A Study of Neonicotinoid Seed Treatments in *Bt* Maize: Insect Resistance Management, Efficacy, and Environmental Fate

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Abstract

Roughly 79-100% of maize in the United States (US) is treated with a neonicotinoid seed treatment (NST), and transgenic (GMO) maize, *Zea mays* L. (Poaceae), that produces insecticidal toxins by way of genes derived from *Bacillus thuringiensis* (*Bt*), occupies more than 75% of maize acreage. Among a variety of secondary pests targeted by NSTs, the primary soil-dwelling pest targeted by *Bt* maize is the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). Transgenic *Bt* technology has dramatically reduced insecticide use for WCR, and insect resistance poses the greatest threat to its utility. To delay resistance to *Bt* traits, in 2010 the US Environmental Protection Agency (EPA) approved a "refuge-in-a-bag" (RIB) insect resistance management (IRM) strategy, where 5% of seeds do not express *Bt* toxins (i.e., "refuge" maize). The RIB strategy is intended to preserve *Bt* trait effectiveness if mating between 'resistant' insects from *Bt* plants and 'susceptible' insects from refuge plants occurs at a high enough frequency. Investigations into the effectiveness of RIB for WCR have shown that beetles emerged from *Bt* plants tend to vastly outnumber beetles emerged from refuge plants, which contributes to low rates of mixed mating. Large proportions of *Bt* beetles is one of several factors that contributes to resistance development, and resistance to all currently-available WCR-*Bt* traits has been documented. I conducted field experiments in two regions (Indiana and Virginia) comparing refuge beetle proportions in NST-treated (NST+) and NST-untreated (NST-) 5% RIB maize, to determine whether NSTs may be limiting refuge beetle emergence. To assess advantages of combining use of *Bt* and NSTs, I compared stand, root injury rating, and yield between NST+, NST-, *Bt* and non-*Bt* maize in both states. I also measured neonicotinoid residues in soil, water, and stream sediment within and surrounding fields of maize, to study the off-site movement and soil residence time of these compounds. I found that 5% seed blends did not produce large populations of refuge beetles in any site-year, and that NSTs showed inconsistent effects on refuge beetle populations. Treatment comparisons showed inconsistent benefits of NSTs when combined with *Bt* traits. I detected neonicotinoid residues in soil matrices throughout the growing season (range: 0 – 417.42 ppb), including prior to planting, suggesting year-round presence of these compounds. My results suggest that, while the effects of NSTs on *Bt* IRM may be inconsistent, the benefits of universally applying NSTs to *Bt* maize for soil pests may not be worth the ecological costs of doing so in all cases.

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Kyle M. Bekelja

General Audience Abstract

About 75% of maize planted in the United States is encoded with genetic traits allowing them to manufacture insecticidal proteins which are toxic to key pests. These insecticidal proteins, known as "*Bt* toxins," are highly specific to targeted insects, and are virtually non-toxic all other animals and non-target insects. One target insect is the western corn rootworm (WCR), which feeds on and damages maize roots. In the past, WCR was controlled by applying millions of kilograms of chemical insecticides every year, at ever-increasing rates, to compensate for reduced effectiveness due to pest resistance. "*Bt*" plants, were released for commercial use in the late 1990s; they reduced the need for growers to apply chemical insecticides for managing key pests. The Environmental Protection Agency established regulations aiming to maintain the effectiveness of *Bt* technology, which they declared have "provided substantial human health, environmental, and economic benefit." This Insect Resistance Management (IRM) strategy, also known as the "refuge" strategy, is meant to preserve *Bt*-susceptible insects so they can pass on their *Bt*-susceptible genes to successive generations. The refuge strategy works by incorporating a certain percentage of non-*Bt* plants per every field of *Bt* plants; if enough insects survive on these "refuge" plants to outnumber "resistant" insects, population-wide *Bt*-susceptibility may be maintained. While this strategy has been effective for other key maize pests, it is failing for WCR, evidenced by WCR populations that have developed *Bt*-resistance. We know current refuge configurations aren't producing enough refuge-WCR to mix sufficiently with resistant WCR, likely due to insufficient refuge sizes. I wanted to know whether the refuge strategy could

be improved, if increasing refuge proportions is not an option. Considering that *Bt* toxins are only effective against a narrow range of insects, seed manufacturers always coat seeds with an insecticide to protect seedlings against other insects. These coatings, or neonicotinoid seed treatments (NSTs), are included on nearly all seed, including those reserved for *Bt* refuges. I conducted two years of field trials to investigate whether removing NSTs from refuge seeds would improve WCR-IRM by providing an insecticide-free "refuge." My results suggest that removing NSTs *may* increase refuge beetle proportions, however my results also show that refuge plant proportions are simply too small to support large enough refuge-WCR populations to delay resistance, regardless of whether NSTs are present or not.

While NSTs may provide extra comfort to growers at little additional cost, questions regarding their necessity at current use patterns have been raised. Several studies have shown inconsistent benefits, and others have shown longer-than-expected persistence in the environment, movement into streams and groundwater, and even alterations to insect and non-insect animal communities associated with their use. I conducted research comparing their relative effectiveness against WCR and non-WCR insect pests in fields of *Bt* maize. Additionally, I scouted for their residues in soil collected in field margins, forests buffering streams, and in water collected from agricultural ditches and waterways neighboring fields. I found that, while NSTs produced higher plant populations, they rarely resulted in greater yields. I detected neonicotinoid residues in soil matrices throughout the growing season, including prior to planting, suggesting year-round presence of these compounds. My results suggest that, while the effects of NSTs on *Bt* IRM may be inconsistent, the benefits of universally applying NSTs to *Bt* maize for soil pests may not be worth the ecological costs of doing so in all cases.

Table of Contents

List of Tables and Figures

Preface/Attribution

Chapter One: Neonicotinoid seed treatments may affect insect resistance management for *Bt* maize targeting western corn rootworm (*Diabrotica virgifera virgifera*, LeConte)

Kyle M. Bekelja conducted and designed all experiments and analyses in fulfilment of experimental objectives outlined in Chapter One. Kathleen M. Miller (M.Sc.) lead design and completion of experiments in Indiana that fulfilled Chapter One objectives. Committee members Dr. Sally V. Taylor and Dr. Christian H. Krupke provided funding and guidance on experimental design and analyses. Dr. Thomas P. Kuhar provided guidance for the design of methods for completion of these objectives in Virginia. All members reviewed manuscript and have provided feedback.

Chapter Two: Detection and quantification of neonicotinoid residues, clothianidin and thiamethoxam, in two corn production regions: Virginia and Indiana

Kyle M. Bekelja conducted and designed all experiments and analyses in fulfilment of experimental objectives outlined in Chapter Two. Kathleen M. Miller (M.Sc.) lead design and completion of experiments in Indiana that fulfilled Chapter Two objectives. Committee members Dr. Sally V. Taylor and Dr. Christian H. Krupke provided funding and guidance on experimental design and analyses. Dr. Sally Entrekin provided guidance on methodology and sample collection for completion of biological compartment sample collection in Virginia. All members reviewed manuscript and have provided feedback.

Introduction

The western corn rootworm (WCR) (Coleoptera: Chrysomelidae: *Diabrotica virgifera virgifera* LeConte) is considered the most economically-damaging soil pest of maize (*Zea mays* L.) in the United States (Wechsler & Smith, 2018). Soil-dwelling larvae feed on root systems, causing nutrient uptake deficiencies (Kahler et al., 1985) and can cause plant lodging in severe infestations (Spike & Tollefson, 1991). Given its reliance on maize, WCR infestations tend to be most severe in fields where maize is grown continuously (i.e., without rotation to non-maize crops). Corn rootworm is the central focus of pest management regimes for maize in the Corn Belt states (Krupke, 2009); it is also a primary pest in continuous maize in southwestern Virginia (Kuhar et al., 1997). Secondary soil-dwelling pests of maize, including wireworms (Coleoptera: Elateridae), white grubs (Coleoptera: Scarabaeidae), seed corn maggots (Diptera: Anthomyiidae, *Delia platura* Meigen), and billbugs (Coleoptera: Curculionidae, *Spenophorus* spp.), are occasional threats to maize production (Sappington et al., 2018).

Preemptive management strategies, such as crop rotation, host plant selection, and insecticide applications are the most reliable way to manage soil-dwelling insects because there are no effective rescue treatments once crops shows signs of injury (Sappington et al., 2018). While WCR will reliably infest fields of continuous maize, other pests cannot be easily monitored, and infestations can be sporadic (Sappington et al., 2018). Crop rotation has remained the most effective management tool for WCR in almost all regions, and is effective against most secondary pests (Taylor, 2022). Choosing maize varieties that are resistant to pests, for example "*Bt* maize," which expresses toxins derived from *Bacillus thuringiensis* (Berliner), are effective strategies against most of the primary pests of maize, but are not effective against other pests. Chemical insecticides can be effective against a wide range of primary and secondary pests.

Given management uncertainties and ease of implementation, as of 2022 seed manufacturers treat nearly 100% of maize seed with a neonicotinoid insecticide to provide assurance against threats of primary and secondary pest injury (Douglas & Tooker, 2015).

A key principle of Insect Resistance Management (IRM) is that over-reliance on any single management strategy or insecticide mode of action will reduce its effectiveness (Sparks et al., 2021), and in the case of chemical insecticides, incidences of unintended exposure to non-target species will increase (Barzman et al., 2015). Agronomic management strategies are not immune to this principle: in the late 1990s, several WCR populations in Illinois showed resistance to crop rotation when fields that had been in maize-soybean rotations were damaged (Levine, 2002). Host-plant resistance strategies can also reduce in effectiveness over time: resistance to all currently-available *Bt* traits for WCR has been documented in select regions (Gassmann, 2011; Gassmann et al., 2014; Gassmann et al., 2019; Gassmann et al., 2020). As for chemical insecticides, growing concerns about neonicotinoid seed treatment use have prompted investigations of their potential to cause harm to pollinators (Krupke & Long, 2015), alter aquatic and terrestrial non-target insect communities (Van Dijk et al., 2013), and result in secondary alterations to food chains as a consequence (Hallmann et al., 2014).

In the United States, millions of acres of *Bt* maize are planted every year (USDA-NASS, 2021). Insect resistance to *Bt* maize is a major concern, and the primary "insect resistance management strategy," the "refuge" strategy, which is aimed at preventing *Bt* resistance from becoming widespread, is failing for WCR in some regions (Gassmann, 2011; Gassmann et al., 2014; Gassmann et al., 2019; Gassmann et al., 2020). Insect "refuges" are failing because they do not produce enough *Bt*-susceptible beetles to outnumber *Bt*-resistant beetles. Other authors have suggested that the failure of insect refuges is related to refuge size: they have inferred that refuge sizes are too small to prevent resistance development (Onstad, 2006; Taylor et al., 2016). Neonicotinoid seed treatments may be another causal factor contributing to small numbers of refuge beetles, given that all seeds, including refuge seeds, have a seed treatment.

Given that WCR resistance to *Bt* traits is evolving, the challenges of soil pest management in general, and concerns regarding ubiquitous use of neonicotinoid seed treatments, this work aims to: 1) determine whether neonicotinoid seed coatings reduce the effectiveness of IRM strategies for WCR-rated *Bt* traits; 2) compare the effectiveness of neonicotinoid seed treatment use in combination with WCR-rated *Bt* traits; 3) compare neonicotinoid concentrations in soil and water matrices between treated and untreated fields; and 4) scout for the presence of neonicotinoid residues in non-agricultural ecological matrices neighboring agricultural areas. Our goal is to inform on the costs and benefits of current neonicotinoid seed treatment use patterns within the scope of IRM goals to preserve *Bt* technology (an effective non-chemical management option for WCR), effectively and efficiently manage soil pests of maize, and to contribute information about their environmental fate.

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Chapter One

Neonicotinoid seed treatments may affect insect resistance management for *Bt* maize targeting western corn rootworm (*Diabrotica virgifera virgifera,* LeConte)

(K. M. Bekelja, K. M. Miller, T. P. Kuhar, C. H. Krupke, S. V. Taylor)

Abstract

Roughly 79-100% of maize in the United States (US) is treated with a neonicotinoid seed treatment (NST), and transgenic (GMO) maize, *Zea mays* L. (Poaceae), that produces insecticidal toxins by way of genes derived from *Bacillus thuringiensis* (*Bt*), occupies more than 75% of maize acreage. Transgenic *Bt* technology has dramatically reduced insecticide use for the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), but insect resistance poses the greatest threat to its utility. The "refuge in a bag" strategy is intended to preserve *Bt* trait effectiveness if mating between 'resistant' insects from *Bt* plants and 'susceptible' insects from refuge plants occurs at a high enough frequency to prevent resistance. Prior work has shown that beetles emerged from *Bt* plants vastly outnumber beetles emerged from refuge plants, which contributes to lower rates of mixed mating, and may contribute to resistance development. Field experiments were conducted in two regions (Indiana and Virginia) comparing refuge beetle proportions in NST-treated (NST+) and NST-untreated (NST-) 5% RIB maize, to determine whether NSTs may be limiting refuge beetle emergence. To assess advantages of combined use of *Bt* and NSTs, plant stand, root injury rating, and yield were compared between NST+, NST-, *Bt* and non-*Bt* maize in both states. We found that 5% seed blends did not produce large populations of refuge beetles in any site-year, and that NSTs showed inconsistent effects on refuge beetle populations. Treatment comparisons showed inconsistent benefits of NSTs when combined with *Bt* traits.

Introduction

The United States (U.S.) is the world's leading maize (*Zea mays* L.) producer with more than 90 million acres planted annually (USDA-NASS, 2022). Almost half of U.S. maize acreage is in the Midwest, a region characterized by relatively homogenous agricultural landscapes of grain crops, largely maize and soybean (Heimlich, 2000). Maize is also grown in the Appalachian region of the mid-Atlantic U.S., a region that contains the largest number of small farms in the U.S. and is characterized by heterogeneous landscapes, small cattle and dairy farms, soybean, and poultry farms (Heimlich, 2000). Maize in this region is generally grown to supply local livestock. Across both regions, there are below-ground insect pests that feed on seeds and root systems.

The relative importance of pests is region-dependent, complicating a universal management plan. Western corn rootworm (Coleoptera: Chrysomelidae, *Diabrotica virgifera virgifera* LeConte) (WCR), the most economically damaging pest, cost U.S. farmers an estimated $2 billion from losses and control costs in 2010 (Wechsler & Smith, 2018). Since WCR is monophagous on maize, it is concentrated where maize is not rotated (i.e., continuous maize). In the Midwest, WCR is the focus of most pest management regimes (Krupke, 2009), whereas WCR is a minor pest in the Appalachian region of the Mid-Atlantic, except in continuous maize where it infests fields at frequencies similar to the Midwest (Kuhar et al., 1997). Other pests include wireworms (Coleoptera: Elateridae), white grubs (Coleoptera: Scarabaeidae), seed corn maggot (Diptera: Anthomyiidae, *Delia platura* Meigen), stink bugs (Hemiptera: Pentatomidae), and billbugs (Coleoptera: Curculionidae, *Spenophorus spp.*) (Sappington et al., 2018). They are generally more problematic in the Mid-Atlantic and are sporadic economic pests in the Midwest. Infestations are difficult to predict and there are no remedial treatments (Sappington et al., 2018).

Prophylactic management tools, such as preventative insecticide applications and host-plant resistance, are widely used (Sappington et al., 2018).

Historically, WCR were managed using broad-spectrum insecticides. These compounds were applied at increasing rates to compensate for resistance evolution (Ball & Weekman, 1962; Meinke et al., 2021). In the mid-1990s, neonicotinoid seed treatments (NSTs) were commercialized and labeled for control of early-season pests including WCR. In 2003, genetically-modified (GM) maize hybrids expressing insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) (Berliner), commonly '*Bt* maize,' targeting WCR were commercialized (Meinke et al., 2021). Currently, NSTs are used on virtually all *Bt* maize acres across U.S. growing regions (Douglas & Tooker, 2015).

