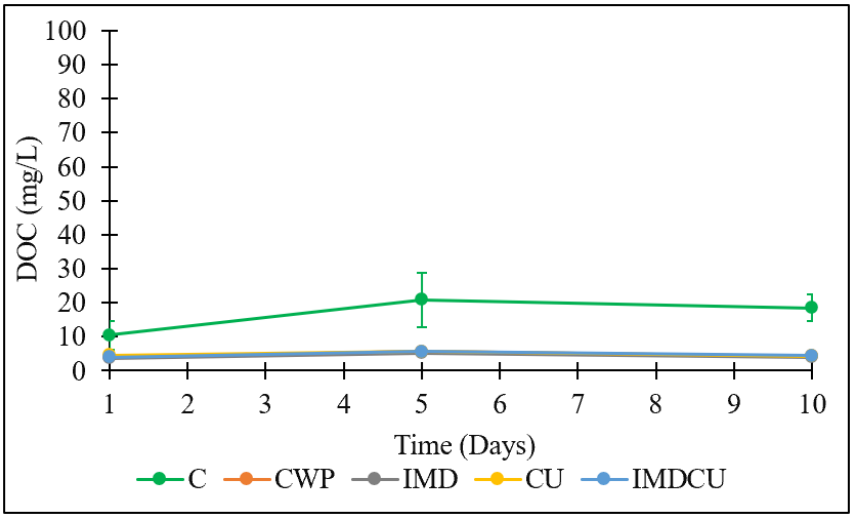

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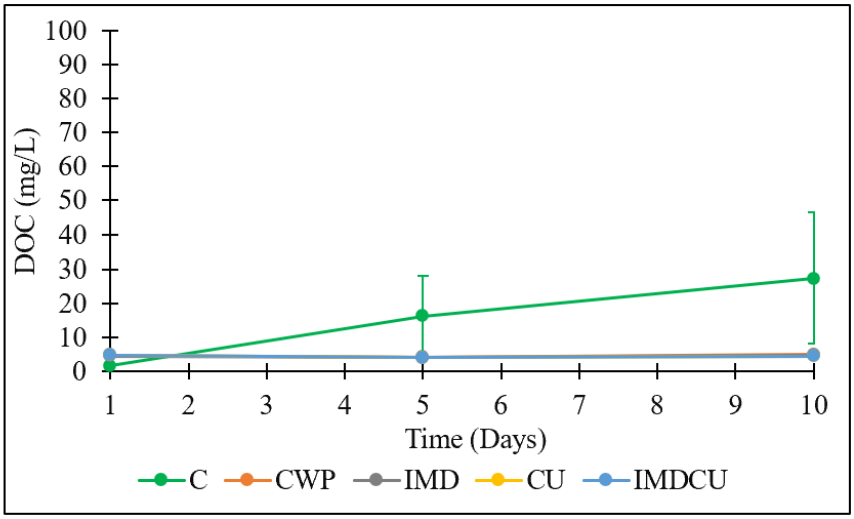

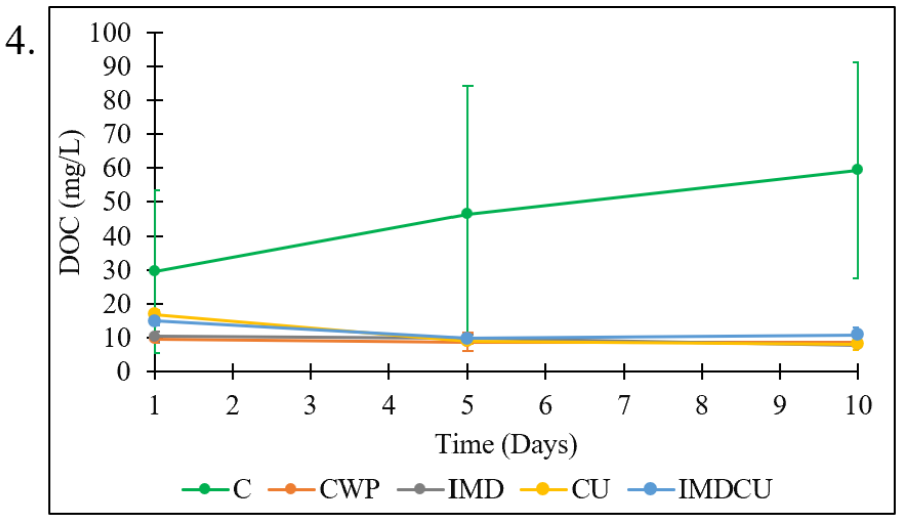

Figure 7. DOC concentrations in mesocosm wetlands over the 10-day sampling period during (a) experiment 1 at 0 ng/L, (b) experiment 2 at 50 ng/L, (c) experiment 3 at 500 ng/L, and (d) experiment 4 at 1,000 ng/L.

There was believed to be an equipment malfunction while analyzing the samples from experiment 1, as the values on day 1 ranged from 30 to 60 mg/L for the planted treatments, which far exceeded the DOC concentrations observed in the other three experiments (0 to 20 mg/L) and in other studies (Opsahl (2005) reported 10 to 40 mgC/L to be high) [61]. Experiments 2, 3, and 4 resulted in expected distributions of DOC concentrations, as shown in Figure 7. The DOC concentrations in the planted mesocosm ranged from 3 to 18 mg/L, while the C had a much wider range of DOC concentrations with values as low as 0 mg/L and as high as 94 mg/L. The planted mesocosms also resulted in more consistent DOC concentrations throughout each experiment, while the C mesocosms were observed to increase in DOC concentrations between days 1 and 10. The DOC concentrations observed in this study were similar to those observed by Messer et al. (2017), who measured DOC concentrations in a mesocosm study comparing organic soils (DOC_{avg} = 39 mg/L) and mineral soils (DOC_{avg} = 9 mg/L) [52]. In a similar

mesocosm study, Han et al. (2020) reported DOC concentrations between 0 and 30 mg/L for their planted mesocosms and 0 and 15 mg/L for their soil control mesocosms [57]. A similar trend was observed within the planted treatments in this study; however, the soil control treatment in this study (C) had much higher DOC values ranging from 0 to 60 mg/L. Han et al. (2020) also observed an increase in DOC values early in the study due to the release of DOC from the inundated sediments, followed by a decrease in DOC levels [57]. In this study similar observations were made, with an increase in DOC concentrations between days 1 and 5 followed by a decrease in DOC between days 5 and 10 in the C treatment during experiment 2.

DOC levels reported in other studies are comparable with the findings of this study. In terms of pesticide application, Simonin et al. (2018b) studied the effects of copper nanoparticles in mesocosm wetlands and found DOC concentrations to range from 5 mg/L to 25 mg/L in their planted mesocosms; similar values were observed in this study [28]. Simonin et al. (2018b) also found DOC concentrations to be 49% higher in mesocosms treated with copper nanoparticles compared to control treatments [28]. In this study, no significant changes were observed in DOC levels based on pesticide application. Coleman et al. (2018) also studied the effects of silver nanoparticles on mesocosm wetlands and reported DOC concentrations ranging from 10 mg/L to 30 mg/L, which are also similar to the concentrations observed in this study [38]. Additionally, Coleman et al. (2018) found no differences in DOC concentrations between treatments with and without silver nanoparticle applications [38]. Keilhauer et al. (2019) and Lindgren et al. (2022) observed much lower DOC concentrations in their FTW mesocosm studies, however both studies used mesocosms that lacked soils and sediments

[47, 48]. Keilhauer et al. (2019) observed DOC levels of approximately 2.4 mg/L in their mesocosms during the spring and summer when temperatures were warmer [47]. Lindgren et al. (2022) measured DOC concentrations of 3.61 mg/L in control treatments and 14.15 mg/L in FTW; the FTW DOC levels are comparable to the DOC levels in the planted treatments of this study; however, the control DOC levels observed by Lindgren et al. were much lower than the ones measured in the control treatment of this study [48].

