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**Evaluating the Non-Target Impacts of Neonicotinoid Seed Treatments in a Three-Year
Corn, Soybean, and Wheat Crop Rotation**

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Background

Insecticide seed treatments are a convenient and economical way to protect a wide variety of crops from insect pests. Cruiser® 5FS (thiamethoxam, Syngenta Crop Protection) and Gaucho 600 Flowable (imidacloprid, Bayer CropScience) are neonicotinoid insecticides that are registered for use as seed treatments on wheat, corn, and soybeans, with application rates varying by the crop and pest targeted. When applied as seed treatments, germinating plants absorb a portion of the active ingredient, but most of the chemical remains in the soil. Studies have shown that neonicotinoid seed treatments have non-target effects on both below and above ground arthropod communities. They may affect earthworms, soil microbial communities and beneficial arthropods, including predators, parasitoids, and pollinators. The chemicals may also be taken up by other plants in the fields, such as winter flowering annuals, which could result in residue exposure to insect pollinators. If used in consecutive crops in the same field, residues from neonicotinoid seed treatments can accumulate in the soil, resulting in higher concentrations than use from a single year alone. In this study, we investigated the repeated use of thiamethoxam and imidacloprid seed treatments in a commonly practiced mid-Atlantic three-year grain crop rotation; specifically, soybean, followed by fall-planted wheat, double-cropped soybean and corn.

Our specific objectives in this study were to: 1) determine the impacts of neonicotinoid seed treatments upon both beneficial and pestiferous foliar and soil organisms, including soil microorganisms; 2) measure how seed treatments impact grain growth and yield; and 3) evaluate whether winter annual flowers within a grain field planted with treated seeds contain neonicotinoid residues. This study is unique in its approach because we evaluated the effects of repeated use of neonicotinoid seed treatments within a common mid-Atlantic grain crop rotation and considered impacts on both arthropods and soil microbes. Overall, this study evaluated whether the seed treatments result in a significant increase in yield in the mid-Atlantic region, and whether use of these treatments impacts beneficial invertebrates and soil health. Results of the study will help producers make informed long-term management decisions regarding seed and seedling protection.

Procedures

The experiment was replicated at two sites, the Wye Research and Education Center (WREC, Wye) in Queenstown, MD, and at the Central Maryland Research and Education Center (BREC, Beltsville) in Beltsville, MD. Four treatments were planted at each site: untreated seeds, fungicide only, fungicide + Gaucho 600 Flowable (imidacloprid), and fungicide + Cruiser® 5FS (thiamethoxam) treated seeds for each crop. At both sites, each treatment was replicated 4 times and treatments were planted in a Latin square design, with each plot measuring 30ft by 50ft (16 total plots).

Before planting, baseline soil measurements were taken for each plot. Measurements included soil compaction, which was measured using a penetrometer, and soil tests for wet aggregate stability, texture, percent soluble salts, soil pH and carbon, hydrogen, and nitrogen content. These measurements were repeated in fall 2017 at the end of this three-year study, to measure and compare the cumulative effects of the crop rotation for each of our seed

treatments. Soil compaction was not measured because soil moisture significantly impacts penetration force, and soil moisture is low in the fall.

2015 Soybean Rotation: Commercially treated soybeans (Variety 93Y8F, Dupont Pioneer) were used at both sites. At Beltsville, soybeans were planted at a 7-inch row spacing with a drill calibrated to 155,000 plants per acre on May 14th into a field that was previously used to grow soybeans. Soybean first emerged around May 26th and were harvested on October 22nd. At Queenstown, soybeans were planted at a 7.5-inch row spacing with a drill calibrated to 150,000 plants per acre into a field that was previously corn on May 26th. First emergence occurred around June 6th, and soybeans were harvested on October 22nd.

Soil samples were taken before planting, one week after soybean emergence (VC-V2), six weeks after planting (V5) and twelve weeks after planting (R3). Solvita tests (Woodbridge laboratories) were used to measure soil respiration immediately after soil collection, and a subsample of the remaining soil was placed in a deep freeze (-80°C) for future analysis of the nitrogen-fixing soil microbial community using quantitative PCR methods and next generation Illumina sequencing. In addition to soil sampling, above ground arthropod abundance was also measured by visually scouting trifoliate early in the season (VC-V2), sweep nets later in the season (V5) and with sticky cards. The epigeal (near the ground surface) invertebrate community was also sampled using pitfall traps and leaf litter extractions. Sticky cards, pitfall traps and litter samples were used at the VC-V2, V5 and R3 stages. Other measurements taken include plant growth, plant germination, and final yield at soybean harvest.