Planting *Bt* maize significantly reduced insecticide use (Benbrook, 2012; Klümper & Qaim, 2014), simplified the logistics of pest management, and reduced the carbon footprint of farming (Perry et al., 2016). Because *Bt* proteins are pest-specific, they have few, if any, non-target effects (Bhatti et al., 2005). Insect resistance is the greatest threat to longevity of *Bt* technology, and as resistance becomes widespread, insecticide use increases to compensate (Benbrook, 2012). Currently, WCR resistance has been documented to all available *Bt* toxins (Gassmann, 2011; Gassmann et al., 2014; Gassmann et al., 2020).

Insect resistance management (IRM) strategies are mandated by the U.S. Environmental Protection Agency (EPA) to forestall or prevent resistance (EPA, 2001). The only approved strategy relies on areas of non-*Bt* plants known as 'refuges.' Refuges are meant to produce large numbers of *Bt*-susceptible individuals to mate with rare, *Bt*-resistant adults (EPA, 2001). Ideally, mating between susceptible and resistant insects produces *Bt*-susceptible offspring that are killed by a "high dose" *Bt* toxin (i.e., twenty-five times the dose necessary to kill susceptible insects)

(EPA, 2001). There are currently no high dose *Bt* toxins targeting WCR. Pyramided or stacked hybrids (i.e., plants expressing two or more pest-specific *Bt* toxins) are used to slow resistance, as insects with resistance to both toxins should be rare (Tabashnik & Gould, 2012).

Blended refuges (i.e., mixtures of *Bt* and non-*Bt* seed created by the manufacturer) are used for pyramided hybrids. Benefits of blended refuges include increased compliance and compatibility with WCR mating biology. In a blended refuge, mixed mating between WCR adults from refuge and *Bt* plants is more likely to occur than in structured refuge arrangements, perhaps a function of *Bt* and refuge plant proximity (Taylor & Krupke, 2018). However, the utility of blended refuges is limited because they do not produce large populations of susceptible WCR realtive to separate, structured block refuges (Tabashnik & Gould, 2012; Taylor & Krupke, 2018).

There is a need to determine why blended refuges do not produce more refuge insects. To our knowledge, there are no studies investigating if insecticides, specifically NSTs, affect emergence of WCR and mating from *Bt* and refuge plants. The ubiquitous use of NSTs on *Bt* maize since its introduction complicates our ability to decipher the relative contribution of each technology to WCR and secondary pest control, and IRM. Overall, the benefits of using NSTs to manage WCR are inconsistent (Oleson et al., 2002; Oleson et al., 2003; Jarvi et al., 2005; Steffey et al., 2005; Oleson et al., 2006; Van Rozen & Ester, 2010; Tinsley et al., 2012; Estes, 2015). There is a need to determine the value, in terms of both crop protection and IRM, of using NSTs on *Bt* maize seed.

Our study was designed to: 1) to measure the effect, if any, of NSTs on WCR emergence from *Bt* and refuge plants in blended refuges; and 2) to measure the effect, if any, of NSTs and *Bt* toxins, combined and separately, on stand, yield, and root injury ratings. Experiments were replicated in Indiana and Virginia to measure effects across different production regions. Our goal was to provide the first empirical assessment of the contributions of these co-occurring pest management approaches to short-term (i.e., in-season damage) and long-term (i.e., *Bt* resistance) management of a key insect pest of maize.

Materials and Methods

Overview

Experiments were conducted in 2018 and 2019 in Virginia, and 2018-2020 in Indiana. Fields were located at Pinney Purdue Agricultural Center (PPAC) in LaPorte Co. and Throckmorton-Purdue Agricultural Center (TPAC) in Lafayette Co., Indiana, and in Montgomery Co., Virginia, including Kentland Farm in Whitethorne, and Hethwood Farm in Blacksburg. Experiments were conducted in fields owned and managed by Purdue University or Virginia Tech. In all years and locations, plots were planted into continuous maize fields. Treatments included *Bt* and refuge seed with and without seed-applied NSTs (herein Bt+, Bt-, refuge+, refuge-).

Seeds

All seed was provided by the manufacturer (Dekalb; Monsanto in 2018 and Bayer CropScience in 2019-2020, St. Louis MO, USA). *Bt* seeds, Genuity SmartStax RIB Complete® (DKC 62-08) expressing *Cry3Bb1* + *Cry34/35Ab1* targeting corn rootworm, and refuge seeds (DKC 62-05) were chosen for their phenotypic similarity because there are no true *Bt*/refuge isolines. In 2018 and 2019, Bt- and refuge- seeds were provided “naked” without any seed coating applied. Bt+ and refuge+ seeds in 2018-2019 and all seeds in 2020 were provided with clothianidin applied at a rate of 0.25 mg/a.i. per seed (Acceleron® Basic). For Bt+ and refuge+ treatments, an additional 1.00 mg of clothianidin was applied by the seed treatment manufacturer (Bayer CropScience in 2018 and BASF in 2019-2020, Research Triangle Park NC, USA) to achieve the rootworm control rate (1.25 mg a.i./seed). In 2020, seeds for Bt- and refuge- treatments were washed to remove coatings using a modified version of a protocol developed by Dr. Joseph

Spencer at the University of Illinois. Seed coating was removed as follows: seeds were partitioned into lots of 5,000 and added to an 18.95 L bucket with 5 L DiH_2O and 20 mL dish soap (Ultra Palmolive Original; Colgate-Palmolive Company, New York, NY, USA). Seeds were agitated by manually stirring for 20 minutes. After stirring, seeds were poured into a sieve and rinsed four times with DiH_2O. Washing and rinsing procedures were repeated three times. Seeds were then blotted with paper towels and dried for 12 h at ambient temperatures. After drying, the seeds were soaked in 10% bleach (Clorox, Clorox Building, Oakland, CA, USA) for 1 h, agitating every 15 minutes. The seeds were then removed from the bleach solution and rinsed 10 times in DiH_2O. After rinsing, the seeds were blotted with paper towels and dried for 24 h. Seed was refrigerated until planting.

Refuge performance

To evaluate refuge performance, we compared the ratio of *Bt* and refuge emerged WCR (herein "*Bt* beetles" and "refuge beetles," respectively) in 5% seed blend refuges with (+) and without (-) NSTs. In Virginia NST+ fields, Bt+ and refuge+ seeds were used. In Indiana NST+ fields, Bt+ and refuge- seeds were used to give refuge beetles the best possible chance of being collected (i.e., by planting insecticide-free refuges). We measured head capsule size and dry weight of emerged beetles to determine their relative reproductive fitness (Murphy & Krupke, 2011). Two replicates of NST+ and NST- fields were planted each year on Virginia Tech's Hethwood fields (2018 and 2019); and one on PPAC and one on TPAC in Indiana (2019 and 2020) (see Table 1.1).

To simulate a 5% blended refuge, only *Bt* seeds were initially planted, and 5% of seeds were chosen for removal using a random number generator (https://www.random.org/). *Bt* seeds were removed and replaced with two refuge seeds, and their location was flagged. The smaller of the two refuge plants was removed following emergence. A subset of plants in each treatment were tested for *Bt*-expression using gene-check strips (Cry3B # AS 015 LS; EnviroLogix, Portland, ME, USA) to ensure that no planting errors were made. In Virginia, all refuge plants inside field cages, and at least two plants immediately adjacent, were tested. In Indiana, three refuge plants from each row were tested. All fields were 30.48 m × 30.48 m, planted on 76 cm row spacing, and at a rate of 70,000 seeds per hectare.

Stable Isotope Labelling

Refuge plants were enriched with ^{15}N (ammonium nitrate, ~98% ^{15}N; Cambridge Isotope Laboratories, Inc. Andover, MA) following methods outlined by Taylor et al. (2016). Briefly, 10 mL of an aqueous solution of ammonium nitrate ^{15}N and distilled water were applied to the root zone of refuge plants at the V2 stage, at the rate of 0.6125 g ammonium nitrate per liter dH_2O. The solution was applied in Virginia using a 10 mL syringe into a 5 cm-deep hole at the base of each plant. Holes were dug using a metal stake, pre-marked for proper hole depth, and angled towards the root system to offer the greatest opportunity for uptake of the stable isotope, and to minimize the potential for leaching into neighboring plants. In Indiana, the ^{15}N solution was applied using a CO_2-pressurized backpack sprayer to a 1-cm-deep hole at the base of every refuge plant. In Virginia, only refuge plants within or adjacent to field cages were labeled; in Indiana, all refuge plants were labeled.

Because ^{15}N application in Indiana was performed on a larger scale, *Bt* plants neighboring labeled plants were tested for ^{15}N concentration to ensure that the solution did not leach. Root tissue from ^{15}N-enriched and unenriched maize plants were sampled approximately four weeks after enrichment. From each plot, five enriched plants and six unenriched plants surrounding each enriched plant were sampled (within row: two plants ahead and two behind; adjacent row: one plant to the left and one to the right). A total of 35 plants were sampled per plot (5 enriched and

30 unenriched). Plants were removed whole, washed of soil particles, and dried in a laboratory oven at 90^{o}C for 24 h. Approximately 4 mg of dried root tissue was removed, ground into a fine powder, placed into a tin capsule (EA Consumables LLC, Marlton, NJ, USA), and weighed. Capsules were placed into non-sterile 96-well plates (Sigma-Aldrich, St. Louis, MO, USA), and shipped on dry ice to the Virginia Tech Department of Geosciences for analysis by mass spectrometry.

Beetle Collection

In Indiana, fields were isolated from neighboring maize by a distance of at least 1.25 km and surrounded by woods or natural areas to minimize WCR immigration and emigration. In Virginia, experimental plots were seeded within larger maize fields because isolation was not possible. To limit movement of beetles, five 3.6-meter × 3.6-meter × 2.13-meter outdoor mesh enclosures (BioQuip Products Inc, Rancho Dominguez, CA, USA Products) were placed over pre-selected areas of each field after planting and enrichment. Frames were custom built using four garden T-posts 2.1-m in each corner, strutted by 60 cm long 38 ×89 mm boards to hold up the edges of the netting. To support the center of the cage netting, an additional 2.4 m T-post was driven into the center of each cage location and was topped with a modified tennis ball (Wilson Sporting Goods, Chicago, IL) to prevent abrasion. All cages were inspected for holes, and edges were buried. Cage locations were chosen to confine as close to a 5% proportion of refuge to *Bt* plants as possible. The average refuge percentage within a cage was 7.4% in 2018 and 5.4% in 2019.

Collecting began at the first adult male appearance. In Indiana, emergence was monitored using pheromone lures (Csal♀m♂N®, Budapest, Hungary) on a yellow sticky trap (Trece Pherocon AM No-Bait trap, Trece Inc. Adair, OK, USA). In Virginia, emergence was monitored by scouting fields. Upon emergence, 23 July 2019 and 17 July 2020 in Indiana, and 5 July 2018 and 1 July 2019 in Virginia, researchers scouted fields during peak mating time: between 0700 and 1100 hours (Marquardt & Krupke, 2009). Fields were checked on a rotating schedule to reduce bias associated with time-dependent peaks in WCR activity. In Indiana, a team of workers scouted eight random rows of each plot. Every WCR found along a selected row was collected. In Virginia, workers sampled cages by inspecting all plants individually, as well as the inside ceilings and walls of every cage. Only WCR found inside cages were collected. Beetles were collected using an aspirator (1135A, BioQuip Products Inc, Rancho Dominguez, CA, USA) and transferred into labeled, clear plastic bags (Ziploc, SC Johnson, Racine, WI, USA). Mating pairs collected were placed into a separate bag and noted as a mating pair. Beetles were stored at 0^{o}C until processing.

Head Capsules, Dry Weights, and ^{15}N Determination

Sex was determined for each beetle by comparing their abdominal distal termini, or, when necessary, by looking for the large planar patch on the first tarsomere of the prothoracic leg, indicating a male (Bekelja et al., 2019). Head capsules widths were measured using a stereo microscope (SM-4B-80S, AmScope, Irvine, CA) with an attached digital camera (MU900, AmScope, Irvine, CA); the distance between the outer edge of each eye was measured to the nearest 0.01 mm. Specimens were then dried in a laboratory oven (Model 40 Lab Oven, Quincy Lab, Chicago, IL) at 100^{o}C for 24 hours. Dry mass was measured to the nearest 0.001 mg using a microbalance (Mettler-Toledo, XP6, Columbus, OH).

All beetles were analyzed for atom percent ^{15}N to differentiate individuals that had fed primarily on labeled refuge plants as larvae, and those that had fed primarily on non-labeled *Bt* plants as larvae. Weighing and ^{15}N analysis by mass spectrometry were performed at the Virginia Tech Department of Geosciences Stable Isotope Laboratory. Beetles were prepared for mass spectrometry using an adaptation of the protocol outlined in Taylor et al. (2016). In short, head capsules and elytra were removed and placed into a tin capsule (EA Consumables LLC, Marlton, NJ, USA), weighed to the nearest 0.001 mg, and crushed. Because males can transfer the ^{15}N label to females through the spermatophore (Murphy et al., 2011), using only elytra and head capsules reduces the chance of contamination. Additionally, sclerotized insect regions are more robust against degradation (Klowden, 2013) and more likely to retain the label. Sample tins were folded and placed into a non-sterile 96-well plate (Sigma-Aldrich, St. Louis, MO, USA).

Stable nitrogen isotope values (δ^{15}N) and %N were determined on an Isoprime 100 isotope ratio mass spectrometer (IRMS) coupled with a vario ISOTOPE elemental analyzer (EA). Stable nitrogen isotope compositions were calibrated relative to the AIR scale with USGS25 and USGS26 via a two-point linear calibration (δ^{15}N = -30.41 ‰ and 53.75 ‰, respectively). We monitored and corrected for linearity and drift when appropriate using a suite of commercial standards (Elemental Microanalysis *urea*, δ^{15}N = -0.30 ‰; *protein*, δ^{15}N = 5.94 ‰; and *wheat flour*, δ^{15}N = 2.85 ‰) interspersed throughout the analytical runs. Analytical uncertainty (1σ), based on the standard deviation of 460 commercial standards, is better than 0.1‰ for δ^{15}N.

Corrected ^{15}N values were used to improve accuracy (Dawson et al., 2002). A series of calculations were used to determine the amount of ^{15}N present to identify a labeled beetle (Taylor et al., 2016; Taylor & Krupke, 2018). First, the ratio of ^{15}N/^{14}N was calculated using the following equation:

$$0.0037\text{*sample corrected } {}^{15}\text{N} / (1000+1),$$

with 0.0037 being a correction factor accounting for the average natural abundance of ^{15}N (the average ratio of ^{15}N/^{14}N in an unlabeled sample is 0.0037 to 1). The following equation was used to determine the percentage of ^{15}N relative to total N in the sample:

$$100*[\text{sample ratio } ^{15}N/^{14}N \ / \ (\text{sample ratio } ^{15}N/^{14}N + 1)].$$

Finally, the following equation was used to determine atom % excess (i.e. the degree to which the sample ^{15}N percentage may have exceeded resident ^{15}N abundance):

$$100*(\text{sample atom } \%^{15}N - 0.3679) \ / \ 0.3679$$

where 0.3679 is the baseline constant atom % of ^{15}N in an unlabeled sample. Beetles that contained ^{15}N in excess of the baseline constant (0.5%) is indication that the beetle fed on a labeled plant. For our study, we used a conservative threshold of three times the baseline, or 1.5% excess, to account for larval movement between plants (Hibbard et al., 2003).

Crop protection

Our second objective was to evaluate the crop protection benefits of *Bt* and refuge seed with and without NSTs. Treatments included Bt+, Bt-, refuge+, and refuge- planted separately (opposed to blended). Four-row plots (12.2 m × 15.24 m) were arranged in a randomized complete block design with four replicates. Plant stand, Oleson node injury rating (0-3) (Oleson et al., 2005), and yield (kg/ha) were measured. Compact method soil sampling (Laub et al., 2018) was used to determine the presence of other pests (e.g., wireworms, white grubs). In Virginia, two separate experiments were planted in different fields at Kentland Farm in Montgomery Co. (2018 and 2019). In Indiana, two separate experiments were planted, one at PPAC and one at TPAC (2018-2020) (see Table 1.2).

