Microbial Communities and Transgenic Crops:

Understanding the Role Transgenic Crops May Play in

the Rise of Antibiotic Resistance

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Antibiotic resistance rates have increased in both clinical and environmental bacteria over the past several decades. While the causes underlying these trends have been investigated in a clinical setting, little work has been done to estimate the contribution of antibiotic resistance genes (ARGs) derived from non-traditional sources such as transgenic (GM) crops. The cultivation and consumption of transgenic crops continues to be a widely debated topic, as the potential ecological impacts are not fully understood. In particular, because ARGs have historically been used as selectable markers in the genetic engineering of transgenic crops, it is important to determine if the genetic constructs found in decomposing or consumed transgenic crops persist long enough in the environment and if they can be transferred horizontally to indigenous microorganisms.

The first objective of this dissertation addresses the question of persistence. Others have also estimated the DNA adsorption capacity of various clays, but have done so by manipulating the surface charge and size of particles tested, which may overestimate sorption and underestimate the DNA available for horizontal transfer. In the present study, isotherms were generated using model Calf Thymus DNA and transgenic maize DNA without surface modification. Montmorillonite, kaolinite, and 3 soil mixtures with varying clay content were used in this study. The adsorption capacity of pure montmorillonite and kaolinite minerals was found to be one to two orders of magnitude less than previously estimated likely due to the distribution of clay particle sizes and

heteroionic particle surface charge. However, it appears that a substantial amount of DNA is still able to adsorb onto these matrices (up to 200 mg DNA per gram of clay) suggesting the potential availability of free transgenic DNA in the environment may still be significant.

Next, the impact of transgenes originating from GM crops was investigated in agricultural soils, microbiomes associated with transgenic maize, and decomposition behavior of transgenic crop biomass. The purpose of this objective was to better understand how transgenic maize may be affecting antibiotic resistance rates among soil bacteria. To this end, bulk soil, rhizosphere soil, and internal endophyte prokaryotic microbiomes associated with conventional and transgenic maize were compared using lllumina MiSeq *16S* amplicon sequencing. Internal endophytes were significantly influenced by time and location within the maize, but not by maize type. Nitrogen-cycling bacteria in the rhizosphere of transgenic pest-resistant (BT) maize experienced a decrease in diversity at day 56 of maize cultivation that was marginally correlated with the level of Cry1Ab protein exudates in soils surrounding transgenic BT maize. Secondly, *bla* ARG expression was tracked across 40 weeks of maize biomass decomposition in soils associated with conventional and transgenic BT maize. *Bla* expression significantly increased in soils associated with BT maize relative to conventional maize soils after 32 weeks or decomposition.

The next phase of research for this dissertation considered the fate of transgenes originating from transgenic crops in wastewater treatment plants (WWTPs). Consumption of transgenic crops and their food products is common in the United States. Once ingested, DNA will be degraded by acids or DNase I and II enzymes found within the digestive system, taken up by cells in the gut (host or bacterial), or pass through the digestive system and excreted partially or wholly intact. In addition to degradation or uptake, transgenic DNA may be excreted as solid waste by the host. As free DNA contained within food matrices is protected from both enzymatic digestion and acid hydrolysis, the genes contained within foods containing GMOs may be allowed to pass through the host's digestive tract intact and enter into WWTP environments. Objective 3 assessed this possibility by screening activated sludge and digester sludge obtained from France and the United States for the following four possible transgenes: *p35* promoter gene, *nos* terminator gene, *nptII* ARG, and *bla* ARG. All wastewater samples were first screened using end-point PCR and all positive results were further analyzed with qPCR to determine the relative concentration of each gene in wastewater. All domestic activated and digester sludge samples were found to contain at least one transgene, with the majority of samples containing all four. However, control wastewater from France, where transgenic crops are neither cultivated nor consumed, contained significantly fewer copies of *p35*, *nos*, and *nptII* genes. Based on these findings, it is possible that some of these genes are derived from partially digested transgenic crop material present in human

waste. This is supported in part by the significant differences in *p35*, *nos*, and *nptII* concentrations in control WWTP systems relative to domestic WWTP systems, as well as additional screening for Cauliflower Mosaic Virus (CaMV) ORF VII genes and larger fragments of possible transformation plasmids. Overall this work concludes that it is possible for genes contained within transgenic crops and processed foods containing their byproducts to survive the human digestive process and enter into WWTP environments, though the source of the detected genes could not be definitively confirmed.

In the final objective, the fate and persistence was characterized in anaerobic digestion. This work is of paramount importance, especially in the case of the *bla* and *nptII* ARGs, as a large fraction of WWTP-generated biosolids is used for land application. Because of the relatively high background levels of antibiotic contaminants in wastewaters there is the further possibility that free ARGs may be horizontally transferred to WWTP bacteria, thus increasing antibiotic resistance in the environment. The ability of WWTP bacteria to take up these transgenes in this environment is a factor of (1) the observed rates of horizontal gene transfer (HGT) in anaerobic environments, (2) the abundance of genes of interest within WWTP anaerobic digesters, and (3) the presence of a selective pressure. To investigate the behavior of free ARG DNA in WWTP environments, anaerobic digesters were constructed and operated at 47°C in quadruplicate for 60 days. The persistence of free DNA was significantly influenced by DNA fragment length. *LUG* genes significantly decreased after day 7, but *nptII* and *bla*

ARGs persisted past 30 days of operation. *Bla* ARGs were only detected in iDNA within the digesters with the highest transgene concentrations (10^6 transgene copies/mL) at day 60 of incubation. It is possible that this increase is due to bacterial uptake of free *bla* in eDNA as a result of an antibiotic-driven selective pressure, inducing the integration of *bla* into bacterial cells although no culturing was performed to confirm this finding. *NptII* genes were below detection in iDNA at all time points after digester construction. The naturally occurring concentrations of *bla* and *nptII* ARGs in the lab scale anaerobic digesters were found to be consistent with those observed in full scale WWTP digesters. However, the existence of these ARGs as primarily eDNA is novel and presently unreported in the literature.

Dedication

For my parents, Robert A. Gardner and Denise K. Gardner. I would not be here without you.

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such a Sisyphean task. Perhaps most importantly, he never (not once) asked, "Do you think you'll graduate this semester?" I am not sure I could have done this without him.

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1. Motivation and Scope of Research

The following chapter outlines the motivation and scope the research completed for this dissertation.

1.1 Introduction and Problem Definition

Antibiotic resistance rates among bacterial communities have increased dramatically over the past several decades. This increase has largely been attributed to widespread use of antibiotics in both medicine and agriculture to aggressively treat bacterial infections [1-4]. These sources are believed to increase antibiotic resistance by providing a significant and consistent selective pressure (i.e.—antibiotics) by which presently antibiotic resistant bacteria are able to propagate antibiotic resistance genes (ARGs). Though these two applications are likely contributing most significantly to increasing rates of antibiotic resistance, smaller non-traditional sources may also be making a significant contribution.

One such source is Genetically Modified Organisms (GMOs), specifically transgenic crops. ARGs have been used traditionally been used as selectable markers in genetic engineering, to allow for easy identification of successfully transformed plant cells. Because these ARGs are located on transformation plasmids within all transformed plant tissues, it is possible that these genes can be released into the environment as GMO biomass decomposes. Therefore, the potential interactions with soil microorganisms are of particular importance. Understanding how these communities are influenced by

transgenic constructs depends upon the ability of these ARGs to enter and persist in the environment. Several previous studies have investigated the DNA adsorption capacities of several clay minerals and environmental soils. However, the experimental conditions of many of these studies have largely overestimated the adsorption capacities of tested matrices. Clay minerals are often only tested based on homogeneous particle size (<2μm) and uniform surface charges, chemically altered to be homoionic with Ca^{2+}, Mg^{2+}, or Na^{+} cations. Environmental soils are often autoclaved to ensure sterility, but this no doubt alters the chemical composition of the tested soils. These experimental conditions do not reflect environmentally relevant conditions, and therefore do not allow researchers to accurately estimate possible GMO DNA adsorption capacities or persistence in soils.

In addition to ARGs, GM crops are transformed to express one or several genes of interest (GOI) that confer varying advantageous traits, including drought resistance, herbicide tolerance, and pest resistance. The most common genes that confer pest resistant include the *cry* gene family. This gene encodes for the production of a toxin protein (eg., *cry1Ab*, *cry1Ac*, etc) that is acutely toxic to lepidopteran, dipteran, and coleopteran insects, some of the most common agricultural crop pests. Several studies have indicated that this protein may also be harmful to sensitive bacteria like Nitrogen-cyclers, which are incredibly important for the agricultural industry[5] . As many lines of GM crops contain this gene, it is possible that the toxicity of its protein product is subtly restructuring the plant's internal and external microbiomes. Neither conventional nor transgenic maize

microbiomes have comparatively characterized, though dominant microbiome genera have been established.

Bacterial exposure to GM crop-derived ARGs is not limited to agricultural environments. Several studies have indicated that human consumption of GM crops or their byproducts results in the breakdown of GM crop biomass and release of GM transgenes within gastrointestinal systems [6, 7]. These free transgenes have two possible fates: 1) they may be taken up by competent bacteria within the digestive tract, thereby increasing the population of antibiotic resistant bacteria within the host organism or 2) may be released from the host body in the form of feces. It is therefore possible that wastewater treatment plants (WWTPs) may harbor GMO-derived transgenes, including ARGs. As a significant portion of wastewater treatment is biologically based (i.e.—activated sludge and anaerobic/aerobic digester tanks), there is a potential risk for WWTP bacteria to come into contact with and take up this free DNA. Any present transgenes that remain in digester tanks may also be transferred to biosolids—50-60% of which is commonly used as agricultural fertilizer annually. As of January 2017, no research has been published that investigates this possible avenue of bacterial exposure to transgenes.

The overall research objective of this project is to better understand the role GMOs, specifically GM crops, may be playing in the rise of antibiotic resistance rates among bacteria in agricultural soils and wastewater treatment facilities. This project will focus on the presence, persistence, and transfer of antibiotic resistance genes derived from GM

maize in the environment. The specific tasks of this project include:

1.) Understand how the behavior and persistence of GM crop-derived DNA varies in different environmental soil matrices.
2.) Characterize the microbiomes associated with conventional and transgenic maize and *bla* gene flow during biomass degradation.
3.) Detect and quantify *p35*, *nos*, *bla*, and *nptII* transgenes in aerobic and anaerobic digesters from wastewater treatment plants across the US.
4.) Observe and quantify GM corn-derived ARGs *bla* and *nptII* persistence in model WWTP anaerobic digesters.

Successful completion of these four objectives will facilitate a better understanding of how non-conventional sources like GMOs may contribute to widespread antibiotic resistance trends. Definitive detection of ARG transfer in a controlled environmental system will be incredibly important as it may aid in the study of HGT in the environment, an area of study that has struggled due to the immense complexity of the matrices and processed involved. A better understanding of the potential lateral transfer of these genes may lead to regulatory changes. Specifically, land application standards of biosolids may need to be adjusted to remove transgenes in order to decrease the chance of gene transfer to soil bacteria.

1.2 Research Hypotheses and Objectives

The overarching hypothesis of this dissertation is that antibiotic resistance genes derived from Genetically Modified Organisms (GMOs), specifically GM crops, affected antibiotic resistance rates among environmental bacteria. To test this hypothesis, the behavior of GM crop DNA, containing ARGs, was assessed in two environments expected to be most impacted by GMO-derived ARGs: agricultural soils and wastewater treatment plants (WWTPs). Specific lines of GM corn (maize) will be chosen based on past cultivation rates and the antibiotic resistance genes they contain. Several permutations of GM maize will be evaluated, including free DNA (Objectives 1 and 4), seed biomass (Objective 1), and maturing plants (Objective 2). Each phase of this research project will be designed to model specific environmental scenarios based on the current gaps in knowledge, as outlined above.

Objective I sought to evaluate the potential adsorption and persistence of GM maize-derived DNA in pure clay minerals and environmental soil matrices. It was hypothesized that (A) pure clay compounds and soils with increasing clay content will have an increasing capacity to bind free DNA. However, it was also expected that the adsorption capacity of untreated pure clays is significantly less than has been previously published (B) DNA will persist longer in soils with higher clay content and lower pH values because of an increased ability to bind free DNA. The tested clay minerals and environmental soils include: kaolinite, montmorillonite, low clay soil (25% sand/silt, 3%

clay, 72% organic matter), sandy loam soil (75.8% sand/silt, 12.7% clay, 8.8% organic matter), and a clay loam soil(38% sand/silt, 39% clay, 22.4% organic matter). To evaluate hypothesis A, adsorption isotherms were constructed using each clay or soil source as well as a free DNA source. The tested DNA sources included Calf Thymus DNA, which has been historically used as a model DNA source for adsorption isotherms, or transgenic BT176 DNA, known to contain the *bla* ARG. To test hypothesis B, sacrificial batch reactors were constructed using each of the three environmental soils at three agriculturally relevant pH levels: 5.0-5.2, 6.0-6.2, and 7.0-7.2. An addition of 1% w/w transgenic MON863 biomass (containing the *nptII* ARG) was added to each batch reactor and allowed to decompose over a period of 18 weeks. The number of *nptII* ARGs present over time was assessed using quantitative real-time PCR (qPCR).

Objective 2 assessed how the bacterial microbiomes associated with conventional and transgenic BT maize differ as maize plants mature as well as the gene flow of the *Bla* ARG as maize biomass decomposes. It was hypothesized that (A) the bacterial microbiomes of transgenic BT maize will differ from those of conventional maize due to the presence of the potentially toxic Cry1Ab protein produced by BT plant tissues and (B) decomposition of BT maize biomass will result in the release of free DNA into soil, which will be taken up by competent bacteria in soils surrounding BT maize and will result in increased *bla* expression. To investigate hypothesis A, conventional and transgenic BT maize was cultivated in triplicate sacrificial pots under greenhouse conditions. Bulk soil,

rhizosphere soil, and corn pith tissue was sampled every 14 days and bacterial community composition was assessed using Illumina Miseq sequencing. To investigate hypothesis B, greenhouse maize was harvested and leaf biomass was applied to the remaining sacrificial pots and allowed to decompose for 40 weeks. Triplicate soil cores were taken every eight weeks, homogenized based on soil depth, and used for RNA extraction. *Bla* expression was then quantified using reverse transcription PCR (RT-PCR).

Objective 3 was concerned with observing and quantifying possible GMO-derived transgenes in wastewater treatment facilities. It was hypothesized that transgenes from GMOs, including ARGs, are present and detectable in wastewater treatment systems across the US at consistent levels. This was assessed by obtaining activated sludge and digester sludge from more than 26 WWTPs across the United States and France. The presence of four common transgenes—*p35* promoter, *nos* terminator, *nptII* ARG, and *bla* ARG—as well as *16S*, which served as a proxy for total bacteria.

Objective 4 aimed to observe and quantify GM corn-derived ARGs *bla* and *nptII* persistence and behavior in WWTP thermophilic anaerobic digesters. It was hypothesized that GM corn-derived ARGs will not be significantly removed during anaerobic digester treatment and will thus risk being transferred to agricultural biosolids. To assess this, anaerobic sludge was obtained from the North Durham WWTP and used to operate quadruplicate thermophilic batch-scale anaerobic digesters. Varying known concentrations of GM maize-derived transgenes were added to each reactor, including

nptII, *bla*, and a corn-specific reference gene was not detected in the source wastewater. Samples were taken over a period of 90 days and DNA was extracted. Transgenes and reference genes were quantified using qPCR. Biogas production in each reactor was also monitored to determine sludge viability for the duration of the experiment.

2. Background and Literature Review

The following chapter contains a brief overview of the current scientific literature that addresses the main topics of this dissertation. Additional, more specific reviews are found in the remaining research chapters.

2.1 Historical Rise in Antibiotic Resistance

Antibiotic resistance rates have increased dramatically in both clinical and environmental bacterial populations over the past several decades and have largely been attributed to the widespread use of antibiotics for medical and agricultural applications [8-11]. Two events must occur for antibiotic resistance to be preserved in bacterial populations: (1) Bacteria must develop antibiotic resistance, either through random mutation or horizontal gene transfer (HGT), and (2) Resistant bacteria must come into contact with the relevant antibiotic(s), which act as a selective pressure and compel bacteria to maintain the antibiotic resistance genes (ARGs). Antibiotic resistance among environmental bacteria has pre-dated the use of antibiotics by humans to combat bacterial infections, as ARGs confer resistance to naturally occurring antibiotics and some heavy metals. Naturally occurring antibiotic resistance is then able to be disseminated among bacterial communities through both physical and biological forces such as rain, wind, biofilm formation, and animal contact [2]. However, an upsurge in the number of bacteria that have acquired immunity to a single antibiotic as well as Multi Drug Resistant (MDR) bacteria has been noted in a wide array of environments, due in part to the widespread

and increased use of antibiotics [1-4]. Resistance genes for tetracyclines, β-lactamases, and several other resistance genes have been shown to have some of the highest rates of increase among soil bacteria [12, 13]. Some forms of β-lactam resistance from the CTX-M family appear to have arisen before the current medical crisis, indicating that antibiotic resistance in some bacteria was derived from other sources [3]. While the causes underlying these trends have been investigated thoroughly in a clinical setting, little work has been done to estimate the contribution of ARGs derived from non-traditional sources.

2.2 GMOs as a Non-Traditional Source of ARGs

Several lines of transgenic crops, also known as genetically modified (GM) crops, are known to contain one or more ARGs including β-lactamase and neomycin resistance genes, which have been traditionally used as selection markers during the plant cell transformation process [14-17]. Since 2000, the cultivation of transgenic crops has increased annually-and will likely continue to do so in years to come. With more than 159.9 million hectares of transgenic canola, maize, soy, and cotton planted globally in 2012 [18], the risk of these genes entering into the environment is noteworthy. Because these antibiotics are still widely used by the medical community to treat a variety of Gram positive and Gram negative bacterial infections [11] it is important to understand how the introduction of GMO-derived ARGs into the environment may have affected the increasing rates of antibiotic resistance among soil bacteria.

2.3 Antibiotic Resistance Genes in Genetic Engineering

Antibiotic resistance genes have been traditionally used as selection markers in the field of genetic engineering and have been used in the transformation of transgenic crops since their development in the 1980s [19]. At the outset of the transformation process, an ARG is ligated into a plasmid vector along with the Gene(s) of Interest (GOI). A promoter gene (*p35*) and terminator gene (*nos*) are also incorporated into the plasmid to ensure that all genes will be functional within the transformed plant cell. This vector is then inserted into plant tissue, typically using either a Gene Gun or *Agrobacterium*-mediated transformation. Plant cells that have been successfully transformed are allowed to develop into mature plants with the GOI and ARG being constitutively expressed throughout all tissues. The two most common ARGs used are *bla,* a gene encoding for β-lactamase enzymes, and *nptII*, a neomycin phosphotransferase gene. *Bla* genes confer resistance to penicillins, cephamycins, carbapenems while *nptII* genes protect against aminoglycoside antibiotics like kanamycin. These two genes have been used in more than 130 unique commercially available lines of transgenic crops, representing crops that were actively cultivated for human consumption [20].