2015/2016 Winter Wheat: Wheat was planted on October 26 at Beltsville and October 27 at Queenstown, with 7-inch (17.8 cm) and 7.5-inch (19.05 cm) row spacings, respectively. The drill was set to a seeding rate of 1.75 million seeds/acre at both sites. The same seed treatments were planted in the same plots used for the soybean rotation, using winter wheat seeds (variety MBX14K297, Mercer) that we treated in a cement mixer. Stand density was measured at emergence and one-week post-emergence, and plant height was measured six weeks post planting. Normalised Difference Vegetation Index (NDVI) was measured twice in the fall and once in the spring (Feekes stages 1, 2 and 5-6) using a Crop Circle optical sensor as another measure of plant growth. Plants were dug up to count tillers in the fall and again in the late spring after wheat had completed tillering (Feekes stages 2 and 5-6). Visual counts for insect pests; primarily aphids and cereal leaf beetle were conducted twice in the fall (Feekes stages 2), and three times in the spring (Feekes stages 5-6, 9-10 and 11).

We continued sampling in the spring after the wheat had completed winter dormancy. At Feekes stages 4, 5-6 and 9-10, soil cores were collected from each plot and used to conduct Solvita respiration tests; a portion of each soil sample was stored at -80°C for later microbial analysis. Additional arthropod sampling was conducted at Feekes stages 6, 10 and 11, through sticky cards, pitfall traps and litter extractions.

In the early spring, we collected buds of winter annual plants that are attractive to pollinators and that grew within the plots. We collected flowers from common henbit (*Lamium amplexicaule*) at Beltsville and from common chickweed (*Stellaria media*) at the Wye. Flowers were collected at both sites on March 18 and were analysed at the USDA Agriculture Marketing Service National Laboratory for imidacloprid, thiamethoxam and clothianidin, a breakdown product of thiamethoxam that is also a registered neonicotinoid insecticide.

Wheat harvest occurred on June 28 at Queenstown and June 30 at Beltsville; yield, moisture and test weight were recorded.

2016 Double-Cropped Soybean: Commercially treated soybean (variety P39T67R, Pioneer) was planted on July 7 at Queenstown with a 7.5-inch row spacing and a seeding rate of 123,000 seeds per acre and July 8 at Beltsville with a 7-inch row spacing and a seeding rate of 200,000 seeds per acre. At emergence (VE Stage), stand count was measured and growth stage was recorded for forty plants per plot. Pitfall traps and sticky cards were set up to sample epigeal and foliar arthropods respectively. Stand counts and growth stage measurements were repeated the following week (VC-V2 stage); plant height was measured at the R1 stage. Foliar insects were visually sampled by examining trifoliates from randomly selected plants at the VC-V2 and R1 stages, and by sweep netting at the R3 stage. Litter was collected, and pitfall traps and sticky cards were deployed at the VC-V2, R1 and R3 stages. Soil was also collected for Solvita respiration tests and stored for qPCR and Illumina sequencing at these stages. Soybean was harvested at both sites on November 2, and yield, moisture and test weight were recorded.

After soybean was harvested, winter annual buds were collected in the spring. We collected flowers from common henbit at the Wye, and common henbit and common chickweed at the Beltsville. Flowers were analysed at the USDA Agriculture Marketing Service National Laboratory for imidacloprid, thiamethoxam and clothianidin.

2017 Corn: Commercially treated field corn was planted on May 4 at Beltsville and May 8 at Queenstown, using TA506-22DPR1b seeds. Corn was planted with a 30-inch row spacing at both sites, with a planting rate of 30,000 seeds per acre at Beltsville and 33,000 seeds per acre at Queenstown. Stand counts were measured shortly after emergence (V3-V4), and plant height was measured at V3-V4 and V7. At the V4 stage, damage by soil pests was assessed by counting the number of plants that showed signs of wireworm, white grub or cutworm damage in four rows per plot. At V7, four sets of five plants were scouted visually for pests and beneficials per plot. At V10-V12, ten plants per plot were scouted destructively, and at V3-V4, a single ear from ten plants was examined for arthropods. Pitfall traps and sticky cards were deployed once before planting, along with litter extraction, and again at the V3-V4, V10-V12 and R3-R4 stages. Soil for Solvita and qPCR was also collected at these times. Corn was harvested on September 27 at Queenstown and on October 5 at Beltsville. The sampling timeline for corn can be seen in **Table 1**.