Plant Stand

Plant stand was measured by counting the total number of plants in 12.19 m (Virginia) or 9.14 m (Indiana) in one of two center rows of each plot.

Root Damage

Root ratings were taken following peak rootworm emergence and ideally, before compensatory root growth could occur (Gray & Steffey, 1998). Five (Virginia) or six (Indiana) randomly chosen non-consecutive plants per plot were excavated using a shovel, with care not to damage root systems. Plant tops were removed, and root systems were placed in pre-labeled bags for transportation. Roots were soaked in water baths and then rinsed to remove soil so that insect feeding injury could be visually assessed. Root damage was estimated using the 0-3 node injury scale (Oleson et al., 2005). Where damage was identified, an estimate of average insect feeding damage per root was determined.

Yield

In Virginia, all ears within 5.3 m row length in one of two center rows of each plot were harvested by hand. Harvested ears were air dried until moisture content reached 10-20%. Grain yield was measured using methods adapted from Bryant et al. (2020). On all harvested ears, we determined: 1) number of kernel rows, 2) number of kernels per row, 3) average kernel weight, and 4) moisture %. Yield was calculated using the following equation:

Yield (kg/hectare) = (# ears/hectare) * (# of kernel rows) * (# of kernels/row) * (average kernel weight)

Kernel weights were standardized to 15.0% moisture. In Indiana, grain yield and moisture were estimated from the two center rows using a Kencaid® 8-XP plot combine equipped with a HarvestMaster GrainGage®.

Additional Soil Pests

Fields were sampled using an adaptation of the compact soil method (CM) (Laub et al., 2018). Twelve 20 cm wide by 15 cm deep holes were excavated and contents sifted for insect larvae. Sample locations were chosen to spatially represent the entire field. In Indiana, fields were sampled for soil pests approximately one week following plant emergence; Virginia fields were sampled shortly after planting in 2018, and immediately prior to planting in 2019 (see Table 1.2). Pest density was estimated using the average number of pests per sample. Insect identifications were confirmed by the Virginia Tech Insect Identification Lab**.**

Data Analysis

All analyses were performed in R Software version 4.2.0 (R Core Team, 2021).

<u>Refuge performance</u>: Refuge performance was assessed by comparing ratios of *Bt* to refuge beetles in NST- and NST+ fields using Fisher's Exact Test. Significance was determined using an alpha level of 0.05. Data were analyzed separately by state and year.

<u>Head capsule and dry weight</u>: Analysis of variance (ANOVA) models were fit using the aov function in R (R Core Team, 2021); analyses were performed using the Anova function in the car package (Fox & Weisberg, 2020). Analyses were performed separately by state. Data from all fields and years were combined due to low refuge beetle emergence. Dry weight data were log transformed to meet assumptions of normality. Virginia models used head capsule size or dry weight as response variables; fixed effects included seed treatment (NST+, NST-), host plant type nested within seed treatment (Bt+, Bt-, refuge+, refuge-), sex (M, F), and interactions treatment*sex and host plant type*sex. Indiana head capsule size and dry weight data were unable to be analyzed using host plant type as a response variable due to small refuge sample sizes and uneven variance. Indiana models used head capsule size or dry weight as the response variables, seed treatment (NST+, NST-), sex (M, F), and the interaction seed treatment*sex as fixed effects. When fixed effects were significant, Tukey's Honestly Significant Difference test compared group means using the TukeyHSD function (R Core Team, 2021).

Crop Protection: Analysis of variance models were fit using the aov function in R (R Core Team, 2021); analyses were performed using the Anova function in the car package (Fox and Weisberg, 2019). Response variables included plant population (plants per hectare), root rating (0-3), and yield (kg per hectare); fixed effects included treatment, field, year, and block (nested within field), and treatment interactions field*year, treatment*field, treatment*year, and treatment*field*year. When fixed effects were significant, Tukey's Honestly Significant Difference test compared group means using the TukeyHSD function (R Core Team, 2021). A cube transformation was applied to Indiana plant population data to meet assumptions of normality. Indiana root rating data were unable to be transformed to meet assumptions. Indiana root ratings were cube-root transformed since it brought the distribution closer to normal than untransformed data. A log10 transformation was applied to Virginia root rating data meet assumptions of normality.

Results

^{15}N Concentration

In Virginia, labeled beetles had a mean percent excess ^{15}N of 11.5% (SE +/- 1.8) and 112.9% (SE +/- 35.6) in 2018 and 2019, respectively. Unlabeled beetles had a mean percent excess of 0.89% (SE +/- 0.07) and 0.47% (SE +/- 0.04) in 2018 and 2019, respectively (see Figure 1.1). In Indiana, labeled beetles had a mean percent excess ^{15}N of 4.67% (SE +/- 0.46) and 11.31% (SE +/- 3.24) in 2019 and 2020, respectively (see Figure 1.2). Unlabeled refuge beetles had a mean percent excess ^{15}N of 0.32% (SE +/- 0.02) and 0.19% (SE +/- 0.02) in 2019 and 2020, respectively.

Refuge Performance

Virginia: In 2018, there were no differences in refuge beetle proportions between NST+ (39%) and NST- (27%) fields (two-tailed Fisher's P = 0.5334). In 2019, *Bt* and refuge beetle proportions differed between NST+ (1.8%) and NST- (17.2%) fields (P = 0.008) (see Figure 1.3).

Indiana: There were no differences in refuge beetle proportions between NST+ (0.91%) and NST- (2.93%) fields in 2019 (two-tailed Fisher's P = 1) or NST+ (4.28%) and NST- (3.82%) 2020 (two-tailed Fisher's P = 1) (see Figure 1.4).

Emergence and Mating Pairs

Virginia, 2018: In NST+ fields, refuge beetles emerged from 6 July to 15 August, and *Bt* beetles emerged from 10 July to 15 August. Forty-four refuge beetles, 70 *Bt* beetles, and five mating pairs (2 *Bt*/*Bt*, 1 *Bt*/refuge, 2 refuge/refuge) were collected. In NST- fields, refuge beetles emerged from 11 July to 29 August, and *Bt* beetles emerged from 16 July to 10 August. A total of three refuge beetles and eight *Bt* beetles were collected (see Figure 1.5).

Virginia, 2019: In NST+ fields, one refuge beetle emerged on 8 August, and *Bt* beetles emerged from 9 to 27 August. One refuge beetle and 48 *Bt* beetles were collected. In NST- plots, refuge beetles emerged from 15 July to 27 August, and *Bt* beetles emerged from 17 July to 27 August. Ten refuge beetles and 52 Bt beetles were collected (see Figure 1.5).

Indiana, 2019: A total of 1098 beetles were collected and a representative sample of 493 beetles was used for analysis. In NST+ fields, refuge beetles emerged from 5 to 7 August, and *Bt* beetles emerged from 23 July to 5 September. Two refuge beetles, 217 *Bt* beetles, and two mating pairs (2 *Bt*/*Bt*, 0 *Bt*/refuge, 0 refuge/refuge) were collected. In NST- plots, refuge beetles emerged from 24 July to 13 August, and *Bt* beetles emerged from 24 July until 5 September. Eight refuge beetles, 266 *Bt* beetles, and 25 mating pairs (24 *Bt*/*Bt*, 1 *Bt*/refuge, 0 refuge/refuge) were collected (see Figure 1.6).

Indiana, 2020: A total of 2901 beetles were collected and a representative subsample of 594 beetles was used for analysis. In NST+ fields, refuge beetles emerged from 16 to 29 July, and *Bt* beetles emerged from 16 July to 18 August. Two refuge beetles, 68 *Bt* beetles, and 26 mating pairs (12 *Bt*/*Bt*, 1 *Bt*/refuge, 0 refuge/refuge) were collected. In NST- fields, refuge beetles emerged from 16 July to 4 August, and *Bt* beetles emerged from 16 July to 20 August. Twenty-one refuge beetles, 503 *Bt* beetles, and 158 mating pairs (70 *Bt*/*Bt*, 8 *Bt*/refuge, 1 refuge/refuge) were collected (see Figure 1.6).

Head capsule and dry weights

Virginia: Beetle head capsule width differed by seed treatment (F = 11.9652, df = 1, P = 0.00065) and sex (F = 26.6272, df = 1, P < 0.0001). Head capsules of beetles from NST+ fields (1.09 mm ± 0.08) were smaller than those from NST- fields (1.12 mm ± 0.06). Females (1.12 mm ± 0.08) had larger head capsules than males (1.07 mm ± 0.06) (see Table 1.3). Dry weight differed only by sex (F = 37.5864, df = 1, P < 0.0001). Females (3.42 mg ± 1.49) were heavier than males (2.34 mg ± 0.81) (see Table 1.4).

Indiana: Head capsule width differed by only by seed treatment (F = 16.2809, df = 1, P < 0.0001). Beetle head capsules from NST+ fields (1.13 mm ± 0.07) were smaller than in NST- fields (1.15 mm ± 0.07) (see Table 1.5). Dry weights differed by seed treatment (F = 36.687, df = 1, P < 0.0001), sex (F = 72.808, df = 1, P < 0.0001), and interaction between seed treatment and sex (F = 13.047, df = 1, P = 0.0003) (see Table 1.6).

Crop Protection

Plant Stand

Virginia: Plant populations differed by treatment (F = 14.964, df = 3, P < 0.0001), field (F = 48.9732, df = 1, P < 0.0001), year (F = 10.7288, df = 1, P = 0.0048) and the interaction of treatment and year was significant (F = 5.8823, df = 3, P = 0.0066) (see Table 1.7)

Indiana: Plant populations differed by treatment (F = 4.1159, df = 3, P = 0.00087), field (F = 10.986, df = 3, P < 0.0001), and the interaction of field and year was significant (F = 3.758, df = 18, P < 0.0001) (see Table 1.8).

Root Injury

Virginia: No variable affected root injury rating. Mean overall root rating for all years and fields was 0.18 (±0.11) nodes (see Table 1.9).

Indiana: Root rating differed by treatment (F = 13.849, df = 3, P < 0.0001) and there was an interaction between field and year (F = 1.892, df = 18, P = 0.02597). Mean overall root rating for all years and fields was 0.02 (±0.06) nodes (see Table 1.10).

Yield

Virginia: Yield differed by field (F = 10.6517, df = 1, P = 0.0043) and treatment (F = 4.0302, df = 3, P = 0.0234) (see Table 1.11).

Indiana: Yield differed by the interaction of field and year (F = 9.006, df = 6, P < 0.0001), but treatment was not significant (F = 0.5199, df = 3, P = 0.66963) (see Table 1.12).

Discussion

Insect Resistance Management

Here, we provide evidence that NSTs can reduce refuge beetle proportions, but this effect is inconsistent, and is neither likely to improve, nor likely to hinder the performance of a 5% blended refuge. Our ^{15}N testing demonstrated that adult beetle populations were heavily dominated by *Bt* beetles in all locations and years, consistent with prior findings (Murphy et al., 2010; Taylor & Krupke, 2018). A 5% seed blend does not produce adequate refuge populations (i.e., large relative to *Bt*) and survival of beetles that fed on *Bt* plants is common with current commercial pyramided hybrids.

Virginia

Refuge beetle proportion varied by year. In 2018, fields were planted between 8-10 May and emergence began on 6 July. Beetle captures were higher in NST+ fields (n = 114) relative to NST- fields (n = 11). Refuge beetles were first to emerge in both treatments, consistent with prior work (Clark et al., 2012; Taylor et al., 2016; Taylor & Krupke, 2018). Despite the difference between overall captures, refuge beetle proportions were consistent between treatments. In 2019, fields were planted between 29-30 May. Our total beetle captures in NST+ (n = 49) and NST- (n = 62) fields were similar and refuge beetles emerged first in both treatments. There were more refuge beetles collected in NST- fields.

Indiana

In both years, total beetle captures were relatively lower in NST+ fields (n = 289) compared to NST- fields (n = 797). Because of unfavorable planting conditions, fields were planted later and therefore closer to the approximate time of rootworm hatch, May to early-June (Krupke, 2009). Despite differences in overall beetle numbers between NST+ and NST- fields, refuge beetle ratios were the same. Removing NSTs from refuge seeds did not increase levels of refuge beetle emergence. It is possible that removing NSTs, a known feeding deterrent (Mullin et al., 2005), increased pressure on refuge seeds and caused population-dependent mortality effects. More research is needed to determine if NSTs affect larval movement between refuge and *Bt* plants.

Two possible (but not mutually exclusive) explanations for the differences between years and locations are heterogenous distributions of WCR larvae across fields (Ellsbury et al., 1998) and or time-dependent mortality attributable to NSTs. Planting timing and insecticide residues from seed coatings could have had an effect on refuge rootworm emergence if higher insecticide concentrations overlapped with early beetle emergence (Alford & Krupke, 2017; Blandino et al., 2017). In Virginia 2019, the majority of rootworm hatch and development in NST+ fields may have occurred outside of the pest management window (i.e., the overlap of an effective titer of insecticide with susceptible stages of the pest). Clothianidin, the NST used in our study, dissipates in root tissue at a rate so that most of the concentration is lost between 15 and 25 days post plant at the highest labeled rate (1.25 mg a.i./seed) (Alford & Krupke, 2017). While intriguing, we cannot state with any certainty that differences in refuge beetle populations were solely a function of planting timing because we relied on natural populations of beetles and did not control for the number of eggs laid in each field. Further investigation is needed to explore the relationship between insecticide concentrations and WCR development (Van Rozen & Ester, 2010; Blandino et al., 2017; Ferracini et al., 2021). Factors such as soil characteristics, plant growth, moisture, and temperature affect residues of seed-applied chemistries, and complicate field studies (Van Rozen & Ester, 2010). Given that neonicotinoids can persist in soil year-long (Goulson, 2013), and that they are applied ubiquitously to seeds of maize (Douglas & Tooker, 2015), future studies should also investigate the possibility of WCR resistance to neonicotinoids, which, to our knowledge, has not been explored.

Crop Protection

Our results were consistent with previous studies that demonstrate little benefit of applying insecticides to *Bt* seed (Oleson et al., 2002; Oleson et al., 2003; Jarvi et al., 2005; Oleson et al., 2006; Alford & Krupke, 2018; Labrie et al., 2020; Smith et al., 2020). We found that plant populations and root injury ratings were different only for refuge- seed and that results were not consistent between years and locations. In Indiana, differences in plant stand and injury ratings did not influence yield, perhaps because of compensatory growth (Coulter et al., 2011). In Virginia 2019, refuge+, refuge-, and Bt+ produced higher yield than Bt- (see Table 1.11). This effect has several possible explanations. Treated and untreated seeds were from different lots and we did not test seed germination pre-plant. Acquiring untreated seed from manufacturers is difficult for researchers and virtually impossible for farmers. Ideally, we would have received all seed from the same lot and applied treatments accordingly. Secondly, Bt+ and refuge+ treatments included a fungicide, which could have protected plants against stand and or yield loss, in addition to providing protection from storage pests. Further, *Bt* and refuge plants are not isolines and are chosen for phenotypic similarities. Hybrids used in the study, DKC 62-08 (*Bt*) and DKC 62-05 (refuge), yielded similarly in 2020-2021 variety trials, but occasionally, DKC 62-05 yields higher (Taylor and Thomason, unpublished data). The purpose of our study was not to measure yield by hybrid, but in the absence of pest pressure, yields are more likely to reflect physiological traits in the plant other than pest resistance.

