

University of Nevada, Reno

**Neonicotinoids: Insecticide Effects On Bacterial Communities  
Of Non-Target *Pieris rapae* Butterflies**

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Bachelor of Science in Biology and the Honors Program

by

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We recommend that the thesis  
prepared under our supervision by

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entitled

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Of Non-Target *Pieris rapae* Butterflies**

be accepted in partial fulfillment of the  
requirements for the degree of

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## Abstract

Neonicotinoids are a commonly used insecticide in agricultural and domestic settings as a form of pest management. While neonicotinoids are looked upon favorably for their specificity towards invertebrates, they also have been implicated in the declines of non-target insect species such as the European honey bee. Recent literature suggests that they may be involved in the decline of various butterfly populations as well, although research has been limited. To further contribute towards an understanding of the effects of neonicotinoids upon butterflies, we performed an experimental study examining the effects of one neonicotinoid insecticide, imidacloprid, on *Pieris rapae* caterpillar performance. Additionally, we extracted and analyzed the microbiota community from each caterpillar to ask if neonicotinoids could also disrupt or alter the bacterial community. Our results show that imidacloprid exposure does have an effect on *Pieris rapae* performance when applied as suggested; however, bacterial composition within the cabbage white gut does not appear to be affected. It is also interesting to note that the bacterial composition obtained differs considerably from a previous study examining the microbiota community of *Pieris rapae*, although different methods were used (Robinson et al. 2010). Thus, further directions for this research, in addition to neonicotinoid exposures to other *Lepidoptera* species, should also include further studies into the composition of the cabbage white bacterial community.

## Acknowledgements

I'd like to express my sincerest gratitude to the artist Beyoncé for her album "4" – combined with a four pack of red bull, her music gave me the drive and motivation to complete this thesis.

On a more serious and genuine note, I'd like to thank the handful of folks that make up the Great Basin Bug Lab, which has graciously allowed me to explore the field of ecology outside of the classroom – and sometimes inside as well. More specifically, I would like to thank Anne Espeset for providing me with the cabbage whites required for conducting these experiments, the protocols for rearing them and her assistance in doing so, and for her contributions towards the design of this project. I am also incredibly grateful for the help of Josh Harrison, who has been there since my first day in the lab to answer any and all questions I've had. He has been my main sounding board since the earliest days of this project and has provided plenty of papers to use as references in addition to performing the initial data analysis and DNA extractions.

Finally, I'd like to express a large amount of appreciation to Dr. Matthew Forister, for his incredible amount of patience and insight. The past three years working in his lab have been a blast and I'm immensely thankful that I had the opportunity to do so. He's taught me more about insects and ecology than I ever thought I would learn during my time up here in Reno and though I have no immediate plans to continue my ecological studies after graduation, the experience and knowledge I've gained under his tutelage translate into an invaluable set of skills applicable to all fields of science. His guidance and mentorship has made my time as an undergraduate much more meaningful and informative than I would have thought otherwise and I am forever grateful for it.

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## Introduction

Ecology can be broadly defined as the interactions of species with each other and with their environment. Specific and sustained interactions between two different species known as symbiosis can be mutually beneficial to each party (mutualism), beneficial to one party and of no effect to the other (commensalism), or beneficial to one and harmful to the other (parasitism); these are not all of the different types of interactions that can take place, but they are some of the most well-studied. Additionally, species can be influenced by multiple other species at the same time. These interactions, combined with the conditions of the environment, can have profound effects on the fitness of a species.

My thesis will be looking at the interactions between the common cabbage white butterfly, *Pieris rapae*, and the several species of bacteria residing within it. Gut bacteria contribute significantly to the growth and development of various insects and confer benefits to their arthropod hosts, such as protection against pathogenic foreign bacteria; in return, these bacteria have a relatively safe place in which to grow and propagate (Dillon & Dillon 2004). Specifically, I will be looking at whether or not these interactions are effected or disrupted by the application of neonicotinoids, a class of insecticides. Neonicotinoids have become widely used as preferred insecticides in recent years due to their selectivity for invertebrates; insects are strongly affected by the neurological effects of neonicotinoids, while vertebrates such as mammals and birds do not appear to be strongly effected (Tomizawa & Casida 2004). However, while vertebrate species may not be effected, non-target insect species (those not viewed as pest species) seem to be suffering drastically and may be experiencing significant declines in their populations (Gilburn et al. 2015).