In summary, DOC concentrations were not influenced by pesticide application. The C treatment had significantly higher DOC concentrations compared to the planted treatments, which further emphasizes the importance of plants in wetland ecosystems. Carbon availability is integral to denitrification processes and is often the limiting factor for denitrification to occur [52]. However, NO_3-N loss and conditions amiable for denitrification were still observed in this study. Therefore, it was unlikely the low DOC concentrations inhibited N cycling.

3.3 Effects of Nanopesticides on Mesocosm Behavior

3.3.1 Imidacloprid

3.3.1.1 Imidacloprid Concentrations

Imidacloprid was observed in experiment 4 at concentrations between 0.02 µg/L and 1.6 µg/L, while in experiments 2 and 3 was not detected regardless of treatment and sampling day as shown in Figure 8. In experiment 4, treatment was found to significantly affect imidacloprid concentrations (p-value = 0.0052), but sampling day was not found to significantly affect imidacloprid concentrations. While steps were taken to minimize cross contamination, the mesocosms were still exposed to wildlife (primarily frogs and

bugs) that might have introduced imidacloprid into control mesocosms. In addition, the plants were purchased from a wholesale retailer as plugs and may have been exposed to imidacloprid prior to establishment in the mesocosms. Further, soils used in the mesocosms were previously used in an agricultural production system of which we did not have pesticide records to determine if they were pre-exposed to these pesticides. Lastly, the added doses (50 ng/L, 500 ng/L, and 1,000 ng/L) were chosen as they represent concentrations previously observed in agroecosystem runoff [3]. These doses were very close to the level of detection (1 ng/L), therefore measurements of imidacloprid and imidacloprid byproducts were difficult to accurately determine.

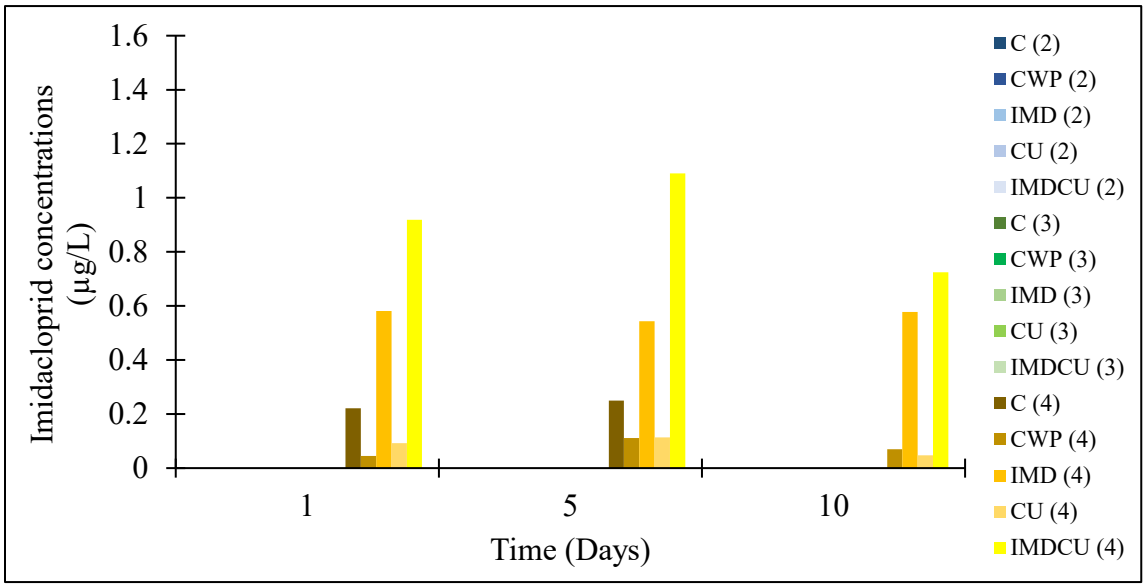

Figure 8. Imidacloprid concentrations for experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L (yellow). Statistical outliers were removed from the graph.

Byproducts of imidacloprid – imidacloprid-urea, desnitro-imidacloprid, and imidacloprid-olefin – were observed in experiments 2, 3, and 4 in almost all treatments. Based on the presence of these byproducts, it was likely the added imidacloprid photodegraded as shown in past studies [62]. Borsuah et al. (2024) studied the

photodegradation of imidacloprid in stream water and found that imidacloprid photodegraded from 1,000 ppb to less than 10 ppb in under 40 hours of light exposure with removal rates between 0.156/hour to 0.623/hour [62]. Ding et al. (2011) also studied the photodegradation of imidacloprid and observed 95% of imidacloprid degrades following 40 hours of photolysis [63]. The prevalence of each imidacloprid byproduct is shown in Figures 9 through 11 below.

Imidacloprid-urea was detected in all experiments (Figure 9). Sampling day significantly influenced imidacloprid-urea concentrations in experiment 3 only (p-value = 0.0401). Treatment was not found to significantly affect imidacloprid-urea levels in any experiment. No consistent trends were observed in imidacloprid-urea levels between days 1, 5 and 10 for each experiment. Imidacloprid-urea was detected in all treatments (including the controls that did not receive imidacloprid doses) which is most noticeable in the large spike in imidacloprid-urea observed in the CWP treatment on day 5 of experiment 2.

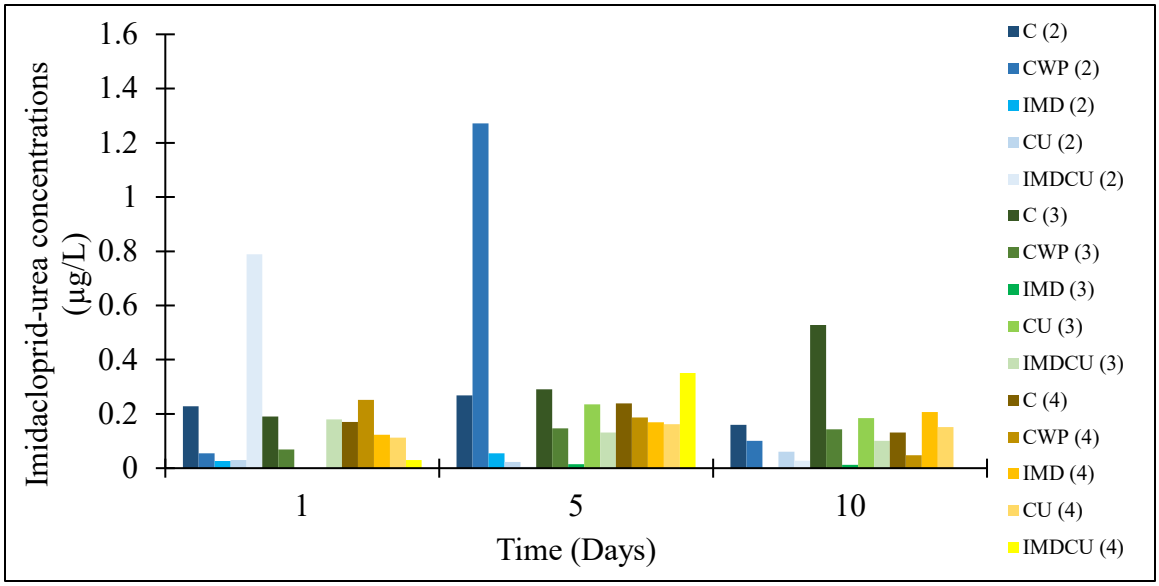

Figure 9. Imidacloprid-urea concentrations for experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L (yellow). Statistical outliers were removed from the graph.