Conclusion

Seed blends are a convenient strategy for IRM because they guarantee grower compliance and simplify farm logistics. Additionally, they are more compatible with WCR mating biology because of limited female dispersal prior to mating (Marquardt & Krupke, 2009) and a more synchronous emergence between *Bt* and refuge beetles compared to separate, structured refuges (Murphy et al., 2010). These factors, in addition to *Bt* and refuge plants being in close proximity (Hughson & Spencer, 2015), contribute to higher rates of mixed mating in seed blends relative to structured refuges (Taylor & Krupke, 2018).

A drawback of seed blends is larval movement between plant types. Meihls et al. (2008) and Head et al. (2014) report greater survival of “late-exposure” WCR larvae on *Bt* maize, that survived on non-*Bt* maize until second or third instar. While larval movement could lead to more rapid evolution of resistance (Murphy et al., 2010), it is also likely a causal factor of synchronous emergence observed in seed blends, thus indirectly facilitating mixed mating (Hibbard et al., 2005;

Murphy et al., 2010; Taylor et al., 2016; Taylor & Krupke, 2018). The cost of larval movement between plants needs to be weighed against the benefits of a higher probability of mixed-mating (Murphy et al., 2010). Onstad (2006) recommends that blends be no smaller than 20% non-*Bt*; this recommendation is supported by more recent findings (Taylor & Krupke, 2018). A concern about increasing refuge sizes is that increasing non-*Bt* plants may result in lower yields. Our results support that, given areas of low pest pressure, refuge plants produce similar yields to *Bt* plants. More refuge yield trials, in addition to the breeding and promotion of refuge hybrids suitable to multiple regions are needed to confidently address this issue (Reisig & Kurtz, 2018). Efforts to promote refuge compliance will require cooperation and promotion by researchers, seed companies, and regulatory agencies

More research is needed on seed blends of various sizes and how they affect refuge beetle emergence and larval movement between plants. We used a threshold to delineate “refuge” versus “*Bt*” beetles in order to account for larval movement between plants. Many individuals in our samples showed ^{15}N data concentrations numerically exceeding the baseline constant while remaining below our threshold for “refuge” beetle determination. We anticipated larval movement between plants, and these individuals had likely fed only minimally on refuge plants (Head et al., 2014). Field cages reduced our sample sizes in Virginia. Open fields, while providing higher overall numbers of rootworms collected, we were unable to account for migrating adults.

Neonicotinoid seed treatments can be effective pest management tools when early-season pests threaten seedling maize (Kabaluk & Ericsson, 2007; Wilde et al., 2007; Jordan et al., 2012; Bryant et al., 2020). They have low mammalian toxicity (Jeschke & Nauen, 2008) and are relatively safe for applicators and non-target mammals. Ecological concerns about their current use patterns have recently come to light (Goulson, 2013). They have been implicated in pollinator declines, and in a host of other direct and indirect alterations to non-target insect and non-insect animal populations (Krupke et al., 2012; Hallmann et al., 2014; Bonmatin et al., 2015; Alford & Krupke, 2019; DiBartolomeis et al., 2019; Krupke & Tooker, 2020). They have also been shown to travel through food webs and up trophic levels (Tooker & Pearsons, 2021). They are prone to leach from seeds and into groundwater (Alford & Krupke, 2019) or be transported into streams (Goulson, 2013), snowmelt (Main et al., 2016) and neighboring plants (Anderson et al., 2015; Alford & Krupke, 2019). Neonicotinoids are a useful pest management tool that should be implemented within an IPM framework, but in the case of field crops, studies that demonstrate benefits of ubiquitous, prophylactic use are rare (Estes, 2015; Tinsley et al., 2016; Alford & Krupke, 2018; Labrie et al., 2020).

We present evidence suggesting that NSTs could interfere with the *Bt* refuge strategy for WCR, however our primary takeaway is that increasing refuge sizes would benefit WCR-IRM more than removing NSTs. Results from our crop protection objectives show that NSTs were not necessary for protecting grain yields in any trial in Indiana, while they may have provided a slight benefit in Virginia. Our study emphasizes the impracticality of universal management plans for managing soil pests of maize across regions. We therefore recommend that NSTs be incorporated into an IPM framework that accounts for the likelihood of yield loss caused by soil pests in different production regions. Local monitoring for soil insect pests led by extension offices and agricultural research and extension centers would be useful to identify zones where soil pest pressures are high enough to warrant seed treatments. Additionally, growers must have the option to purchase seed without NSTs if developing such a framework is to be made possible.

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Tables and Figures

Table 1.1 Summary of Locations, Key Dates, and Planting Methods for Fields Used for IRM Trials in Virginia and Indiana

		Field Coordinates				**^{15}N**	***Bt* seed**	
Year	**Location**	**N**	**W**	**Field ID**	**Planted**	**Label**	**planting**	**Refuge seed planting**
2018	VA	37°20’92’’	-80°42’95’’	HCU	8 May	26 May	Hand-planted (95% *Bt*)	Hand-planted refuge seeds at 345 (5% of total seeds) random locations
		37°20’89’’	-80°43’08’’	HCT	10 May	25 May		
		37°21’15’’	-80°44’11’’	H21U[a]	8 May	6 June		
		37°20’97’’	-80°44’47’’	H21T	9 May	25 May		
2019	VA	37°20’96’’	-80°43’02’’	HCU	29 May	N/A[b]	Machine-planted (100% *Bt*)	Hand planted refuge seeds at 345 (5% of total seeds) random locations; nearest *Bt* seed removed
		37°20’86’’	-80°43’04’’	HCT	29 May	11 June		
		37°21’20’’	-80°44’16’’	H21U	30 May	7 June		
		37°20’95’’	-80°44’43’’	H21T	30 May	6 June		
	IN	40°17’45”	-86°54’31”	TPAC T3	4 June	17 June	Machine-planted (100% *Bt*)	Hand-planted two refuge seeds at 345 random locations; smaller of two refuge plants and nearest *Bt* plant removed after germination
		40°18’00”	-86°53’43”	TPAC T6	3 June	17 June		
		41°26’31”	-86°55’31”	PPAC L3	6 June	26 June		
		41°26’46”	-86°56’29”	PPAC E3	6 June	26 June		
2020	IN	40°17’45”	-86°54’31”	TPAC T3	26 May	12 June	Machine-planted (100% *Bt*)	Hand-planted two refuge seeds at 345 random locations; smaller of two refuge plants and nearest *Bt* plant removed after germination
		40°18’00”	-86°53’43”	TPAC T6	26 May	12 June		
		41°26’31”	-86°55’31”	PPAC L3	2 June	16 June		
		41°26’46”	-86°56’29”	PPAC E3	2 June	18 June		

[a] H21U was planted 100% *Bt* then refuge seeds were planted in field cages in 2018

[b] HCU was lost to bird feeding on seeds in 2019

Table 1.2. Summary of Locations, Key Dates, and Planting Methods for Fields Used for Crop Protection Trials in Virginia and Indiana

		Field Coordinates			**Planting**	**Planting**	**Soil Pest**	**Stand**	**Root**	
Year	**State**	**N**	**W**	**Field ID**	**Date**	**Method**	**Sampling**	**Counts**	**Ratings**	**Yield**
2018	VA	37°19’09’’	-80º58’16”	BOT	22 May	Hand-planted	26 May	14 June	30 July	N/A[a]
		37°19’00’’	-80º58’54”	TOP	23 May					
	IN	40°17’45’’	86º54’31”	TPAC T3	22 May	Machine-planted	6 June	12 June	26 June	29 Oct
		40°18’00’’	86º53’43”	TPAC T6	17 May		24 May	30 May	26 July	
		41°26’31’’	86º55’31”	PPAC L3	4 June		20-21 June	25 June	30 July	
		41°26’46’’	86º56’29”	PPAC E3	4 June		20-21 June	25 June	27 July	
2019	VA	37°19’09’’	-80º58’16”	BOT	29 May	Machine-planted	15 May	19 June	23 July	29 Aug
		37°19’00’’	-80º58’54”	TOP						
	IN	40°17’45’’	86º54’31”	TPAC T3	4 June	Machine-planted	28 June	28 June	13 Aug	4 Nov
		40°18’00’’	86º53’43”	TPAC T6	3 June		27 June	27 June	13 Aug	4 Nov
		41°26’31’’	86º55’31”	PPAC L3	18 June		1 July	1 July	20 Aug	5 Nov
		41°26’46’’	86º56’29”	PPAC E3	6 June		1 July	1 July	12 Aug	5 Nov
2020	IN	40°17’45’’	86º54’31”	TPAC T3	26 May	Machine-planted	22 June	22 June	5 Aug	2 Nov
		40°18’00’’	86º53’43”	TPAC T6	26 May		19 June	19 June	5 Aug	3 Nov
		41°26’31’’	86º55’31”	PPAC L3	2 June		25 June	25 June	6 Aug	11 Nov
		41°26’46’’	86º56’29”	PPAC E3	2 June		25 June	25 June	6 Aug	11 Nov

[a] Yield was not harvested in Virginia in 2018 due to deer feeding

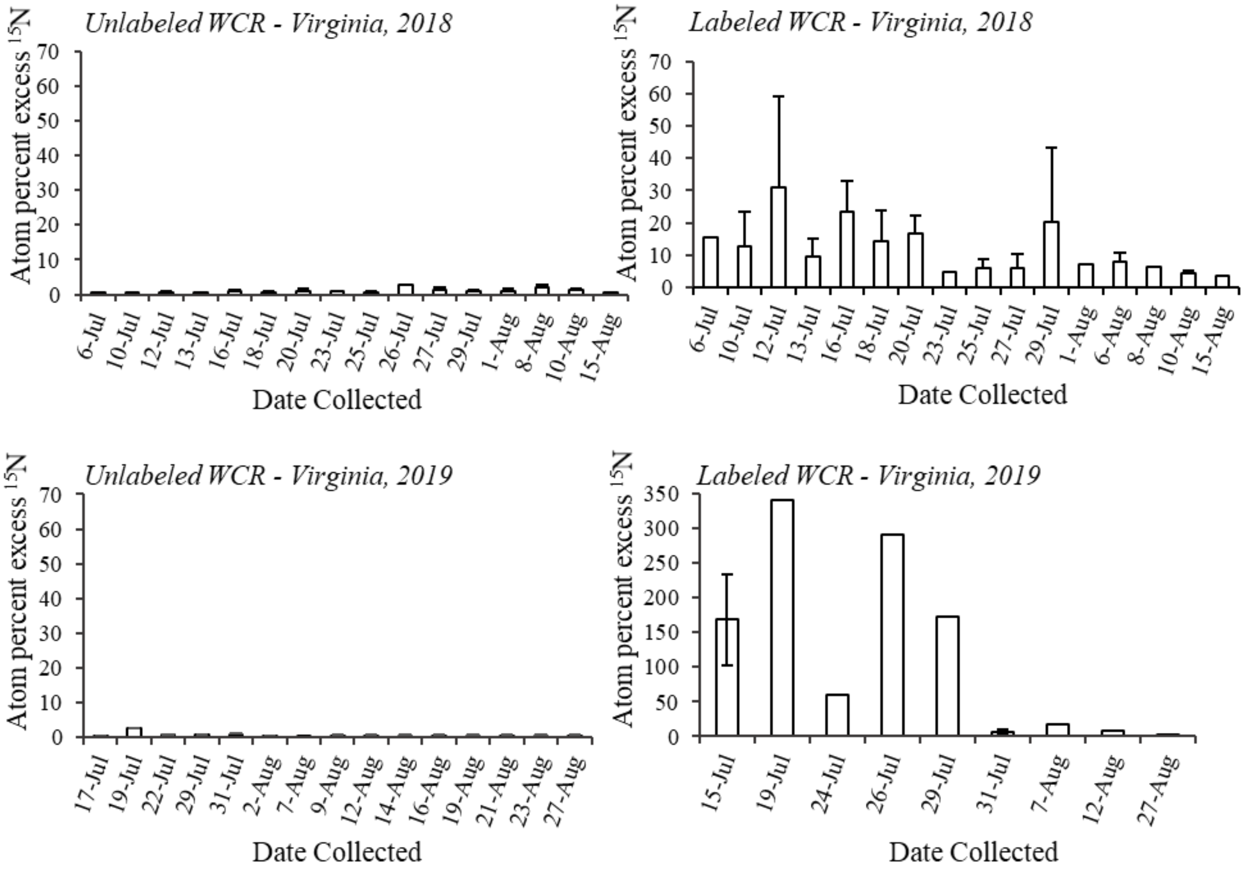

Figure 1.1. Bar graphs showing average atom percent ^{15}N in unlabeled (*top left*, *bottom left*) and labeled (*top right*, *bottom right*) beetles captured in Virginia, 2018-2019. Beetles were “labeled” if their atom percent excess ^{15}N exceeded a conservative threshold of 1.5%. This threshold differentiates beetles that had fed primarily on refuge plants versus those that may have been exposed only briefly.

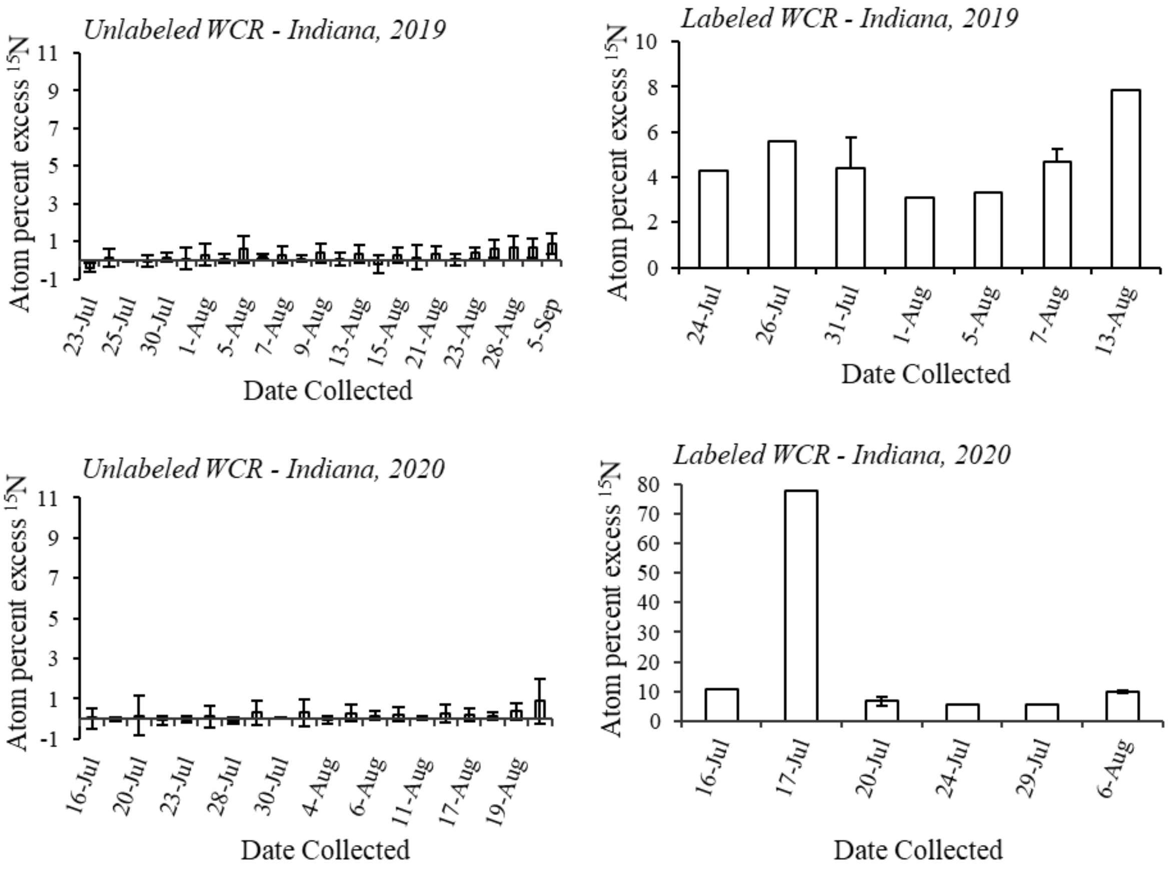

Figure 1.2. Bar graphs showing average atom percent ^{15}N in unlabeled (*top left*, *bottom left*) and labeled (*top right*, *bottom right*) beetles captured in Indiana, 2019-2020. Beetles were “labeled” if their atom percent excess ^{15}N exceeded a conservative threshold of 1.5%. This threshold differentiates beetles that had fed primarily on refuge plants versus those that may have been exposed only briefly.