β-Lactams are still one of the most widely used class of antibiotics, and resistance to these antibiotics is a severe threat because they have low toxicity and are used to treat a broad range of infections [13]. The mechanisms of β-lactam resistance include inaccessibility of the antibiotics to their target enzymes, modifications of target enzymes,

and/or direct deactivation of the antibiotics by β-lactamases [21, 22]. In Gram negative bacteria, the primary resistance mechanism is enzymatic inactivation through the cleavage of the β-lactam ring by β-lactamases. A wide variety of *bla* genes have been discovered that encode for approximately 400 β-lactamase enzymes, making them one of the most effective known ARGs [23]. The *nptII* gene allows for the transcription of the *nptII* enzyme, which inactivates aminoglycoside antibiotics through phosphorylation once the antibiotic enters the bacterial cell. Aminoglycosides are no longer as effective as in previous years due to an increase in resistance, and are not prescribed frequently as a result. However, it is still important to understand how the commercial use of these genes may have contributed to the rise in antibiotic resistance because of the extent of their use as selectable markers in engineering transgenic crops.

2.4 Cultivation of Genetically Modified Crops

Transgenic crops were first developed for human consumption by 1976, though the first successful transgenic crop, antibiotic-resistant tobacco, was not planted until 1983. Between 1996 and 2013, the number of acres dedicated to the cultivation of transgenic crops increased 100 times over. This represents an increase in acreage from approximately 4.2 million total acres to over 430 million total acres [24]. By 2014, transgenic crop varieties made up 94% of soybean acreage, 93% of maize acreage, and 96% of cotton acreage in the US alone, according to the US Department of Agriculture. As of 2013, transgenic crops were cultivated for human use in 27 countries, including eight

developed nations and 19 developing nations. In total more than 18 million farmers grow transgenic crops, of whom 54% live in developing nations (Figure 1).

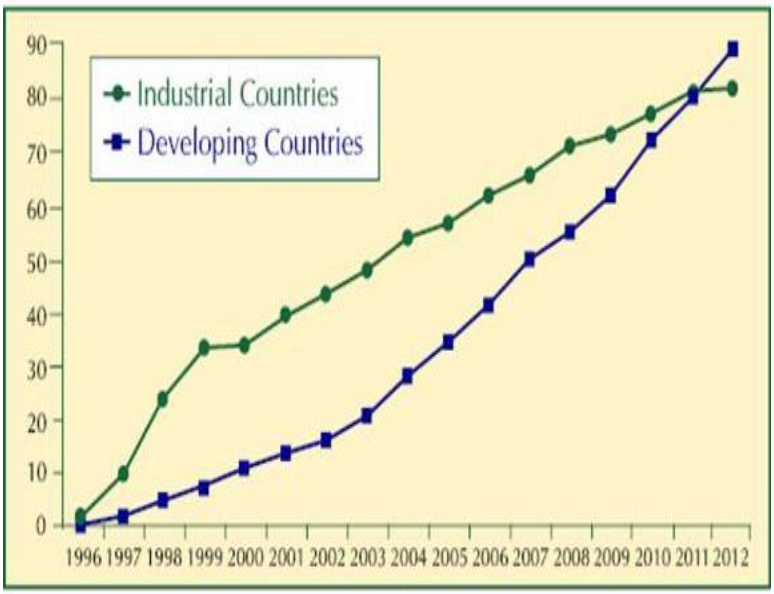

Figure 1: Agricultural land used for all GM crop cultivation among developed and developing nations, from 1996-2013. The Y-axis denotes the percent of agricultural land dedicated to the cultivation of transgenic crops in millions of hectares. Adapted from J. Clive [25].

The most popular traits based on total planted acreage include herbicide-resistance ("HT") and pest-resistance ("BT"), which have been incorporated into many crops including, but not limited to, soybeans, cotton, and maize [26]. These transgenic varieties have quickly replaced conventional varieties as the dominant cash crops since they were first planted (Figure 2). Cultivation of BT and HT maize increased dramatically, accounting for an average only 2% of total maize acreage in 1996 but jumping to 68.5% of total acreage by 2011. At least 28 lines of BT and HT maize contain either *bla* or *nptII*

marker genes, the largest share of ARG-containing GMOs used for human consumption [20]. HT soybeans, BT soybeans, HT cotton, and BT cotton follow a similar pattern of increase in cultivation over the same period of time.

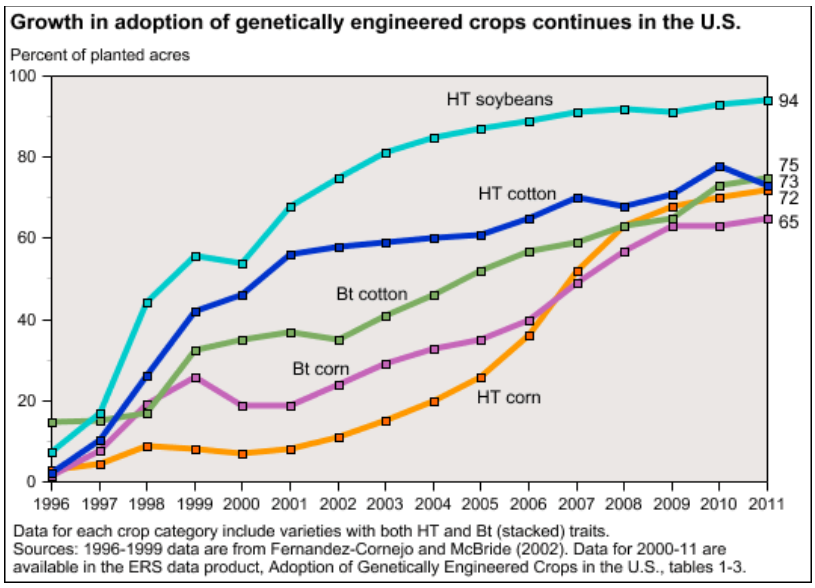

Figure 2: Percent of total land used for pest-resistant (BT) and herbicide-resistant (HT) lines of GM cotton, maize, and soybeans from 1996-2011. Adapted from Fernandez-Cornejo et al. [27].

2.5 Gene Flow into the Environment

2.5.1 Gene Flow in Agriculture

The quantity of transgenes that persist in any given environment will be largely dependent on both the amount of GMO biomass that is able to decompose and the chemistry of the surrounding soil. The most obvious route of bacterial exposure to ARGs contained within transgenic crops is that of direct exposure in agricultural fields. In this

scenario, transgenes from any given transgenic crop may enter the soil environment from both above and below ground (Figure 3). As the above ground biomass matures, plant matter will naturally accumulate and decompose on the soil surface to a variety of natural factors. Transgenes will enter the soil as plant matter is broken down, and may be taken up by competent soil bacteria. Upon harvesting, a significant amount of corn stover is commonly left in the field to decompose. Depending on the harvest method, up to 54.3 bushels/acre/year may be left to decompose in the field [28]. If the land is then tilled prior to the next season's planting, transgenes will become homogenized in multiple layers of soil; if no tillage occurs, the transgene will likely remain more concentrated at the soil surface.

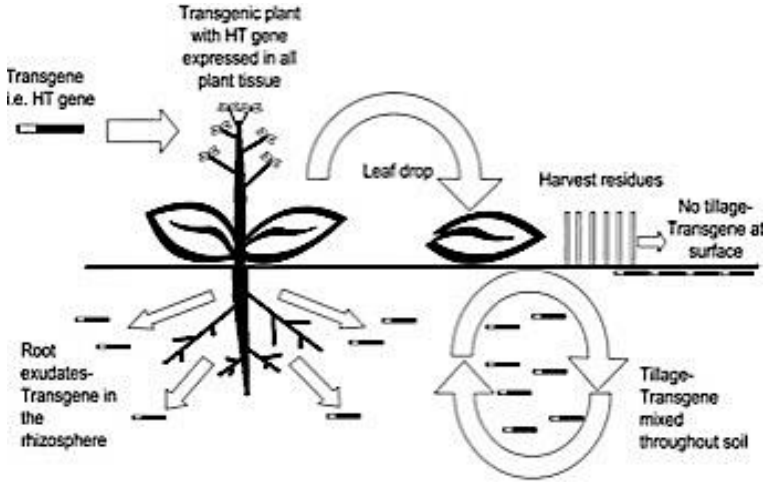

Figure 3: Schematic showing the potential avenues of exposure between the GM crop transgenes and soil bacteria. An Herbicide Tolerance (HT) gene is used as a model gene. Adapted from Dunfield and Germida [16].

Living crop biomass found below the soil surface has also been shown to be a source of GM transgenes [29-31]. Because transgene cassettes are constitutively expressed in all plant tissues, all cells are actively transcribing these genes and producing their respective proteins. In the case of BT crops, the insecticidal Cry toxin protein is produced in all tissues, most notably the root system. These proteins are then exuded by the plant and can persist in the surrounding soil [29, 32]. BT maize, potato, and rice have been shown to produce these exudates, and may adversely affect sensitive nutrient cycling bacteria [5]. This could impact the nutrient availability for crops and other living organisms in and around the soil.

Genetic material from GM crops has been found to be stable and not readily degraded in a variety of environments, from incredibly complex soil environments to the plants themselves [33-35]. Soil chemistry will have the most observable effect of the persistence of GM transgenes once they are introduced into an environment. Clay compounds have a well-documented ability to sequester large amounts of DNA in soil environments [35-38]. The DNA-clay complexes formed are incredibly robust and provide free DNA significant protection against DNases [39-41]. Several studies have tested the adsorption capacity of pure clay compounds like montmorillonite, citing values in excess of 9,000μg/g and 30,000μg/g depending upon the surface charge of the clay particles [37]. Bound DNA is also up to 100 times more resistant to DNase I activity than free DNA [40], implying that bound DNA is able to persist for a significant amount of time in soils.

2.5.2 Gene Flow in Wastewater Treatment Plants

Traditional municipal wastewater treatment plants have been shown to contain both antibiotic resistant bacteria and antibiotic chemicals. The levels of resistance and ARGs found here is one of the highest outside of hospital wastewater settings [23, 42-44]. Resistance to vancomycin, cefazolin, and penicillin was found to be 11%, 39%, and 30% among total bacteria in WWTP activated sludge basins, respectively. The effluent of the same WWTPs, which is commonly discharged into nearby rivers, still showed significant resistance rates of 15%, 39%, and 20%, respectively. When taking into account the number of CFUs per mL, these levels of resistance are significantly higher than those found in either drinking water or surface water sources (Table 1).

Table 1: Percentage of detected antibiotic-resistant bacteria in hospital effluent, municipal sewage activated sludge, municipal sewage effluent, surface water, and drinking water. Adapted from Schwartz et al. [43].

	Hospital Effluent	Activated Sludge (Municipal WWTP)	Effluent (Municipal WWTP)	Surface Water	Drinking Water
Vancomycin	6.8 (± 5.0)	11 (± 3.8)	15 (± 10)	2.3 (± 0.5)	20 (± 10)
Ceftazidime	45 (± 21)	44 (± 17)	27 (± 17)	11 (± 1.6)	5.1 (± 2.4)
Cefazolin	58 (± 23)	39 (± 16)	39 (± 20)	8.1 (± 0)	48 (± 27)
Penicillin G	71 (± 25)	30 (± 8.0)	20 (± 6.7)	31 (± 3.3)	43 (± 26)
Imipenem	8.1 (± 3.5)	2.8 (± 0.2)	0.6 (± 0.4)	0.4 (± 0.1)	0

Well-documented sources of antibiotics in the environment include animal husbandry, household disposal, medical applications, and the chemical production of antibiotics. A wide variety of antibiotics including -lactams, macrolides, and

aminoglycosides have been found at low levels in a variety of soil environments (Table 2). These residual antibiotics likely act as a selection pressure, compelling antibiotic resistant bacteria to maintain ARGs because they confer a selective advantage.

Table 2: Concentration and degradation kinetics of antibiotics naturally found in a variety of soils environments. Adapted from Thiele-Bruhn [45].

Class	Compound	Concentration	Sample Soil: texture/pH	Degradation (%)	Time (days)
Tetracycline	chlortetracycline	5.6 μg/g	Sandy Loam/6.1	88	30
		4.7 μg/kg	Soil	0	180
	tetracycline	50-300 μg/kg	Soil	0	180
		10 μg/g	Poultry Manure	65	84
		10 μg/g	Manure-Amended Soil	100	14
Sulfonamide	sulfanilamide		Soil	0	14
	sulfabenzamide	250-1000 μg/L	Manure/Sludge	0/50	28/0.4
	sulfadiazine	250-1000 μg/L	Manure/Sludge	0/50	28/0.4
	sulfameter	250-1000 μg/L	Manure/Sludge	0/50	28/0.4
	trimethroprim	500 μg/L	Sewage Sludge	50	22-41
Aminoglycoside	streptomycin	5.6 μg/g	Sandy Loam/6.1 + Manure	0	30
β-lactam	penicillin	5.6 μg/g	Sandy Loam/6.1 + Manure	0	30
	ceftiotur		Clay Loam	50	22.2
			Sand	50	49
			Silty Clay Loam	50	41.5
	mecillinam	500 μg/L	Sewage Sludge	50	0.5-0.7
Macrolide	erythromecin	5.6 μg/g	Sandy Loam/6.1 + Manure	70	78
	tylosin	5.6 μg/g	Sandy Loam/6.1 + Manure	0	30

The sources of the ARGs themselves are difficult to track due to the sheer number of potential sources and the complexity of the WWTP system inputs. Understanding how non-traditional sources of ARGs, like GM crops, enter WWTP systems is therefore equally complicated, if not more so. However, it has been documented that genetic elements from

the food we consume, including GM crops, have the ability to pass through the digestive system intact and not degraded [46-48]. This would provide a relatively simple route to explain the exposure of WWTP bacteria to GMO-derived ARGs. Most transgenes that enter a WWTP will likely be removed through settling and end up in digester sludge tanks. Approximately 50% of all digester sludge is transformed into biosolids, which are then land applied as agricultural fertilizer. Because no regulations currently exist regarding the level of ARGs in biosolids these genes are allowed to remain is biosolids, providing another avenue from which GM-derived ARGs can come in contact with soil bacteria.

2.5.3 Detection of *bla* and *nptII* Antibiotic Resistance Genes

The gene flow of GMO transgenes is not limited to their immediate environments. Multiple forms of the *bla* gene have been documented in municipal wastewater, hospital wastewater, dairy and swine farm lagoons, and surface water [49-52]. *NptII* genes have also been detected in multiple sources including soil, groundwater, municipal wastewater [53-57]. This suggests that these genetic elements are much more mobile than initially thought and have the potential to affect the microbial communities in several different microbiomes.

2.6 Selection and Justification of GM Maize Varieties

When designing this project and planning out the experimental methods, the relevancy of the crop was one of the most important issues to consider. Arguably, the

ideal crop in this case could be defined as the variety that had both a high number of acres planted annually and a high percentage of lines that contained ARGs. Because transgenic crop genes have been shown to survive the digestive tract in humans and possibly end up in municipal WWTPs, selecting a crop that is typically grown for human consumption would allow us to investigate multiple routes of bacterial exposure to ARGs. The two most popular crops that fit these conditions were found to be soybean and maize. Though the percentage of transgenic soybean grown in the US is greater than that of maize (Figure 2), the total area devoted to the cultivation of maize is significantly higher than that of soybeans—92.3 million acres vs. 75.2 million acres [58]. This, coupled with the high percentage of transgenic maize lines that contain ARG selectable markers, have led us to target transgenic maize over other transgenic crops. Looking into the specific lines of commercially available transgenic maize, MON863 (Monsanto) and BT maize lines have been cultivated on an international scale and contain *nptII* and *bla* genes, respectively. Based on these characteristics, these two lines of transgenic maize were selected to investigate the aims outlined in Chapter 1.

3. Behavior and Persistence of GMO-Derived DNA in Different Environmental Soil Matrices

The following chapter describes the work completed to address Objective I. This work was accepted in February 2017 for publication in *Chemosphere*, Volume 175 pages 45-51.

3.1 Introduction

Acquisition of antibiotic resistance has increased dramatically in both clinical and environmental bacterial populations over the past several decades and has largely been attributed to the widespread use of antibiotics for medical and farming applications [8-11]. An upsurge in the number of bacteria that have acquired immunity to a single antibiotic as well as multi drug resistant (MDR) bacteria has been noted in a wide array of environments ranging from hospital settings to wastewater treatment plants (WWTPs) and groundwater [2, 4] In particular, antibiotic resistance genes (ARGs) for tetracyclines and β-lactamases have been shown to have some of the highest rates of increase among soil bacteria [12, 13]. Some forms of β-lactam resistance from the CTX-M family appear to have arisen before the current medical crisis, suggesting that some bacteria may have acquired resistance from sources other than exposure to antibiotics [3]. While the causes underlying these trends have been investigated thoroughly in a clinical setting, little work has been done to estimate the contribution of ARGs derived from non-traditional sources such as those from transgenic crops.

Several lines of transgenic crops are known to contain one or more ARGs including β-lactamase and neomycin resistance genes. These genes have been traditionally used as selection markers during the plant cell transformation process, and are most commonly found in first and second generation transgenic crops [14, 17]. With more than 159.9 million hectares of transgenic canola, maize, soy, and cotton planted globally in 2012 [18] the risk of these genes entering into the environment is noteworthy. Several lines of pest-resistant BT maize contain the *bla* gene, encoding resistance to penicillins, cephamycins, and carbapenems [49]. However, it is worth noting that these genes are only contained in a fraction of GM crop lines and ARGs are being used as selection markers in transgenic crops less and less frequently. As these same β-lactam antibiotics are still used by the medical community to treat a variety of Gram positive and Gram negative bacterial infections, it is important to understand how the current and historical use of transgenic crops containing ARGs may have contributed to the increasing rates of antibiotic resistance among soil bacteria. To this end, an accurate assessment of how much transgenic DNA is entering into the environment and persisting must be obtained.

It has been well established that nucleic acids can persist in soils for a significant amount of time, especially in soils with elevated clay content [35, 37, 38]. Previous studies have estimated the capacity of several pure clay materials [36, 39, 40] as well as environmental soils and sediments [38, 59] to adsorb model DNA such as calf thymus or herring sperm DNA. Some of these experiments were unable to find a maximum

adsorption capacity, citing values in excess of 9,000 μg calf thymus DNA/g dry clay or 30,000 μg herring sperm DNA/g clay [37]. They have also demonstrated that the resulting DNA-clay complexes have the ability to shield bound DNA from significant DNAse activity [60], likely increasing DNA persistence in environmental soil. However, many of the studies that examined the DNA adsorption capacity of pure clay minerals calculated the adsorption capacities of specific clays and soils after separating particles based on size, often only using particles < 2 μm, and making clays homoionic to Ca^{2+}, Na^{+}, or Mg^{2+}. This pretreatment is not relevant to any known environmental condition and has likely resulted in an overestimation of the DNA adsorption capacity of tested soils and clays. These studies have also only measured total DNA adsorption to clay surfaces making no distinction between ssDNA and dsDNA, which may have varying microbial transformation potential. Thus, in this Objective, several adsorption isotherms were constructed to evaluate the natural ability for pure kaolinite, a 1:1 clay mineral, and Montmorillonite, a 2:1 clay mineral, as well as three environmental soil samples with varying clay content to adsorb model calf thymus DNA and transgenic maize DNA. The resulting isotherms were compared to past literature in order to determine each mineral's natural ability to adsorb DNA, without the introduction of particle charge or size biases. The adsorption capacity of calf thymus DNA was compared to transgenic maize DNA to test the effect of source DNA GC content on the formation of DNA-clay complexes.