Desnitro-imidacloprid was detected at low concentrations during experiments 3 and 4 with concentrations up to 1.5 µg/L detected (Figure 10). Sampling day was not found to significantly affect desnitro-imidacloprid concentrations, and treatment was only found to significantly influence concentrations in experiment 4 (p-value = 0.0128). No consistent trends in desnitro-imidacloprid levels were observed in experiments 3 and 4.

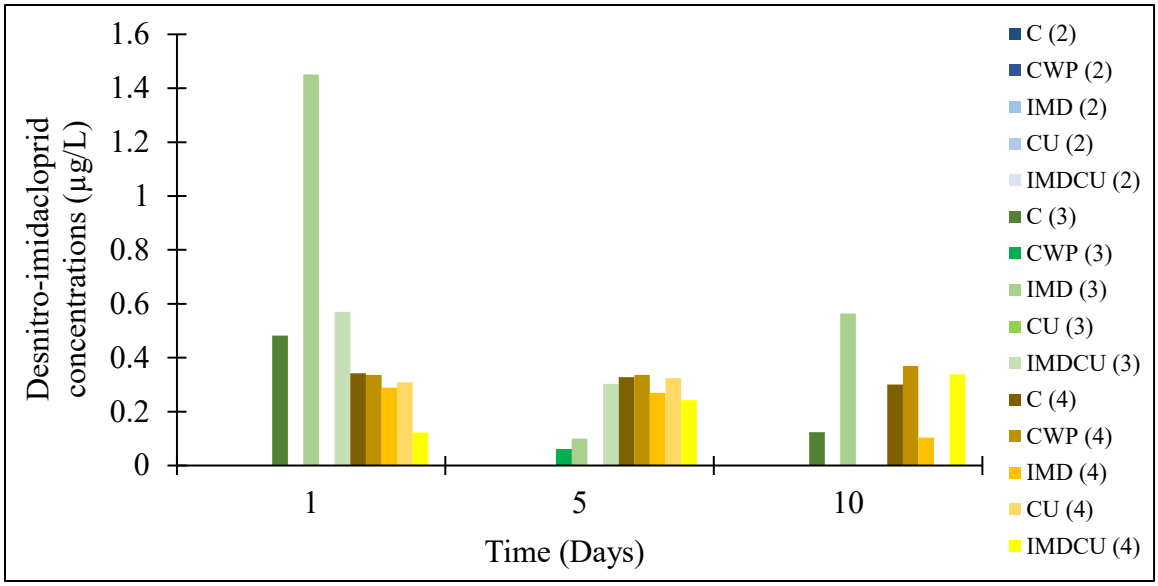

Figure 10. Desnitro-imidacloprid concentrations for experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), experiment 4 at 1,000 ng/L (yellow). Statistical outliers were removed from the graph.

Imidacloprid-olefin was detected in experiments 2, 3, and 4 (Figure 11). Imidacloprid-olefin was only detected at concentrations up to 0.18 µg/L in experiment 2 but experiment 3 exhibited much higher concentrations with some treatments exceeding 3 µg/L. Experiment 4 had median concentrations ranging from 0.03 µg/L to 1 µg/L. Sampling day was not found to significantly affect imidacloprid-olefin levels, and treatment was significant only in experiment 2 (p-value = 0.0234). Imidacloprid-olefin levels were not observed to consistently increase or decrease over time in each experiment and treatment.

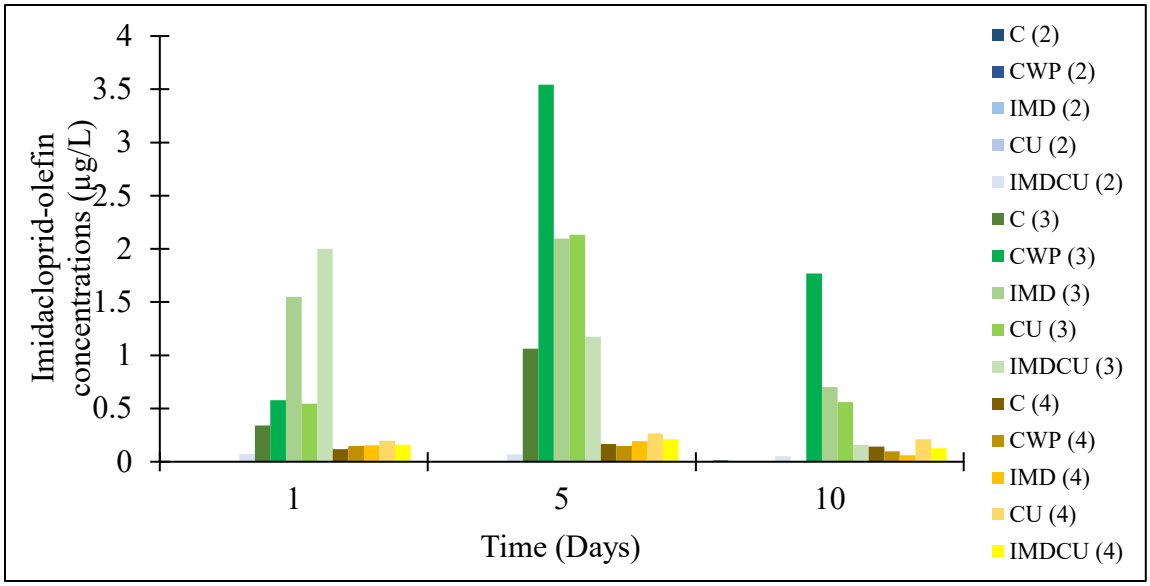

Figure 11. Imidacloprid-olefin concentrations for experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L. Statistical outliers were removed from the graph.

Based on the presence of imidacloprid-urea, desnitro-imidacloprid, and imidacloprid-olefin it is likely that the imidacloprid was undergoing photodegradation or microbial degradation [64]. Both Todey et al. (2018) and Liu et al. (2006) found that imidacloprid-urea was the most common byproduct of imidacloprid observed following photolysis [65, 66]. The breakdown of imidacloprid to desnitro-imidacloprid was especially concerning due to toxicity concerns. Klarich Wong et al. (2019) found that selective toxicity protects mammals from being affected by parent neonicotinoid insecticides; however, the byproducts of neonicotinoid insecticides have toxicological profiles that are not readily rejected by the mammalian nicotinic acetylcholine receptors [64]. Because of desnitro-imidacloprid's different toxicological profile, it has been found to be 317 times more toxic to mammals [64]. Research has found that imidacloprid-olefin is also more toxic than its parent compound (approximately 10 times) to non-target invertebrates [67]. Due to ecotoxicity concerns, the US Environmental Protection Agency

(EPA) has set aquatic life benchmarks for imidacloprid to be 114,500 µg/L (acute) and 9,000 µg/L (chronic) for freshwater vertebrates and 0.386 µg/L (acute) and 0.01 µg/L (chronic) for freshwater invertebrates [68]. Imidacloprid-urea also has an acute aquatic life benchmark for freshwater invertebrates of 47,400 µg/L [68]. Imidacloprid concentrations observed in this study did not exceed the aquatic life benchmarks for freshwater vertebrates; however, they did exceed both the acute and chronic benchmarks for freshwater invertebrates in experiment 4. The imidacloprid-urea levels observed did not surpass the aquatic life benchmarks in any experiment.