In the mid-2000s, colonies of the European honey bee, *Apis mellifera*, began dying off in mass numbers; the honey bee is an important pollinator for agricultural business and is responsible for pollinating more than \$200 billion worth of crops worldwide (Fairbrother et al. 2014). More than a decade's worth of research point to neonicotinoid insecticides as being an important factor in their decline and show that non-target pollinator species are at risk of being affected by these compounds (Fairbrother et al. 2014, Blacquière et al. 2012).

The loss of key pollinator species would have major impacts on society and its ability to produce enough food to support a growing global population. Thus, research into neonicotinoids and its effects on other non-target insect species is of key importance to conservationists. Honey bees are not the only pollinators in nature, however; butterflies also contribute to the pollination of various plants and research into the effects of neonicotinoids upon their numbers is far less in comparison to the studies conducted on honey bees. To date, there is currently only one experimental study (Pecenka & Lundgren 2015) regarding the effects of neonicotinoids on butterflies – specifically, monarch butterflies (*Danaus plexippus*) – and one observational study on butterfly species in the U.K. (Gilburn et al. 2015).

Given this limited amount of information, further research into the effects of neonicotinoid exposure upon non-target Lepidoptera species is required in order to more precisely understand what effects, if any, neonicotinoids have upon butterfly performance and other areas of interest such as gut bacteria. Specifically, questions that we hope to answer with this study are whether or not neonicotinoid exposure has an effect on butterfly development, as suggested by the previous study on monarch butterflies, and whether gut bacteria composition is effected as a result of exposure to insecticide.

## Literature Review

This study will build upon the research concerning neonicotinoid insecticides and non-target butterfly species. Neonicotinoids have already been implicated as a strong contributor to the decline of the European honey bee, specifically through potential weakening of honey bees to the point where they are more susceptible to illness and cold (Fairbrother et al. 2014). Although evidence suggests that neonicotinoids may be to blame for the plight of the bees, the literature surrounding the effects of neonicotinoids on butterfly species is limited – to be more precise, research in this area consists of a single experimental study regarding monarchs (Pecenka & Lundgren 2015) and an observational study on butterfly species in the U.K. (Gilburn et al. 2015), although pest Lepidoptera (specifically certain moths) have been studied.

Pecenka & Lundgren (2015) studied the lethal and sub-lethal effects of clothianidin – a neonicotinoid – on monarch larvae and show that clothianidin exposure at realistic levels throughout their larval lives can have lethal consequences. Gilburn et al. (2015) modeled butterfly population indices from 1985 to 2012 for 17 pervasive butterfly species in the United Kingdom; their models show a negative correlation in 15 of the 17 indices with neonicotinoid usage; these negative associations with butterflies are most apparent in England, where neonicotinoid usage is highest while butterfly numbers seem to be stable in Scotland where insecticide usage is low. However, the Gilburn et al. study does not show whether or not there is a casual link between neonicotinoid usage and butterfly population decline.

This study looks to replicate the results from the Pecenka & Lundgren study using a different neonicotinoid insecticide – imidacloprid – in another butterfly species, *Pieris*

*rapae*, which is also one of the 17 species modeled by the Gilburn et al. study. In doing so, this work will seek to confirm results provided by both prior studies while answering questions that both pose for further research.

## Methodology

Cabbage white eggs were obtained from Carolina Biological Supply Co. and reared on a laboratory bench top at ambient temperature (approximately 20 degrees C). Caterpillars were exposed to one of four insecticide treatments ten days after hatching. The insecticide used was Merit 75WP (active ingredient – imidacloprid | 75%) and a whole treatment was considered to be the full suggested amount of application (18mg/500 mL water) from the bottle; caterpillars either received a whole treatment, half (9mg/500 mL water), one-tenth (1.8mg/500 mL water), or a control treatment (500 mL water).