Table 1: Timeline for 2017 field corn

Sampling type	1-week pre planting	1-week post emergence (V3-V4)	4 weeks post planting (V7)	6 weeks post planting (V10-V12)	8 weeks post planting (R1)	10-12 weeks post planting (R3-R4)
Stand density		x				
Plant height		x	x			
Visual insect counts			x		x	x
Soil pests		x				
Sticky cards	x	x		x		x
Pitfall Traps	x	x		x		x
Litter samples	x	x		x		x
Soil samples	x	x		x		x

Statistical Analysis: Analysis of variance was used to test for differences in stand count, yield, plant height, and insect abundance, using the Fit Model platform of JMP 13.1.0 (SAS)

Institute Inc., Cary, NC). Data from both sites was combined for all analyses. The assumption of normality was tested using a Shapiro-Wilk test, and data was transformed as necessary. The assumption of homoscedasticity was tested using Levene's test, and weighted least squares methods (Weighting factor: (residual variance)⁻¹ of the fixed effect that most deviated from homoscedasticity) were used when needed. When effect differences were statistically significant ($P < 0.05$), Tukey's HSD means comparisons were used to compare treatment effects.

Results

Foliar Insect Abundance

2015 Full Season Soybean: Because the overall number of arthropods was very low, insects were combined into plant feeders and beneficials for analysis. Plant feeding insects included flea beetles, leafhoppers, plant-feeding thrips, caterpillars, weevils and aphids, and were significantly less abundant in the plots treated with Cruiser as compared to the control and fungicide treatments; plots treated Gaucho had significantly lower insects than fungicide, but not control plots (Treatment $F_{3,15.89} = 9.9742$, $P = 0.0034$). Beneficial insects included minute pirate, predatory, and lacewings, and Cruiser plots had significantly lower numbers of beneficial insects compared to fungicide treated or control plots (Treatment $F_{3,18.66} = 3.8676$, $P = 0.0261$).

2015-2016 Winter Wheat: Insect abundance was measured through visual inspection twice in the winter and three times in the spring. The two winter dates were combined for analysis, and the three spring dates were combined for analysis separately. In the winter, only aphids were present; in the spring, aphids and cereal leaf beetle were both observed, and their numbers were combined for analysis. The only beneficials that were present were parasitized aphid mummies; however, their numbers were too low for analysis. In the winter, the number of aphids was higher on the second sampling date (Date $F_{1,43} = 4.6846$, $P = 0.0360$). Aphid abundance was significantly lower in the Cruiser and Gaucho treatments as compared to the control and fungicide treatments (Treatment $F_{3,43} = 12.3011$, $P < 0.0001$) (**Figure 1**).

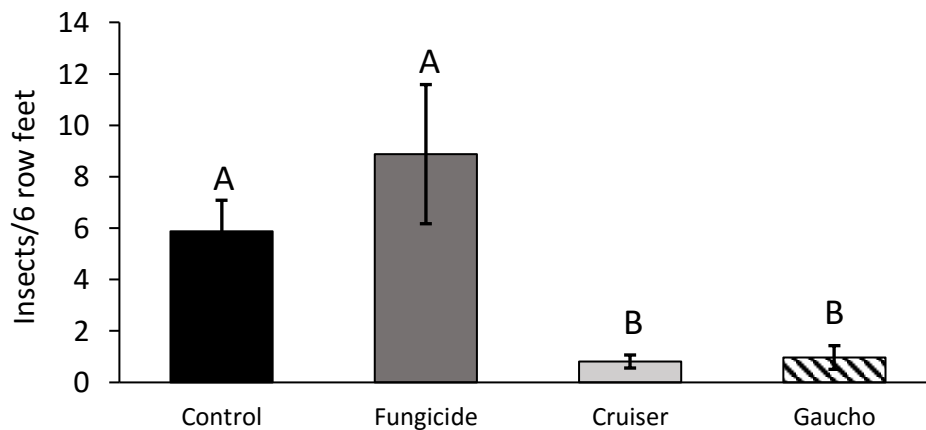


Figure 1: Mean number of aphids per 1.8 row meters in the winter. The mean number of aphids was lower in the Cruiser and Gaucho treatments than in the control and fungicide treatments (Treatment $F_{3,43} = 12.3011$, $P < 0.0001$). Significant differences indicated by letters; N.S. = no significance; error bars indicate standard error.

In the spring, the number of plant-feeding insects (aphids and cereal leaf beetle) was significantly lower on the third sampling date compared to the first and second (Date $F_{2,71} = 47.7823$, $P < 0.0001$). There was no significant difference between the control, Cruiser

and Gaucho treatments, although the fungicide only treatment had a significantly higher number of aphids than the Cruiser treatment (Treatment $F_{3,71}=2.9207$, $P=0.0399$).