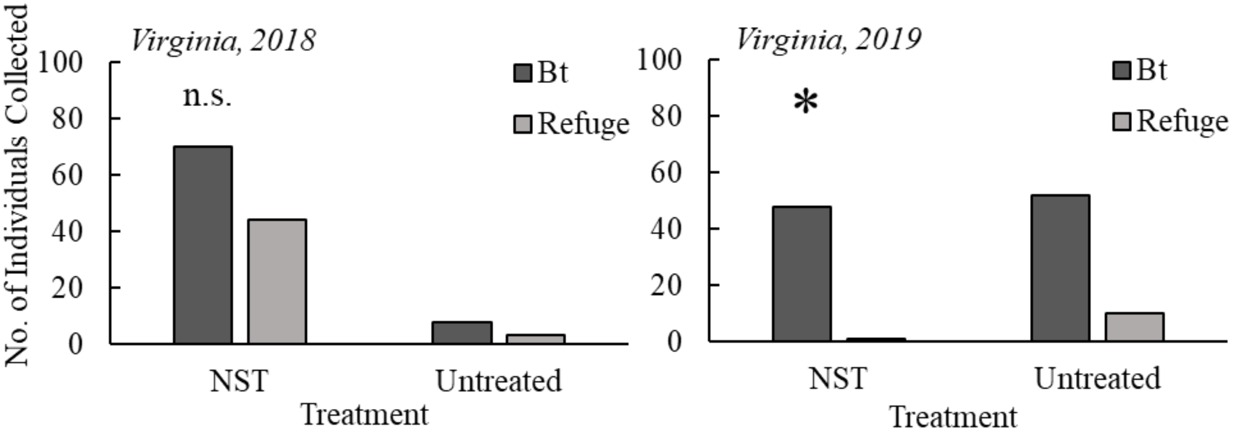

Figure 1.3. Bar graphs showing proportions of *Bt* and Refuge insects collected from NST+ and NST- plots in Virginia, 2018 (*left*) and 2019 (*right*). Proportions were compared using Fisher's exact test (α = 0.05).

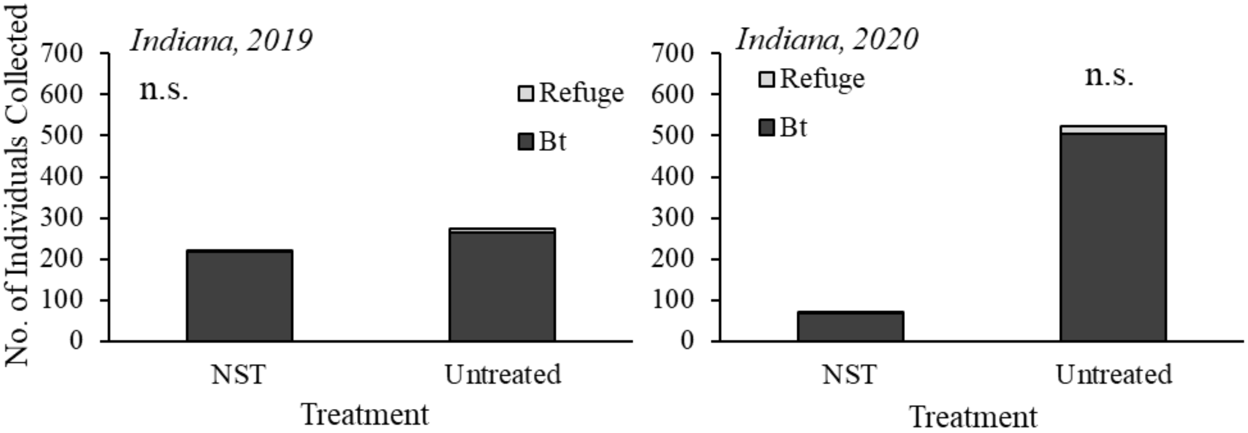

Figure 1.4. Bar graphs showing proportions of *Bt* and Refuge insects from NST+ and NST- plots in Indiana, 2019 (*left*) and 2020 (*right*). Proportions were compared using Fisher's exact test (α = 0.05).

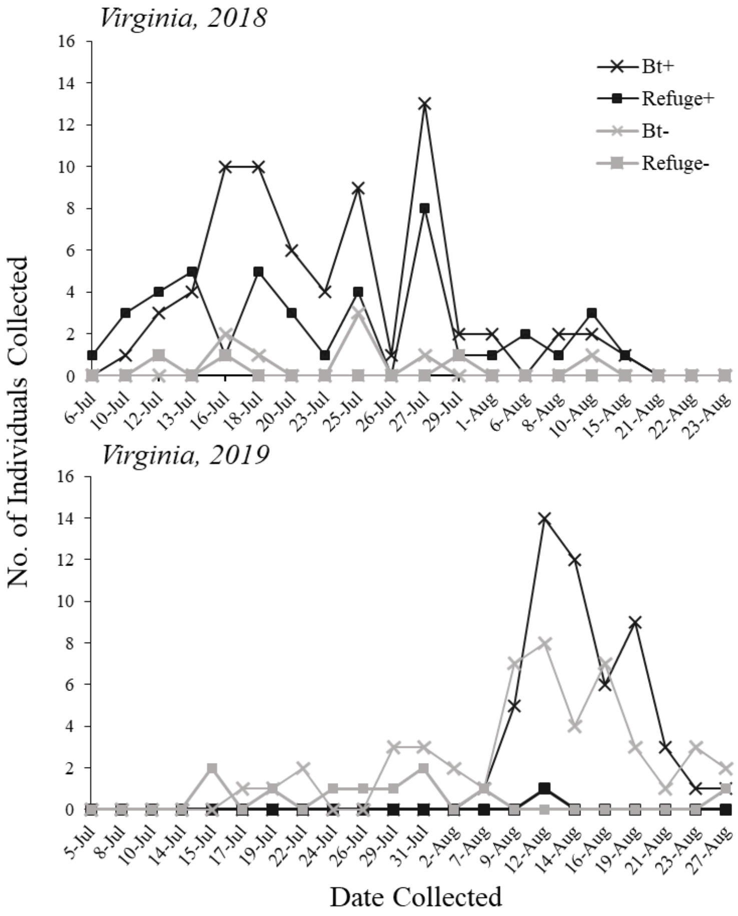

Figure 1.5. Line graph showing rootworm emergence by sampling date and host plant type for Virginia, 2018 (*top*) and 2019 (*bottom*).

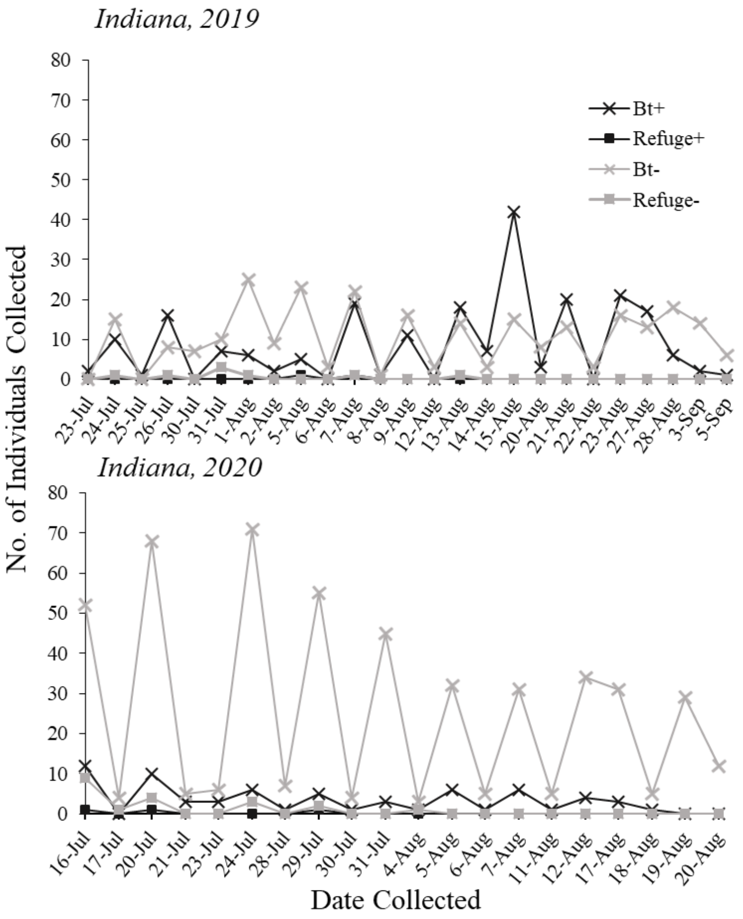

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Figure 1.6. Line graph showing rootworm emergence by sampling day and host plant type for Indiana, 2019 (top) and 2020 (bottom).

Table 1.3. Two-Way Analysis of Variance of Beetle Head Capsule Width by Seed Treatment, Sex, Host Plant Type, and Seed Treatment × Host Plant Type, Virginia 2018-2019

Source	**Factor**	**df**	**F**	***p***
Head Capsule Width (mm)	**Seed Treatment**	**1**	**11.9652**	**0.00065**
	Sex	**1**	**26.6272**	**<0.0001**
	Host Plant (Seed Treatment)	2	2.8692	0.05880
	Seed Treatment x Sex	1	0.0114	0.91518
	Host Plant (Seed Treatment) x Sex	2	1.5367	0.21730

Treatment	**n**	**Mean**	**SD**
NST+	167	1.09	0.08
NST-	69	1.12	0.06
M	92	1.07	0.06
F	144	1.12	0.08

Table 1.4. Two-Way Analysis of Variance of Beetle Dry Weight by Seed Treatment, Sex, Host Plant Type, and Seed Treatment × Host Plant Type in Virginia, 2018-2019

Source	**Factor**	**df**	**F**	***p***
Dry Weight (mg)	Seed Treatment	1	1.2396	0.26670
	Sex	**1**	**37.5864**	**<0.0001**
	Host Plant (Seed Treatment)	2	0.9433	0.39090
	Seed Treatment x Sex	1	0.8279	0.36380
	Host Plant (Seed Treatment) x Sex	2	0.8181	0.44260

Treatment	**n**	**Mean**	**SD**
M	92	2.34	0.81
F	144	3.42	1.49

Table 1.5. Two-Way Analysis of Variance of Beetle Head Capsule Width by Seed Treatment, Sex, and Seed Treatment × Sex, Indiana 2019-2020

Source	**Factor**	**df**	**F**	***p***
Head Capsule Width (mm)	**Seed Treatment**	**1**	**16.2809**	**<0.0001**
	Sex	1	1.3230	0.2503
	Seed Treatment x Sex	1	0.7378	0.3905

Treatment	**n**	**Mean**	**SD**
NST+	289	1.13	0.07
NST-	797	1.15	0.07

Table 1.6. Two-Way Analysis of Variance and Post-hoc Tukey Test of Beetle Dry Mass by Seed Treatment, Sex, and Seed Treatment × Sex, Indiana 2019-2020

Source	**Factor**	**df**	**F**	***p***
Dry Weight (mg)	**Seed Treatment**	**1**	**36.687**	**<0.0001**
	Sex	**1**	**72.808**	**<0.0001**
	Seed Treatment x Sex	**1**	**13.047**	**0.00032**

Post-hoc Tukey test for treatment and sex

Treatment	**n**	**Mean**[a]	**SD**
(NST+) M	62	2.87 *a*	0.69
(NST+) F	227	4.34 *b*	1.5
(NST-) M	326	2.87 *c*	0.62
(NST-) F	471	3.62 *d*	1.60

[a] Values not connected by the same letter are significantly different ($\alpha < 0.05$)

Table 1.7. Two-Way Analysis of Variance and Post-hoc Tukey Test of Plant Population by Treatment, Field, Year, Block, and Interactions, Virginia, 2018-2019

Source	**Factor**	**df**	**F**	***p***
Plant Population (pph)	**Treatment**	**3**	**14.6940**	**<0.0001**
	Field	**1**	**48.9732**	**<0.0001**
	Year	**1**	**10.7288**	**0.00476**
	Block	6	0.6679	0.67691
	Treatment x Field	3	1.3317	0.29900
	Treatment x Year	**3**	**5.8823**	**0.00663**
	Field x Year	1	1.3646	0.25985
	Treatment x Block	18	0.9654	0.53227
	Treatment x Field x Year	3	1.0848	0.38379

Treatment means and post-hoc Tukey test

Treatment	**n**	**Mean**[a]	**SD**
2018			
Bt+	6	70,293 a	9,615
Bt-	6	63,479 abd	10,972
Refuge+	6	65,631 ab	15,547
Refuge-	6	51,644 cd	9,575
2019			
Bt+	8	63,748 abd	7,838
Bt-	8	44,381 c	10,832
Refuge+	8	61,193 abd	7,077
Refuge-	8	55,006 bcd	3,012

[a] Values not connected by the same letter are significantly different (α < 0.05)

Table 1.8. Two-Way Analysis of Variance and Post-hoc Tukey Test of Plant Population by Treatment, Field, Year, Block, and Interactions, Indiana, 2018-2020

Source	**Factor**	**df**	**F**	***p***
Plant Population (pph)	**Treatment**	**3**	**4.1159**	**0.00869**
	Field	**3**	**10.9858**	**<0.0001**
	Year	2	0.6235	0.53831
	Block	**12**	**2.0454**	**0.02858**
	Treatment x Field	**9**	**4.7535**	**<0.0001**
	Treatment x Year	**6**	**17.0626**	**<0.0001**
	Field x Year	**6**	**4.6789**	**0.00034**
	Treatment x Block	36	1.2182	0.22446
	Treatment x Field x Year	**18**	**3.7582**	**<0.0001**

Post-hoc Tukey test for Treatment x Year

Treatment	**n**	**Mean**[a]	**SD**
2018			
Bt+	15	67,206 *abc*	5,691
Bt-	15	70,547 *c*	5,795
Refuge+	15	67,363 *abc*	8,283
Refuge-	15	61,568 *abd*	8,712
2019			
Bt+	16	67,912 *bc*	8,115
Bt-	16	66,387 *abc*	8,747
Refuge+	16	67,643 *abc*	6,931
Refuge-	16	59,390 *ad*	12,369
2020			
Bt+	16	61,981 *ab*	10,601
Bt-	16	51,935 *d*	12,708
Refuge+	16	68,887 *c*	9,167
Refuge-	16	72,206 *c*	7,025

[a] Values not connected by the same letter are significantly different (α < 0.05)

Table 1.9. Two-Way Analysis of Variance of Root Injury Rating by Treatment, Field, Year, Block, and Interactions, Virginia, 2018-2019

Source	**Factor**	**df**	**F**	***p***
Root Ratings (0-3)	Treatment	3	0.3353	0.80000
	Field	1	1.0468	0.32148
	Year	1	1.4883	0.24016
	Block	6	2.2119	0.09585
	Treatment x Field	3	1.0406	0.40139
	Treatment x Year	3	0.7699	0.52756
	Field x Year	1	0.0099	0.92202
	Treatment x Block	18	0.7876	0.68944
	Treatment x Field x Year	3	0.6201	0.61213

Treatment	**n**	**Mean**	**SD**
Bt+	14	0.168	0.09
Bt-	14	0.191	0.16
Refuge+	14	0.169	0.93
Refuge-	14	0.184	0.06

Table 1.10. Two-Way Analysis of Variance and Post-hoc Tukey Test of Root Injury Rating by Treatment, Field, Year, Block, and Interactions, Indiana, 2018-2020