For application of their assigned treatment, caterpillars were reared on 6 cm<sup>2</sup> leaf discs harvested from cabbage purchased from a grocery store. Leaf discs were soaked in an insecticide treatment and left to dry for 15 minutes before being flipped over and left to dry for an additional 15 minutes (for a total of 30 minutes drying time). Caterpillars were switched over to new leaf discs every two days and were reared under lights set to an 18 hour light/6 hour dark schedule.

Caterpillars from each treatment group were selected at random for frozen preservation throughout the study. To prepare for DNA extraction, samples were surface sterilized for 30 seconds in 95% EtOH followed by submergence in a 10% bleach solution and a 70% EtOH solution for 2 minutes each; samples were then rinsed with distilled water. Following surface sterilization, DNA was extracted from caterpillar samples and shipped off to the Genomic Sequencing and Analysis Facility at the University of Texas at Austin; sequencing was performed on the Illumina MiSeq platform and targeted the 16s locus.

After sequencing was completed, a one-way Analysis of Variance (ANOVA) was performed using the R statistical programming language to analyze the effects of

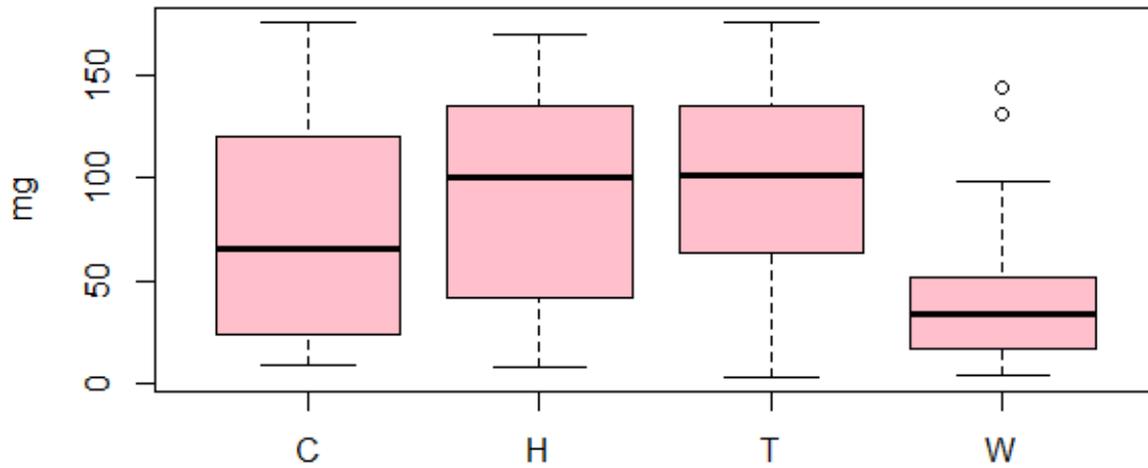
insecticide treatment on caterpillar performance; ordination plots of the bacterial communities were also produced.