2016 Double Cropped Soybean: Foliar insects were visually assessed twice, at the V2 and R1 stages, by evaluating the mean number of insects per 10 trifoliates. The most prevalent plant feeding insects were leafhoppers, plant-feeding thrips and caterpillars, and a smaller number of weevils, aphids and spotted cucumber beetles. While there was a significantly higher number of plant feeding insects present on the second date, (Date $F_{1,43}=37.9665$, $P<0.0001$), plant feeding insects did not differ significantly between treatments (Treatment $F_{3,43}=2.4211$, $P=0.0790$). Beneficial insects included minute pirate bugs, predatory thrips, and lady beetles, which were present in higher numbers on the second sampling date than the first (Date $F_{1,43}=19.4815$, $P<0.0001$). However, beneficial insects did not differ significantly between treatments (Treatment $F_{3,43}=2.3794$, $P=0.0829$).

2017 Corn: At the V4 stage, we counted the number of plants showing signs of soil pest damage, which did not differ significantly between treatments (Treatment $F_{3,15}=1.0504$, $P=0.3992$). Visual counts for insects on plant were conducted three times. Because the sampling method was different each time, data from the three dates were analyzed separately. The first set of samples were taken at the V7 stage. The most abundant plant feeding insects were plant thrips, followed by leafhoppers, aphids and flea beetles. Plant feeding insects were not significantly impacted by treatment (Treatment $F_{3,15}=0.2970$, $P=0.8270$) (**Figure 2**).

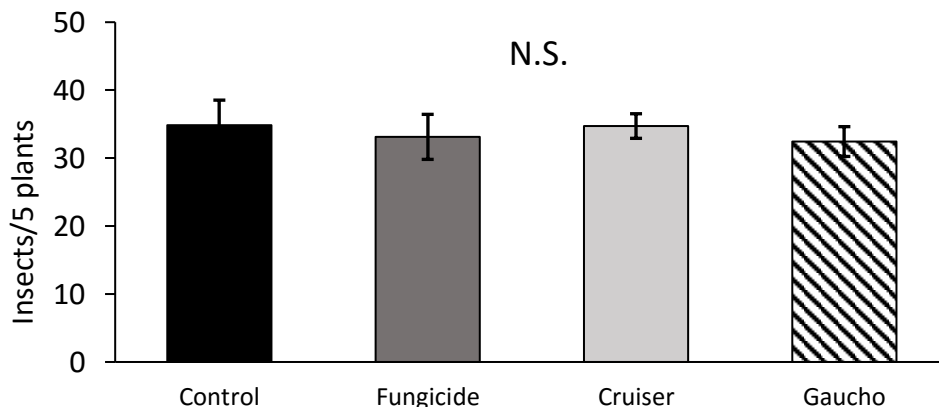


Figure 2: Plant feeding insects in corn during the V7 stage. Plant feeding insects were not significantly impacted by treatment (Treatment $F_{3,15}=0.2970$, $P=0.8270$). Significant differences indicated by letters; N.S.= no significance; error bars

The only beneficial arthropods observed were lady beetles and spiders. Beneficial arthropods also did not differ significantly between treatments (Treatment $F_{3,15}=0.3360$, $P=0.7796$) (**Figure 3**)

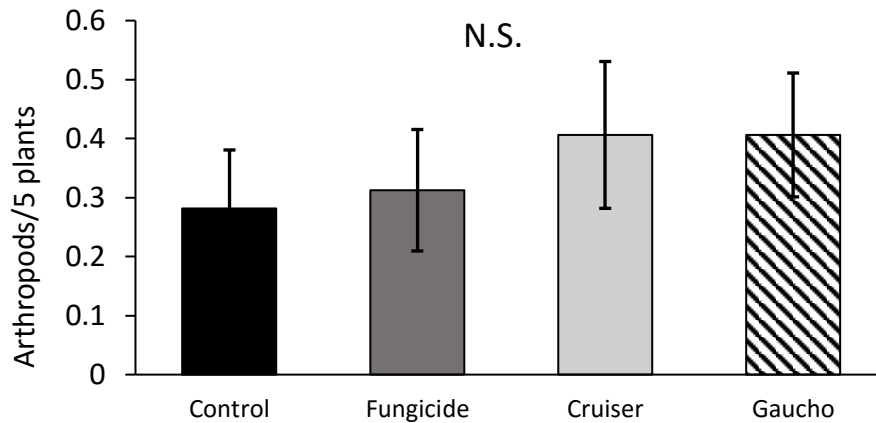


Figure 3: Beneficial arthropods in corn during the V7 stage Beneficial arthropods did not differ significantly between treatments (Treatment $F_{3,15}=0.3360$, $P=0.7796$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

At the R1 stage, we counted the number of arthropods per 10 plants through destructive sampling. Primary pests observed were plant hopper and plant bugs, which did not differ significantly between treatments (Treatment $F_{3,15}=0.2972$, $P=0.8268$) (Figure 4).