Source	**Factor**	**df**	**F**	***p***
Root Ratings (0-3)	**Treatment**	**3**	**13.8495**	**<0.0001**
	Field	**3**	**12.2134**	**<0.0001**
	Year	**2**	**10.1668**	**0.00010**
	Block	**12**	**2.4384**	**0.00842**
	Treatment x Field	9	0.8962	0.53208
	Treatment x Year	6	2.1607	0.05386
	Field x Year	**6**	**7.0259**	**<0.0001**
	Treatment x Block	36	0.9750	0.51983
	Treatment x Field x Year	**18**	**1.8920**	**0.02597**

Post-hoc Tukey test for Treatment

Treatment	**n**	**Mean**^{a}	**SD**
Bt+	47	0.006 *a*	0.007
Bt-	47	0.012 *a*	0.017
Refuge+	47	0.014 *a*	0.019
Refuge-	47	0.050 *b*	0.125

^{a} Values not connected by the same letter are significantly different (α < 0.05)

Table 1.11. Two-Way Analysis of Variance and Post-hoc Tukey Test of Grain Yield by Treatment, Field, Block, and Interactions for Virginia, 2019

Source	**Factor**	**df**	**F**	***p***
Yield (kg/ha)	**Treatment**	**3**	**4.0302**	**0.023445**
	Field	**1**	**10.6517**	**0.004313**
	Block	**6**	**2.7048**	**0.047312**
	Treatment x Field	3	1.6747	0.207967

Post-hoc Tukey test for treatment

Treatment	**n**	**Mean**[a]	**SD**
2019			
Bt+	8	6,301 *a*	1,004
Bt-	8	5,299 *b*	875
Refuge+	8	6,263 *a*	698
Refuge-	8	5,766 *ab*	952

[a] Values not connected by the same letter are significantly different (α < 0.05)

Table 1.12. Two-Way Analysis of Variance of Grain Yield by Treatment, Field, Year, Block, and Interactions, Indiana, 2018-2020

Source	**Factor**	**df**	**F**	***p***
Yield (kg/ha)	Treatment	3	0.5199	0.66963
	Field	**3**	**109.2310**	**<0.0001**
	Year	**2**	**12.9730**	**<0.0001**
	Block	**12**	**9.0098**	**<0.0001**
	Treatment x Field	9	1.7482	0.08918
	Treatment x Year	6	1.9951	0.07426
	Field x Year	**6**	**9.0057**	**<0.0001**
	Treatment x Block	36	0.5500	0.97729
	Treatment x Field x Year	18	0.8176	0.67540

Treatment	**n**	**Mean**	**SD**
Bt+	47	10,004	2,479
Bt-	47	9,848	2,479
Refuge+	47	10,168	2,684
Refuge-	47	9,912	2,422

Chapter Two

Detection and quantification of neonicotinoid residues, clothianidin and thiamethoxam, in two corn production regions: Virginia and Indiana

(K. M. Bekelja, K. M. Miller, S. Entrekin, C. H. Krupke, S. V. Taylor)

Abstract

Neonicotinoids are the most widely-used class of insecticides in the world, in part because of their low mammalian toxicity, effectiveness at low concentrations, and spectrum of possible uses. Roughly 79-100% of maize in the United States (US) is treated with a neonicotinoid seed treatment (NST). Neonicotinoids provide maize seedlings with early-season protection from insect attack both below and above-ground. The same chemical properties that make neonicotinoids readily systemic also give them a tendency to leach off seeds and into ground and surface water. On maize seed, a maximum of 1.34% of active ingredient applied to the seed coating is detectable in plant tissues; thus, the remaining 98% has potential to contribute to environmental residues in soil and water. Studies conducted in other regions have found evidence of year-long persistence of residues in soil, and have detected concentrations exceeding acute and chronic toxicity benchmarks for aquatic organisms. Herein, we scouted for neonicotinoid residues in soil, water, and stream sediment within and surrounding fields of maize in Virginia and Indiana, to study the off-site movement and soil residence time of these compounds. We compared neonicotinoid concentrations in agricultural soils planted in maize with and without NSTs, and soils collected from field edges versus riparian forest buffers. Neonicotinoid residues were detected in soil matrices throughout the growing season, including prior to planting, suggesting year-round presence of these compounds. Water samples showed neonicotinoid residues exceeding chronic toxicity thresholds in 96.9%, 71.0%, and 66.7% of samples in 2018, 2019, and 2020, respectively.

Introduction

The United States (U.S.) is the world's leading producer of maize (*Zea mays* L.) with more than 90 million acres planted annually and a market value over $61 billion USD in 2020 (USDA-NASS, 2021). Insect pests can pose an economic threat to the productivity of maize, and insects that feed on plants below-ground can be difficult to manage, and infestations can be difficult to predict (Sappington et al., 2018). Following the introduction of neonicotinoids in the mid-1990s, the proportion of acres treated with insecticides, largely on seed coatings, has increased from <50% (1950-1990) to nearly 100% by 2011 (Osteen & Fernandez-Cornejo, 2013; Douglas & Tooker, 2015; Tooker et al., 2017).

Neonicotinoids are the most widely-used class of insecticides in the world, in part because of their low mammalian toxicity, effectiveness at low concentrations, and spectrum of possible uses (Jeschke & Nauen, 2008; Sparks, 2013). In addition to being broadly used in agriculture, neonicotinoids are applied on lawns and gardens, in households for roaches and ants, and on companion animals and livestock to treat parasites (Jeschke et al., 2011). Their chemical properties make them versatile; they can be applied as foliar sprays, soil drips or drenches, and as seed coatings (Elbert et al., 2008). They have high water solubility and a relatively small molecule size, making them readily systemic and translaminar (Jeschke et al., 2011).

Neonicotinoid seed treatments provide logistic and safety benefits to pesticide applicators because seed can be purchased with insecticide applied, and rates are generally lower, on an area basis, than spray and soil drench applications (Jeschke et al., 2011). They provide seedlings with early-season protection from insect attack both below and above-ground (Elbert et al., 2008; Jeschke et al., 2011; Bryant et al., 2020). Neonicotinoid seed treatments are applied prophylactically as "insurance" against soil-dwelling insects that are notoriously difficult to predict

(Sappington et al., 2018). As of 2022, nearly 100% of commercial maize and cotton seed sold in the U.S. includes a neonicotinoid insecticide in its seed coating (Douglas & Tooker, 2015).

Since these compounds are so ubiquitously applied, there have been many studies investigating their effects on non-target animals across a range of habitats. Neonicotinoids are under specific scrutiny as one several possible causes of honeybee populations decline worldwide (VanEngelsdorp et al., 2008; Goulson, 2013; Hristov et al., 2020). The same chemical properties that make neonicotinoids readily systemic also give them a tendency to leach off seeds and into ground and surface water. On maize seed, a maximum of 1.34% of active ingredient applied to the seed coating is detectable in plant tissues; thus, the remaining 98% has potential to contribute to environmental residues in soil and water (Alford & Krupke, 2017).

Residues have been detected in groundwater (Hladik et al., 2014; Huseth & Groves, 2014; Kurwadkar et al., 2014; Wettstein et al., 2016; Bradford et al., 2018; Alford & Krupke, 2019), surface water (Hladik et al., 2014; Johnson & Pettis, 2014; Hladik & Kolpin, 2016; Benton et al., 2017; Hartz et al., 2017; Struger et al., 2017; Hladik, Main, et al., 2018), neighboring plants (Alford & Krupke, 2019; Knight et al., 2021), and snowmelt (Main et al., 2016). Their use in crops can disrupt biological control and cause unintended yield losses. For example, non-target pests such as slugs and caterpillars can sequester residues in their bodies from treated food sources and intoxicate predators (Douglas et al., 2015). Neonicotinoids have also been implicated in indirect alterations to food webs: Hallmann et al. (2014) showed declines in insectivorous bird populations associated with areas of higher concentrations in surface waters, likely resulting from pesticide-mediated declines in aquatic macroinvertebrates (Van Dijk et al., 2013). Neonicotinoids are highly toxic to some aquatic macroinvertebrates (Van Dijk et al., 2013; Morrissey et al., 2015; Pisa et al., 2015; Bartlett et al., 2018). Kreutzweiser et al. (2007) showed that trees treated for emerald ash borer at an intentionally high dose of imidacloprid expressed insecticide concentrations in fallen leaves up to 80 parts per million (ppm), and that feeding effects on stream-dwelling insect detritivores occurred at just 1.3 ppm.

Neonicotinoid use is higher in U.S. regions, such as in Indiana where maize is grown on millions of acres every year, compared to the Appalachian Valley and Ridge regions, where maize is grown on a smaller scale, largely to supply local livestock operations (Heimlich, 2000; USDA-NASS, 2021). Both regions are characterized by areas of karst topography (Parvinder Sethi, 2014), where surface water is in close connection with groundwater (Packman, 2022; VA-DCR, 2022). Physiographic regions with karst features leave little time for sunlight to degrade organic and inorganic contaminants before reaching below-ground springs, increasing the persistence of pesticides and other agricultural chemicals (Packman, 2022). Some studies examined movement from seed treatments into waterways and soil in Indiana (Alford & Krupke, 2017; Alford & Krupke, 2019) and Pennsylvania (Frame et al., 2021), however there is a dearth of information surrounding the environmental fate of these compounds in the limestone karst soils of Appalachia.

The objective of this study was to measure clothianidin and thiamethoxam residue levels throughout the growing season in agricultural soils and surface water in two different maize production environments. We collected soil samples throughout the growing season from fields planted with maize seed with and without neonicotinoid seed coatings. We collected water samples from sources adjacent to agricultural fields to determine if compounds were detectable in surface waters, and if so, when and at what concentrations. In Virginia, we also collected soil from field edges and riparian forest buffers, sediment, and water samples from two stream reaches upstream and downstream from continuous maize fields to compare residues between agricultural and non-agricultural environments. We hypothesized that samples from fields planted with neonicotinoids and samples collected adjacent to maize fields will have higher concentrations than fields planted without neonicotinoids and non-agricultural lands. Our goal is to document environmental residues in multiple locations to inform on the environmental fate of these compounds to aid in a cost/benefit analysis of a ubiquitously-used insecticide.

Methods

Virginia Corn Fields and Forested Reference Site Samples

We collected soil samples from within fields planted with maize seeds coated with and without the neonicotinoid clothianidin at 1.25 mg a.i./seed. Maize fields were located at Hethwood Farm in Blacksburg, Va. (37°20’97.85’’N, -80°44’47.85’’W). Fields were continuous maize and not cultivated for a least eight years prior to our study; soil types were Groseclose and Poplimento soils, a well-drained mix formed by weathered sandstone, shale, and limestone that is common to Virginia, Tennessee, and Kentucky (USDA-NRCS, 2003). Seeds were planted at a rate of 68,419 seeds per hectare. Three different locations were sampled: 1) a field planted with untreated maize seed (DKC 62-08, Dekalb, Monsanto, St. Louis, MO, USA); 2) a field planted with clothianidin-treated maize seed (DKC 62-08 with Acceleron® Basic and additional 1.00 mg a.i./seed applied); and 3) a forested reference site with no known neonicotinoid use. Treated seeds were purchased with a rate of 0.25 mg a.i./seed applied by the manufacturer. An additional 1.00 mg a.i./seed was applied by the seed treatment manufacturer (Bayer CropScience, RTP, NC, USA) for a total clothianidin rate of 1.25 mg a.i./seed. Fields were planted on 22-23 May. Soil was collected from five representative locations (chosen to represent the entire field) using a 10 cm diameter x 15 cm deep soil core. Samples were combined in an 18.95 L bucket and mixed using a garden trowel. A 250 g subsample was taken and stored in paper bags labeled with location and date. Samples were stored at 0°C until extractions. Soil sample collections occurred at specific intervals: the day before planting, the day after planting, 1 week, 3 weeks, 6 weeks, and 12 weeks post-planting.

Virginia Field Edges and Riparian Zones (2019-2020)

Sampling locations are shown in Figure 2.1. We collected soil from locations along agricultural field edges, and from within adjacent riparian forest buffers. Samples were collected from within segments of Tom's Creek at Kentland Farm in Montgomery Co. For samples taken on the southeastern side of the creek, soil type was Ross-Purdy, a very deep, well-drained soil formed in loamy alluvium on flood plains and low terraces, common to Virginia, Missouri, Ohio and Indiana (USDA NRCS, 2003). Western side samples were Guernsey soils, which are deep, moderately well-drained soils formed from interbedded siltstone, shale, and limestone, and are common to southern Ohio, Virginia, West Virginia, and Pennsylvania (USDA-NRCS, 2003). Accessibility, in part, determined sample locations because of unwalkable steep grades and cliffs. On the southeastern edge of Tom's Creek, soil sample locations were adjacent to a grass strip approximately 20 m in width, beyond which were agricultural fields that contained various vegetables and hemp. Soil samples along the western edge of Tom's creek were taken from the west side of the creek along the southern edge of a maize field, located 600 m upstream from southeast samples. During each sampling event, a golf cup cutter (10 cm deep x 10.7 cm diameter) was used to extract three soil cores from each location. Soil cores were added to an 18.95 L bucket and contents were mixed with a garden trowel. A 250 g subsample was transferred into two prelabeled Ziploc bags. Ziploc bags were placed into a cooler on ice, then stored at -15^{o}C until extractions. Sampling times are summarized in Table 2.1.

Virginia Within-Stream Samples (2019-2020)

Sampling locations are shown in Figure 2.1. We collected within-stream biological compartment samples (e.g., water, sediment) at various timepoints to determine whether residues could be detected in pattern with concentrations in agricultural field edges and forest buffers.

Samples were collected from a deemed “agricultural” (37.203679, -80.566112) and “non-agricultural” (37.212336, -80.553634) stream catchment along Tom’s Creek, which were delineated using Unites States Geological Survey (USGS) National Hydrography Dataset (NHDPlus HR). Timepoints for sample collection were based on planting times for nearby maize fields, weather, and water levels in Tom’s Creek. Collections occurred at three locations (labeled “A”, “B”, and “C”) within one pre-defined stream reach per catchment. Reference samples, to serve as potential negative controls, were collected from a third site, located approximately 8.1 km away from Tom’s Creek catchment samples.

Water: Three pre-labeled 200 mL amber glass jars (140-0250NC, Thermo Scientific, Waltham, MA) were used to collect three subsamples at each location (“A”, “B”, and “C”) within each stream reach at each timepoint. Each jar was rinsed in stream water three times before collection. Jars were immediately placed in a cooler on ice and stored at -15^{o}C until processing.

Sediment: Three surface sediment samples were collected using a PVC ring (8cm in diameter) pre-marked to 2 cm depth. Sampling locations were dictated by stream conditions, i.e., sediment samples were not necessarily able to be collected from the same exact location for every sampling event. When a location with sufficient sediment deposits was identified, the PVC ring was pressed into the sediment up to the pre-marked depth line, then a joint knife (Hyde Tools Inc., Southbridge, MA) was slid underneath the ring and remained flush with the ring bottom while the sample was lifted above the water surface. Excess water was poured off, and the remaining sediment was placed in a 200 mL amber glass jar (140-0250NC, Thermo Scientific, Waltham, MA). Jars were placed in a cooler on ice and stored at 0^{o}C until processing.