Finally, the adsorption potential of transgenic maize DNA was further tested on three environmental soil matrices.

A second important factor when considering the risk of bacterial exposure to transgenic crop-derived ARGs is the ability of free DNA to persist in soil environments. Environmental soil matrices are known for being notoriously complex and heterogeneous, making the tasks of determining influential physicochemical characteristics difficult. However, weight of literature indicates that there are several factors associated with environmental soil that are mostly likely to affect DNA persistence potential, including soil pH, presence of DNase enzymes, and soil clay composition. The DNA adsorption capacity and pH of soils appear to be negatively correlated, with more DNA adsorbing and persisting in soils with lower pH values [41, 61]. Romanowski et al. [40], for example, reported an eight-fold increase in the soil DNA adsorption capacity when decreasing pH from 9 to 5. Further studies have determined that maximum DNA adsorption and persistence occurs around pH 3.0 for Montmorillonite, kaolinite, and goethite minerals, likely due to increased DNA denaturation [62]. However, this pH is outside the range of agriculturally relevant soil acidity, as the optimum soil pH for most crops is ranges from pH 5 to pH 8[63]. The presence of DNase enzymes produced and excreted by soil bacteria would also affect extracellular DNA persistence [38, 64, 65]. Many classes of DNase have been identified in soils, each with varying affinities for the sequences and structures of free DNA [66]. However, free DNA bound to clay minerals

and other soil particles are offered significant protection from DNase activity and are able to persist in soils despite the presence of these enzymes [67]. Finally, increasing clay content in environmental soils is expected to increase overall DNA persistence, and has been discussed extensively in previous sections of this chapter.

Overall, it is expected that DNA from transgenic maize will be most persistent in soils containing elevated clay content and least persistent in soils containing minimal clay content because of clay's ability to adsorb free DNA. DNA will also persist longer in soils with lower pH values because of an increased ability to bind free DNA. To test this, soil batch reactors were constructed in triplicate containing either low clay soil, sandy loam soil, or clay loam soil at three agriculturally relevant pH ranges, as well as a defined addition of transgenic MON863 maize biomass, which contains the *nptII* ARG. Total copies of *nptII* remaining in soil batch reactors will be quantified over time, allowing for the evaluation of the persistence of free DNA released from decomposing MON863 biomass.

3.2 Methods

3.2.1 DNA Adsorption Capacities

3.2.1.1 Clay Minerals and Soils

Montmorillonite, kaolinite, and acid washed sand were obtained from Sigma (St. Louis, MO). Sandy loam, clay loam, and silt loam soil matrices (Table 3) were obtained

through Sands and Soils (Durham, NC). Soil mixtures were homogenized and ground to pass through an 80 mesh sieve. In addition, the pH of kaolinite and montmorillonite was found to be 6.82 and 4.01, respectively. All characterization of soil physico-chemical properties was performed by the North Carolina Department of Agriculture and Consumer Sciences Division in accordance with standard protocols. These soils were not autoclaved to preserve the natural soil chemistry and structure.

Table 3: Physico-Chemical Properties of Soils Used for Adsorption Isotherms

Soil Type	% Clay	% Humic Matter	CEC (mequiv/100g)	pH
Clay Loam	39.0	0.09	6.7	5.3
Sandy Loam	12.7	0.04	2.0	5.5
Low Clay Soil	3.0	0.22	5.8	5.2

3.2.1.2 DNA Preparation

Calf thymus DNA (Sigma-Aldrich, St. Louis, MO) with an approximate GC content of 41.9% was obtained and resuspended in molecular grade nuclease-free water to a stock concentration of 500 μg/mL. High quality genomic DNA from transgenic maize with an average GC content of 42.3-55.4% [68] was isolated using the following optimized CTAB isolation protocol. First, 500 μL CTAB Buffer (100mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone, 2% hexadecyl trimethyl-amonium bromide (CTAB) (w/v), and 0.5% β-Mercaptoethanol (v/v)) was added to 100 mg maize biomass and incubated at 60°C for two h. Samples were removed from heat and 1.0 μL RNAse A (10 mg/mL) was added to the mixture and incubated at 37°C for 30 min to

remove RNA and increase the accuracy of spectrophotometric measurements. Seven hundred μL phenol:chloroform:IAA was added, tubes were vortexed briefly and centrifuged at 13,000x g for 60s. This step was repeated until no debris could be seen at the interface. Then, 700 μL chloroform was added to the aqueous phase and tubes were centrifuged at 13,000x g for 2 min. The aqueous phase was transferred to a new tube and 0.08 volumes of cold 5 M ammonium acetate and 0.54 volumes of isopropanol were added, mixed by inversion, and incubated on ice for 45 min. DNA was then purified by ethanol precipitation and the resulting pellet was resuspended in 50 μL TE Buffer. DNA concentration and quality were determined using a Qubit fluorometer 2.0 for dsDNA only and NanoDrop spectrophotometry for total DNA. As NanoDrop instruments are known for significantly overestimating DNA concentrations, the degree of DNA overestimation was calculated using a series of DNA solutions with known DNA concentrations. The authors found the NanoDrop to overestimate total DNA by an average factor of 2.957. Thus, the raw NanoDrop readings obtained by the authors were divided by a factor of 2.957 to calculate the true concentration of total DNA (μg/mL)[69]. The concentration of ssDNA was estimated by subtracting dsDNA QuBit concentrations from total DNA NanoDrop measurements. Only extracted DNA with a 260/280 of 1.8-2.0 and a 260/230 of 2.0-2.2 were used for adsorption isotherms. RNA contamination in both DNA sources was determined to be negligible. This was assessed using the Qubit RNA HS kit (Thermo Fisher Scientific).

3.2.1.3 Adsorption Isotherms

Adsorption isotherms were constructed using a batch slurry method using siliconized 15 mL glass centrifuge tubes. A total weight of 0.5 g clay or soil was added to 1.0 mL nuclease free dH_2O containing a DNA concentration ranging from 0 to 150 μg/mL. Suspended DNA was not found to be significantly denatured by the lack of ions over the course of the isotherm experiments. Calf Thymus DNA was used as a source of DNA material for kaolinite and montmorillonite adsorption isotherms only. Because Calf Thymus DNA has been used historically as a DNA source in adsorption experiments it will allow direct comparisons between the clay adsorption findings in this study and those of older studies. Transgenic maize DNA was used as a DNA source for both pure clay isotherms and all three environmental soil isotherms because of its increased environmental relevance and potential to harbor antibiotic resistance genes. All isotherms were performed in quadruplicate. Tubes were mixed at 80 rpm for two h and then centrifuged at 6,000x g at 4°C for 30 min. Supernatants were collected and centrifuged again at 11,000x g 4°C for 20 min. The DNA concentration in the resulting final supernatant was analyzed using a NanoDrop and Qubit 2.0 according to manufacturer instructions for total DNA and dsDNA concentrations, respectively.

3.2.1.4 DNase Detection

Soil substrates and pure clay minerals were tested for the presence of DNAse proteins using DNase Alert kits (IDT, Coralville, IA). As no standard method for quantifying DNase proteins in soils matrices were known to the research team samples were prepared in multiple ways to optimize the kit-based protocol and determine the best method for detecting DNases. Soils were pretreated using the following protocols: (1) 0.025g of soil was added to 200 μL dH_2O and was shaken at 120 rpm at room temperature for 30 min. Samples were collected every 5 min and centrifuged at 13,000x g for 10 min and supernatants were collected and measured for the presence of DNases according to kit manufacturer instructions. (2) 0.025 g of soil was added to 200 μL dH_2O and was shaken at 120 rpm at room temperature for 30 min. This slurry was measured for DNases every 5 min without centrifugation. Positive and negative controls were used for each sample set. All tests for the presence of DNases in tested clays and soils using the DNase Alert kit were performed in triplicate and found to be negative. Therefore, we assumed that the relative contribution of DNases to the sorption values obtained in this study was minimal and no adjustments were made.

3.2.1.5 Statistical Analysis

The resulting concentrations were evaluated using the Langmuir equation (Equation 1)

$$Q = \frac{Qmax\, K_l\, Ce}{1 + K_l\, C_e} \quad (Equation\ 1)$$

where Q_{max} is the maximum adsorption capacity (μg/g), Q is the adsorbed DNA concentration (μg/g), C_e is the DNA concentration in solution at equilibrium (μg/mL), and K_l is the Langmuir constant (mL/μg). Each isotherm was linearized as C_e/q_e vs. C_e to determine the Langmuir constants and coefficient of determination (R^2) values. Statistically significant differences among isotherms were assessed using one-way analysis of variance (ANOVA) and paired t-tests.

3.2.2 Environmental Persistence of GM Maize DNA

3.2.2.1 Environmental Soil Reactor Construction

MON863 seed biomass, containing the *nptII* ARG, was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Tested soils included Minimal Clay, Sandy Loam, and Clay Loam soils (Sands and Soils Co., Durham, NC) described in Section 3.2.1.1 above. Again, these soils were not autoclaved to preserve the natural soil chemistry and structure as well as any microbial activity that may be relevant as the maize biomass decomposes. Ten grams of soil was added to sterile Ehrlenmeyer flasks, and soil pH was buffered to the following pH ranges using $CaCO_3$: 5.0-5.2, 6.0-6.2, and 7.0-7.2. Once the soil pH reached the desired level, 1% (w/w) MON863 biomass was added to each reactor. Reactors were sealed with aluminum foil and kept in a sterile secondary container. Each condition was tested in triplicate. Samples of 0.25g of soil sampled from each reactor every in triplicate every 21 days. Samples were stored at -80°C until DNA could be extracted.

3.2.2.2 DNA Isolation and *nptII* Quantification

Due to the higher clay content of the Clay Loam soil, several pretreatment steps were necessary in order to ensure the release of all DNA bound to clay surfaces. Clay soils were washed with the following pretreatment solutions: triplicate washes with 100mM Tris-HCl buffer to neutralize soil pH; One wash with 100mM NaCl; One wash with 100mM NaPO3 to allow for phosphate group substitutions; One wash with desorption solution, adapted from Pietramellara et al. [70](2% SDS; 17 mM lactic acid, 3 mM KH_2PO_4, 27 mM Na_2HPO_4, 0.23 mM $MgSO_4$, 11 mM NH_4Cl, 19 μM $CaCl_2$, 0.5 μM $FeSO_4$, 86 mM sodium pyrophosphate, 57 mM EDTA). DNA was then isolated from each sample using the PowerLyzer PowerSoil kit (MoBio Laboratories Inc., Vancouver, CAN) according to manufacturer instructions. Eluted DNA was quantified using a QuBit 2.0 (Thermo Fischer Scientific, Waltham, MA) and stored at -20°C for downstream analysis.

DNA was screened for the number of *nptII* genes using the following primer and TaqMan®™ probe sequences: F: 5′- AGGAAGCGGTCAGCCCAT-3′, R: 5′- GCGTTGGCTACCCGTGATAT-3′, Probe: FAM-5′- CGCCGCCAAGCTCTTCAGCAA-3′- TAMRA [71]. QPCR assays were performed on a Stratagene Mx3000P (Agilent, Santa Clara, CA) using the following conditions: Initial denaturation at 95°C for 60s, followed by 40 cycles of 95°C for 30s and 60°C for 30s. The copy number of *nptII* per sample was then normalized to copies per gram of dry soil. Background levels of *nptII* were obtained

from control soils containing no added MON863 biomass and were subtracted from each corresponding experimental soil.

3.3 Results and Discussion

3.3.1 DNA Adsorption to Unaltered Clays

3.3.1.1 Clay Adsorption Capacity

The adsorption trends of calf thymus DNA was tested using untreated kaolinite and montmorillonite clays (Figures 4-5). For all tested materials, the adsorption capacities were reached when the adsorbed DNA concentrations (Q_e) failed to increase while the DNA concentration remaining in the supernatant (C_e) continued to increase suggesting that all available adsorption sites on the clays had been saturated. In general, calf thymus DNA adsorption occurred rapidly onto both clays and continued to increase. Adsorption rates started to decrease at approximately 200 μg total DNA/g dry kaolinite and 40 μg total DNA/g dry montmorillonite. The adsorption of dsDNA was favored over ssDNA for both clays, with a higher observed fraction of ssDNA in the supernatants remaining after equilibrium had been reached. A statistically significant difference also was detected between the adsorption capacities of untreated montmorillonite and kaolinite clays for calf thymus DNA (p=0.047).

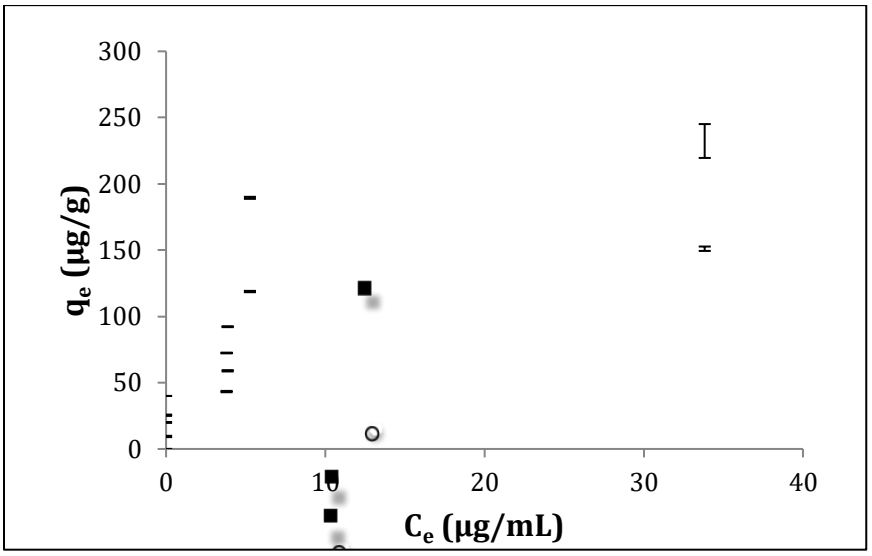

Figure 4: Adsorption of model Calf Thymus DNA to pure Kaolinite clay. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

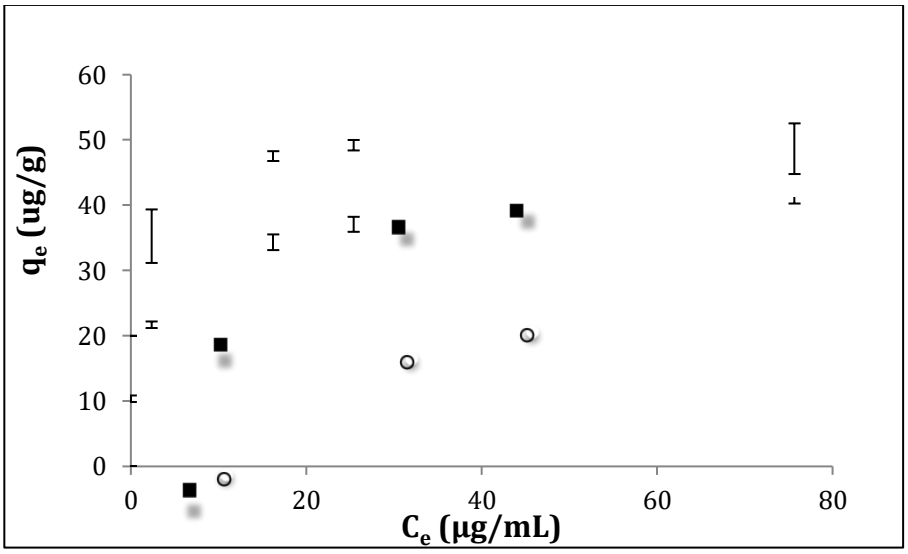

Figure 5: Adsorption of model Calf Thymus DNA to pure Montmorillonite clay. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

The adsorption of transgenic maize DNA was also tested (Figures 6-7). As with the calf thymus DNA isotherms, the adsorption of transgenic maize DNA onto pure kaolinite and montmorillonite started rapidly. Kaolinite adsorption trends continued to increase up to 40 μg total DNA/g kaolinite, where the adsorption rate began to lessen. Montmorillonite adsorption of DNA saturated at approximately 30 μg DNA/g montmorillonite.

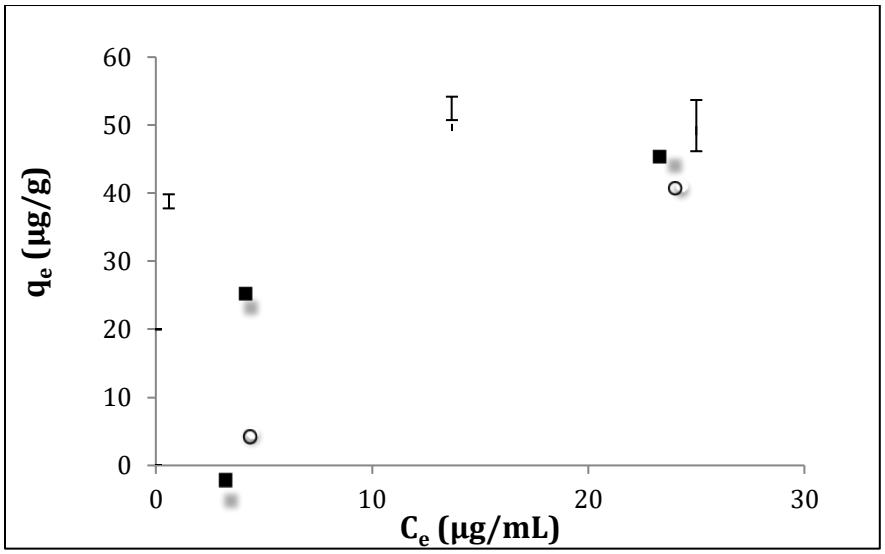

Figure 6: Adsorption of transgenic BT maize DNA to pure Kaolinite clay. Q_e: amount of DNA adsorbed (μg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (μg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

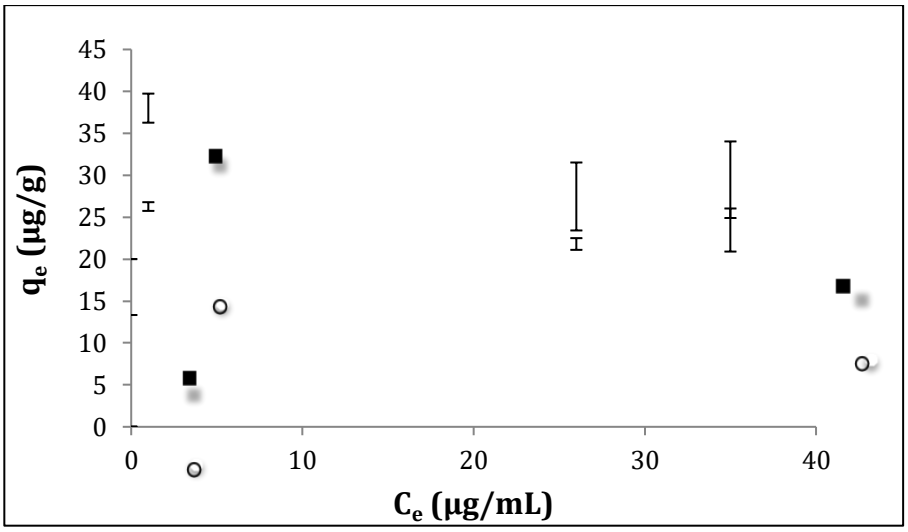

Figure 7: Adsorption of transgenic BT maize DNA to pure Montmorillonite clay. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

The adsorption trends of single and double stranded transgenic maize DNA were similar to those observed when testing the adsorption of calf thymus DNA, as there was a significant difference in the adsorption capacities of each mineral (p=0.043). Though it was only marginally statistically significant, there was also a slight difference in the adsorption of calf thymus DNA and transgenic maize DNA onto kaolinite particles (p=0.062), with more Calf Thymus DNA able to adsorb. However, no significant difference was measured between the adsorption of the two DNA sources on montmorillonite (p=0.34)

3.3.2 DNA Adsorption to Environmental Soils

3.3.2.1 Soil Adsorption Capacity

Transgenic maize DNA had limited ability to adsorb to the low clay soil, reaching a saturation plateau by the addition of an initial concentration of 5 μg/mL (Figure 8). Adsorption trends for ssDNA were similar to those of dsDNA for this soil matrix. Sandy loam soil adsorption increased in a nearly linear fashion and leveled off slowly. Clay loam soil had the most rapid adsorption of all tested environmental soil matrices, though it still was considerably slower than the rates associated with kaolinite and montmorillonite. Adsorption of transgenic maize DNA rapidly increased until it reached a plateau at more than 60 μg/g. For both sandy loam and clay loam soil matrices, adsorption of dsDNA was again favored over the adsorption of ssDNA (Figure 9-10).