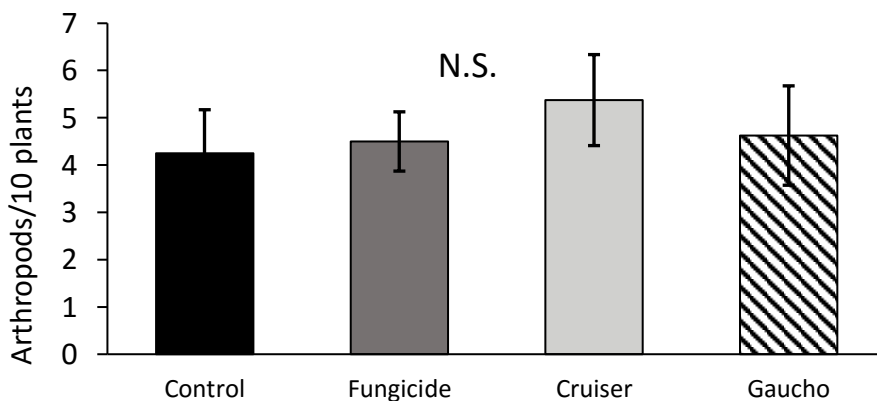


Figure 4: Pests in corn during the R1 stage. Pests did not differ significantly between treatments (Treatment $F_{3,15}=0.2972$, $P=0.8268$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

Predators observed at R1 stage included minute pirate bugs, spiders, lacewing eggs and lady beetles. Predators were not significantly different between treatments (Treatment $F_{3,15}=2.7294$, $P=0.0807$) (Figure 5).

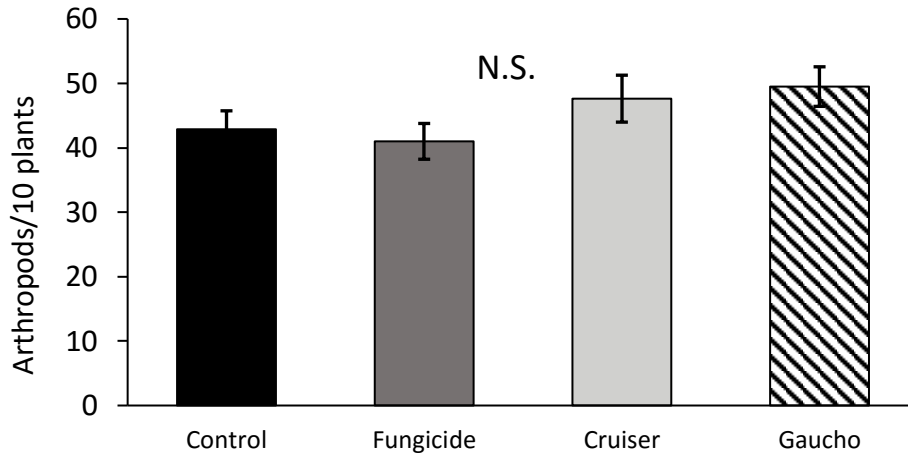


Figure 5: Predators in corn during the R1 stage. Predators did not differ significantly between treatments (Treatment $F_{3,15}=2.7294$, $P=0.0807$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

At the R3-R4 stage, pests and predators were scouted visually for one ear each from ten plants per plot. The pests observed were plant thrips, sap beetles, dirt-colored seed bugs and corn earworm. The number of pests was significantly higher in the Cruiser treatment than the fungicide treatment, but did not differ significantly between the other treatments (Treatment $F_{3,15}=3.3996$, $P=0.0455$) (Figure 6).