Indiana Corn Fields (2018-2020)

We collected soil and water samples from two fields at both Throckmorton Purdue Agricultural Center (TPAC) in Lafayette Co. (40°17’48’’N, 86°54’13’’W) and Pinney Purdue Agricultural Center (PPAC) in LaPorte Co. (41°26’35’’N, 86°55’49’’W), and a forested control site. Fields within each location were approximately 1.25 km and 1.4 km apart, respectively. Fields were continuous maize and not cultivated for a least two years prior to our study. Soil type was Tecumesh and Tracy series, a well-drained soil. Seeds were planted at a rate of 68,419 seeds per hectare. Treated seeds in 2018-2019 were purchased with a rate of 0.25 mg a.i./seed applied by the manufacturer. An additional 1.00 mg a.i./seed was applied by the seed treatment manufacturer (Bayer CropScience in 2018 and BASF in 2019, RTP, NC, USA) for a total clothianidin rate of 1.25 mg a.i./seed. Because all seeds were provided in 2020 with clothianidin applied, untreated seeds were washed to remove the seed coating using a protocol developed by Dr. Joseph Spencer at the University of Illinois (personal communication). Briefly, seeds were partitioned into lots of 5,000, added to an 18.95 L bucket with 5 L DiH2O and 20 mL dish soap (Ultra Palmolive Original; Colgate-Palmolive Company, New York, NY, USA), and manually stirred for 20 minutes. Seeds were then poured into a sieve and rinsed four times with DiH2O. Washing and rinsing was repeated twice more. Seeds were then blotted with paper towels, air-dried for 12 h, and then soaked in 10% bleach solution for 1 h to prevent mold and bacterial growth, agitating every 15 minutes. Seeds were removed from the bleach solution and rinsed 10 times in DiH2O. After rinsing, the seeds were blotted with paper towels and air-dried for 24 h. All washed seed was stored at 0℃ until planting to prevent germination. Treatments were planted in a randomized block design with four blocks per replicate. Fields were planted on 17 May, 22 May, and 4 June in 2018; 4-6 June and 18 June in 2019; and 26 May and 2 June in 2020.

Soil: Samples were collected from 3.04 m x 12.19 (TPAC) or 3.04 m x 9.14 m (PPAC) plots planted with four different treatment combinations: 1) untreated maize seed (DKC 62-08), herein “Bt-” ; 2) clothianidin-treated maize seed (DKC 62-08) herein “Bt+”, 3) untreated maize seed (DKC 62-05, Dekalb, Monsanto in 2018 and Bayer CropScience in 2019-2020, St. Louis, MO, USA) herein “Refuge-”, and 4) clothianidin-treated maize seed (DKC 62-05) herein “Refuge+”. Soil was collected from four random locations per plot using a 10 cm diameter x 15 cm deep soil core. Samples were combined in an 18.95 L bucket and mixed using a garden trowel. A 250 g subsample was taken and stored in paper bags labeled with location and date. Samples were stored -20℃ until extractions. Soil sample collections occurred at specific intervals: the day before planting, the day after planting, one week, three weeks, six weeks, and 12 weeks post-planting.

Water: Surface water samples were collected from ditches and waterways near fields. Water samples were collected in 500 mL Nalgene amber bottles (Thermo Scientific, Waltham, MA) and stored at -20°C until extraction. Water sample collections occurred at specific intervals: the day before planting, the day after planting, 1 week, 3 weeks, 6 weeks, and 12 weeks post-planting.

Data Analysis

All analyses were performed using R software (R Core Team, 2021).

Virginia: Maize field, field edge, and riparian forest buffer soil samples were analyzed separately by year using two-way analysis of variance (ANOVA) with repeated measures via the “anova_test” function in the rstatix package (Kassambara, 2021). All data were transformed using a log(x+1) transformation to achieve better model fit. Models used insecticide concentration (e.g., clothianidin) as a response variable, with type (e.g., field edge, forest buffer, control), or treatment (e.g., treated, untreated, control) and sample timing as fixed effects, with an additional factor containing an individual/subject identifier. When treatment effects were observed ($\alpha = 0.05$), a

TukeyHSD test compared treatment means. Insecticide residues were not detected in a sufficient proportion of sediment and water samples to perform statistical analyses; therefore values are reported.

Indiana: Maize field and water samples were analyzed separately by year using a two-way ANOVA with repeated measures via the "anova_test" function in the rstatix package. For soil samples, outliers were identified using the "quantile" function, then removed using the "subset" function before analysis (R Core Team, 2021). All data were transformed using a log(x+1) transformation to achieve better model fit. Models used insecticide concentration (e.g., clothianidin or thiamethoxam) as response variables, with treatment (i.e., maize hybrid and treatment combinations) and sample week as fixed effects, with an additional factor containing an individual/subject identifier. Where treatment effects were observed ($\alpha = 0.05$), a TukeyHSD test compared treatment means.

Results

Limits of detection (LODs) for clothianidin were 0.1 ppb, in soil and 0.004 – 0.03 ppb in water; limits of quantification (LOQs) were 0.1 ppb in soil and water. Limits of detection for thiamethoxam were 0.01 ppb in soil and 0.0007 ppb in water; limits of quantification were 0.05 ppb in soil and 0.002 ppb in water.

Virginia Corn Fields and Forested Reference Site (2018)

In all fields, clothianidin detection frequencies were 97.4% with the median of 3.634 ppb (range: 0.175 – 417.42). Clothianidin residues did not differ by sample week (df = 5, F = 0.780, $p = 0.178$) or field type (df = 2, F = 2.041, $p = 0.1590$) (Table 2.3). Mean clothianidin residue levels were 6.89 (SD = 6.90) ppb in field with clothianidin-treated seeds, 6.00 (SD = 2.84) ppb in fields without clothianidin-treated seeds, and 0.57 (SD = 0.33) ppb for the forested reference site (see Table 2.4).

Virginia Field Edges and Riparian Zones

2019: Overall detection frequencies for clothianidin were 75.6%. Median concentrations were 0.2723 ppb (range: 0 – 10.156) and 0.178 ppb (range: 0 – 5.402) for field edge and riparian buffer soil samples, respectively. Clothianidin concentrations differed only by sampling event timing (df = 4, F = 8.179, $p < 0.001$) (see Table 2.5). Concentrations were highest at sampling event 1 (M = 2.95, SD = 3.00) and declined until sampling event 4 (M = 0.46, SD = 0.78) (see Table 2.6).

2020: Overall detection frequencies for clothianidin were 30%. Median concentrations were 0 ppb (range: 0 – 2.161) and 0 ppb (0 – 0.538) in field edge and riparian buffer soil samples, respectively. Clothianidin concentrations differed only by location (df = 1, F = 6.132, $p = 0.0160$), where field edge (M = 0.26, SD = 0.51) had significantly higher overall mean concentration than forest buffer (M = 0.05, SD = 0.12) (see Table 2.5 & Table 2.6).

Virginia Within-Stream Samples (2019-2020)

In total, only five sediment samples (6.25%) in 2019 contained detectable levels of clothianidin. These samples were collected from the non-agricultural catchment on 5 June. Mean concentration from location A was 0.13 (SD = 0.12) ppb, and from location B 0.10 (SD = 0.03). There were no detectable levels of clothianidin in any water sample.

Indiana Corn Fields

Soil: *2018*. Overall detection frequencies were 99.5% clothianidin and 86% thiamethoxam. Median concentrations were 4.674 ppb (range: 0.184 – 65.880) clothianidin and 0.141 ppb (range: 0 – 3.376) thiamethoxam. *2019*. Overall detection frequencies were 100% clothianidin and 80.7% thiamethoxam. Median concentrations were 5.807 ppb (range: 0.2 – 367.091) clothianidin and

0.0917 ppb (range: 0 – 1.445) thiamethoxam. *2020*. Overall detection frequencies were 100% clothianidin and 65.7% thiamethoxam. Median concentrations were 5.238 ppb (range: 0.668 – 131.679) clothianidin and 0.042 ppb (range: 0 – 1.052) thiamethoxam.

Clothianidin and thiamethoxam residues differed by treatment in all years (Table 2.7 & Table 2.8). Clothianidin concentrations were highest in plots planted with seed treatments. Thiamethoxam detections showed less consistent trends because concentrations did not always differ between treated plots. Clothianidin and thiamethoxam residues differed by sample week in all years. In 2018, clothianidin residues were generally highest three weeks after planting; in 2019, they peaked immediately at post-planting; in 2020, highest concentrations were detected at pre-planting and post-planting, then decreased through the remaining sampling weeks. Thiamethoxam residues were highest at the earlier sampling weeks in all years.

<u>Water</u>: *2018*. Overall detection frequencies were 100% clothianidin and 90.6% thiamethoxam. Median concentrations were 0.239 ppb (range: 0.455 – 36.351) clothianidin and 0.007 ppb (range: 0 – 5.911) thiamethoxam. *2019*. Overall detection frequencies were 100% clothianidin and 87.1% thiamethoxam. Median concentrations were 0.016 ppb (range: 0.019 – 7.426) clothianidin and 0.003 ppb (range: 0 – 0.150) thiamethoxam. *2020*. Overall detection frequencies were 100% clothianidin and 91.4% thiamethoxam. Median concentrations were 0.147 ppb (range: 0.009 – 7.857) clothianidin and 0.007 ppb (range: 0 – 2.422) thiamethoxam.

Clothianidin and thiamethoxam residues did not differ by sample week or treatment in any year (Table 2.7 & Table 2.8).

Discussion

Our results demonstrate that neonicotinoid residues are consistently detectable in agricultural soils, as we measured residues prior to planting and throughout the growing season from both treated and untreated plots. In Virginia, clothianidin residues were detected in field edge soil (57%) and forest buffer soil (34.5%), providing further evidence for offsite movement of neonicotinoids in soil matrices. Interestingly, the only concentrations measured in sediments occurred in the non-agricultural catchment, indicating potential sources of neonicotinoid inputs upstream. In Indiana, we detected residues in almost 100% of water samples from waterways adjacent to fields.

In Indiana, we consistently measured neonicotinoids in water samples collected from ditches, ponds, and creeks near maize fields. Clothianidin residues at these sampling sites, which were absent of riparian buffers, exceeded EPA-established chronic exposure benchmarks for ecotoxicity in 96.9%, 71.0%, and 66.7% of samples in 2018, 2019, and 2020, respectively. Both our overall detection frequency (clothianidin: 94.9%; thiamethoxam: 18%) and overall median concentrations (clothianidin: 0.149 ppb, range = 0.009 – 36.35; thiamethoxam: 0.005 ppb, range = 0 – 5.911) were relatively high. Hallmann et al. (2014) showed that concentrations in surface water exceeding 0.02 ppb resulted in declines in insectivorous bird populations as a result of alterations made to invertebrate communities; 94.9% and 18.2% of our samples exceeded this threshold for clothianidin and thiamethoxam, respectively. Barmentlo et al. (2021) showed that emergence of most orders of aquatic insects was absent at 10 ppb, and diversity of most species decreased by 50% at 1 ppb. Several studies report alterations to aquatic insect communities occurring at concentrations even lower than regulatory thresholds and limits of quantification for standard analytical methods (Schepker et al., 2020; Kuechle et al., 2022). Alford and Krupke (2019) report clothianidin concentrations in leachate as high as 3.37 ppb, peaking at 4-weeks post-planting. In 2018 and 2020, our clothianidin residues in water peaked early in the season: pre-planting and 1-week post-planting, respectively; in 2019, water residues peaked 6-weeks post-planting.

In Virginia, within-stream samples (e.g., water and sediment) were collected from sites with forested riparian buffers ≥ 15 meters in width; neonicotinoid residues were not detected in any water sample. This may be evidence of the effectiveness of riparian forest buffers at limiting insecticide inputs from agricultural fields into surface waters (Main et al., 2015; Satkowski, 2016; Chandler et al., 2020), or indication that our methods were insufficient to measure residues. Concentrations measured in soil collected from riparian zones nearby water collection sites would support this hypothesis, as well as Satkowski (2016), who shows that neonicotinoids bind more tightly to riparian soils due to higher organic matter content. More research would be needed to confirm whether riparian zones prevented the contamination of our study stream from reaching concentrations within our limits of detection. Differences in topography, soil type, and intensity of agricultural land use between Indiana and Virginia study areas likely influenced detection frequencies between states (Hladik, Corsi, et al., 2018).

In both states, our mean pre-plant soil sample neonicotinoid concentrations (Indiana: M = 8.2 ppb; Virginia: M = 4.99 ppb) were greater than or consistent with those reported by Schaafsma et al. (2015) (M = 4.0 – 5.6 ppb), who suggest that if seed treatments are used consistently in maize-soybean-winter wheat rotations, residues tended to plateau around 6 ppb in their study area. Reported half-lives for clothianidin and thiamethoxam range from 148-6931 days and 7-353 days, respectively, therefore it is conceivable that residues from prior plantings can remain present throughout the growing season (Goulson, 2013). Detections of neonicotinoids in untreated plots throughout the growing season may be indicative of leftover residues from prior plantings (DeCant & Barrett, 2010; Goulson, 2013; Schaafsma et al., 2015), lateral leaching from surrounding NST+

maize (Radolinski et al., 2019), and or surface transport via erosion rills (Stehle et al., 2016). Year-long persistence of neonicotinoid residues is likely to have undesirable ecological consequences on resident invertebrate and vertebrate communities (Hallmann et al., 2014; Disque et al., 2019), and agricultural drawbacks may include evolution of insect resistance via chronic exposure to compounds at low doses (Gressel, 2011). Persistence in soil can also cause gradual leaching of neonicotinoids into waterbodies year-round (Hladik et al., 2014; Hladik, Corsi, et al., 2018).

The purpose of this study was to compare neonicotinoid concentrations in soil planted with treated and untreated maize seed, and to measure residues in non-target soil and water matrices adjacent to agricultural fields in two states. Our hypothesis that neonicotinoid residues would be in higher concentrations in soil in treated versus untreated plots was supported only in Indiana. Virginia samples showed similar concentrations between treated and untreated fields. The reason for season-long neonicotinoid detections in untreated fields in Virginia remains unclear, but lateral leaching and surface transport of residues from treated plots, in additional to several years of planted treated maize seed are possible causal factors. Water detections did not support our hypothesis that neonicotinoid residues would be higher in water from agricultural streams. We measured neonicotinoids in water samples from all sites in Indiana, and their concentrations were similar despite surrounding landscape differences.

Off-site movement of pesticides has been documented for several decades and for numerous classes. For example, pesticides, including insecticides, have been documented in Chesapeake Bay oysters before the rapid adoption of neonicotinoids (Lehotay et al., 1998). Documenting temporal and geographic trends in environmental residues are important data when performing risk analyses and determining research priorities for different habitats and their associated biodiversity. Unfortunately, neonicotinoid use as seed treatments in maize is no longer tracked by pesticide use surveys, making it difficult or impossible to associate these compounds with any theoretical ecological consequence of their use. Future studies should look for off-site effects of these compounds, such as changes to macroinvertebrate diversity, abundance, and emergence.

To our knowledge, our study was the first to compare residues in soil and water in two different farm resource regions: Indiana, being part of the U.S. Heartland region where the majority of maize is produced, and southwestern Virginia, where maize is grown on a smaller scale, often with less frequent rotation to other crops (Heimlich, 2000). Neonicotinoids protect plants against early-season and soil pests of maize (Andersch & Schwarz, 2003; Elbert et al., 2008; North et al., 2018; Bryant et al., 2020), but several regions report low pest pressures and inconsistent benefits of use, especially in combination with transgenic *Bt* toxins, which target the key pests of corn in the US (Krupke et al., 2017; Alford & Krupke, 2018; Labrie et al., 2020; Smith et al., 2020). While neonicotinoids have been shown to be important drivers of alterations to aquatic insect communities in some studies (Hallmann et al., 2014; Cavallaro et al., 2019), it remains important to consider them within the context of other agricultural inputs, and to keep in mind that interactions between aquatic ecosystem endpoints and ecological stressors can be complex (Ormerod et al., 2010). We propose that considerations be made to change use patterns towards a need-based integrated pest management framework, rather than preventative applications on nearly all seeds, regardless of location.