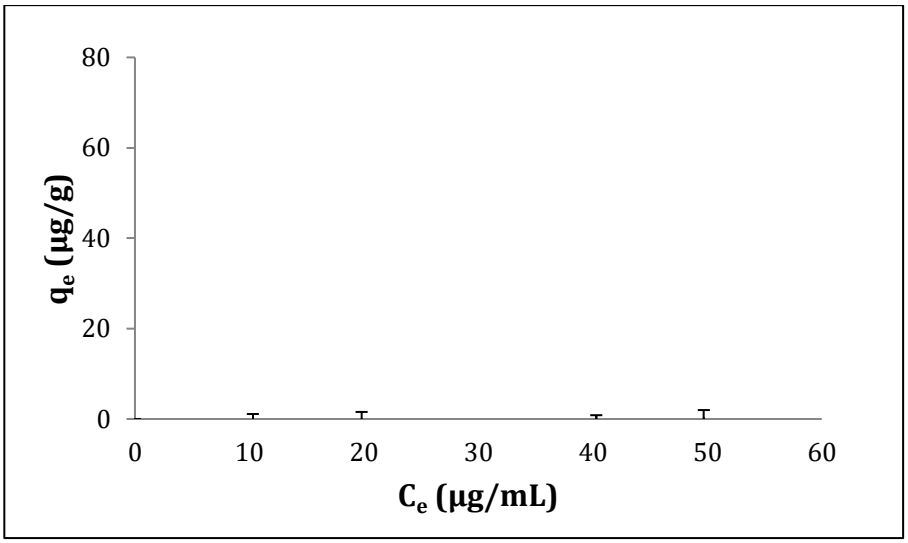

Figure 8: Adsorption of transgenic BT maize DNA to Low Clay Soil. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

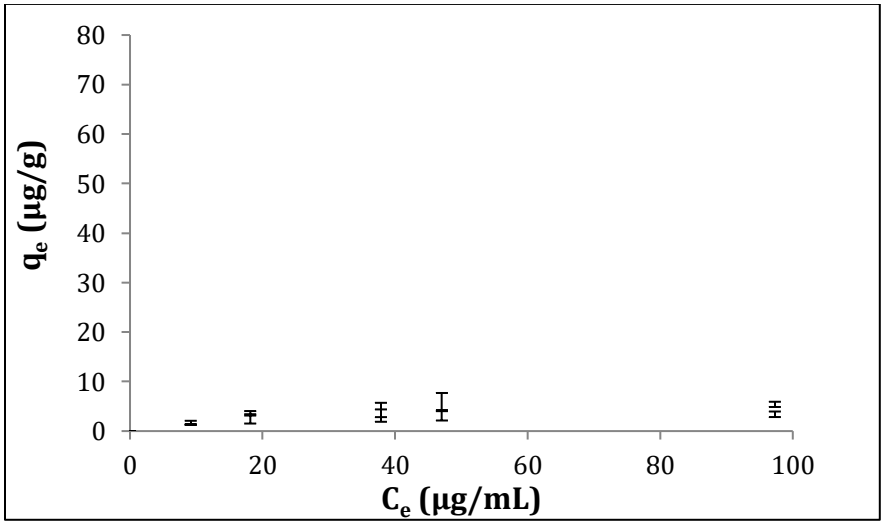

Figure 9: Adsorption of transgenic BT maize DNA to Sandy Loam Soil. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

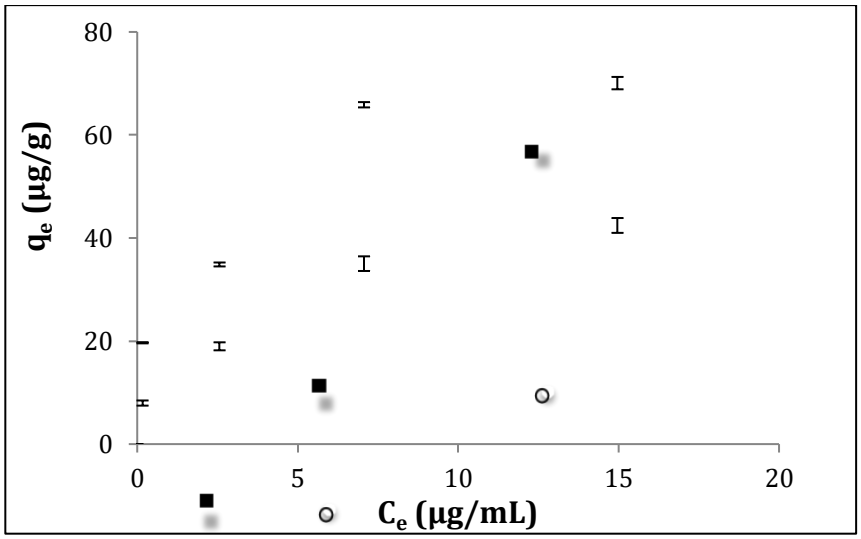

Figure 10: Adsorption of transgenic BT maize DNA to Clay Loam Soil. Q_e: amount of DNA adsorbed (µg DNA per g dry clay). C_e: Concentration of DNA remaining in solution after equilibrium had been reached (µg DNA per mL dH_2O). Square data points correspond to total DNA (dsDNA + ssDNA) measurements while circle data points correspond to dsDNA only.

The three tested environmental soils offer significantly different adsorption potentials for transgenic maize DNA, as determined though a one-way ANOVA. When analyzed individually using a paired t-test, there was a statistically significant difference between transgenic maize DNA adsorption to low clay soil and sandy loam soil (p=0.033), low clay and clay loam soils (p=0.032), and sandy loam and clay loam soils (p=0.021). The Langmuir adsorption constant (K_L) and maximum adsorption capacity (Q_{MAX}) values for all isotherms can be found in Table 4.

Table 4: Isotherm Properties for All Tested Soils

Soil Matrix	DNA Source	K_L (mL/μg)	Q_{MAX} (μg/g)	R^2
Kaolinite	Calf Thymus	0.0060	244	0.92
Montmorillonite	Calf Thymus	0.26	48.8	0.99
Kaolinite	Transgenic Maize	0.0061	55.6	0.99
Montmorillonite	Transgenic Maize	0.023	28.7	0.99
Minimal Clay	Transgenic Maize	8.6	0.805	0.05
Sandy Loam	Transgenic Maize	10	6.10	0.94
Clay Loam	Transgenic Maize	0.97	73.5	0.97

The Langmuir adsorption constant K_L was determined experimentally and calculated using the Langmuir isotherm equation. The R^2 value quantifies the correlation strength between the linearized C_e/Q_e vs. C_e values and the overall suitability of the Langmuir Isotherm model for each isotherm.

A small amount of DNA, approximately 0.8-3.1 μg/mL, was found to be present in control supernatants for both the low clay and sandy loam soils, and appears to be highly correlated to the amount of organic matter present in the soils (Figure 11).

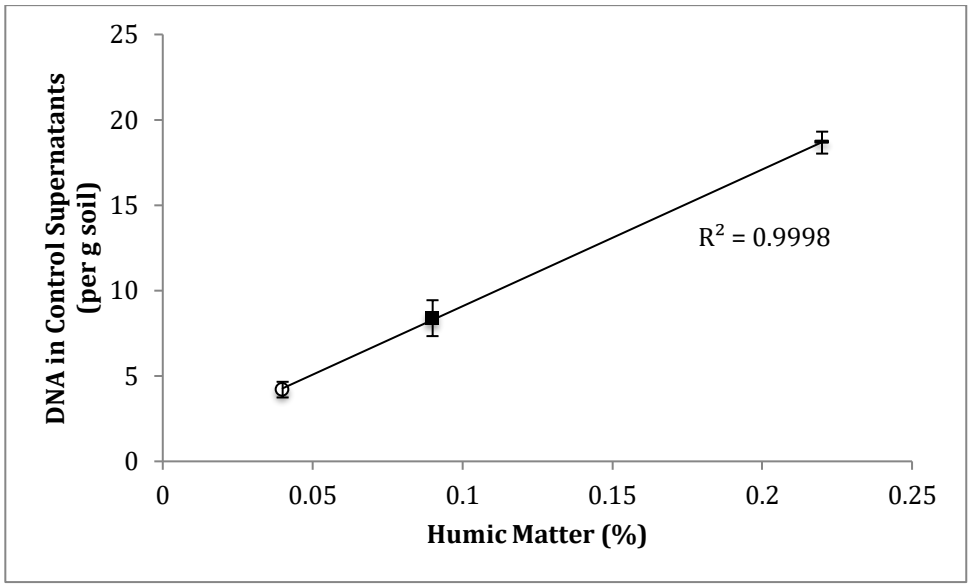

Figure 11: DNA found in the supernatants of control adsorption isotherms as a function of the percent humic matter (HM) in each soil type. Hollow circle data points correspond to sandy loam soils (0.04% HM), Black squares correspond to clay loam soils (0.09% HM), and dashed data points correspond to low clay soils (0.22% HM). DNA found in control supernatants appeared to be highly correlated to levels of humic matter (R^2=0.9938).

For these samples, the amount of DNA present in the control supernatants after equilibrium was subtracted from the amount of DNA present in experimental supernatants to get a final clay-adsorbed DNA for each treatment. The amount of DNA present in control supernatants was highly correlated to the amount of organic matter present in each soil (R^2=0.99), with sandy loam having the lowest concentration of DNA and sandy soil having the highest concentration of DNA.

While the correlation between high adsorptive capacity and higher soil clay content was expected, the difference in adsorption between kaolinite and montmorillonite

was not. This disparity is particularly noticeable when comparing the adsorption of calf thymus DNA to the two clay minerals. One explanation for this disparity is the difference in mineral structure between these two clays. Kaolinite ($Al_2Si_2O_5(OH)_4$) is a 1:1 clay mineral and is composed of one tetrahedral sheet bound to one octahedral sheet. The outer surface of the tetrahedral sheet is bound by O^{2-} atoms, while the outer surface of the octahedral sheet is bound by OH^- groups. The OH^- groups likely provide ample adsorption sites for the phosphate-based DNA backbone to become bound, either through O^{2-} substitution or the formation of an additional hydrogen bond between the kaolinite OH^- group and the PO_4^{2-} of the DNA [72]. Montmorillonite ($(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2 \cdot nH_2O$) on the other hand is a 2:1 clay mineral consisting of two tetrahedral sheets sandwiching one octahedral sheet. Unlike kaolinite, the top and bottom layers of this mineral consist entirely of O^{2-} atoms [73]. The mechanisms available for free DNA to adsorb onto montmorillonite is likely limited to O^{2-} substitution between the O^{2-} of the tetrahedral sheets and the PO_4^{2-} groups of the DNA. This process may be less energetically favorable, making adsorption more difficult. Similarly, transgenic maize DNA displayed a higher affinity for adsorbing to the surface of kaolinite over montmorillonite minerals. The relatively high percentage of GC content in maize genomic DNA may account for this discrepancy in adsorption capacity. The different C-G nucleotide structures may have a different binding affinity for kaolinite than A-T structures. Thus, this may have contributed to the discrepancies in adsorption capacities

for these different DNA sources, though further studies are necessary to determine the exact mechanisms responsible.

The systems tested appeared to have a slight preference for the adsorption of dsDNA over ssDNA, as ssDNA was much more common in the final isotherm supernatants containing unbound DNA. The relative proportion of ssDNA and dsDNA used in the isotherm experiments may be dependent upon the DNA extraction method used and, thus, may vary from method to method. However, because only one method was used to extract transgenic maize DNA, this ratio is expected to be consistent in our experiments. The structural nature of the bound DNA (i.e, ssDNA vs. dsDNA) may be important as it may affect the ability of bound DNA to be taken up by soil bacteria and functionally integrated into bacterial genomes. For example, dsDNA may be easier for bacteria to integrate into their genomic DNA through homologous recombination or a similar mechanism as ssDNA would have the added difficulty of finding a complementary region to adhere to. Others have shown that the uptake efficiency of ssDNA is 200 fold lower than that of dsDNA of a comparable length and sequence homology [74].

This may have downstream effects on transformation rates in natural systems. Transformation is conventionally defined as the uptake of foreign DNA in the environment by a bacterium and the integration of that DNA into the genome of the host bacterium the process of homologous recombination. More than 60 bacterial species

distributed among multiple taxonomic groups are known to display natural competence [75], and most of these species contain homologous recombination genes and pathways that are highly similar to one another [76]. One of the most well studied pathways is the SOS pathway and the RecA protein involved in this system. As dsDNA is taken up by competent bacteria and transported across the cell membrane through type IV pili and type II secretion systems [69, 77], nucleases degrade one strand thereby converting it into ssDNA. Once ssDNA reaches the cytoplasm, the DprA protein transports the ssDNA to RecA proteins within the cell [78]. RecA binds to ssDNA, forming a RecA-DNA complex, and searches for an area of high sequence similarity on the dsDNA of the bacterial genome. RecA then catalyzes several reactions to complete the process of recombination, integrating the foreign ssDNA into the bacterial genome. This process is slightly altered between Gram positive and Gram negative bacteria, as their cell membrane structures vary significantly. However, most competent bacteria share the same general RecA-dependent transformation process as well as highly similar proteins.

Because dsDNA is required to be converted into ssDNA within the cell before homologous recombination can occur, it would be logical to assume that ssDNA would be preferred for transformation events. However, Miao *et al*. [74] found that dsDNA is preferentially taken up over ssDNA; this may have evolved as a defense mechanism against single-stranded viral DNA. In addition, it is likely that most ssDNA in the environment is less stable and would be degraded by DNases and unable to be taken up

by competent bacteria. GC content and length are the most reliable predictors of stability of dsDNA in any environment. DNA fragments containing lower percentages (<45%) of GC sequences are less stable as dsDNA and are more likely to be degraded into ssDNA [79, 80]. Based on the presented data, dsDNA appears to also be preferentially adsorbed by several clay minerals, thereby increasing the amount of persistent DNA in the environment that competent bacteria may have access to. In addition, because maize DNA has an estimated GC content of 42.3%-55.4% (with any variations occurring because of the number of redundant transposon sequences within the maize genome) it is possible that transgenic maize DNA would be more stable in the environment, allowing for added protection from DNase degradation and more opportunity to find and adsorb onto clay particles.

3.3.2.2 *NptII* Persistence in Environmental Soils

The ability of *nptII* genes from decomposing MON863 biomass to persist in natural soils was assessed using three soils containing varying clay content, each at three different pH conditions that reflect a natural pH range of agricultural soils: 5.0-5.2, 6.0-6.2, and 7.0-7.2. The number of *nptII* genes in each soil type at each pH level was found to be relatively consistent at the first sampling time (Figure 12-14), ranging from 1.47 $x10^5$ to 2.59 $x10^4$ copies detected per gram of dry soil. All soil treatments exhibited the same downward trend in *nptII* detection over time, indicating that MON863 biomass decomposition and DNA breakdown was occurring. Significant levels of *nptII* genes

were found to persist in soils 18 weeks after biomass began decomposing. However, the degree of breakdown appeared to be specific to both soil type and soil pH.

The persistence trends of the *nptII* in Sandy Loams soils appeared similar to those observed in Low Clay Soils (Figure 12). Sandy Loam soils at pH 5, 6, and 7 were initially found to have 1.38 $x10^5$, 1.12 $x10^5$, and 9.70 $x10^4$ copies of *nptII* per gram soil, respectively. These levels significantly decreased at week 6, but rebounded at week 9 to 1.74 $x10^4$, 2.91 $x10^4$, and 5.51 $x10^5$ copies for soils at pH5 through 7, respectively. Detected copies of *nptII* decreased again at week 12 to 1.35 $x10^3$, 1.50 $x10^3$, and 7.17 $x10^4$ copies (pH 5 through 7, respectively) and decreased further at week 18 to be 3.04E $x10^2$, 1.88 $x10^3$, and 3.37 $x10^3$ copies (pH 5 through 7, respectively).

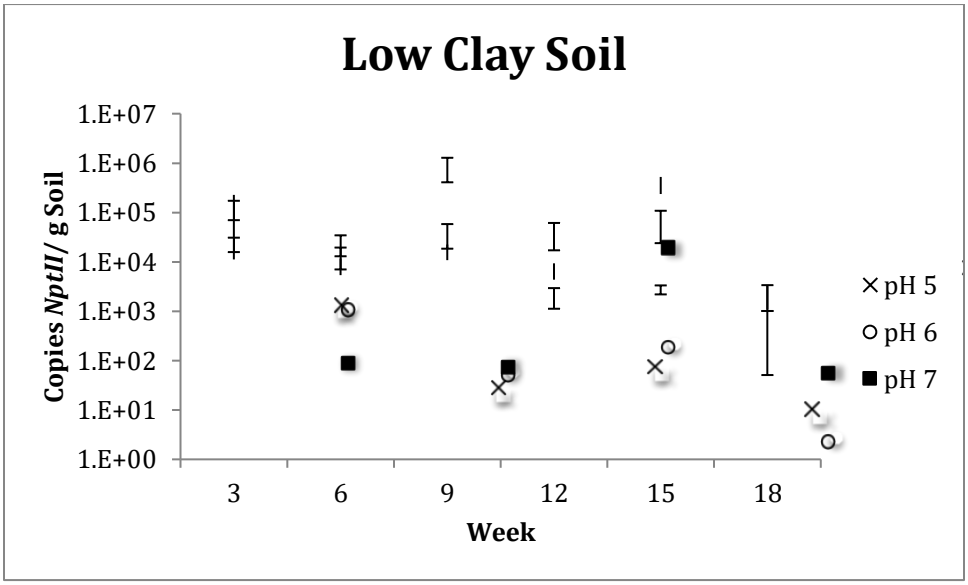

Figure 12: The persistence of *nptII* derived from decomposing MON863 seed material in Low Clay soil (25% sand/silt, 3% clay, 72% organic matter). Persistence was assessed at three different soil pH levels: pH=5.0-5.2 (black crosses), pH=6.0-6.2 (hollow circles), and pH=7.0-7.2 (black squares). Error is reported as standard deviation (n=9).