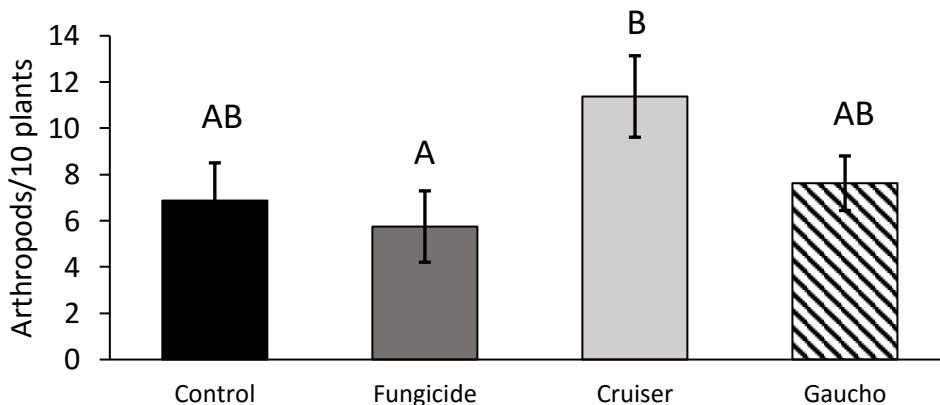


Figure 6: Pests in corn during the R3-R4 stage. Pests were significantly higher in the Cruiser treatment compared to the fungicide only treatment (Treatment $F_{3,15}=3.3996$, $P=0.0455$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

The predatory arthropods observed at the R3-R4 stage were minute pirate bugs, lacewing eggs, lady beetles and spiders. Predators were not significantly different between treatments (Treatment $F_{3,15}=0.3295$, $P=0.8041$) (Figure 7).

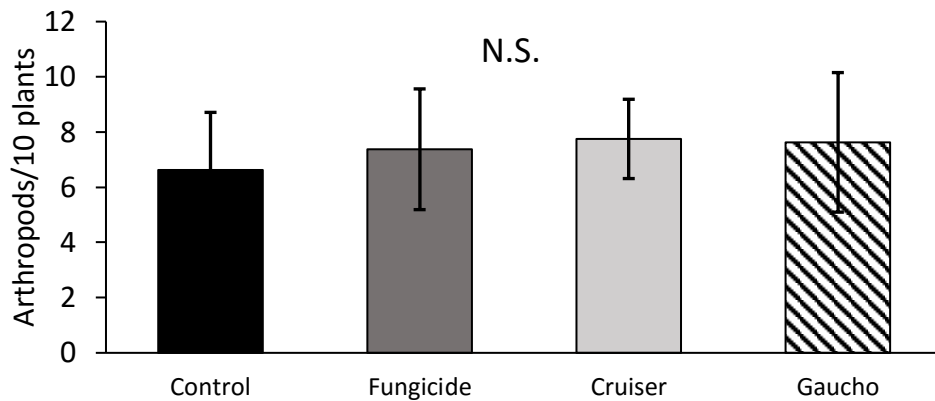


Figure 7: Predators in corn during the R3-R4 stage. Predators did not differ significantly between treatments (Treatment $F_{3,15}=0.3295$, $P=0.8041$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

2015 Full Season Soybean: Initial soybean stand counts showed that plots with the Gaucho treatment had a significantly higher stand count compared to Cruiser, fungicide and control plots (Treatment $F_{3,15}=15.1055$, $P<0.0001$). Plant height measurements revealed no significant difference between seed treatments (Treatment $F_{3,15}=0.7884$, $P=0.5190$). Overall yield, corrected to 13% moisture, also failed to demonstrate a significant treatment effect (Treatment $F_{3,15}=0.6266$, $P=0.6089$) (**Figure 8**).

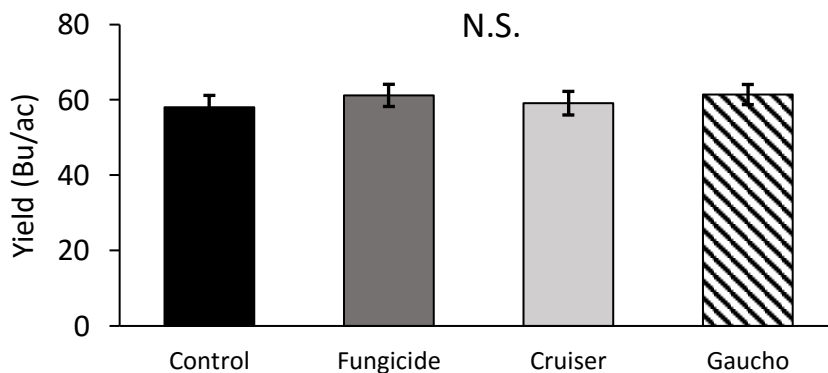


Figure 8: Mean yield of 2015 soybean, corrected to 13% moisture. There was no significant difference in yield between treatments combined across both sites (Treatment $F_{3,15}=0.6266$, $P=0.6089$). Significant differences indicated by letters; N.S.= no significance; error bars indicate standard error.