3.3.1.2 Effects on Nutrient Cycling

Findings from this study exhibited changes in NO_3-N and PO_4-P removal rates following pesticide application, but no changes in NH_4-N, and DOC levels in wetland habitats. Significant differences in N uptake in below ground biomass were also observed between control mesocosms and those treated with nanopesticides, therefore it is likely the nanopesticides also affect plant uptake. Lindgren et al. (2022) also studied the effects of imidacloprid and another neonicotinoid insecticide (thiamethoxam) on mesocosm wetlands and observed no significant differences in NO_3-N concentrations between the mesocosms with and without neonicotinoid applications [48]. Additionally, that study observed denitrification potential was not negatively affected by the presence of imidacloprid at the mesocosm scale [48].

The imidacloprid concentrations observed in this study do not show consistent decreases over time in the planted mesocosms likely due to the low concentrations assessed in this study. Therefore, due to the inconsistency in concentration and the presence of byproducts, imidacloprid likely underwent photodegradation as a primary

removal mechanism. This is inconsistent with Lindgren et al.'s study, where imidacloprid was shown to be significantly reduced in FTW mesocosms due to plant uptake [48]. However, mesocosms were covered with foil in those experiments to limit photodegradation of the pesticides. Further, imidacloprid byproducts (desnitro-imidacloprid and imidacloprid-urea) at concentrations in the study were negligible to those of the parent compound, which was not observed in this study (namely imidacloprid-olefin, which was detected at concentrations exceeding 3.5 µg/L, over three times the applied imidacloprid concentration), once again supporting photodegradation in that study [48]. Lv et al. (2016) also observed planted mesocosms to significantly reduce pesticides (imazalil and tebuconazole) compared to unplanted controls [40]. Lv et al. (2016) concluded microbial degradation, plant uptake, adsorption, photolysis, and hydrolysis all contributed to the removal of pesticides [40].

Plant and soil samples were collected before and after pesticide application; however, due to time and budget constraints the soil and plant samples were not analyzed for imidacloprid uptake. Other studies have outlined the importance of plant uptake and soil adsorption in managing pesticide loads. Lindgren et al. (2022) found imidacloprid to accumulate in above and below ground biomass in their study of neonicotinoids in FTW mesocosms [48]. Lv et al. (2016) also found plants to accumulate 5-6% of pesticides applied, and sediments to accumulate 1-7% of pesticides applied [40]. Bonmatin et al. (2005) detected imidacloprid in the stems and leaves (4.1 µg/kg), panicles (6.6 µg/kg), and pollen (2.1 µg/kg) of maize crops over a four-year study [69]. A better understanding of plant uptake and soil adsorption of imidacloprid is necessary for guiding the design of constructed wetlands in agroecosystems. Plant uptake and sedimentation are two proven

removal methods for neonicotinoids such as imidacloprid [40]. As such, understanding how plants and soils are able to remove pesticides from the water column will provide guidance on the design and development of constructed wetlands for agroecosystems with high pesticide loads. Additionally, findings regarding imidacloprid concentrations within plants can improve our understanding of the potential exposure to wildlife and ecotoxicity concerns.

3.3.2 Copper Hydroxide

3.3.2.1 Copper Hydroxide Concentrations

All of the mesocosms used in this study were watered with tap water provided by Kentucky American Water in Lexington. A 2020 study found copper (Cu) concentrations up to 0.232 mg/L in the water supply, which is over 200 times the maximum concentration of Cu applied to the mesocosms in the form of Kocide 3000 [69]. Therefore, the results of the $Cu(OH)_2$ analysis were inconclusive. Graphs showing the concentrations of Cu in the undigested and digested water samples are included in Appendix D; however, the Cu concentrations measured exceeded 30 µg/L even at the lowest dose of 50 ng/L.

3.3.2.2 Plant Uptake and Sedimentation

Above and below ground biomass were analyzed for Cu concentrations after pesticide application; mean concentrations are shown in Tables 13 and 14. Cu concentrations were not found to be significantly different between treatments in both the above ground biomass (p-value = 0.4503) and below ground biomass (p-value = 0.8212).

Table 13. Cu concentrations in above ground biomass post-experiment.

	Cu content (gCu/m^2)
CWP	2,882.61 ± 864.92
IMD	1,509.27 ± 619.49
CU	3,567.37 ± 2,931.86
IMDCU	2,088.43 ± 498.03

Table 14. Cu concentrations in below ground biomass post-experiment.

	Cu content (gCu/m^2)
CWP	97,111.63 ± 18,615.84
IMD	82,623.61 ± 37,305.65
CU	102,407.8 ± 35,546.50
IMDCU	100,407.80 ± 11,160.41

Soil samples collected pre- and post-experiment were analyzed for metal concentrations and compared. The average Cu concentrations from each treatment before and after pesticide application are found in Table 15; all treatments had an increase in Cu concentrations at a significant level, most likely due to the Cu already present in the water.

Table 15. Cu concentrations in soil samples pre- and post-experiment.

	Pre-experiment (gCu/m^2)	Post-experiment (gCu/m^2)	T-test p-value
C	0.31 ± 0.02	0.46 ± 0.04	0.0204
CWP	0.31 ± 0.02	0.50 ± 0.01	0.0097
IMD	0.31 ± 0.02	0.48 ± 0.02	0.0109
CU	0.31 ± 0.02	0.49 ± 0.02	0.0161
IMDCU	0.31 ± 0.02	0.47 ± 0.03	0.0278

Multiple studies analyzed the effects of metal nanoparticles on mesocosm wetlands, many of which outline the importance of plant uptake in the removal of metals. For example, two studies evaluating silver nanoparticles in mesocosm observed duckweed (*lemnoideae*) and waterweed (*Egeria densa*) had higher concentrations of silver following the application of silver nanoparticles [35, 38]. Simonin et al. (2018a)

studied the effects of repeated $Cu(OH)_2$ exposure on wetland habitats, where Cu concentrations in plant biomass doubled after three applications of $Cu(OH)_2$ with roughly 3-7% of copper originating from $Cu(OH)_2$ being recovered from the biomass [9]. In another similar study, Cu concentrations were found to be significantly higher in plant biomass following $Cu(OH)_2$ exposure [28]. High Cu concentrations were observed in plant material in this study; therefore, its likely plant uptake is occurring.

3.3.2.3 Effects on Nutrient Cycling

Similar to imidacloprid, the $Cu(OH)_2$ application was found to increase NO_3-N removal rates and decrease PO_4-P removal rates, though the influence of $Cu(OH)_2$ was not found to be statistically significant. $Cu(OH)_2$ was not found to significantly impact NH_4-N and DOC concentrations as well, and no significant differences in plant growth and survival were observed. Other studies that focused on $Cu(OH)_2$ had similar findings; for example, Simonin et al. (2018b) observed P concentrations were not significantly influenced by pesticide applications [28]. The same study also reported 30-60% increases in N and DOC concentrations (attributed to algae and macrophyte mortality) following $Cu(OH)_2$ exposure and using higher concentrations [28]. However, substantial increases in N and DOC concentrations were absent throughout the course of this study. Another study focusing on $Cu(OH)_2$ application in mesocosm wetlands by the same lead author, Simonin et al. (2018a), concluded $Cu(OH)_2$ applications did not negatively affect plant yields in wetland habitats; in fact, the authors suggested $Cu(OH)_2$ led to increased stimulation of plants, and when coupled with fertilization the fertilizer is able to alleviate the stress typically caused by $Cu(OH)_2$ on plant communities [9]. Simonin et al. (2018a)

did note $Cu(OH)_2$ may still have “unintended detrimental effects” to soil processes, such as those involved in nutrient cycling [9].