The persistence trends of the *nptII* in Sandy Loams soils appeared similar to those observed in Low Clay Soils (Figure 13). Sandy Loam soils at pH 5, 6, and 7 were initially found to have 1.38 $x10^5$, 1.12 $x10^5$, and 9.70 $x10^4$ copies of *nptII* per gram soil, respectively. These levels significantly decreased at week 6, but rebounded at week 9 to 1.74 $x10^4$, 2.91 $x10^4$, and 5.51 $x10^5$ copies for soils at pH5 through 7, respectively. Detected copies of *nptII* decreased again at week 12 to 1.35E $x10^3$, 1.50 $x10^3$, and 7.17 $x10^4$ copies (pH 5 through 7, respectively) and decreased further at week 18 to be 3.04 $x10^2$, 1.88 $x10^3$, and 3.37 $x10^3$ copies (pH 5 through 7, respectively).

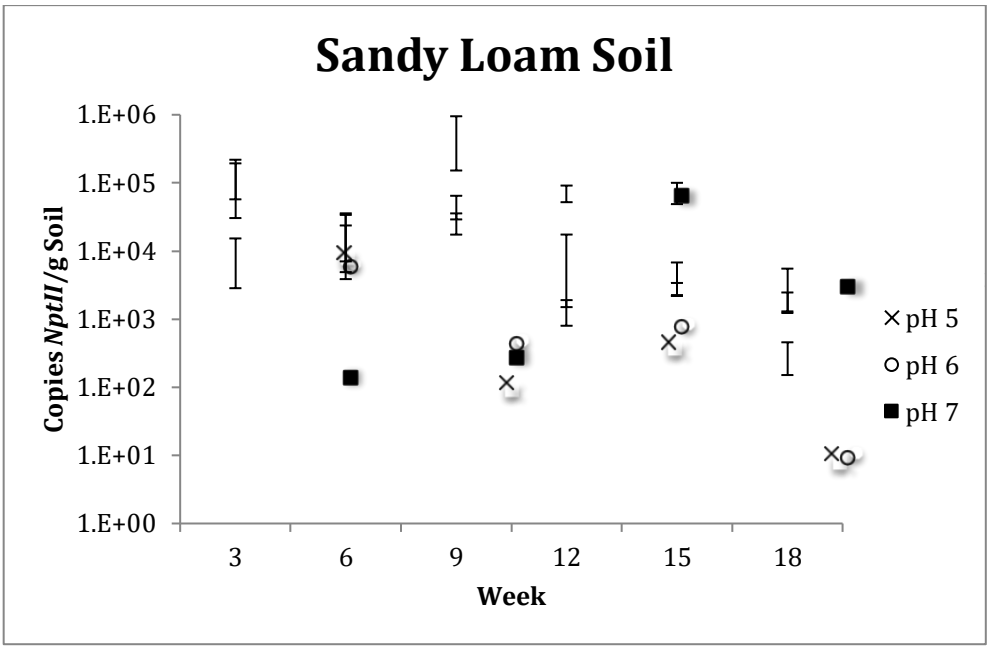

Figure 13: The persistence of *nptII* derived from decomposing MON863 seed material in Sandy Loam soil (75.8% sand/silt, 12.7% clay, 8.8% organic matter). Persistence was assessed at three different soil pH levels: pH=5.0-5.2 (black crosses), pH=6.0-6.2 (hollow circles), and pH=7.0-7.2 (black squares). Error is reported as standard deviation (n=9).

Clay loam soils at pH 5, 6, and 7 were initially found to have concentrations of *nptII* that were similar to both Low Clay and Sandy Loam soils: 9.43E x10^4, 2.16 x10^5, and 3.28 x10^4 copies of *nptII* per gram soil, respectively (Figure 14). *NptII* levels were similar in week 6 but decreased in weeks 9 and 12 to 4.61 x10^3, 1.93 x10^3, and 8.49 x10^3 copies for soil pH 5, 6, and 7. By week 18, the level of *nptII* gene in each gram of soil was found to be 2.63E x10^3, 1.46 x10^3, and 3.37 x10^3 copies for soil pH 5, 6, and 7, respectively.

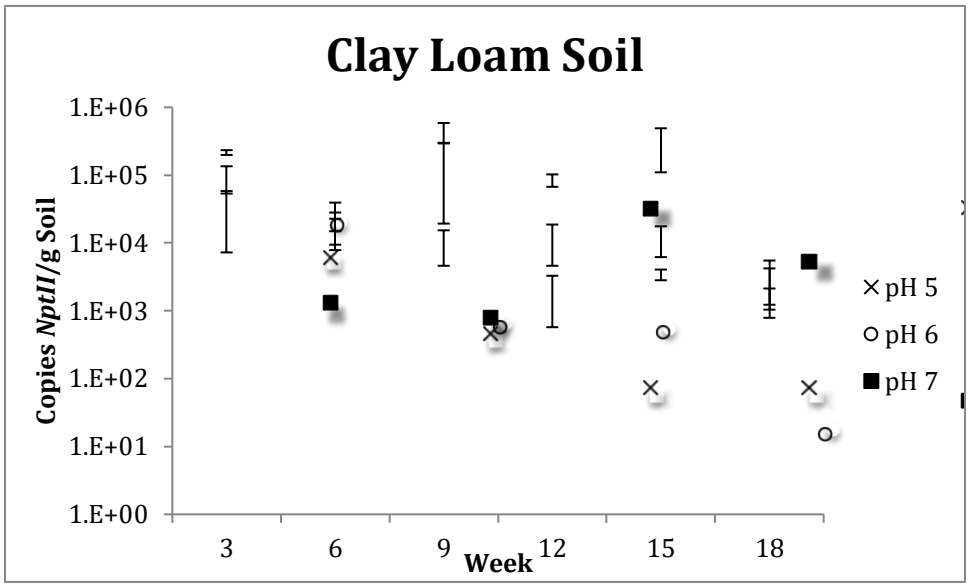

Figure 14: The persistence of *nptII* derived from decomposing MON863 seed material in Clay Loam (38% sand/silt, 39% clay, 22.4% organic matter). Persistence was assessed at three different soil pH levels: pH=5.0-5.2 (black crosses), pH=6.0-6.2 (hollow circles), and pH=7.0-7.2 (black squares). Error is reported as standard deviation (n=9).

The persistence of *nptII* genes appeared to be more dependent upon the soil pH than upon the clay content of the soil used. The rates of *nptII* decay, or disappearance, in each reactor is shown in Table 5. Decay rates were lowest for Low Clay and Clay Loam Soils at pH 5, and were found to be approximately -822.1 copies per week and -624.4 copies per week, respectively. These negative decay rates reflect a net increase in the detected number of *nptII* genes per gram soil in batch reactors over the course of 18 weeks. However, the net increases in *nptII* genes under either condition were not found to be statistically significant. This indicates that the net negative decay rate does not reflect a true increase in *nptII* copy number, but rather the lack of a true net decrease. This implies that free DNA released from decomposing MON863 biomass is better able to persist in

soils matrices at pH 5. The rates of decay between sandy loam soils at pH 5 and 6 were not statistically significant, though pH 6 had the lowest rate of decay among sandy loam soils.

Table 5: Decay Rates of Free DNA Released from Decomposing MON863 Biomass in Three Environmental Soils. Negative rates of decay are denoted by (*) and reflect a net increase in the detected levels of *nptII* across 18 weeks of sampling.

Soil Type	pH	Rate of Decay (copies/week)
Low Clay	5	-822.1*
Low Clay	6	763.4
Low Clay	7	4578.7
Sandy Loam	5	860.8
Sandy Loam	6	797.1
Sandy Loam	7	990.6
Clay Loam	5	-624.4*
Clay Loam	6	1776
Clay Loam	7	659.889

Overall, pH was found to have a moderately significant impact on the ability of *nptII* to persist in soils (p=0.051) was determined by multiple linear aggression analysis to significantly persist. Soil clay content was found to have a less significant effect (p=0.56). Among tested soil pH ranges, pH 5 had a moderately significant ability to sequester free DNA (p=0.052) compared to pH 6 (p=0.085) and pH 7 (p=0.29). This is likely due to increased protonation of soil mineral surfaces because of the abundance of free H^+ ions. The negatively charged phosphate backbone of ssDNA and dsDNA would have an

increased ability to bind to soil and clay particles with an overall cationic surface charge, as opposed to an anionic surface charge. In addition, the negative charges associated with these phosphate groups is likely decreased in more acidic environments (i.e.—pH 5) as the isoelectric point of DNA is approximated as 5 [41]. This would make adsorption to nearby soil particles easier in acidic soil environments. Increased adsorption to soils particles also allows for protection for DNases, as mentioned above, leading to the increased persistence seen in pH 5 soils.

NptII levels at weeks 9 and 15 increased relative to levels at weeks 6 and 12 for all samples. It is possible that this occurred as a result of compounded errors associated with soil sampling, DNA extraction efficiency, and minor natural variations that often occur among separate qPCR assays. This is supported by the standard deviations for each data point, which are typically small and therefore do not indicate significant errors in data associated with *nptII* quantification. Many researchers have noted in past research that tracking the *nptII* gene from GM crops is a difficult task. The main obstacles to tracking *nptII* appeared to be non-specific primer-binding and a high level of background "noise" in environmental samples. To combat the first issue, a Taqman®™ Probe-based qPCR assay was selected over a SYBR Green chemistry assay because the probe allows us to make a more confident assertion that any non-specific primer binding was minimal or non-existent.

The acquisition of antibiotic resistance occurs naturally in the environment as a result of the horizontal gene transfer of ARGs. Selective pressures in the form of

background concentrations of several antibiotics are known to be present in the environment as a result of antibiotic-producing fungi or the disposal of pharmaceuticals, which act as a selective pressure to induce the uptake, transfer, and maintenance of ARGs by sensitive bacterial communities. However, there are several additional avenues through which bacterial communities may have gained access to ARGs and developed antibiotic resistance. Such avenues include the misuse of antibiotic prescriptions for illnesses, the widespread use of concentrated animal feeding operations (CAFOs), and historical use of ARGs as selection markers in some lines of GM crops. Our results show that transgenic maize DNA is comparable to previous model DNA systems in terms of its adsorption capacity for clay minerals and soils. This adsorbed DNA could potentially persist in soils, especially in clay matrices commonly used in agriculture. It should be noted that we did not investigate the integrity of the maize DNA after the DNA extraction protocol to determine if its incorporation into a bacterial genome led to the acquisition of the antibiotic resistant phenotype. We also could not control for the size or GC composition of the extracted DNA fragments in our experiments. However, we determined through end-point PCR that some fragment of the *bla* ARG contained within the transgenic maize line we chose to investigate was present in the final DNA elution, which was then used to construct the adsorption isotherms. In conclusion, the overall adsorption capacities of montmorillonite and kaolinite appear to be significantly lower than the values cited in previous literature by one to two orders of magnitude. Despite

these differences, the amount of transgenic maize DNA that can potentially adhere to soil matrices in the environment is still significant, approximately 70 μg of DNA per dry gram of soil. We would therefore conclude that the risk of microbial exposure to ARGs contained within transgenic maize lines, and any uptake thereof, should be investigated more thoroughly.

4. Characterization of the Microbiomes Associated with Maturing Conventional Maize

The following chapter describes the research used to address Objective II.

4.1 Introduction

Transgenic crops have become a ubiquitous component of the agricultural industry in the United States over the past 30 years. They typically exhibit desirable traits such as drought tolerance, herbicide resistance, or pest resistance and have been incorporated into many markets, notably as a source of biofuels, human food, and animal feed. The most abundant traits based on total planted acreage include herbicide resistance ("HT") and pest resistance ("BT"), which have been incorporated into many crops such as soybeans, cotton, and maize [27]. These transgenic varieties have quickly replaced conventional varieties as the dominant cash crops since they were first planted (Figure 2). Cultivation of BT and HT maize increased dramatically in the past 20 years, accounting for an average of only 2% of total maize acreage in 1996 but increasing to 68.5% of total acreage by 2011. HT soybeans, BT soybeans, HT cotton, and BT cotton follow a similar pattern of increase in cultivation over the same time period [27].

In the current work, we focus on characterizing the microbiomes naturally associated with maturing conventional maize. This knowledge would provide baseline information and assist in closing current knowledge gaps regarding how the microbiomes associated with developing maize are formed. In the present study, we carry out our experiments under environmentally relevant but controlled conditions (i.e., cultivating

crops in greenhouses) to help minimize confounding variables within the system. The main objective of this work was to characterize the bacterial microbiomes associated with conventional maize and determine what community shifts, if any, were significantly associated with major maize growth stages.

4.2 Material and Methods

4.2.1 Maize Microbiome Characterization

4.2.1.1 Soil Conditions

Maize cultivars were grown under controlled greenhouse conditions in order to characterize resulting microbial communities found in the bulk soil, rhizosphere soil, and internal stalk tissue. Samples were taken every two weeks for eight weeks to correspond with important growth stages of maize (Figure 15) [90].

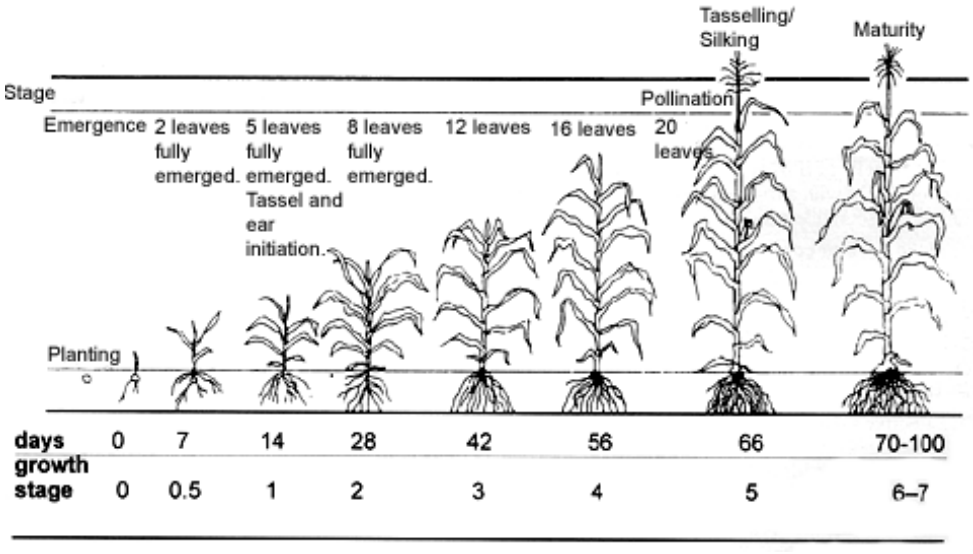

Figure 15: Development stages of *Zea mays* during the first 100 days of growth. Soil and endophyte samples will be collected at growth stages 0, 0.5, 1, 2, 3, and 4 for microbial community analysis. Adapted from www.dpi.nsw.gov.au.

Soil from a fallow maize field was obtained with permission from Duke University and homogenized before a sample was sent to the North Carolina Department of Agriculture and Consumer Sciences lab for analysis. Based on their recommendations 140 lb/acre Nitrogen as Urea fertilizer (46-0-0, Hummert International) and 20 lb/acre Sulfur as $CaSO_4$ was applied and the soil was homogenized for even distribution of the amendments. As soil pH was measured at 6.4, a suitable pH level for growing maize, no pH adjustment was made. Amended soil was added at a consistent depth to 8 L plastic planters and placed in the Duke University Greenhouse.

Seeds from a single maize cultivar were surface sterilized in a 3% H_2O_2 solution for 15 min before being transferred to sterile petri dishes containing damp germination paper. Seeds were allowed to germinate away from direct sunlight at room temperature for 72 h before planting. Individual seedlings were planted in sacrificial pots, approximately two inches from the soil surface. Planters were watered immediately before and after planting, and as needed for the duration of the experiment.

4.2.1.2 Maize Sampling Methods

Samples were obtained from sacrificial planters of maize in triplicate every two weeks for eight weeks. From each planter, soil samples were taken in triplicate from bulk soil and rhizosphere soil using a soil corer of approximately 1 in. diameter. Samples were homogenized and stored at -80°C until DNA was extracted. To collect endophyte samples,

maize stalks were cut down, surface sterilized by washing with tap water for 30 s, dilute NaClO for 30 s, and dH_2O for 30s. Stalks were then split, and maize pith biomass was excised every 12 in. using a sterile razor blade. These samples were homogenized using a mortar and pestle and stored at -80°C until DNA could be extracted.

Bacterial DNA from rhizosphere and bulk soil samples was extracted in triplicate using the PowerLyzer PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer instructions. Homogenized maize pith biomass was suspended in 1X PBS in a sterile 1.5 mL centrifuge tube and shaken on a horizontal vortex for 45 min to remove bacteria from the plant material. Maize biomass was then removed from the tube and the tube was centrifuged at 13,000x g for 5 min to obtain a cell pellet. The supernatant was decanted, and DNA was extracted from the resulting pellet using the UltraClean Microbial DNA kit (MoBio Laboratories, Inc.), according to kit manufacturer instructions. All extracted DNA was quantified using a Qubit 2.0 fluorometer (Waltham, MA) and stored at -20°C until further analysis.

4.2.1.3 Illumina Miseq Sequencing and Data Analysis

The DNA extracted from bulk soil bacteria, rhizosphere soil bacteria, and endophytes was then prepared for metagenomic sequencing. *16S* rRNA primer sequences 314F and 805R (5'-CCTACGGGNBGCASCAG-3' and 5'GACTACNVGGGTATCTAACC-3', respectively) were taken from Takahashi et al. [91]. These primers span the V3-V4 *16S* rRNA regions and have increased degeneracy to target both bacteria and archaea.

Samples were prepared according to the "*16S* Metagenomic Sequencing Library Preparation" workflow outlined by Illumina (Illumina Inc., San Diego, CA), with the following exception: The initial amplicon PCR was performed in triplicate for each sample. These triplicate PCR reactions were then pooled (75 μL total), and 25 μL of that pool was used for all subsequent steps. Each sample was dual-indexed with unique nucleotide barcodes sequences based on Nextera XT N7XX and N5XX chemistry. Samples were normalized, pooled, and run on a paired-end MiSeq platform using V3 sequencing technology at the Duke University Sequencing facility (Durham, NC).

The metagenomic data for each sample were demultiplexed, trimmed, and filtered for quality using the Illumina BaseSpace App (Illumina Inc., San Diego, CA). The resulting data were then imported into the *16S* Metagenomics App, where taxonomy was assigned for each operational taxonomical unit (OTU) using the GreenGenes May 2013 database. The resulting taxonomic tables were imported into R, PAST3, and GraphPad Prism (version 7) statistical programs for bioinformatics analysis [92]. Due to elevated nucleotide sequence similarities between Cyanobacteria and chloroplast, additional analysis was required to verify the identity of Cyanobacteria in maize endophyte samples [93, 94]. Raw *16S* sequences were obtained from FASTQ files of each endophyte sample and imported into BLAST [95], and aligned with Cyanobacteria and chloroplast DNA to determine sequence overlap. *16S* sequence identity was assigned if sequences were at least 97% similar.