## Results

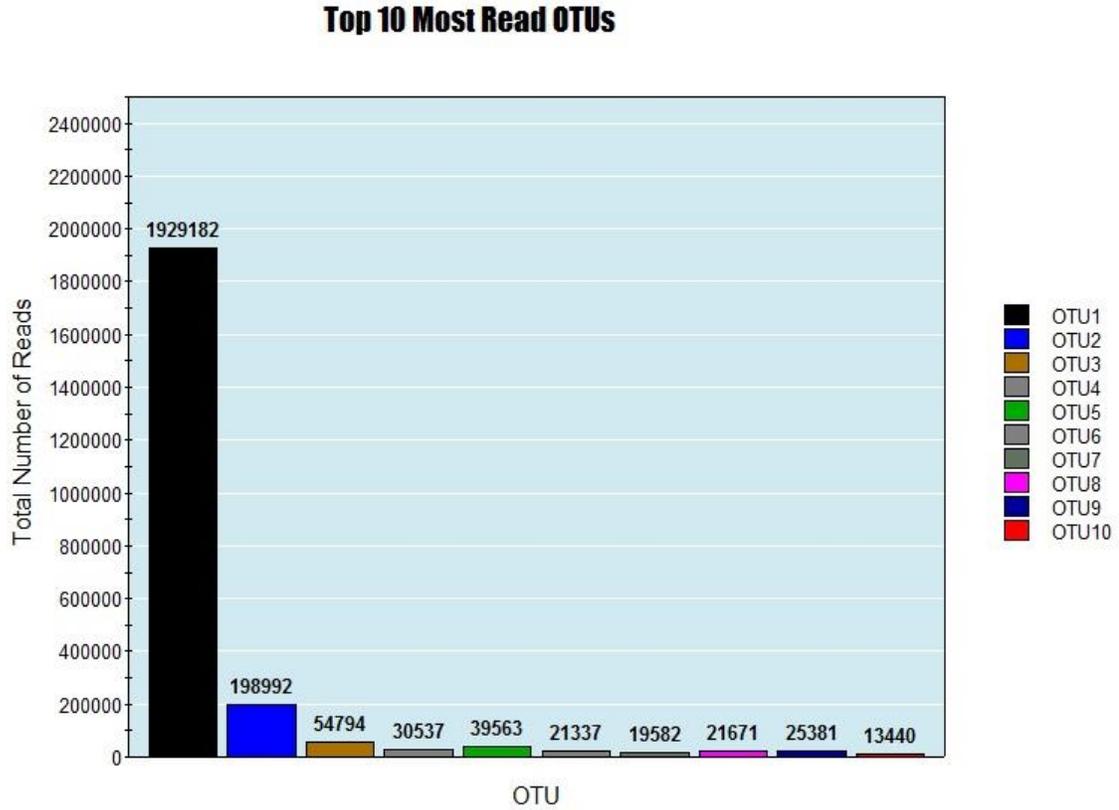
Forty-eight hours after exposure to an insecticide treatment, caterpillars were weighed as a measure of performance. Significant differences were found in the weights of caterpillars that had been treated with a whole standard dose of insecticide (one-way ANOVA,  $F = 6.29$ ,  $df = 3,105$ ,  $P < 0.00058$ ; Fig. 1) in comparison to the other three treatments.

Sequencing analysis of the DNA extracted from caterpillar guts resulted in almost 1900 operational taxonomical units (OTUs), with each OTU representing a putative, distinct bacterial species. The most common OTU, with almost 2 million reads among all caterpillars, belonged to the genus *Enterococcus* – for comparison, the 2<sup>nd</sup> most common OTU had about 200,000 reads and was a member of the genus *Klebsiella* (Fig. 2). In examining the OTUs that were most abundant, we were able to identify the most common bacterial species residing within *Pieris rapae* as those belonging to the two genera above as well as *Asaia*, *Pseudomonas*, *Propionibacterium*, *Streptophyta*, *Serratia*, and *Acinetobacter*.