Regarding N plant uptake, the CWP treatment showed higher N concentrations in below ground biomass compared to the CU and IMDCU treatments. Thus, it is likely the Cu nanoparticles present in $Cu(OH)_2$ inhibited N uptake in plants. More specifically, the presence of Cu may have harmed N-fixing bacteria surrounding the root system. All treatments were exposed to Cu through the water, however the two treatments exposed to $Cu(OH)_2$ (CU and IMDCU) were observed to have the lowest N concentrations in below ground biomass post-experiment. This is most likely due to the different properties of metallic nanoparticles; nanoparticles have been shown to interact with plants differently than metal ions, and also have been shown to have different dissolution rates [37]. Two similar studies by Stegemeier et al. (2022) and Ward et al. (2022) outlined the effects of silver nanoparticles on wetland habitats. The two studies found silver to accumulate primarily in the root tissue and observed silver nanoparticle application to negatively affect microbial populations and diversity in wetland ecosystems, respectively [36, 37]. Carley et al. (2020) also found Cu concentrations to be significant drivers in microbial community composition in wetland mesocosms treated with $Cu(OH)_2$ [16].

Due to the pre-existing Cu in the water, no removal of Cu was observed in the mesocosms in this study. However, in other studies, removal of $Cu(OH)_2$ (and other metals) has been attributed to plant uptake, sedimentation, and dissolution. As stated previously, Simonin et al. (2018b), Yuan et al. (2018), and Colman et al. (2018) observed uptake of silver nanoparticles in their mesocosm wetlands [28, 35, 38]. Sedimentation was observed in this study, as Cu concentrations in the soil significantly increased after

the experiments were conducted. In terms of dissolution, Vencalek et al. (2016) observed at low concentrations $Cu(OH)_2$ has a half-life of 1-8 hours and is readily able to dissolve into the water column; this extended half-time also allows more time for plant uptake to occur, further removing metals from the water column [34]. Similarly, Simonin et al. (2015b) observed $Cu(OH)_2$ to have a dissolution half-life of approximately 8 hours and to rapidly dissolved in the water column [28].

CHAPTER 4. CONCLUSIONS AND FUTURE WORK

As stated previously, this project had two objectives: 1) to determine how nanopesticides affect nutrient cycling and 2) to determine if constructed wetlands are viable BMPs for agroecosystems with high pesticide loads. In terms of nutrient cycling, nanopesticide application was shown to accelerate NO_3-N removal rates and reduce PO_4-P removal rates in constructed wetland mesocosms. Additionally, both $Cu(OH)_2$ and imidacloprid exposure inhibited N uptake in below ground biomass. Regarding the use of constructed wetlands as treatment BMPs for pesticides, limited samples had imidacloprid and $Cu(OH)_2$ present in water samples in the mesocosm wetlands following enrichment. Imidacloprid was only detected at 1,000 ng/L and $Cu(OH)_2$ was unable to be accurately detected due to the pre-existing presence of Cu in the water. The imidacloprid mesocosms had byproducts of imidacloprid in all three experiments, therefore it can be assumed that the imidacloprid was photodegrading. Cu concentrations in the water column were inconclusive; however high Cu concentrations in below ground biomass and soil samples indicate plant uptake and sedimentation were occurring. Therefore, due to the low concentration enrichments, source water, and the potential for rapid photodegradation of the imidacloprid, this study cannot conclude that the constructed wetlands successfully removed the nanopesticides through biogeochemical processes. Regardless, imidacloprid and $Cu(OH)_2$ applications were administered at low concentrations and were not found to severely impact the survival and success of constructed wetland plants and NO_3-N following one growing season of exposure.

Due to the limited time period of this project, additional analysis of plant and soil samples needs to be completed for multiple years of exposure. Additional soil sample

analyses (specific analyses shown in Table 16) need to be completed on the post-experiment soil samples to better understand how pesticide application affects microbial communities throughout the soil profile. Additionally, plant and soil samples pre- and post-experiment have yet to be analyzed for imidacloprid concentrations and the results are anticipated to be received in the summer of 2024. Determining the change in imidacloprid concentrations in plant and soil samples will show the persistence of imidacloprid in downstream wetland ecosystems as well as provide insight on the potential negative effects to wildlife in these ecosystems.

Table 16. Additional soil analyses.

Test Name	**Analytes Reported**
Soil Texture	Sand, Silt, Clay, Textural Class
Cation Exchange Capacity and exchangeable bases	CEC, Base Saturation, Exchangeable Ca, Mg, K, and Na
Water Holding Potential	Filed capacity H2O, wilting point H2O, plant available H2O
Macronutrients	B, Mn, Cu, Fe

APPENDICES

A. SAMPLING SOP

Mesocosm materials
15 100-gallon Rubbermaid stock tanks
Local topsoil, 30 cm depth in each mesocosm
Cattail (Typha latifolia), 36 total, 3 per mesocosm
Soft stem bulrush (Schoenoplectus tabernaemontani), 48 total, 4 per mesocosm
Pickerel weed (Pontederia cordata), 36 total, 3 per mesocosm
15 Onset dataloggers

Chemicals
Kocide 3000 – 0, 50, 500, 1,000 ng/L
Imidacloprid – 0, 50, 500, 1,000 ng/L
Potassium nitrate – 10 mg/L

Sampling materials
3 sampling days x 15 samples – 40 mL glass vials (TOC/DOC – *meso*Lab)

- Per experiment: 45
- Total: 180

7 sampling days x 30 samples – 20 mL glass scintillation vials (NO_3/NH_4/PO_4 – *meso*Lab)

- Per experiment: 210
- Total: 840

3 sampling days x 15 samples – 20 mL amber scintillation vials (IMD contaminants – WWU)

- Per experiment: 45
- Total: 180

3 sampling days x 15 samples – 60 mL amber plastic bottles *UNFILTERED, ACIDIFIED, DIGESTED (Nano Cu contaminants – WWU)

- Per experiment: 45
- Total: 180

3 sampling days x 15 samples – 60 mL amber plastic bottles *FILTERED, ACIDIFIED, DIGESTED (Nano Cu contaminants – WWU)

- Per experiment: 45
- Total: 180

3 sampling days x 15 samples – 30 mL amber plastic bottles *UNFILTERED, UNACIDIFIED, UNDIGESTED (Nano Cu contaminants – WWU)

- Per experiment: 45
- Total: 180

2 sampling days x 12 samples – plant composite samples for above ground and below ground biomass

2 sampling days x 12 samples – soil composite samples for above ground and below ground biomass
Handheld YSI Meter – 7 sampling days x 15 samples – DO, ORP, pH, temperature, conductivity, TSS

Miscellaneous materials
Nitrile Gloves
Cooler / Ice packs
Ziplock bags
Sharpie and Labeling tape
Syringes
Filter holders
GF/F filters (25 mm)

Pre-Experiment
- Establish mesocosms (fall 2022)
- Inundate wetlands (spring 2023)
- Collect plant samples prior to pesticide application
- Place and program HOBOs in each mesocosm

Sample Days: 0, 1, 2, 3, 5, 7, 10

Day	**Bottle size**	**Storage**	**0**	**1**	**2**	**3**	**5**	**7**	**10**
DOC	40 mL filtered + 1 drop sulfuric acid	Fridge (28 days)		X			X		X
NO3/PO4	20 mL filtered	Fridge (2 days)	X	X	X	X	X	X	X
NH4	20 mL filtered + 1 drop sulfuric acid	Fridge (28 days)	X	X	X	X	X	X	X
Nanopesticides (Imidacloprid)	20 mL filtered	Fridge		X			X		X
Nanopesticides (Nano cu)	60 mL (unfiltered and acidified) 60 mL (filtered and acidified) 30 mL (unfiltered and unacidified)	Fridge		X			X		X
Handheld YSI			X	X	X	X	X	X	X
Soil composite	Ziplock bags	Freezer	Before exp 1, after exp 4						
Plant composite	Ziplock bags	Fridge	Before exp 1, after exp 4						