4.2.2.3 Statistical Analysis

The resulting data was normalized to gene copies per gram of soil. The copy number of *bla* transgenes was also compared the copy number of *16S* genes per gram of soil. Significant differences in *bla* expression were determined via ANOVA and multiple regression analysis.

4.3 Results and Discussion

4.3.1 Diversity of Soil and Endophyte Communities

The maize microbiomes were characterized by sampling maturing sacrificial corn cultivars grown under controlled conditions. In addition to minimizing the potential confounding effects of extraneous variables, greenhouse growing conditions were chosen over field conditions for two main reasons: 1) Risks associated with crop loss from herbivory or unfavorable growing conditions were too great, given the limited supply of maize seeds; 2) Greenhouse temperatures reflected natural growing conditions for both maize and mesophilic soil bacteria. As mesophilic bacteria make up the bulk of soil bacteria, greenhouse conditions were not likely to dramatically shift bacterial populations in the proposed sampling areas [97].

More than 15,882,620 high quality sequences were identified and classified across 90 soil and maize tissue samples (30 samples sequenced in triplicate). Taxonomy (≥97% similarity) was assigned to sequences in each sample using the GreenGenes 2013 database. A total of nine replicate samples were excluded from further analysis; three failed to

properly sequence and six did not have satisfactory rarefaction curves, indicating species diversity was not adequately captured. Bulk soil and rhizosphere soil communities were dominated by 29 and 30 phyla, respectively, across all sampling times (Figure 50). Core phyla were largely similar among bulk soils associated with maize and were dominated by *Proteobacteria* (60.3% ± 2.31%), *Firmicutes* (9.05% ± 2.31%), *Bacteroidetes* (9.53% ± 1.42%), *and Actinobacteria* (5.57% ± 0.949) across all sampling times. *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were found to offer the greatest contributions to dissimilarities among bulk soil and rhizosphere soil bacterial communities at the phylum level (Figure 16). Overall, bulk soil prokaryotic community structure at the phylum level did not significantly shift from those in the initial bulk soil prior to maize planting (p=0.73), as determined by an analysis of similarity (ANOSIM) [98], and were unaffected by time (p=0.16). Rhizosphere soils were dominated by similar phyla: *Proteobacteria* (51.71% ± 2.03%), *Firmicutes* (14.14% ± 3.12%), *Actinobacteria* (7.07% ± 0.403%), and *Bacteroidetes* (5.75% ± 1.07%). Additionally, rhizosphere soil phyla did not shift significantly over 56 days (p=0.16), determined via ANOSIM. Observed rhizosphere phyla were marginally influenced by time (p=0.065).

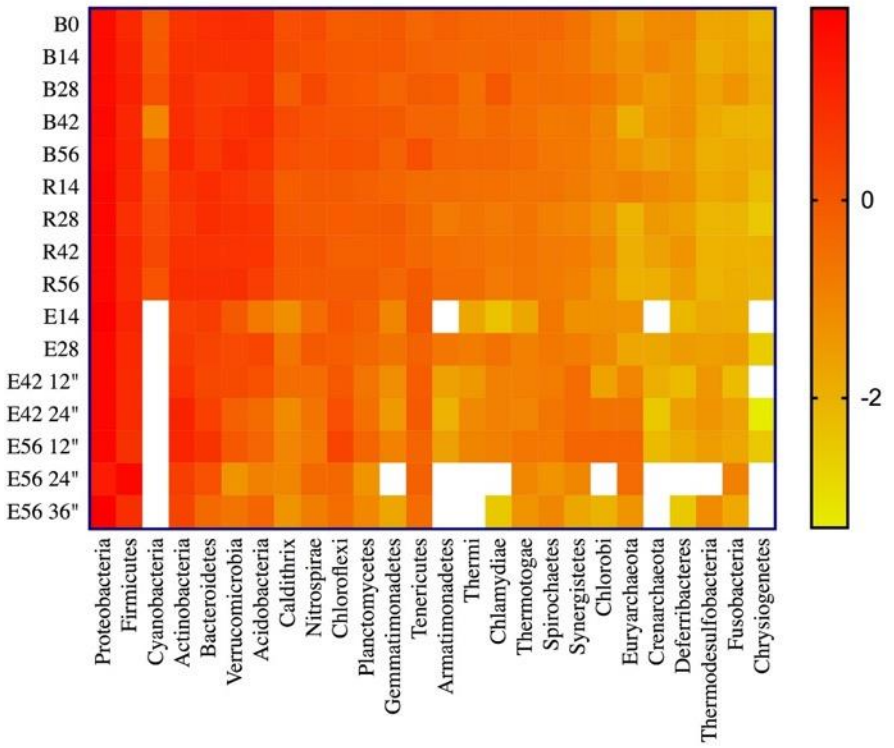

Figure 16: Heatmap of log-transformed phylum-level taxonomic data with unsupervised clustering for endophyte, bulk soil, and rhizosphere soil communities associated with conventional maize during the first 56 days of maize cultivation. Yellow squares indicate weak contributors to sample dissimilarity, while red squares indicate strong contributors to sample dissimilarity. White squares refer to taxa that were not detected in a given sample. Each sample is defined by the following notation: (1) First letters in each sample refer to sample type—Bulk Soil ("B"), Rhizosphere Soil ("R"), or Endophyte ("E"); (2) Numbers referred to the sampling date ('0', '14', '28', '42', '56') or endophyte sampling depth ('12" ', '24" ', '36" ').

Endophyte communities were dominated by 27 phyla. These communities were more variable relative to time but were largely dominated by *Proteobacteria* (54.5% ± 16.1%), *Firmicutes* (18.3% ± 18.9%), *Actinobacteria* (9.91% ± 4.40%), *and Bacteroidetes* (4.48% ± 2.48%) across all sampling times. These phyla also offered the greatest contributions to overall diversity within endophyte communities. Observed phyla did not significantly

shift across 56 days of growth, determined via ANOSIM (p=0.31). No statistically significant differences between endophyte phyla were observed with respect to sampling depth (p=0.24). However, sampling time had a significant impact on endophyte structure at the phylum level (p=0.042). All OTUs classified as *Cyanobacteria* were further investigated within endophyte samples due to significant doubts regarding their true sequence identity. A significant fraction of these OTU sequences were more similar to chloroplast 16S rRNA than *Cyanobacteria* 16S rRNA—more than 90% of raw sequences in some samples. As chloroplasts are evolutionarily derived from bacteria, this high degree of sequence overlap is to be expected [93, 99-101]. However, because these sequences are likely not representative of the true endophytic maize microbiome, these OTUs were excluded from further analysis.

The relative abundance of prokaryotic classes was also characterized in all samples. Sixty-one core bacterial classes were identified in bulk soils associated growing maize. *Alphaproteobacteria* were the most abundant observed class (21.6% ± 2.08%), followed by *Betaproteobacteria* (10.8% ± 1.46%), *Gammaproteobacteria* (9.53% ± 1.24%), *Clostridia* (9.06% ± 2.08%), *Deltaproteobacteria* (7.15% ± 1.34%), and *Bacilli* (4.91% ± 1.64%), averaged across all sampling times (Figure 17). These classes accounted for more than 62% of observed taxa. Alpha diversity was generally high among both bulk soils and rhizosphere soils (Figure 51). Rhizosphere soils were dominated by 59 bacterial classes, many of which overlapped with those observed in bulk soils. *Alphaproteobacteria* were

again the most abundant across all sampling times (20.3% ± 3.26%), followed by *Betaproteobacteria* (20.3% ± 6.79%), *Gammaproteobacteria* (11.6% ± 3.18%), *Sphingobacteriia* (6.39% ± 0.53%), *Clostridia* (5.82% ± 1.82%), and *Actinobacteria* (5.53% ± 0.96%). Similar to those of bulk soils, these classes accounted for more than 69% of observed taxa in rhizosphere soils. Internal maize endophyte communities were most distinct from bulk and rhizosphere soil communities. Alpha-diversity was generally lower among these samples (Figure 51), which were dominated by 58 classes of bacteria. *Alphaproteobacteria* (33.6% ± 22.1%), *Bacilli* (16.9% ± 24.7%), *Clostridia* (11.4% ± 7.29%), *Betaproteobacteria* (8.95% ± 5.70%), *Actinobacteria* (7.34% ± 5.67%), and *Gammaproteobacteria* (5.04% ± 3.84%) dominated these communities. These taxa comprised more than 83% of all bacterial classes detected in maize tissue. A marginally significant difference was detected in the relative abundance of *Nitrospira* associated with maize tissues (0.44% ± 0.29%) (p=0.079). Endophyte communities were more dynamic than either bulk or rhizosphere soils, which is reflected in the standard deviation associated with each taxa. However, the standard deviations for each set of sample triplicate are much smaller. All classes belonging to the phylum *Cyanobacteria* were excluded from further analysis in maize endophyte samples.

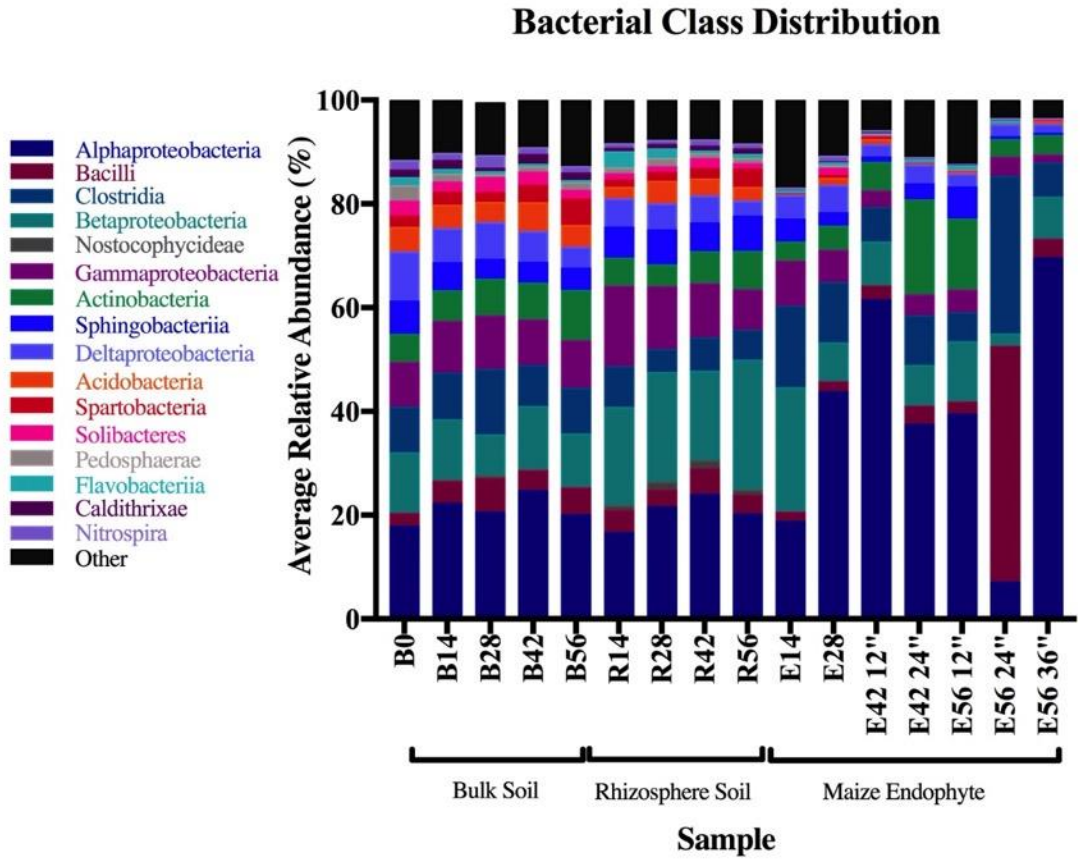

Figure 17: Relative abundance of prokaryotic classes distributed among bulk soil, rhizosphere soil, and corn endophyte communities. Each sample is defined by the following notation: (1) First letters in each sample refer to the sample type—Bulk Soil ("B"), Rhizosphere Soil ("R"), or Endophyte ("E"); (2) Numbers referred to the sampling date ('0', '14', '28', '42', '56') or endophyte sampling depth ('12" ', '24" ', '36" ').

Bulk soil nitrogen cycling communities did not significantly shift during 56 days of maize cultivation, determined via ANOSIM. This was confirmed through multiple linear regression analysis, which showed that neither sampling date (p=0.16) nor corn type (p=0.23) significantly affected prokaryote community composition. This result was not unexpected, as bulk soil prokaryotic communities grown under stable greenhouse

conditions are unlikely to be exposed to any significant selection pressure. Endophyte communities were moderately influenced by the growth stage of the maize (p=0.099) and the relative location of the endophytes within the maize stalk (p=0.056). Rhizosphere communities were more significantly influenced by the growth stage of maize cultivars (p=0.00080). These communities shifted with maize development stage, likely in response to specific maize root exudates and chemical signaling [102].

The community structures of nitrogen cycling bacteria were also characterized at the genus level across all bulk and rhizosphere soil samples associated with maturing maize (Figure 18). Nitrogen cycling bacteria are essential for maintaining the necessary balance of nitrogen chemical species in agricultural soils as well as larger ecological environments. In agricultural environments, nitrifying bacteria are primarily responsible for converting ammonia (NH_3) or ammonium (NH_4^+) to nitrate (NO_2^-) via the oxidation of nitrite (NO_2^-), while denitrifiers convert nitrite to gaseous nitrogen (N_2). Nitrogen fixers fix atmospheric nitrogen into usable inorganic compounds necessary for plant growth. Each type of nitrogen cycling bacteria was identified within each soil and maize sample. Total nitrogen cycling genera—nitrifiers, denitirifers, and nitrogen fixers—in soils accounted for a maximum of 7.12% ± 0.647% and 6.74% ± 0.608% in bulk and rhizosphere soils, respectively (Figure 18A). *Azospirillum* (4.02% ± 0.469%), *Clostridium* (2.78% ± 0.463%), and *Nitrospira* (2.75% ± 0.488%) genera were most abundant in bulk soils associated with conventional maize across all time points. No significant differences were

observed in total nitrogen cycler presence or abundance across 56 days of maize cultivation. *Denitrobacter* bacteria were also noticeably abundant in bulk and rhizosphere soils, though these bacteria were less stable across samples and appeared most abundant in bulk soils at days 14, 28, and 42. *Azospirillum* (3.02% ± 0.845%), *Clostridium* (1.01% ± 0.702%), and *Nitrospira* (0.685% ± 0.211%) were the most abundant nitrogen cyclers in rhizosphere soils. Nitrifying bacteria were most abundantly found in bulk and rhizosphere soils of maize, with a small percentage found in internal endophyte communities (Figure 18B). *Nitrospira* (1.32% ± 0.702), *Nitrosococcus* (0.144% ± 0.039%), and *Nitrosovibrio* (0.113% ± 0.039%) genera dominated bulk soil communities consistently among all time points. Nitrifying bacteria steadily increased in abundance in the rhizosphere soil across 42 days of maize growth, before decreasing slightly at day 56. Nitrifying bacteria were overall less abundant among endophytes, but *Azospirillum* remained the dominant genera among these samples across all time points. Nitrogen fixing bacteria comprised the largest fraction of total nitrogen cyclers in bulk soils, rhizosphere soils, and endophyte tissues (Figure 18C). Nitrogen fixer abundance and structure remained consistent in bulk soils associated with maize across 56 days of maize growth. These communities were dominated by *Azospirillum*, *Clostridium*, and *Frankia* genera. Nitrogen fixing bacteria in rhizosphere soils were more dynamic, though these communities were still dominated by *Azospirillum*, *Clostridium*, and *Rhizobium*. Shifts in nitrogen fixers correlated strongly with maize growth stage (p=0.0076. Nitrogen fixing

bacteria were noticeably different in endophyte communities (Figure 18D). *Clostridium* (4.12% ± 9.87%) was by far the most abundant genera among these samples, followed by *Azospirillum* (0.269% ± 0.414%). *Rhizobium* and *Frankia* were consistently detected within these samples, but at lower levels than either *Clostridium* or *Azospirillum*. Average relative abundance of these genera was strongly correlated with sampling date and depth.

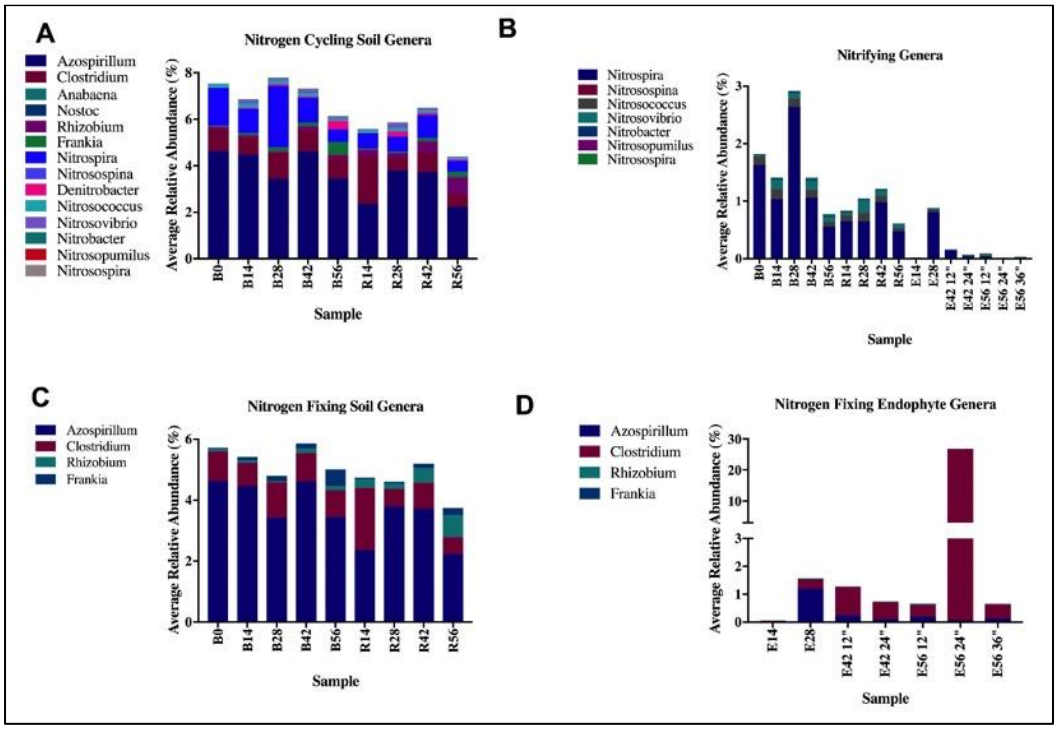

Figure 18: A) Relative abundance of nitrogen cycling genera in bulks soils and rhizosphere soils associated with conventional maize (nitrogen fixers, nitrifiers, and denitrifiers). B) Relative abundance of nitrifying bacteria in soils and endophytes associated with conventional maize. C) Relative abundance of nitrogen fixing bacteria in soils associated with conventional maize. D) Relative abundance of nitrogen fixing bacteria associated with conventional maize endophytes. Each sample is defined by the following notation: (1) First letters in each sample refer to the sample type—Bulk

Soil ("B"), Rhizosphere Soil ("R"), or Endophyte ("E"); (2) Numbers referred to the sampling date ('0', '14', '28', '42', '56') or endophyte sampling depth ('12" ', '24" ', '36" ').