2015-2016 Winter Wheat: Stand density was measured at emergence and one-week post emergence at both field locations. While stand count was significantly higher on the second sampling date (Date $F_{1,43.85}=45.8290$, $P<0.0001$), it was not significantly impacted by treatment (Treatment $F_{3,43.85}=1.2540$, $P=0.3018$). Plant height, measured six weeks post planting, was not impacted by treatments (Treatment $F_{3,18.79}=1.0949$, $P=0.3757$). Yield, corrected to 14% moisture, was not significantly different between treatments (Treatment $F_{3,15}=2.6362$, $P=0.0877$) (**Figure 9**).

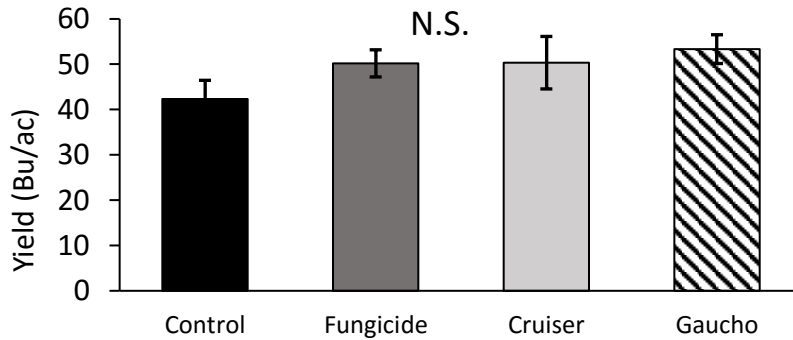


Figure 9: Mean yield of winter wheat, corrected to 14% moisture. There was no significant difference in yield between treatments combined across both sites ($F_{3,15}=2.6362$, $P=0.0877$). Significant differences indicated by letters; N.S. = no significance; error bars indicate standard error.

2016 Double cropped soybean: Stand density was measured at emergence and one-week post emergence as the number of plants per two row meters. Stand density was not significantly impacted by date (Date $F_{1,43.99}=3.3860$, $P=0.0725$) or treatment (Treatment $F_{3,43.99}=1.4873$, $P=0.2311$). Plant height was measured at the R1 growth stage. There was no significant difference in height between treatments (Treatment $F_{3,15}=1.2284$, $P=0.3340$). Yield, corrected to 13% moisture, did not differ significantly between treatments (Treatment $F_{3,15}=0.3051$, $P=0.8213$) (**Figure 10**).

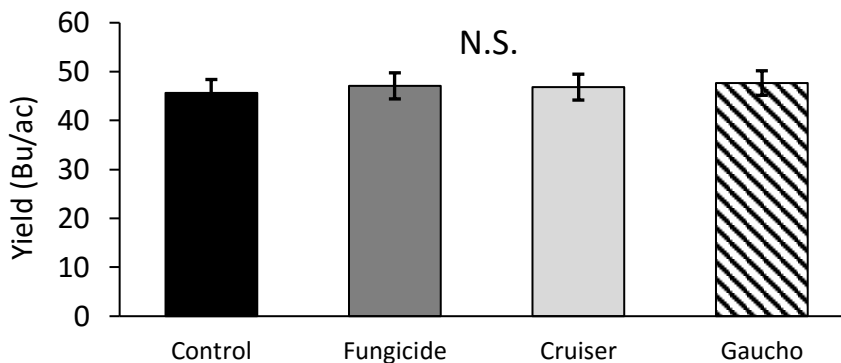


Figure 10: Mean yield of double cropped soybean, corrected to 13% moisture. There was no significant difference in yield between treatments combined across sites (Treatment $F_{3,15}=0.3051$, $P=0.8213$). Significant differences indicated by letters; N.S. = no significance; error bars indicate standard error.

2017 Corn: Stand density was measured as the number of plants per 2 row meters shortly after emergence. Gaucho and Cruiser treatments had significantly higher stand density than the control, but not the fungicide treatment (Treatment $F_{3,15}=5.9607$ $P=0.0070$) (**Figure 11**). Yield, corrected to 15.5% moisture, did not differ significantly between treatments (Treatment $F_{3,15}=0.5558$ $P=0.6522$) (**Figure 12**).