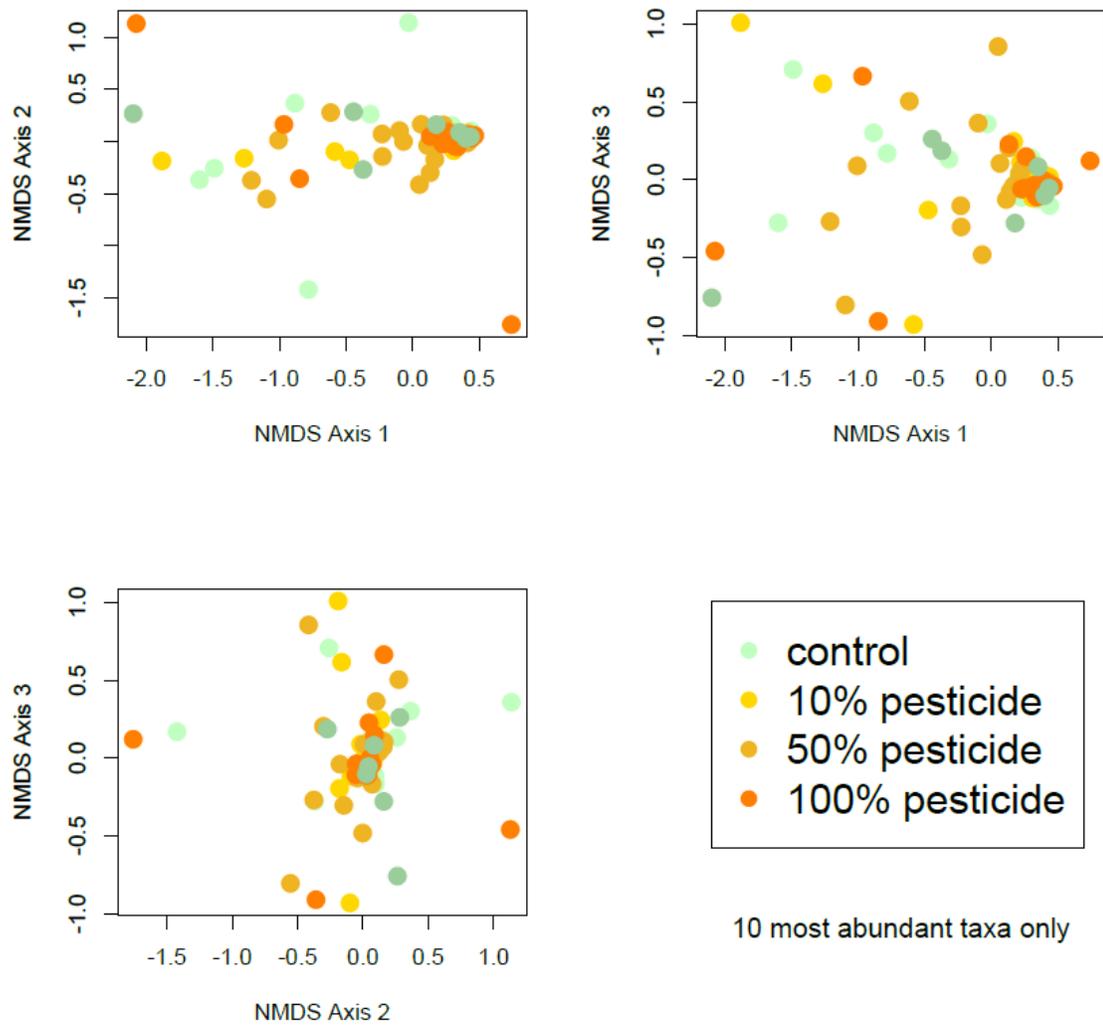
To identify whether or not bacterial gut composition was affected by insecticide treatment, NMDS plots were generated using the ten most common taxa mentioned above as well as all OTUs (Figs. 3 & 4). These plots suggest that bacterial composition did not change as a result of insecticide treatment. This is further supported by analysis of bacterial richness and abundance. Species richness is the number of different species present while abundance is the total number of individuals present per species; neither of these metrics were significantly affected by insecticide treatment (Fig. 5 & 6).