Azospirillum, a nitrogen-fixing bacterium previously recognized to be closely associated with crops and grasses [103], was identified as the most abundant nitrogen cycler across all samples and comprised approximately 4.0% ± 0.49% and 2.8% ± 0.84% of total prokaryotes across bulk and rhizosphere soils, respectively. *Azospirillum* was found to be the greatest contributor to dissimilarities among nitrogen cycling bacteria, as confirmed through a correlation analysis (Figure 19). *Nitrospira* and *Clostridium* were also dominant in bulk and rhizosphere soils, constituting 0.9% and 0.8%, and 1.2% and 0.6% of total prokaryotes, respectively. *Nitrospira* was found to have second-greatest effect on dissimilarities among nitrogen cycling bacteria. *Rhizobium*, *Frankia*, and *Denitrobacter* genera were also found to have significant impacts on dissimilarities among soils, though this was not observed in every sample.

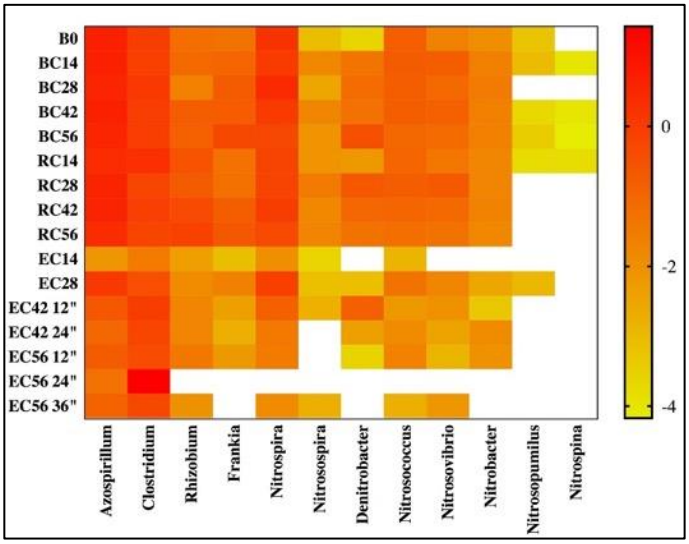

Figure 19: Heatmap of log transformed relative abundance counts with corresponding histogram of nitrogen cyclers in bulk soil, and rhizosphere soil communities associated with conventional maize during the first 56 days of maize cultivation. Yellow squares indicate weak contributors to sample dissimilarity, while red squares indicate strong contributors to sample dissimilarity. White squares refer to taxa that were not detected in a given sample. Each sample is defined by the following notation: (1) First letters in each sample refer to sample type—Bulk Soil ("B"), Rhizosphere Soil ("R"), or Endophyte ("E"); (2) Numbers referred to the sampling date ('0', '14', '28', '42', '56') or endophyte sampling depth ('12" ', '24"', '36" ').

As noted above, a small significant difference in community structure was seen in rhizosphere soils at day 56 (p=0.065). Differences in diversity were visually confirmed with Non-Metric Dimensional Scaling analysis (Figure 20). Most bulk soil and rhizosphere communities at the genus level were similar overall and formed a single cluster. Dissimilar samples included conventional maize rhizosphere soil at days 14 and 56.

In conclusion, this work presents a full characterization of the community structures of bacterial endophyte and rhizosphere communities associated with

conventional maize across 56 days of maize development. Though no significant differences among phyla were detected, the maize microbiome characterization presented in this dissertation may advance current research in related fields, such as the use of maize for bioethanol production or phytoremediation.

5. Track GMO-Derived Transgenes in Wastewater Treatment Plants Across the US and France

The following chapter describes the research used to address Objective III. The presented work is in preparation and will be submitted for review to the journal of *Water Research* in May 2017.

5.1 Introduction

5.1.1 Transgenic Crop Constructs

Transgenic crops have become increasingly popular relative to their conventional counterparts since their initial development in the 1980s. As of 2012, nine crops have been approved for cultivation and human consumption in the United States, including soybean, maize, cotton, canola, potatoes, alfalfa, papaya, sugar beets, squash and tomatoes [109]. Many individual lines of each of these crops containing one or more genetically engineered traits (e.g., herbicide tolerance, pest-resistance, drought tolerance, and virus resistance) have been approved, amounting to hundreds of lines released for cultivation in the United States alone. The most popular traits are pest-resistance (BT lines)

herbicide-tolerance (HT lines), or stacked pest- and herbicide-resistance. More than 130 lines of transgenic crops have also been documented to contain one or more ARGs, including β-lactamase and neomycin resistance genes [20]. These genes have traditionally been used as selection markers during the plant cell transformation process, and are most commonly found in first- and second-generation transgenic crops [19]. This practice has fallen out of favor in recent years due to the controversies surrounding the use of ARGs as selection markers and their potential to contribute to the rise in environmental antibiotic resistance [14-16, 109].

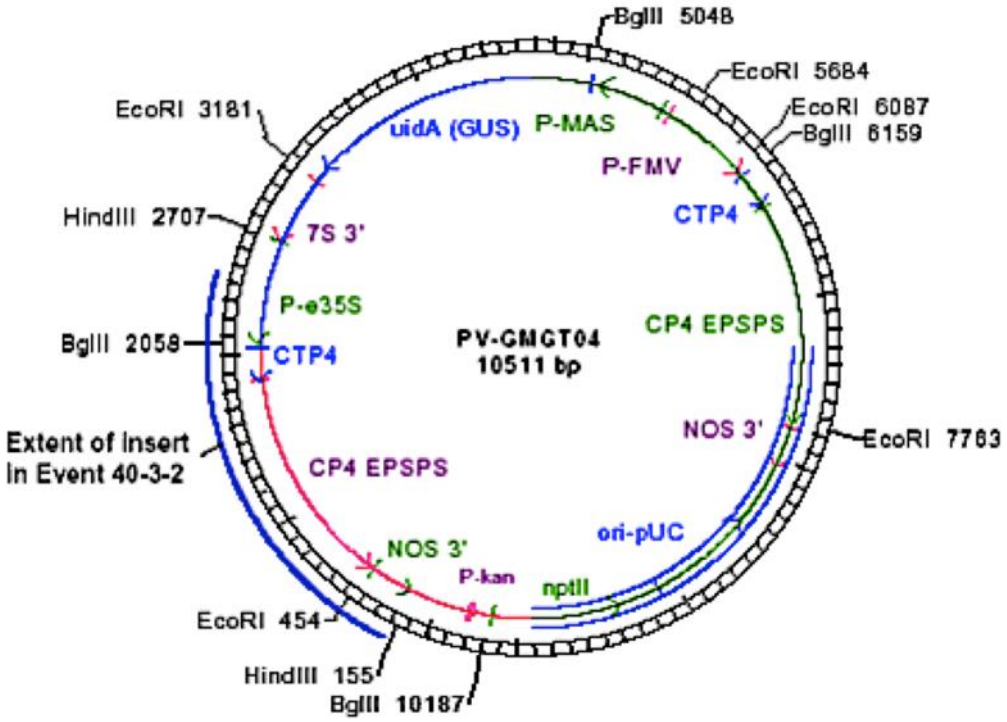

Figure 20: Plasmid map of the construct (10,511bp) used in the transformation of Monsanto RoundUp Ready (RR) soybean line 40-3-2. EcoRI, HindII and BgIII restriction endonuclease sites are denoted in black. Adapted from Taylor et al. [110].

An example transformation plasmid is shown in Figure 28. This plasmid construct was used in the transformation of Monsanto RoundUp Ready (RR) soybean line 40-3-2 and contains genetic elements from vector PV-GMGT04. *P-e35S* promoter genes and *nos* terminator genes are both ubiquitously used to ensure gene regulation and transcription of inserted genes of interest (GOI) in transformed eukaryotic cells. This plasmid also contains *EPSPS* pesticide tolerance genes and aminoglycoside and kanamycin resistance genes (*nptII* and *P-kan*). Multiple copies of individual genes may be found on a single transformation plasmid such as that shown in Figure 28, which contains duplicate copies of *EPSPS* and *nos* genes.

5.1.2 Transgenic Crop Cultivation and Consumption Trends

From 1990 and 2015 the percentage of agricultural land used for transgenic crop cultivation increased 100-fold from 17,000km^2 to 1,797,000 km^2 [111]. By 2014, only 6% of total agricultural area in the United States was dedicated to the cultivation of conventional soybeans, 4% to conventional cotton, and 7% to conventional maize [26]. This trend has been observed outside of developed nations as well, with 54% of the world's transgenic crops being produced by farmers in 19 developing countries [25]. The top producers of transgenic crops include the United States, Brazil, Argentina, and India (Table 6).

Table 6: Global Production of Transgenic Crops Ranked by Individual Country (Millions of Hectares) in 2014 and 2015. Adapted from James, 2005 [111].

Rank	Country	Cultivated Area (Million Hectares) 2014	Cultivated Area (Million Hectares) 2015
1	USA	73.1	70.9
2	Brazil	42.2	44.2
3	Argentina	24.3	24.5
4	India	11.6	11.6
5	Canada	11.6	11.0
6	China	3.9	3.7
7	Paraguay	3.9	3.6
8	Pakistan	2.9	2.9
9	South Africa	2.7	2.3
10	Uruguay	1.6	1.4
11	Bolivia	1.0	1.1
12	Philippines	0.8	0.7
13	Australia	0.5	0.7
14	Burkina Faso	0.5	0.4
15	Myanmar	0.3	0.3
16	Mexico	0.2	0.1
17	Spain	0.1	0.1
18	Colombia	0.1	0.1
19	Sudan	0.1	0.1
20	Honduras	<0.1	<0.1
21	Chile	<0.1	<0.1

22	Portugal	<0.1	<0.1
23	Vietnam	<0.1	<0.1
24	Czech Republic	<0.1	<0.1
25	Slovakia	<0.1	<0.1
26	Costa Rica	<0.1	<0.1
27	Bangladesh	<0.1	<0.1
28	Romania	<0.1	<0.1
	Total	181.5	179.7

In addition to being sold in supermarkets as the crop product itself, these crops may be further processed into other forms of food. For transgenic maize this includes grits, cornmeal, flour, cereals, and some fermented products. Genetically modified crops are also further broken down to obtain derivative products that are incorporated into other processed foods. These products include but are not limited to corn starches (e.g., maltodextrin, dextrose, high fructose corn syrup, and sugar alcohols), soy lecithin, sugar (from sugar beets), and vegetable oils [112]. These byproducts can then be incorporated into other foodstuffs, including baked goods, breads, baby foods, and other snack foods.

5.1.3 Breakdown and Behavior of Transgenic DNA During Digestion

Consumption of transgenic crops and crop byproducts is extremely common in the United States; the widespread cultivation and incorporation into foodstuffs makes this almost inevitable [113]. Once ingested, DNA within transgenic crops can conceivably behave in three ways: 1) DNA will be degraded by acids or DNase I and II enzymes found within the digestive system, 2) DNA will be taken up by cells in the gut (host or bacterial),

or 3) DNA will pass through the digestive system and be excreted partially or wholly intact [114]. Because transgenic crops contain genetic cassettes containing foreign DNA—including antibiotic resistance genes—controversy exists regarding the ability of transgenes to be transferred to surrounding gut microflora within the host [115]. All transgenic crop lines intended for human or animal consumption are subject to rigorous review for approval—including tests for allergenicity, toxicity, and other acute adverse health outcomes. However, these assessments are often only performed on animal models and employ classic toxicological endpoints (e.g., NOAEL or LOAEL) [116]. Assessments also typically last for a period of 90 days, which should detect most significant acute health outcomes but may not observe subtle health effects or those associated with chronic ingestion of transgenic crops [109]. Uptake of the transgenes within transgenic crops by the host or gut microbiome would only be observed, if at all, under such chronic ingestion conditions. It has been widely established that free DNA in any environment is highly susceptible to degradation by DNase I and II enzymes—this susceptibility would only be heightened in gastrointestinal systems with the added presence of digestive acids [117]. However, free DNA contained within food matrices appears to be protected from both enzymatic digestion and acid hydrolysis and can pass intact through a host's digestive tract intact [118]. DNA fragments released during the digestive process may be absorbed by the host via two primary mechanisms: 1) directly by epithelial cells or antigen-presenting cells within the intestinal mucosa, or 2) specialized M-cells that continuously

sample the host's gut contents in an effort to protect against infection [6, 7].

Several studies suggested that there is evidence of gene transfer from ingested transgenic crop biomass to host. Fragments up to 1600bp in size of a transgenic rubisco gene were detected in the digestive system as well as a 180bp fragment of a second transgene was also detected in both spleen and liver tissues of mice reared on transgenic soybean leaves [118]. Studies have also noted that the ingestion of transgenic crop biomass can be linked to observable health perturbations in the small intestine, caecum, and gastric mucosa of the digestive system of model rats [119]. However, it remains unclear whether these phenotypic changes are due to the uptake and expression of transgenes and their corresponding proteins by gut microflora or by the transgenic crop. Free DNA from ingested food material has also been detected in tissues of fetuses and newborns, suggesting a possible placental route of exposure [120]. It has also been reported that bacteria located within the small bowel are capable of taking up and maintaining crop transgenes in immune-compromised individuals and individuals with ileostomies [46]. This phenomenon has been observed at similar rates for both conventional and transgenic plants [117]. Because several lines of previously commercially available transgenic crops contain one or more ARGs [20], the consequences associated with uptake of transgenic crop DNA within the gut microbiome may be more significant.

5.1.4 Transgenes in Wastewater Treatment Plants

In addition to degradation or uptake, transgenic DNA may be excreted as solid waste by the host. As noted above, free DNA contained within food matrices appears to be highly protected from outside degradation. It is therefore possible that transgenes derived from transgenic crops may be introduced into municipal wastewater treatment plant (WWTP) systems. With approximately 16,000 in operation in the United States, municipal WWTPs are responsible for treating the wastewater produced by more than 75% of US households [121]. Individual plants may vary, but generally consist of primary treatment, secondary treatment, and tertiary treatment [122]. All incoming waste is first treated by primary treatment. This involves phase separation, allowing solids and sludge to settle to the bottom of holding tanks and fats/oils/greases to rise to the top. Waste then moves on to secondary treatment, which is designed to biologically degrade suspended and dissolved organic compounds (i.e., biological oxygen demand) [123, 124]. This typically takes the form of activated sludge tanks, rotating biological contactors, aerated lagoon, or constructed wetlands and is performed by aerobic aquatic bacteria that function optimally between 4°C and 32°C. Most of the wasted solids from biological treatment tanks may be further treated via aerobic or anaerobic digestion to produce biosolids. More than 50% of biosolids produced by WWTPs in the US are used for downstream agriculture or land applications [125]. Tertiary treatment, if performed, functions to improve water quality with additional disinfection (e.g., chlorine, ozone, or

UV treatment) and the removal of micropollutants [123]. As described above, two major processes in the wastewater treatment scheme are biologically based: secondary treatment and sludge digestion. This suggests that any partially or wholly intact free DNA contained within human waste may be exposed to a range of microbes within those treatment processes.

Given the high rates of transgenic crop cultivation and consumption, as well as the noted possibility of crop transgenes being able to survive the digestive process, it is possible that partial or full length transgenes derived from GM crops may be present in WWTPs. To investigate this, activated sludge and digester sludge were screened for transgenes. Monitoring digester sludge for transgenes may provide some insights into of GMO-derived ARGs that are incorporated into biosolids prior to their land application as agricultural fertilizers. The genes selected for screening include *p35* (promoter sequence), *nos* (terminator sequence), *bla* (ARG), and *nptII* (ARG). Both *p35* and *nos* genes are incorporated into a wide array of GM crop lines, including canola, wheat, tomato, BT and HT maize, BT and HT cotton, and BT and HT soy. To our knowledge, this is the first study quantifying transgenes in WWTPs.

5.2 Materials and Methods

5.2.1 Wastewater Sludge Collection and DNA Isolation

Activated sludge samples were obtained from twenty-six wastewater treatment plants located in North Carolina, Ohio, Oregon, Pennsylvania, Washington, West

Virginia, Missouri, Kansas, and Iowa. Control samples were obtained from France (Figure 29). These WWTPs were selected because their treatment capacities covered a broad range (0.3-155 MGD) and they were geographically distributed (Table 7). Samples were shipped overnight on dry ice. Upon receipt, samples were stored at -80°C until DNA was extracted.

Figure 21: Map of the WWTP activated sludge and digester sludge collection sites in the US. Samples were obtained from WWTPs in North Carolina, Ohio, Oregon, Pennsylvania, Washington, West Virginia, Missouri, Kansas, and Iowa. Control WWTP samples were obtained from France. DNA was isolated from each sample and used to detect possible genes derived from digested transgenic crops.

Table 7: Size and location characteristics of each sampled WWTP. WWTPs are reported anonymously by location and size was measured in terms of influent flow rate in millions of gallons per day (MGD).

WWTP Number	WWTP Location	Size (MGD)
1	France	58.1
2	NC	20.0
3	NC	2.5
4	WV	10.0
5	OH	35.0
6	OH	155.0
7	PA	10.0
8	PA	68.6
9	PA	2.13
10	PA	0.30
11	PA	7.2
12	PA	18.5
13	OR	9.7
14	WA	1.44
15	MO	0.30
16	MO	10
17	KS	1.5
18	KS	2.5
19	KS	2.0
20	KS	1.8
21	IA	2.0
22	KS	2.5
23	WA	21.0
24	WA	56.0
25	OR	9.7
26	PA	4.5

Samples were stored at -80°C until DNA was extracted. Approximately 10mL of each sample was filtered and DNA was isolated using the PowerWater DNA Isolation Kit (MoBio Laboratories, Inc.) according to kit manufacturer instructions. Eluted DNA was quantified using a QuBit 2.0 (Life Technologies) and stored at -20°C for downstream analysis.

Of the twenty-six WWTPs mentioned above, ten facilities from the following areas also provided digester sludge: North Carolina, Missouri, Kansas, Iowa, and Oregon. Control digester samples were obtained from France. Aliquots of 5mL were centrifuged in triplicate to obtain biosolids for DNA extraction using the PowerLyzer PowerSoil kit (MoBio Laboratories, Inc.) according to manufacturer instructions. Eluted DNA was quantified using a QuBit 2.0 (Life Technologies) and stored at -20°C for downstream analysis.

5.2.2 End-Point PCR and qPCR Conditions

DNA was then screened using qualitative end-point PCR for *p35*, *nos*, *bla*, and *nptII* genes using the primers found in Table 10. The sequences used to code for *bla* and *nptII* ARGs are highly conserved among transgenic crop lines and are included in at least 130 lines of GM crops [20]. Therefore, one would expect that a single primer set for each of these genes would be able to detect the corresponding ARG from multiple sources. Taqman®™ Probe-based qPCR assays were selected over SYBR Green chemistry assays

because they provide a higher level of precision when screening environmental samples and the likelihood of non-specific primer binding is minimal.