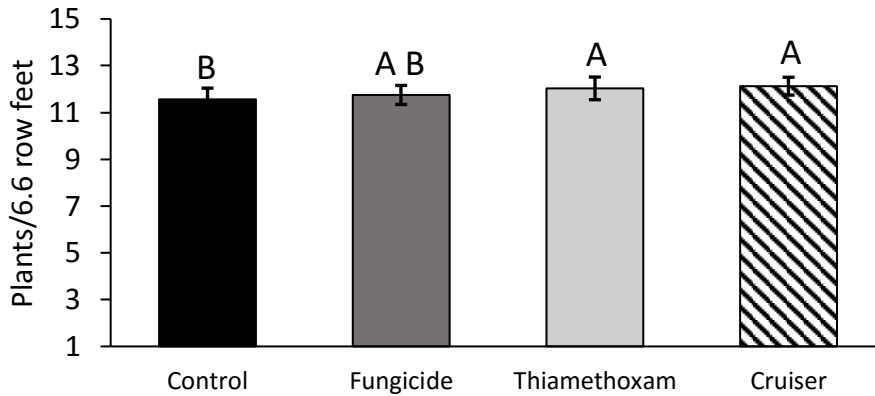


Figure 11: Mean stand density of corn per 2 row meters. Gaucho and Cruiser treatments had significantly higher stand density than the control, but not the fungicide treatment (Treatment $F_{3,15}=5.9607$ $P=0.0070$). Significant differences indicated by letters; N.S. = no significance; error bars indicate standard error.

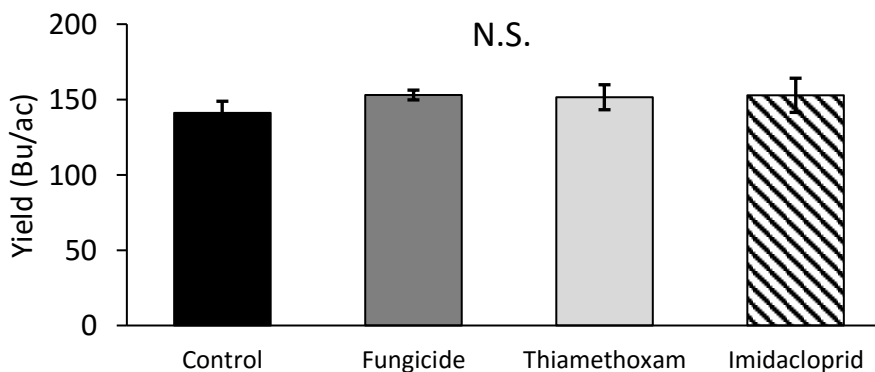


Figure 12: Mean yield of corn, corrected to 15.5% moisture. There was no significant difference in yield between treatments pooled across sites (Treatment $F_{3,15}=0.5558$, $P=0.6522$). Significant differences indicated by letters; N.S. = no significance; error bars indicate standard error.

Winter Annual Flowers

2016: Neonicotinoid residues were not found in any of the samples from 2016.

2017: No neonicotinoid residues were found in the chickweed samples from Queenstown or the henbit samples from Beltsville. Trace amounts (unquantifiably low amounts reported as “trace” by the testing lab) of imidacloprid were found in four of the chickweed samples from Beltsville. Given that the chemical was present in plots of all four treatments in very low concentrations, its presence does not appear to be correlated to the experiment.

Arthropod Community Composition

Insect collected through pitfall traps, litter extraction, sticky cards and sweep netting are being identified and analysed. Results will be presented to the board when analysed.

Soil Quality and Microbial Analyses

Samples collected after corn harvest will be sent to the Cornell Nutrient Analysis Lab for measurement of soil quality parameters (pH, wet aggregate stability, etc.). DNA is being extracted from soil samples that were stored for microbial analysis. After DNA extraction is completed, the abundance and community composition of soil microbes will be evaluated

using quantitative PCR and Illumina sequencing. Results will be presented to the board when analysed.

Conclusions

Visual insect sampling and yield – The use of NSTs reduced early season pest abundance in full season soybean and wheat, but not in double cropped soybean or corn. NSTs also reduced beneficial insects in full season soybean. Stand count and plant height measurements indicate that the use of NSTs did not improve plant growth through pest protection or direct stimulation in most cases. Pest abundance was low throughout the study; in the absence of serious pest pressure, the neonicotinoid seed treatments did not improve yield in any of the crops.

Winter annual flowers – In this case, neonicotinoid residues from NSTs were not taken up by winter annual flowers and did not pose a threat to pollinators. However, persistence of neonicotinoids in soil and uptake by flowering plants could vary based on factors such as temperature, soil type and flowering plant species.

A final report will be submitted after results from arthropod sampling, soil quality measurements and soil microbial analyses are completed. This will provide more insight into the non-target impacts of NSTs. In terms of yield and pest suppression, this study suggests that there is no economic benefit associated with using neonicotinoid treated seeds when pest pressure is low.