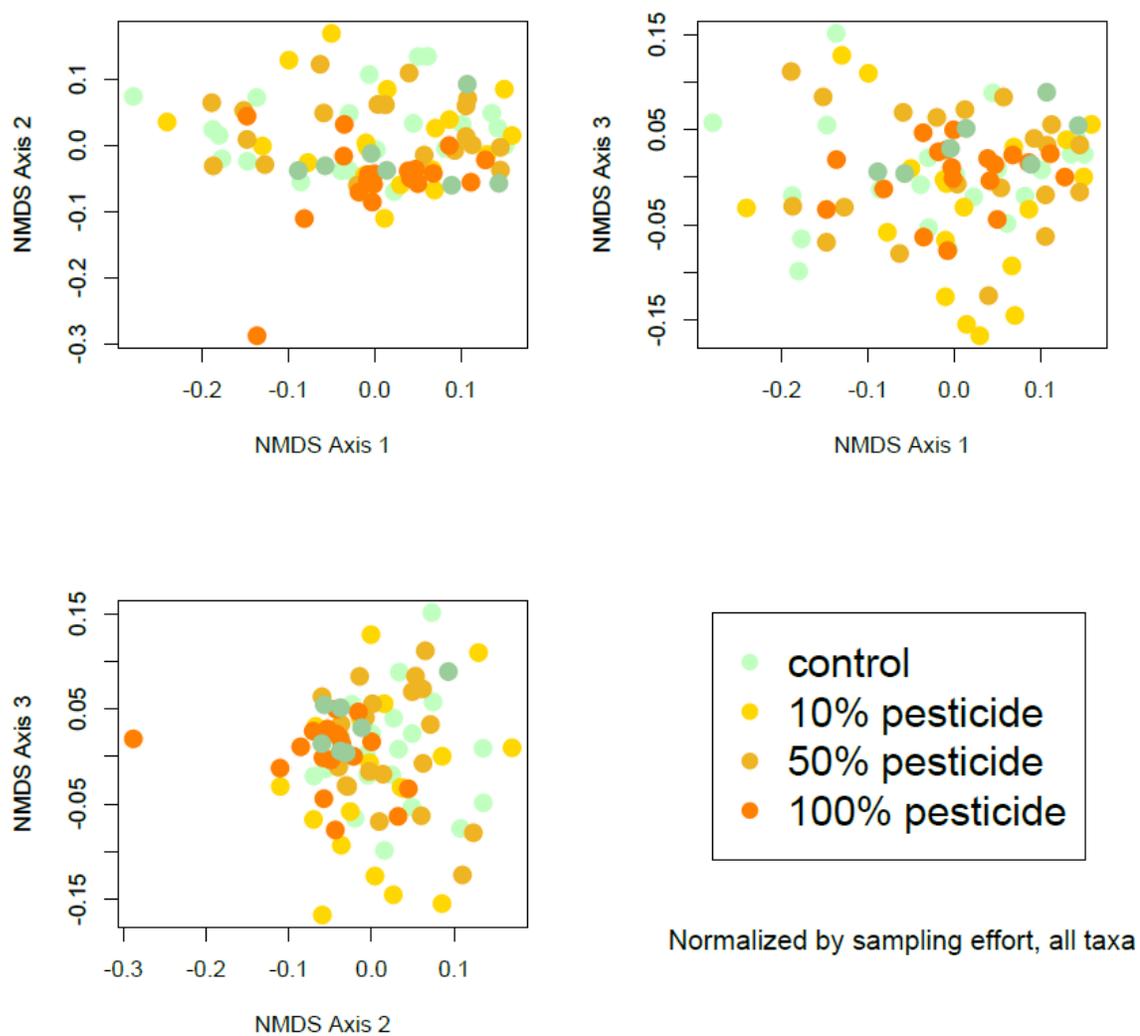
**Figure 1.** Boxplots illustrate the median weights (and upper and lower quantiles) in each treatment group (C = Control, H = Half, T = Tenth, W = Whole). The weights of all the caterpillars exposed to a whole treatment of an insecticide is significantly lower than weights in the other three treatments.