Material Checklist

	0	1	2	3	5	7	10
Gloves							
Cooler							
Ice packs							
Sharpie							
Labelling tape							
YSI							
YSI binder							
Pen/pencils							
Syringes (15)							
Filter holders (15)							
GF/F filter papers (15)							
0.45 filters	X		X	X		X	
20 mL glass scintillation vials (30)							
40 mL glass TOC vials (15)	X		X	X		X	
20 mL amber scintillation vials (15)	X		X	X		X	
60 mL amber plastic bottles (30)	X		X	X		X	
30 mL amber scintillation vials (15)	X		X	X		X	

Field sampling

Two days before experiment:

- Drain tanks
- Refill with water up to 19.5” (approximately 140 L)
- Label samples bottles with date, sampler initials, and mesocosm ID
- Pre-acidify NH4, DOC, and CU bottles

On day 0:

- Top off tanks to 19.5” if needed
- Add nitrogen, Kocide and/or IMD to designated mesocosms
- Stir mesocosms for 1 minute using a meter stick

Days 0 – 10:

- Measure and record water depth
- Stir mesocosms for 30 seconds each using a PVC stir rod
- Place YSI in mesocosm until fully submerged; wait to stabilize then record DO, ORP, pH, temperature, conductivity, TDS in binder
- Use syringe to pull water out of mesocosm; add filter and filter holder; push water through to saturate the filter
- Filter water into vials
    - NO3/PO4/NH4 and filtered CU samples are filtered with GF/F filters
    - DOC vials are filtered with 0.45 filters
- Repeat until all sample bottles/vials are filled
- Place bottle/vials into cooler immediately after filled

On day 10:

- Upload HOBO data

Collecting plant and soil samples:

- Above ground biomass samples include the portion of bulrush and cattail above the soil surface
- Roots are dug up and rinsed to remove soil
- Plant and root samples are placed in gallon ziplock bags and stored in a freezer
- Soil samples are dug up and placed in UK Regulatory Service bags and stored in a freezer

B. PLANT AND SOIL DATA

Table B.1. Pre-experiment Soil Data

Sample	pH	Buffer pH	P (lb/ac)	K (lb/ac)	Ca (lb/ac)	Mn (lb/ac)	Zn (lb/ac)	Soil-Water pH
A1	6.8	7.24	483	559	12182	410	13.6	7.53
A2	6.77	7.25	477	503	11136	379	13.7	7.5
A3	6.69	7.26	478	504	11503	392	13.3	7.43
B1	6.73	7.25	532	597	9451	374	14.3	7.46
B2	6.75	7.27	513	588	10434	386	20.9	7.48
B3	6.77	7.28	517	563	9541	367	15.3	7.5
C1	6.75	7.25	541	572	9387	356	12.3	7.48
C2	6.78	7.26	543	600	9537	388	16.7	7.51
C3	6.74	7.29	519	561	9840	371	16.1	7.47
D1	7.01	7.36	491	635	13194	419	19.8	7.72
D2	7.01	7.37	488	639	14147	426	21	7.72
D3	7.05	7.37	479	650	12931	418	23.9	7.76

Table B.2. Pre-experiment soil data (continued).

Sample	Boron	Plant available water	Cation exchange capacity	Base saturation	Organic matter	Field capacity	TN	Wilting Point
A	1.4	23.16	18.54	161.98	4.68	40.2	0.22	17.04
B	1.24	24.28	19.66	149.62	4.3	42.15	0.24	17.86
C	0.76	24.43	18.69	154.97	4.16	42.02	0.23	17.59
D	0.92	22.85	18.83	181.18	5.4	40.26	0.25	17.41

Table B.3. Pre-experiment Soil Data (continued).

Sample	Exchange K	Exchange Ca	Exchange Mg	Exchange Na	Cu (lb/ac)	Mn (lb/ac)	Fe (lb/ac)	Texture
A	0.78	27.6	1.62	0.03	2.64	170	434	Silt loam
B	0.83	27	1.56	0.02	2.74	200	450	Silt loam
C	0.79	26.6	1.55	0.02	2.76	224	450	Silt loam
D	0.9	31.5	1.7	0.03	3.02	212	408	Silt loam

Table B.4. Post-experiment soil data.

Sample	pH	Buffer pH	P (lb/ac)	K (lb/ac)	Ca (lb/ac)	Mn (lb/ac)	Zn (lb/ac)	Soil-Water pH
C1	6.59	7.16	331	753	8457	539	10	7.34
C2	7.12	7.36	298	856	14161	783	15	7.82
C3	7.1	7.37	309	866	13192	734	14	7.8
CWP1	6.97	7.31	482	356	13973	463	16.1	7.68
CWP2	7.13	7.36	414	333	14990	461	18.7	7.83
CWP3	6.92	7.28	494	324	10755	395	13.7	7.64
IMD1	6.79	7.23	520	320	9914	405	15.6	7.52
IMD2	6.93	7.3	501	301	11921	426	14.3	7.65
IMD3	6.95	7.3	450	285	12043	448	13.4	7.66
CU1	6.85	7.24	400	338	11292	476	12.6	7.57
CU2	7.02	7.31	446	336	13641	435	17.3	7.73
CU3	6.89	7.28	452	306	10890	407	11.4	7.61
IMDCU1	7.07	7.32	446	306	12948	477	17.1	7.77
IMDCU2	6.99	7.31	466	276	12791	509	13.8	7.7
IMDCU3	7.04	7.36	437	286	15042	551	15.2	7.75

Table B.5. Post-experiment soil data (continued).

Sample	Organic matter	TN	Cu (lb/ac)	Mn (lb/ac)	Fe (mg/kg)	Al (mg/kg)
C1	4.18	0.223	3.8	580	343	993
C2	5.09	0.27	4.46	390	306	841
C3	4.75	0.257	4.18	290	321	853
CWP1	5.09	0.24	4.5	542	235	870
CWP2	5.56	0.229	4.58	520	211	805
CWP3	4.28	0.219	4.38	524	250	944
IMD1	4.61	0.24	4.16	418	275	989
IMD2	4.64	0.247	4.42	546	231	903
IMD3	4.35	0.215	4.2	514	252	901
CU1	4.47	0.219	4.5	1028	289	923
CU2	4.95	0.235	4.34	484	189	842
CU3	4.16	0.197	4.2	538	261	927
IMDCU1	4.51	0.228	4.18	620	195	865
IMDCU2	4.68	0.225	4.4	628	199	883
IMDCU3	4.83	0.231	3.94	624	169	797

Table B.6. Pre-experiment above ground biomass data.

Sample	% N	% P	% K	% S	% Ca	% Mg	ppm Zn	ppm Fe	ppm Mn	ppm Cu	ppm B	ppm Mo	%C
P1	0.929	0.32	3.55	0.247	1.178	0.125	23	484	1776	5.9	18.6	3.33	44.31
P2	0.695	0.541	3.66	0.227	1.348	0.162	29	9723	2041	5.5	32.2	1.98	35.67
P3	1.336	0.751	2.69	0.251	2.217	0.275	56	12487	2190	7.7	37.3	3.03	34.79

Table B.7. Post-experiment above ground biomass data.