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Tables and Figures

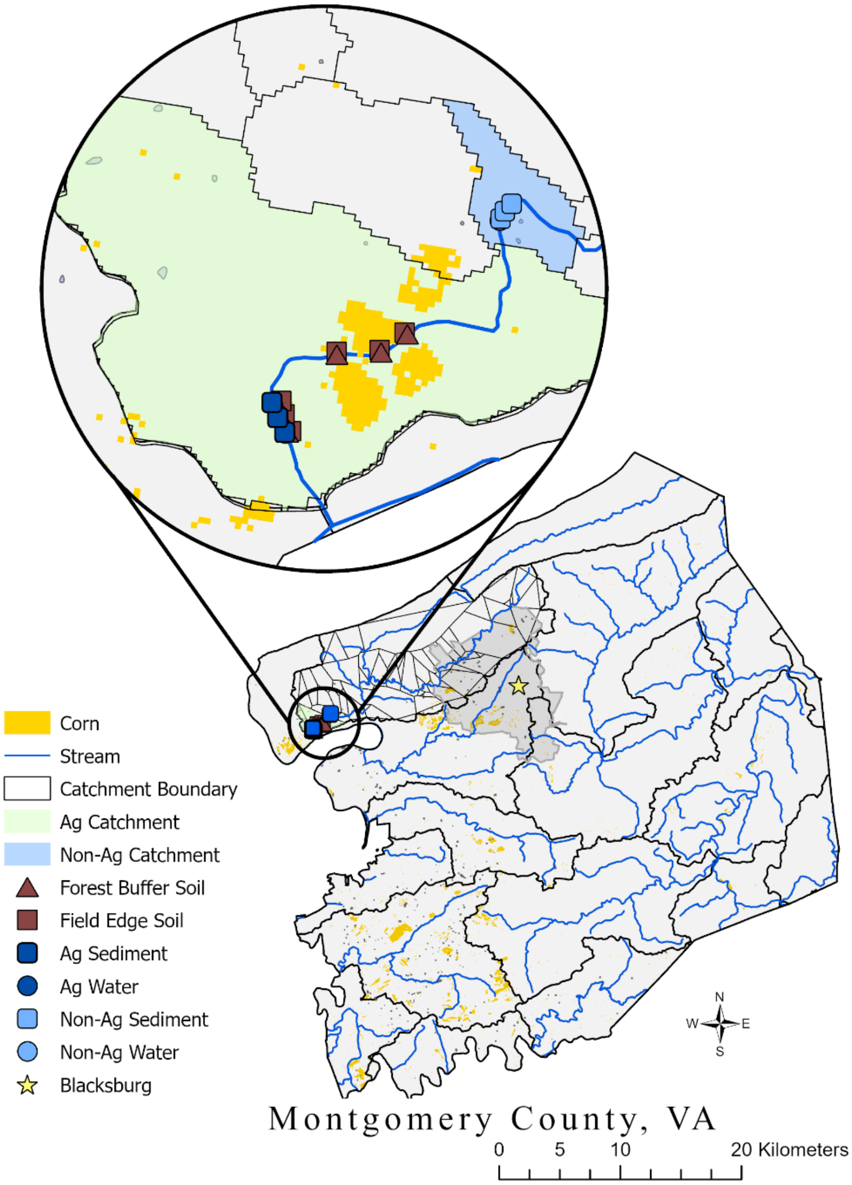

Figure 2.1. Map showing agricultural and non-agricultural catchments for field edge and riparian forest buffer soil, in-stream sediment, and water samples in Virginia, 2019-2020.

Table 2.1. Sampling Events and Dates for Field Edge and Forest Buffer Soil Samples in Virginia, 2019-2020

Year	**Sampling Event**	**Date**
2019	1	6 June
	2	11 June
	3	18 June
	4	3 July
	5	20 August
2020	1	14 May
	2	1 June
	3	15 June
	4	17 July
	5	24 July
	6	7 August

Table 2.2. Sampling Events and Dates for Water and Sediment Samples in Agricultural and Non-Agricultural Catchments in Virginia, 2019-2020

Year	**Sampling Event**	**Date (Catchment Type)**
2019	1	5 May (Ag)
	2	20 May (Ag)
	3	27 May (Ag)
		28 May (non-Ag)
	4	4 June (Ag)
		5 June (non-Ag)
	5	18 June (Ag)
	6	6 August (Ref)
	7	20 August (Ag)
		20 August (non-Ag)
2020[a]	1	5 May (Ag)
	2	1 June (Ag)
	3	15 June (Ag)
	4	17 July (Ag)

[a] The non-agricultural catchment was sampled in 2020, but samples were not processed due to low frequency of residue detection

Table 2.3. Two-Way Analysis of Variance with Repeated Measures of Clothianidin Residue in Virginia Corn Field Soil by Sample Week and Treatment

Sample	**Year**	**Compound**	**Factor**	**df**	**F**	***p***
Soil	2018	Clothianidin	Sample week	5	0.780	0.1780
			Treatment	2	2.041	0.1590

Table 2.4. Mean Clothianidin Residues in Soil by Sample Week and Treatment in Virginia, 2018

Sample	**Year**	**Compound**	**Factor**		**Mean (ppb)**	**SD**
Soil	2018	Clothianidin	Sample Week:	Pre	5.785	1.841
				Post	8.867	9.353
				1 wk	3.487	3.700
				3 wk	3.979	1.957
				6 wk	5.215	5.776
				12 wk	6.194	3.221
			Treatment:	NST+	6.888	6.906
				NST-	6.002	2.839
				Control	0.572	0.334

Table 2.5. Two-Way Analysis of Variance with Repeated Measures of Clothianidin Residues in Soil by Sampling Event and Type in Virginia, 2019-2020

Year	**Factor**	**df**	**F**	***p***
2019	Sampling Event	4	8.179	**<0.001**
	Type	1	0.014	0.9060
	Sampling Event × Type	4	0.695	0.6010
2020	Sampling Event	5	0.666	0.6510
	Type	1	6.132	**0.0160**
	Sampling Event × Type	5	0.940	0.4620

Table 2.6. Mean Clothianidin Residue by Sampling Event and Type for Field Edge and Riparian Buffer Soil Samples for Virginia, 2019-2020

Year	**Compound**	**Factor**		**Mean (ppb)**[a]	**SD**
2019	Clothianidin	Sampling Event	1	2.948 b	3.002
			2	0.567 a	1.226
			3	0.114 a	0.136
			4	0.462 a	0.781
			5	0.026 a	0.065
		Type	Edge	1.214	2.531
			Forest Buffer	0.967	1.576
2020	Clothianidin	Sampling Event	1	0.107	0.179
			2	0.124	0.182
			3	0.292	0.607
			4	0.076	0.263
			5	0.288	0.653
			6	0.076	0.108
		Type	Edge	0.263 a	0.513
			Forest Buffer	0.050 b	0.124

[a] Values not connected by the same letter are significantly different ($\alpha < 0.05$)

Table 2.7. Two-Way Analysis of Variance with Repeated Measures of Neonicotinoid Residues by Sample Week and Treatment in Indiana, 2018-2020

Sample	**Year**	**Compound**	**Factor**	**df**	**F**	***p***
Soil	2018	Clothianidin	Sample week	5	3.807	**0.0020**
			Treatment	4	36.954	**>0.001**
		Thiamethoxam	Sample week	5	13.495	**>0.001**
			Treatment	4	3.682	**>0.001**
	2019	Clothianidin	Sample week	5	5.256	**>0.001**
			Treatment	4	49.247	**>0.001**
		Thiamethoxam	Sample week	5	8.577	**>0.001**
			Treatment	4	3.390	**0.0100**
	2020	Clothianidin	Sample week	5	14.558	**>0.001**
			Treatment	4	33.954	**>0.001**
		Thiamethoxam	Sample week	5	5.476	**>0.001**
			Treatment	4	1.648	0.1620
Water	2018	Clothianidin	Sample week	5	1.035	0.2420
			Treatment	2	0.866	0.4330
		Thiamethoxam	Sample week	5	0.8730	0.5140
			Treatment	2	0.7710	0.4740
	2019	Clothianidin	Sample week	5	1.264	0.3130
			Treatment	2	1.072	0.3590
		Thiamethoxam	Sample week	5	1.266	0.3120
			Treatment	2	1.112	0.3460
	2020	Clothianidin	Sample week	5	0.935	0.4740
			Treatment	2	0.770	0.4720
		Thiamethoxam	Sample week	5	0.899	0.4950
			Treatment	2	0.746	0.4840

Table 2.8. Mean Neonicotinoid Residues in Soil by Sample Week and Treatment in Indiana, 2018-2020

Year	**Compound**	**Factor**		**Mean Conc. (ppb)**[a]	**SD**
2018	Clothianidin	Sample Week:	Pre	4.4.947 *b*	3.663
			Post	5.612 *ab*	3.813
			1 wk	4.398 *b*	1.990
			3 wk	6.736 *a*	3.775
			6 wk	6.688 *ab*	5.076
			12 wk	6.559 *ab*	5.788
		Treatment:	Bt-	4.306 *c*	1.784
			Bt+	7.037 *a*	4.693
			Refuge+	8.587 *a*	5.801
			Refuge-	4.973 *c*	3.178
			Control	0.521 *b*	0.597
	Thiamethoxam	Sample Week:	Pre	0.516 *d*	0.566
			Post	0.229 *bc*	0.171
			1 wk	0.513 *cd*	1.230
			3 wk	0.207 *bc*	0.144
			6 wk	0.129 *ab*	0.101
			12 wk	0.079 *a*	0.074
		Treatment:	Bt	0.194 *ab*	0.192
			Bt+	0.398 *a*	1.049
			Refuge+	0.309 *a*	0.409
			Refuge-	0.223 *ab*	0.184
			Control	0.000 *b*	0.000
2019	Clothianidin	Sample Week:	Pre	6.349 *a*	4.554
			Post	7.964 *b*	4.453
			1 wk	5.673 *a*	3.179
			3 wk	6.227 *a*	4.928
			6 wk	5.625 *a*	4.098
			12 wk	5.345 *a*	4.200
		Treatment:	Bt	4.647 *c*	2.667
			Bt+	9.128 *a*	5.191
			Refuge+	8.173 *a*	5.294
			Refuge-	5.365 *c*	2.887
			Control	0.401 *b*	0.174
	Thiamethoxam	Sample Week:	Pre	0.201 *bc*	0.226
			Post	0.197 *abc*	0.128
			1 wk	0.481 *c*	1.074
			3 wk	0.072 *a*	0.053
			6 wk	0.077 *ab*	0.071
			12 wk	0.115 *ab*	0.167
		Treatment:	Bt	0.248 *ab*	0.755
			Bt+	0.168 *ab*	0.206
			Refuge+	0.093 *a*	0.080
			Refuge-	0.216 *b*	0.301

			Control	0.000 *a*	0.000
2020	Clothianidin	Sample Week:	Pre	8.194 *cd*	5.245
			Post	8.313 *c*	5.040
			1 wk	5.863 *bd*	2.884
			3 wk	5.792 *ab*	3.813
			6 wk	5.202 *ab*	3.510
			12 wk	4.474 *a*	2.769
		Treatment:	Bt	5.749 *bd*	4.168
			Bt+	8.959 *a*	3.589
			Refuge+	6.103 *b*	3.664
			Refuge-	4.910 *d*	3.805
			Control	1.900 *c*	1.905
	Thiamethoxam	Sample Week:	Pre	0.274 *b*	1.178
			Post	0.095 *ab*	0.094
			1 wk	0.052 *a*	0.036
			3 wk	0.038 *a*	0.033
			6 wk	0.033 *a*	0.038
			12 wk	0.021 *a*	0.028
		Treatment:	Bt	0.169	0.915
			Bt+	0.045	0.067
			Refuge+	0.046	0.042
			Refuge-	0.055	0.063
			Control	0.031	0.027

[a] Values not connected by the same letter are significantly different ($\alpha < 0.05$)

Table 2.9. Mean Neonicotinoid Residues in Water by Sample Week and Treatment in Indiana, 2018-2020

Year	**Compound**	**Factor**		**Mean Conc. (ppb)**	**SD**
2018	Clothianidin	Sample Week:	Pre	5.914	13.476
			1 wk	1.184	1.602
			3 wk	0.262	0.154
			6 wk	0.497	0.840
			12 wk	0.095	0.075
		Treatment:	Treated Ag	0.204	0.149
			Untreated Ag	0.535	0.661
			Control	3.211	9.272
	Thiamethoxam	Sample Week:	Pre	0.890	2.215
			1 wk	0.039	0.043
			3 wk	0.030	0.061
			6 wk	0.005	0.005
			12 wk	0.003	0.004
		Treatment:	Treated Ag	0.018	0.042
			Untreated Ag	0.016	0.023
			Control	0.431	1.517
2019	Clothianidin	Sample Week:	Pre	0.087	0.104
			1 wk	0.073	0.050
			3 wk	0.211	0.286
			6 wk	3.183	3.518
			12 wk	0.523	0.494
		Treatment:	Treated Ag	0.811	1.849
			Control Creek	0.938	2.195
			Control Pond	0.198	0.176
	Thiamethoxam	Sample Week:	Pre	0.005	0.005
			1 wk	0.005	0.007
			3 wk	0.019	0.044
			6 wk	0.002	0.002
			12 wk	0.008	0.009
		Treatment:	Treated Ag	0.007	0.006
			Control Creek	0.005	0.007
			Control Pond	0.021	0.052
2020	Clothianidin	Sample Week:	Pre	0.619	0.830
			1 wk	1.654	3.073
			3 wk	0.694	0.958
			6 wk	0.074	0.106
			12 wk	0.106	0.160
		Treatment:	Treated Ag	0.677	0.877
			Control Creek	0.082	0.096
			Control Pond	1.162	2.240
	Thiamethoxam	Sample Week:	Pre	0.013	0.009
			1 wk	0.413	0.984

	3 wk	0.088	0.237
	6 wk	0.003	0.003
	12 wk	0.003	0.004
Treatment:	Treated Ag	0.080	0.239
	Control Creek	0.006	0.006
	Control Pond	0.219	0.694

Conclusion

This work aimed to characterize the costs and benefits of NST use within the context of *Bt*-IRM, crop protection benefits, and neonicotinoid environmental fate. The preceding chapters outlined experiments which: 1) compared the performance of the *Bt*-Insect Resistance Management "refuge" strategy between fields with and without NSTs; 2) compared the relative crop protection benefits of *Bt* varieties and NSTs used alone and in combination; 3) compared neonicotinoid residues in soil and water in maize fields planted with and without NSTs; and 4) quantified neonicotinoid residues in off-site soil, water, and stream sediment in a stream adjacent to agricultural fields.

We present evidence suggesting that NSTs could interfere with the *Bt* refuge strategy for WCR, however our primary takeaway is that increasing refuge sizes would benefit WCR-IRM far more than removing NSTs. Our work shows that even in the absence of seed treatment, IRM refuges sizes are likely too small to facilitate *Bt* trait preservation by way of WCR refuge beetle production.

Results from our crop protection objectives show that NSTs were not necessary for protecting grain yields in any trial in Indiana, while they may have provided a slight benefit in Virginia. Our study emphasizes the impracticality of universal management plans for managing soil pests of maize across regions. We therefore recommend that NSTs be incorporated into an IPM framework that accounts for the likelihood of yield loss caused by soil pests in different production regions. Nation-wide monitoring for soil insect pests would useful in developing such a framework.

Results from corn field and offsite soils, sediment, and water sampling demonstrate that neonicotinoid residues are consistently detectable in both agricultural and nearby non-agricultural soils, as well as in waterways adjacent to fields in Indiana. In both states, we detected clothianidin residues in soil samples from treated and untreated plots consistently throughout the growing season, including prior to planting. In Virginia, clothianidin residues were detected in field edge soil (57%) and forest buffer soil (34.5%), providing further evidence for offsite movement of neonicotinoids in soil matrices. In Virginia, we did not detect clothianidin residues in water samples. This may be evidence of the effectiveness of riparian forest buffers at limiting insecticide inputs from agricultural fields into surface waters, but future research at our study sites would be needed to confirm this. Our only detections in sediments occurred in a stream catchment within which no obvious sources of neonicotinoid inputs could be identified, indicating sources of neonicotinoid inputs upstream. To our knowledge, this work was the first to compare residues in soil and water in two different farm resource regions: Indiana, being part of the U.S. Corn Belt where the majority of corn is produced, and southwestern Virginia, where corn is grown on a smaller scale, often with less frequent rotation to other crops.