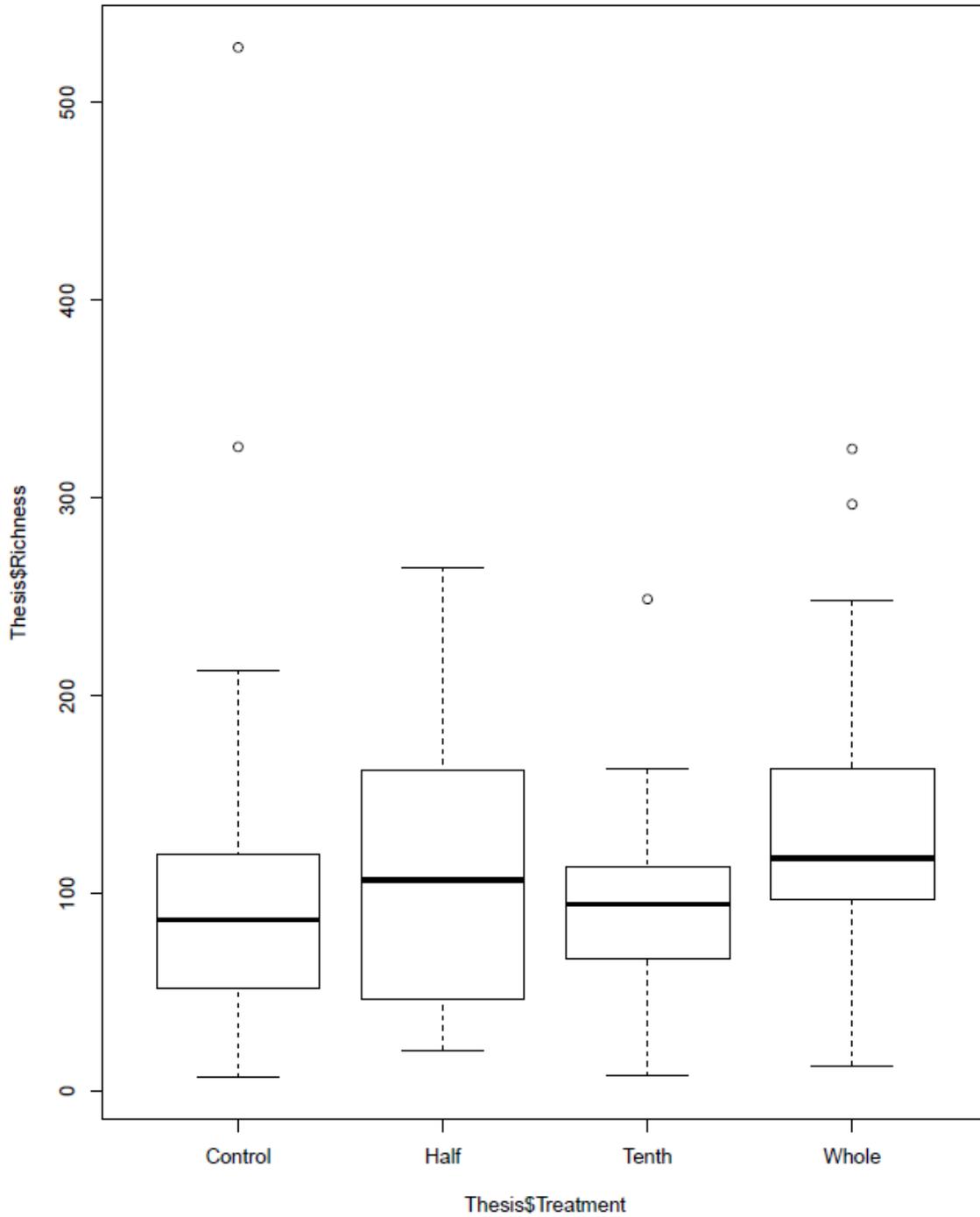
**Figure 2.** Bars show the number of reads for each of the ten most abundant OTUs. OTU1 has more than 10 times the amount of reads than OTU2, the 2<sup>nd</sup> most detected OTU.