Sample	% N	% P	% K	% S	% Ca	% Mg	ppm Zn	ppm Fe	ppm Mn	ppm Cu	ppm B	ppm Mo	%C
CU1	0.721	0.133	1.63	0.332	1.158	0.106	9	121	2598	1.1	12.8	3.82	43
CU2	0.572	0.15	1.73	0.201	0.703	0.096	10	86	984	1.3	15.3	1.8	44.14
CU3	0.838	0.276	1.72	0.358	0.764	0.151	15	127	2078	5.1	19.9	3.03	43.03
IMDCU1	0.709	0.161	2.01	0.16	0.944	0.101	11	63	2014	1.3	15.5	6.13	43.77
IMDCU2	0.74	0.159	1.81	0.196	1.072	0.081	9	62	2362	1.7	13.8	2.96	43.73
IMDCU3	0.576	0.167	1.52	0.37	1.16	0.113	11	61	2143	1	13.6	2.46	43.18
CWP1	0.697	0.169	1.99	0.351	0.898	0.089	10	61	1702	2.5	9.1	2.99	43.56
CWP2	0.64	0.15	1.51	0.355	0.928	0.108	9	52	1541	1.3	11.7	2.39	43.35
CWP3	0.64	0.173	1.62	0.217	1.172	0.118	8	46	2066	1.7	11.8	3.93	42.39
IMD1	0.667	0.163	1.6	0.268	0.772	0.096	9	50	1847	1.4	9.9	1.65	43.09
IMD2	0.665	0.129	1.68	0.224	0.926	0.088	9	77	2894	0.9	14.8	3.14	43.76
IMD3	0.464	0.107	1.24	0.175	0.888	0.073	8	49	2363	0.8	7	2.99	43.64

Table B.8. Pre-experiment below ground biomass data.

Sample	% N	% P	% K	% S	% Ca	% Mg	ppm Zn	ppm Fe	ppm Mn	ppm Cu	ppm B	ppm Mo	%C
P1	0.362	0.831	0.62	0.227	8.303	0.671	59	24060	4733	5.3	51.2	1.48	13.97
P2	0.521	1.405	0.78	0.252	5.051	0.33	65	21432	3672	8.4	48.8	0.92	22.52
P3	0.431	0.398	2.6	0.249	2.54	0.23	51	5670	2574	8	24.5	1.77	35.24

Table B.9. Post-experiment below ground biomass data.

Sample	% N	% P	% K	% S	% Ca	% Mg	ppm Zn	ppm Fe	ppm Mn	ppm Cu	ppm B	ppm Mo	%C
CU1	0.341	0.239	0.83	0.159	1.149	0.181	47	6542	1201	4.2	16.5	2.1	36.28
CU2	0.217	0.296	0.66	0.214	11.26	0.456	39	11833	1597	8.5	24.2	0.54	18.87
CU3	0.256	0.251	1.55	0.154	0.771	0.129	17	3026	1157	4.7	9.6	0.64	38.7
IMDCU1	0.33	0.252	0.75	0.09	0.65	0.139	183	1902	757	7.2	7.6	5.28	32.37
IMDCU2	0.234	0.37	0.65	0.137	2.664	0.348	34	10475	1203	5.3	21.3	0.61	15.54
IMDCU3	0.314	0.473	0.79	0.16	1.79	0.387	49	13115	1978	6	27.8	1.1	17.58
CWP1	0.336	0.364	0.96	0.111	1.692	0.193	38	10856	1268	4.9	23.6	1.26	24.71
CWP2	0.42	0.26	0.97	0.116	0.785	0.202	37	3874	969	5.3	10.2	2.38	29
CWP3	0.455	0.199	1.21	0.116	0.401	0.107	28	1836	540	4	6.8	1.69	35.94
IMD1	0.339	0.37	1.84	0.202	1.396	0.209	34	8559	1118	7.5	19.9	0.91	27.95
IMD2	0.257	0.377	0.98	0.208	1.31	0.22	31	7764	1264	10.1	17.4	0.64	36.22
IMD3	0.443	0.233	1.5	0.089	0.457	0.106	51	2223	656	3.2	8.5	4.31	36.83

C. STATISTICAL ANALYSIS CODE AND RESULTS

Nutrient Analysis Code - Example

```
proc contents;
run;

proc print;
run;

proc sort; by Treatment;
proc means maxdec=2 n mean min max std range alpha=0.05 clm median;by Treatment;
var NO3 PO4 NH4 DOC IMD UREA OLE DES CU CU_DF CU_DU;
run;

proc sort; by Sampling_Day;
proc means maxdec=2 n mean min max std range alpha=0.05 clm median; by
Sampling_Day; var NO3 PO4 NH4 DOC IMD UREA OLE DES CU CU_DF CU_DU;
run;

/********************************** NO3 ********************************/

proc anova;
class Sampling_Day;
model NO3 = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model NO3 = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model NO3=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model NO3=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
```

```
lsmeans Sampling_Day*Treatment;
run;

/****************************** PO4 ******************************/

proc anova;
class Sampling_Day;
model PO4 = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model PO4 = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model PO4=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model PO4=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/****************************** NH4 ******************************/

proc anova;
class Sampling_Day;
model NH4 = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model NH4 = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;
```

```
proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model NH4=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model NH4=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/********************************** DOC ********************************/

proc anova;
class Sampling_Day;
model DOC = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model DOC = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model DOC=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model DOC=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;
```

```
/******************************** IMD ********************************/

proc anova;
class Sampling_Day;
model IMD = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model IMD = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model IMD=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model IMD=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/******************************** UREA ********************************/

proc anova;
class Sampling_Day;
model UREA = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model UREA = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
```

```
ods trace on;
class Treatment Sampling_Day;
model UREA=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model UREA=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/********************************** OLE **********************************/
proc anova;
class Sampling_Day;
model OLE = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model OLE = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model OLE=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model OLE=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;
```

```
/********************************* DES *********************************/

proc anova;
class Sampling_Day;
model DES = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model DES = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model DES=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model DES=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/********************************* CU *********************************/

proc anova;
class Sampling_Day;
model CU = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model CU = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
```

```
class Treatment Sampling_Day;
model CU=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model CU=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;

/****************************** CU_DF ******************************/

proc anova;
class Sampling_Day;
model CU_DF = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model CU_DF = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model CU_DF=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model CU_DF=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;
```

```
/****************************** CU_DU ********************************/

proc anova;
class Sampling_Day;
model CU_DU = Sampling_Day;
means Sampling_Day /  tukey;
run;

proc anova;
class Treatment Sampling_Day;
model CU_DU = Treatment Sampling_Day Treatment*Sampling_Day;
means Treatment / tukey;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Treatment Sampling_Day;
model CU_DU=Treatment|Sampling_Day;
lsmeans Treatment*Sampling_Day/slicediff=Sampling_Day;
lsmeans Treatment*Sampling_Day;
run;

proc glimmix data=WORK.IMPORT4;
ods trace on;
class Sampling_Day Treatment;
model CU_DU=Sampling_Day|Treatment;
lsmeans Sampling_Day*Treatment/slicediff=Treatment;
lsmeans Sampling_Day*Treatment;
run;
```

Biomass and Soil Analysis Code – Example

```
proc sort; by Treatment;
proc means maxdec=2 n mean min max std range alpha=0.05 clm median;by Treatment;
var N P Cu;
run;

proc anova;
class Treatment;
model N = Treatment;
means Treatment /  tukey;
run;

proc anova;
class Treatment;
model P = Treatment;
means Treatment /  tukey;
run;

proc anova;
class Treatment;
model Cu = Treatment;
means Treatment /  tukey;
run;
```

Nutrient Removal Analysis Code – Example

```
proc contents;
run;

proc print;
run;

proc anova;
class Experiment;
model N_Removal = Experiment;
means Experiment /  tukey;
run;

proc anova;
class Treatment Experiment;
model N_Removal = Treatment Experiment Treatment*Experiment;
means Treatment / tukey;
run;
```