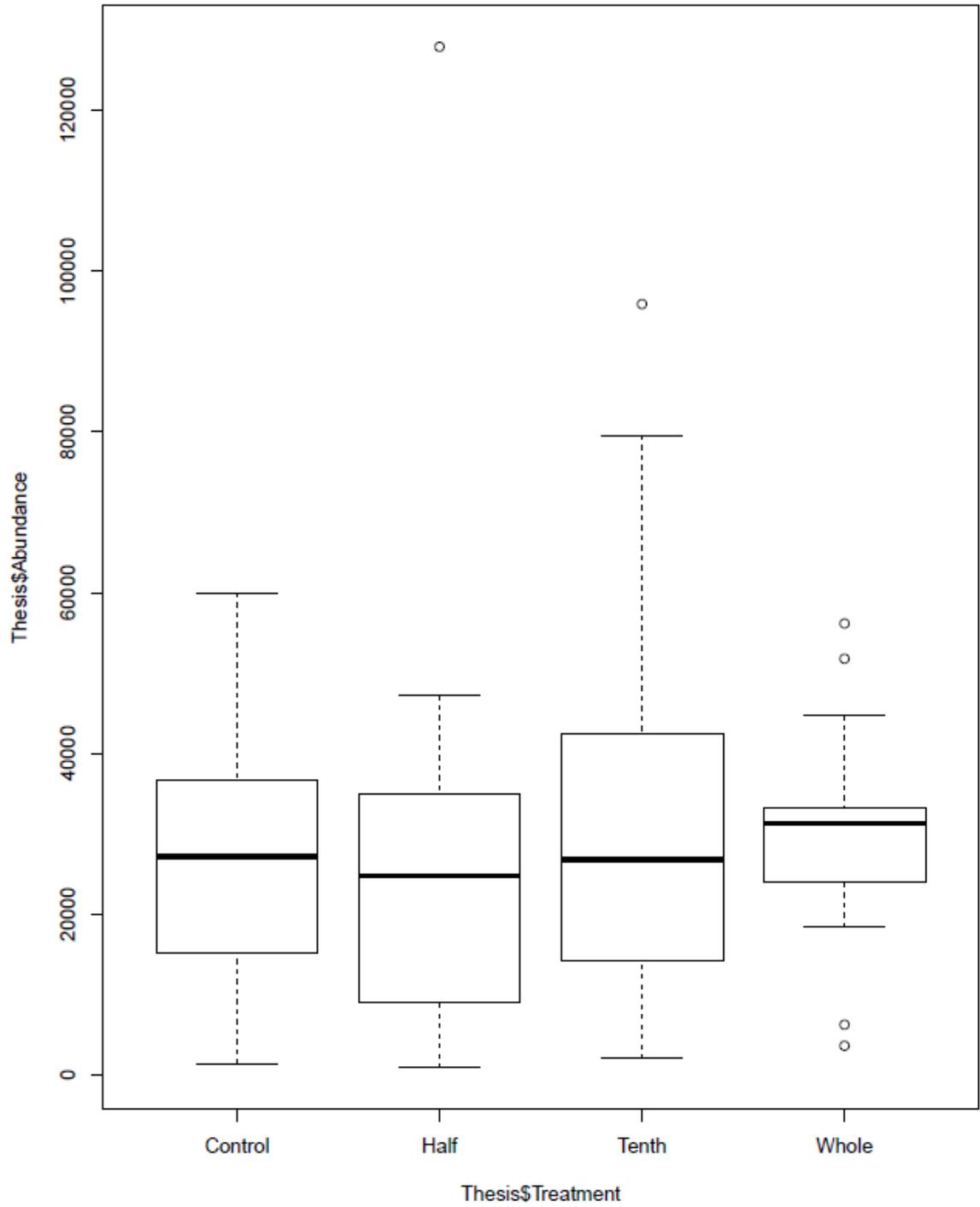
**Figure 3.** Ordination (NMDS plots) of the 10 most common taxa; each dot represents a caterpillar and the color of the dot represents the treatment received. The cluster of colors as opposed to a separation into distinctive groups implies that pesticide treatment has no effect on bacterial composition.



**Figure 4.** Ordination (NMDS plots) of all bacterial taxa; each dot represents a caterpillar and the color of the dot represents the treatment received. The cluster of colors as opposed to a separation into distinctive groups implies that pesticide treatment has no effect on bacterial composition.



**Figure 5.** Boxplots of bacterial species richness for each treatment group. Richness does not vary significantly among groups.



**Figure 6.** Boxplots of number of bacterial individuals (abundance) per treatment group, which do not differ among groups.

## Discussion

Our results show that neonicotinoids do adversely affect the development of *Pieris rapae* caterpillars; however, this decline in performance is not tied to any disruption in their microbiota composition. Interestingly enough, Robinson et al. (2010) also examined the bacterial community within the *Pieris rapae* midgut but obtained results that varied from this study – specifically, their species richness ranged from 6-15 while our caterpillars had anywhere from 7-265 different OTUs observed. This difference in species richness may be a result of variation in rearing techniques of Carolina Biology Supply Company, from which cabbage white eggs were obtained for both studies, as well as differences in the methodology for DNA extraction and sequencing. Further studies are required to understand variation in bacterial composition of the *Pieris rapae* butterfly; in particular, studies into the microbiota composition of cabbage whites obtained in the wild from different areas, and as opposed to those reared in the lab.

The broader implications of this include the need for more careful review of the usage and regulation of neonicotinoids. Mortality was not recorded for this study as a measure of performance since caterpillars were intentionally sacrificed via cryopreservation at different stages of their development for DNA sequencing of their microbiota. However, all caterpillars made it to the forty-eight hour mark for weighing and the differences in mean weights are consistent with previous findings of the adverse effects of neonicotinoids upon non-target species. Further research could explore non-lethal effects that might be expressed by adult butterflies reared on different neonicotinoid treatments, as well as further research into mortality effects (which were not assayed in this study) on cabbage whites and other butterflies.

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