D. COPPER CONENTRATIONS IN THE WATER COLUMN

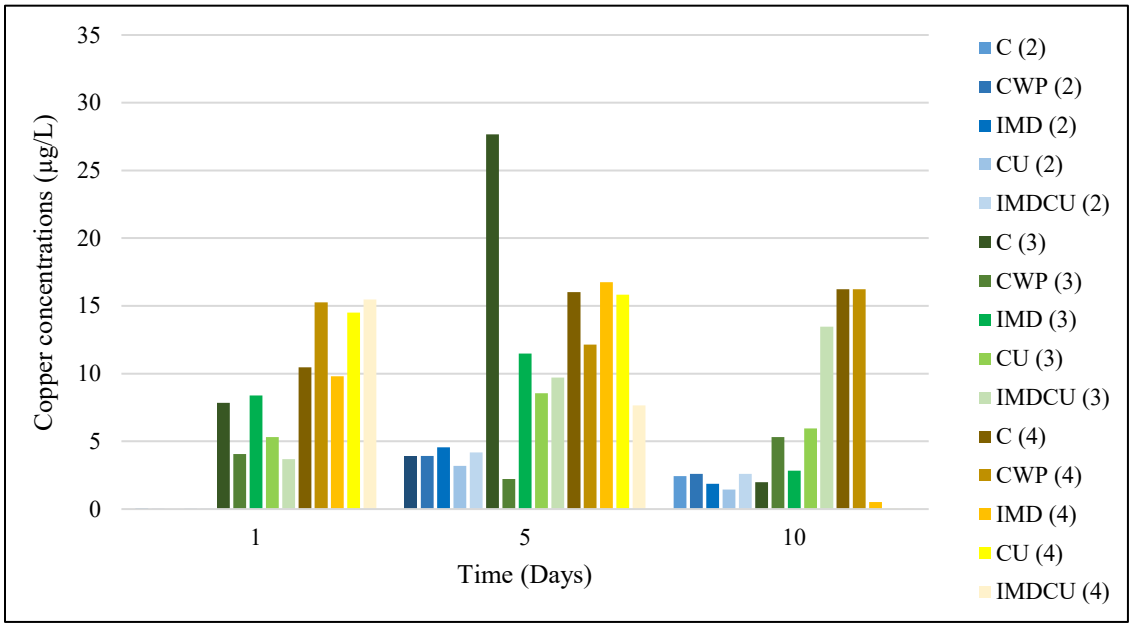

Figure E.1. Undigested copper concentrations in experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L (yellow).

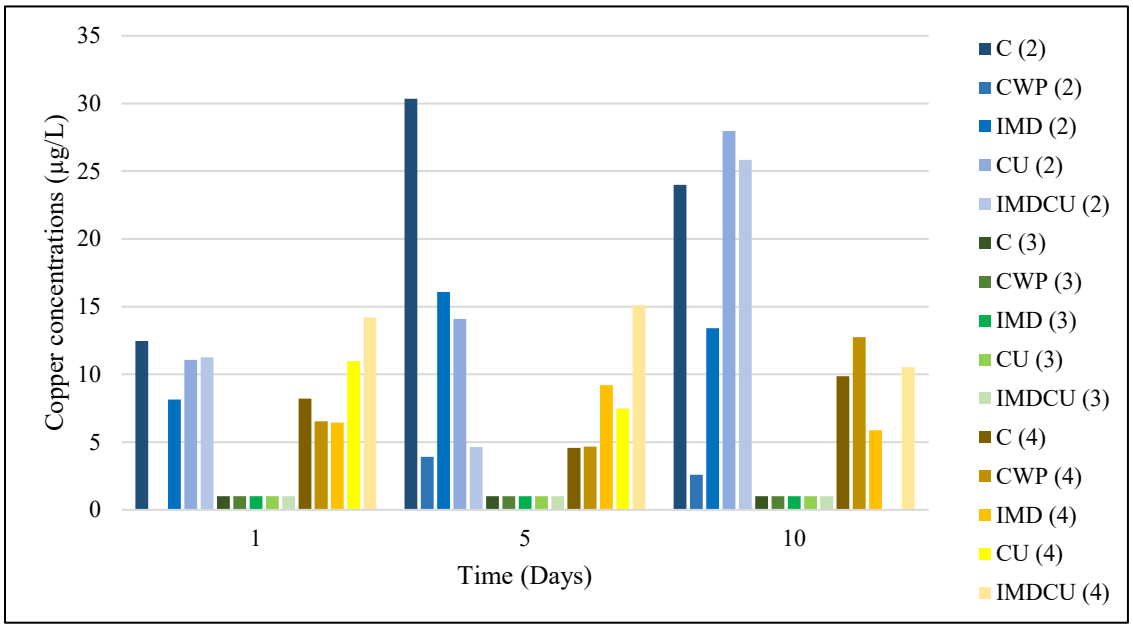

Figure E.2. Digested, filtered copper concentrations in experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L (yellow). Samples C1-IMD1 of experiment 3 were not analyzed for Cu on days 1,5, and 10.

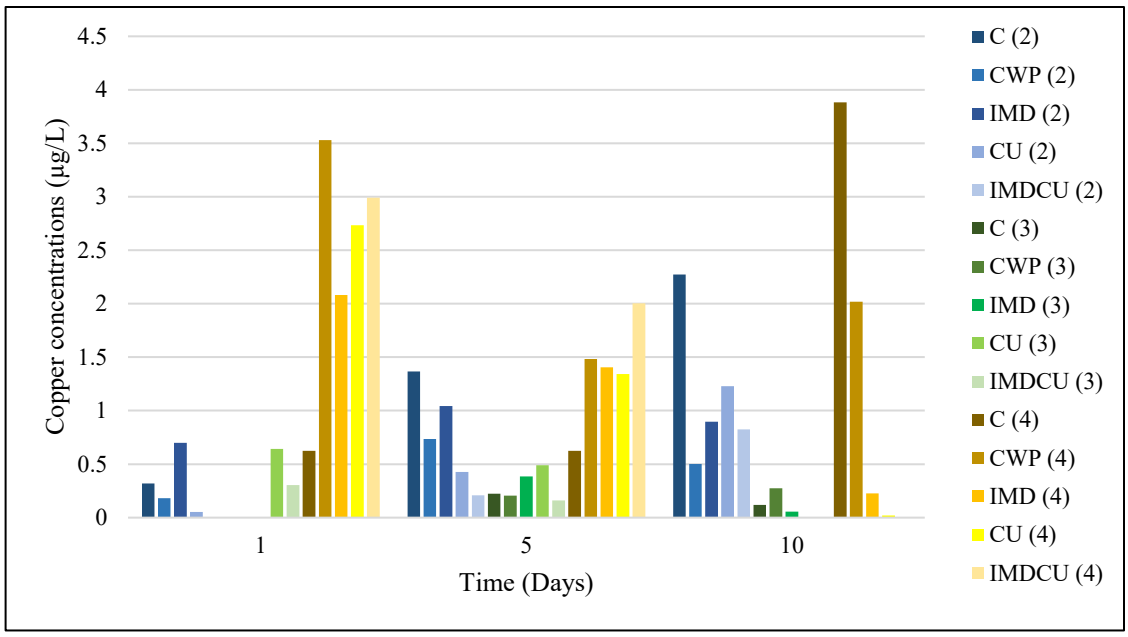

Figure E.3. Digested, unfiltered copper concentrations in experiment 2 at 50 ng/L (blue), experiment 3 at 500 ng/L (green), and experiment 4 at 1,000 ng/L (yellow).

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