In addition, DNA was also screened for the presence of Cauliflower Mosaic Virus (CaMV) ORF VII region to determine whether the detected *p35* genes were viral in origin. The primers used for the ORF VII region amplify a 141 bp region of the virus genome and are as follows [126]: F: 5′- AGCGGTCAAAATATTGCTTA-3′; R-5′- AACTTACCGTATGCTAGATTACCT-3′. Mixed primer end-point screenings using the Forward *p35* primer and Reverse *nos* primer were also performed in an attempt to identify larger fragments of potential transformation plasmids. Negative and positive controls were incorporated into each PCR run. All samples yielding a positive band were then analyzed using the Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA) quantitative real-time PCR (qPCR) thermocycler. Results were normalized to *16S* (Table 8) for each transgene in each sample [127].

Table 8: Primer and TaqMan®™ Probe Sequences for qPCR Assays

Target Gene	Primer Sequence (5′-3′)	TaqMan®™ Probe (5′-3′)	Amplicon Length (bp)	Source
p35	F: GCCTCTGCCGAC AGTGG T R: AAGACGTGGTT GGAAC GTCTTC	FAM-CAAAGATGG ACCCCCACCCACG-TAMRA	82	[128]
nos	F: GTCTTGCGATGAT TATCATATAATTTCT G R: CGCTATATTTTGTT TTCTATCGCGT	FAM- AGATGGGTT TTTATGATTAGAG TCCCGCAA-TAMRA	120	[129]
bla	F: TGCGCGGAACCCC TATT TGT R: GCGGCGACCGAG TTGCTCTT	N/A	382	[130]
nptII	F: AGGAAGCGGTCA GCCCAT R: GCGTTGGCTACC CGTGATAT	FAM-CGCCGCCAA GCTCTTCAGCAA-TAMRA	60	[71]
16S	F: TCCTACGGGAGG CAGCAG R: GGACTACCAGGGT ATCTAATCCTGTT	FAM- CGTATTACC GCGG CTGCTGGC AC-TAMRA	466	[131]

QPCR amplification using SYBR Green chemistry was performed for *bla* under the following conditions: Initial denaturation at 95°C for 3min, followed by 40 cycles of 95°C for 45s, 55°C for 60s, and 72°C for 30s. Fluorescent measurements were taken during

elongation. The dissociation curve was assessed at the end of the run under the following conditions: 95°C for 60s, 55°C for 30s, and 95°C for 30s.

QPCR amplification using TaqMan®™ Probe chemistry was performed for *p35*, *nos*, and *nptII* using the following conditions: Initial denaturation at 95°C for 60s, followed by 40 cycles of 95°C for 30s and 60°C for 30s. The following conditions were used for *16S*: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30s and 60°C for 60s. Fluorescence measurements for each assay were taken during annealing steps for all qPCR assays.

5.2.3 Statistical Analysis

The data were normalized to gene copies per mL of wastewater. The copy number of each transgene was also compared the copy number of *16S* genes per mL of wastewater (number of bacteria per mL). Multiple linear regression analyses and correlation analyses were performed to investigate the relationships between gene concentration and WWTP characteristics.

Averages and standard deviations of all data were calculated using Microsoft Excel 2007. All other statistical tests-including t-tests and ANOVAs-were conducted in RStudio 1.0.136. Multiple linear regressions were also performed to reveal relevance of WWTP size on the abundance of the detected genes.

5.3 Results and Discussion

5.3.1 Qualitative PCR Screening Results

Activated sludge obtained from 26 WWTPs and digester sludge from 10 WWTPs was screened for the presence of transgenes derived from digested transgenic crops. All of the selected transgenes (i.e., *p35*, *nos*, *nptII*, and *bla*) were detected during the preliminary screening of WWTP activated sludge and digester sludge using end-point PCR. Results of this preliminary qualitative screening of WWTP activated sludge can be seen in Table 9, and results for WWTP digester sludge can be seen in Table 10. All but three samples contained at least one transgene, and many samples contained all four transgenes. However, extracted DNA from the three WWTPs with no detected transgenes appeared highly degraded when run out on an agarose gel, both pre- and post-PCR. End-point PCR screenings for *16S* genes also failed to amplify. This suggests that the DNA within the sludge may have become damaged in transit and therefore the negative qualitative PCR results do not necessarily indicate a lack of transgenes present in these samples. These samples were excluded from further analysis. Additional screenings for CaMV ORF VII genes were negative in all WWTP samples.

Table 9: End-point PCR screening results for WWTP activated sludge samples: (+) indicates a positive screening result; (-) indicates that the corresponding transgene was not detected.

WWTP Location	*p35*	*nos*	*bla*	*nptII*	Total
1	-	-	+	+	2
2	+	+	-	-	2
3	+	+	+	+	4
4	+	+	-	-	2
5	+	+	+	+	4
6	+	+	+	+	4
7	+	+	-	+	3
8	+	+	-	-	2
9	+	-	-	+	2
10	+	+	+	+	4
11	+	+	+	+	4
12	+	+	-	+	3
13	+	+	+	+	4
14	+	+	-	-	2
15	+	+	+	-	3
16	+	+	+	+	4
17	+	+	+	+	4
18	+	+	+	+	4
19	+	+	+	+	4
20	+	+	+	+	4
21	+	+	+	+	4
22	-	-	+	-	1
23	-	-	+	+	2
24	-	-	-	-	0
25	-	-	-	-	0
26	-	-	-	-	0

Table 10: End-point PCR screening results for WWTP digester sludge samples: (+) indicates a positive screening result; (-) indicates that the corresponding transgene was not detected.

WWTP Location	*p35*	*nos*	*bla*	*nptII*	Total
1	+	+	+	+	4
2	+	+	+	+	4
15	+	+	+	+	4
17	-	-	+	+	2
18	-	-	+	-	1
19	+	+	+	+	4
20	+	+	+	+	4
21	+	+	+	+	4
22	+	+	+	+	4
25	-	-	-	-	0

5.3.2 Additional Qualitative PCR Screening Results

The *p35* promoter gene found in many transgenic crops is derived from the Cauliflower Mosaic Virus (CaMV), a unique feature among most transgenes. DNA extracted from WWTP activated sludge and digester sludge was also screened for CaMV ORF VII genes, though none were detected by end-point PCR. This suggests that the detected *p35* genes have not been extracted from virus sources and thus exist either as eDNA or within iDNA bacteria. However, the exact source has yet to be identified.

Sludges were also screened for larger plasmid fragments using the Forward *p35* primer and Reverse *nos* primer described in Table 8. The results of these screenings can be found in Figures 30-32.

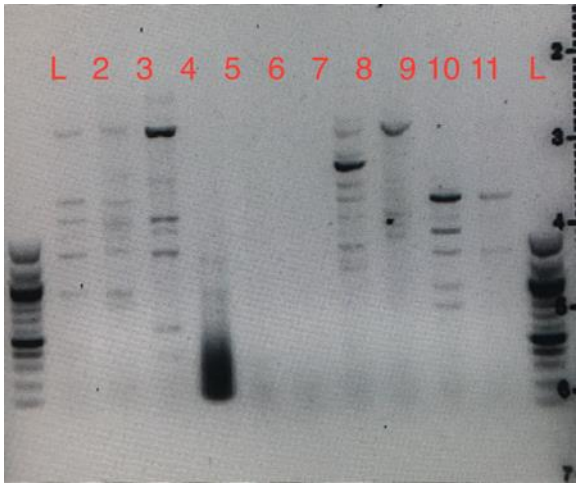

Figure 22: PCR products resulting from mixed *p35F*/*nosR* primer screenings for WWTPs 2-11. Lanes 1 and 12 contain 100 bp ladder (max fragment size 2,000bp) obtained from New England BioLabs (Ipswich, MA).

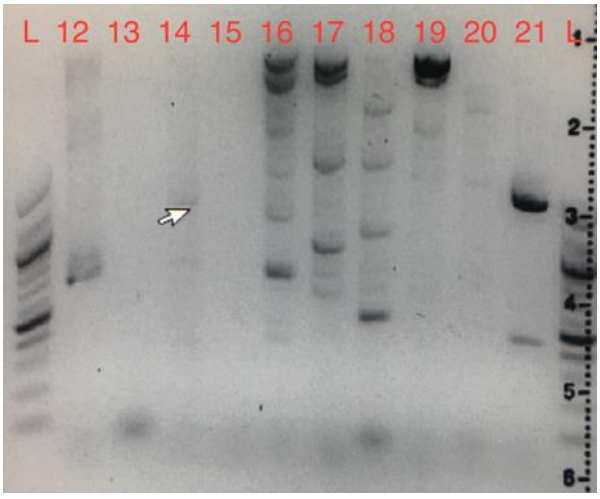

Figure 23: PCR products resulting from mixed *p35F*/*nosR* primer screenings for WWTPs 12-21. Lanes 1 and 12 contain 100 bp ladder (max fragment size 2,000bp) obtained from New England BioLabs (Ipswich, MA).

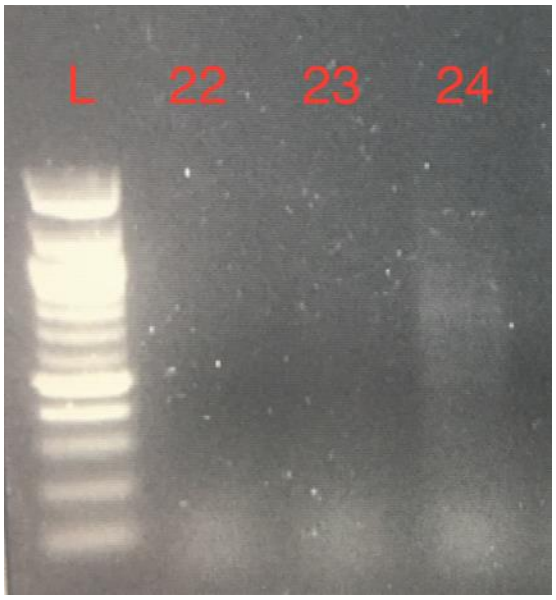

Figure 24: PCR products resulting from mixed *p35F*/*nosR* primer screenings for WWTPs 22-24. Lane 1 contains 100 bp ladder (max fragment size 2,000bp) obtained from New England BioLabs (Ipswich, MA).

Nested PCRs using the PCR products obtained in the *p35*/*nosR* screening were also performed with the *bla* primers detailed in Table 8 (Figure 33-34).

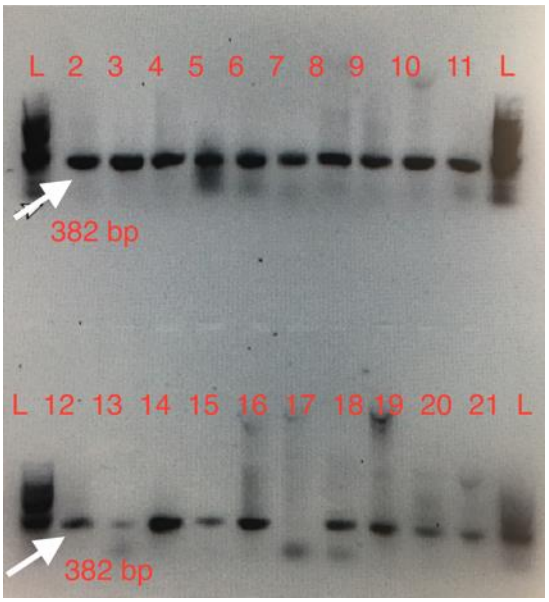

Figure 25: PCR products resulting from *bla* nested PCRs using *p35F/nosR* PCR products as template DNA for WWTPs 2-21. *Bla* target amplicons are 382bp. Lanes 1 and 12 of each row contain 100 bp ladder (max fragment size 2,000bp) obtained from New England BioLabs (Ipswich, MA).

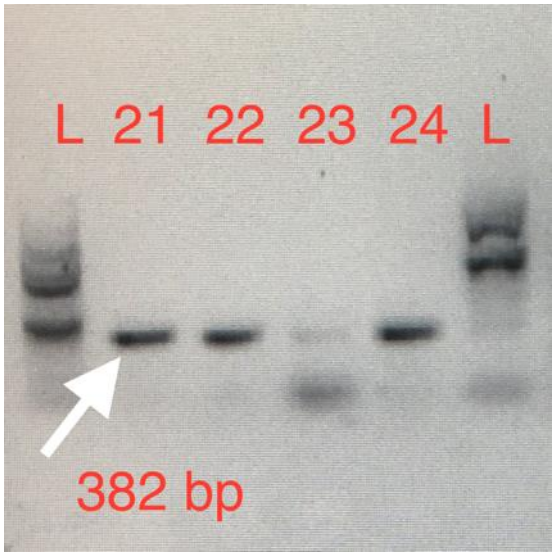

Figure 26: PCR products resulting from *bla* nested PCRs using *p35F/nosR* PCR products as template DNA for WWTPs 21-24. *Bla* target amplicons are 382bp. Lanes 1 and 5 of each row contain 100 bp ladder (max fragment size 2,000bp) obtained from New England BioLabs (Ipswich, MA).

These additional PCR screening also support the idea that DNA from transgenic crops can be found in WWTP environments. *P35F/nosR* primer screenings resulted in the amplification of multiple large gene fragments in all WWTP samples. Some of these bands were found to contain the *bla* ARG when performing a nested PCR with the *p35F/nosR* PCR products from each WWTP sample. If some of these fragments do originate from transgenic crops, the multiple band sizes are easily explained by the example transformation plasmid outlined in Figure 28. Multiple copies of promoters, terminators, genes of interest, and selection markers are often incorporated into a single transformation plasmid [110], and could therefore generate multiple bands using a single PCR primer set. However, as copyright and intellectual property laws often protect the sequence information for transformation plasmids of transgenic crops, the identity and source of these bands could not be verified.

5.3.3 Quantitative PCR Screening Results

Samples that tested positive for potential transgenes during the preliminary screening described in Section 5.3.1 were then further analyzed using qPCR to determine the abundance of each transgene in wastewater sludges. The standard curve conditions for each qPCR assay can be found in the Chapter 5 Appendix (Table 16).

Results of the secondary quantitative screening of WWTP activated sludge are shown in Figures 28-31. Copy numbers of the *p35* promoter gene in domestic wastewater samples ranged from 2.92 $x10^2$ to 3.61 $x10^4$ copies per mL of wastewater, with an average of 1.12 $x10^4$ copies/mL (Figure 35). The abundance of *p35* in activated sludge was not significantly different among wastewater treatment facilities sampled in North Carolina, Ohio, Pennsylvania, and West Virginia (WWTPs 1-14). The abundance of *p35* detected in activated sludge from facilities sampled in Missouri, Iowa, and Kansas was more variable (WWTPs 15-21); *p35* levels were significantly lower in WWTPs 15 and 18 (p=.0014). Overall copy numbers among wastewater digesters were lower, but the same trends were observed. *P35* abundance ranged from 2.53 $x10^2$ to 2.28 $x10^3$ copies per mL of wastewater, with an average of 1.13 $x10^3$ copies per mL. *P35* levels were significantly higher in activated sludge compared to digester sludge obtained from domestic WWTPs (p=0.0040). The concentration of *p35* genes in control activated sludge obtained from France (WWTP 1) was significantly lower compared to that of domestic wastewaters (p=0.00040).

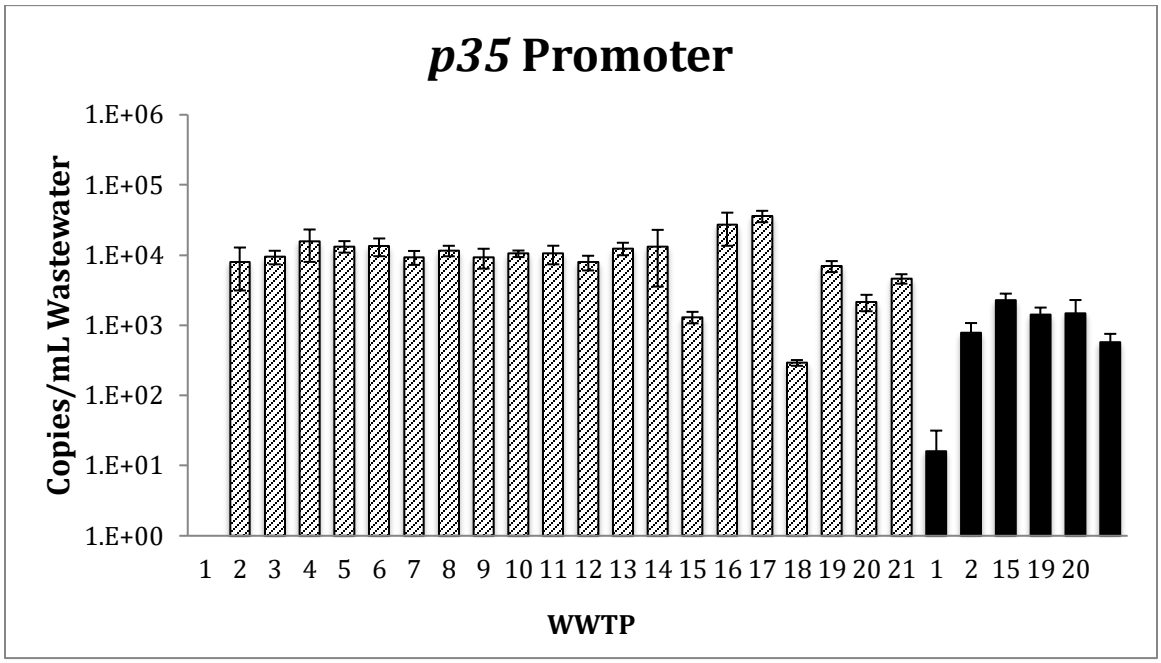

Figure 27: Quantitative detection of promoter gene *p35* in wastewater activated sludge and digester sludge. The observed copy number of *p35* for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

Similar trends were observed in the detected copy numbers of the *nos* terminator genes in domestic wastewater samples (Figure 36). *Nos* terminator copy numbers ranged from 2.26×10^2 to 4.78×10^4 copies per mL of wastewater, with an average of 1.15×10^4 copies per mL. Similarly to the *p35* promoter genes, the abundance of *nos* in activated sludge was not significantly different among wastewater treatment facilities sampled in North Carolina, Ohio, Pennsylvania, and West Virginia (WWTPs 1-14). The abundance of *nos* detected in activated sludge from facilities sampled in Missouri, Iowa, and Kansas was

more variable (WWTPs 15-21); *nos* gene concentrations were significantly lower in WWTPs 15 and 18 (p=0.00030). Detected *nos* values in WWTP 20 were only marginally significantly lower than in other WWTPs (p=0.093). Lower *nos* copy numbers were observed in digester sludge samples than in activated sludge samples. In these samples, *nos* abundance ranged from 4.19 $x10^1$ to 7.95 $x10^3$ copies per mL of wastewater, with an average of 2.77 $x10^3$ copies per mL. *Nos* gene copy numbers were highest in WWTPs 2, 15, and 20, but these levels were not significantly different from other digesters (p=0.052). *Nos* copy numbers were also not significantly different between activated and digester sludge (p=0.058). Again, the concentration of *nos* genes in control activated and digester sludge samples was significantly lower compared with those from domestic wastewaters (p=0.040).

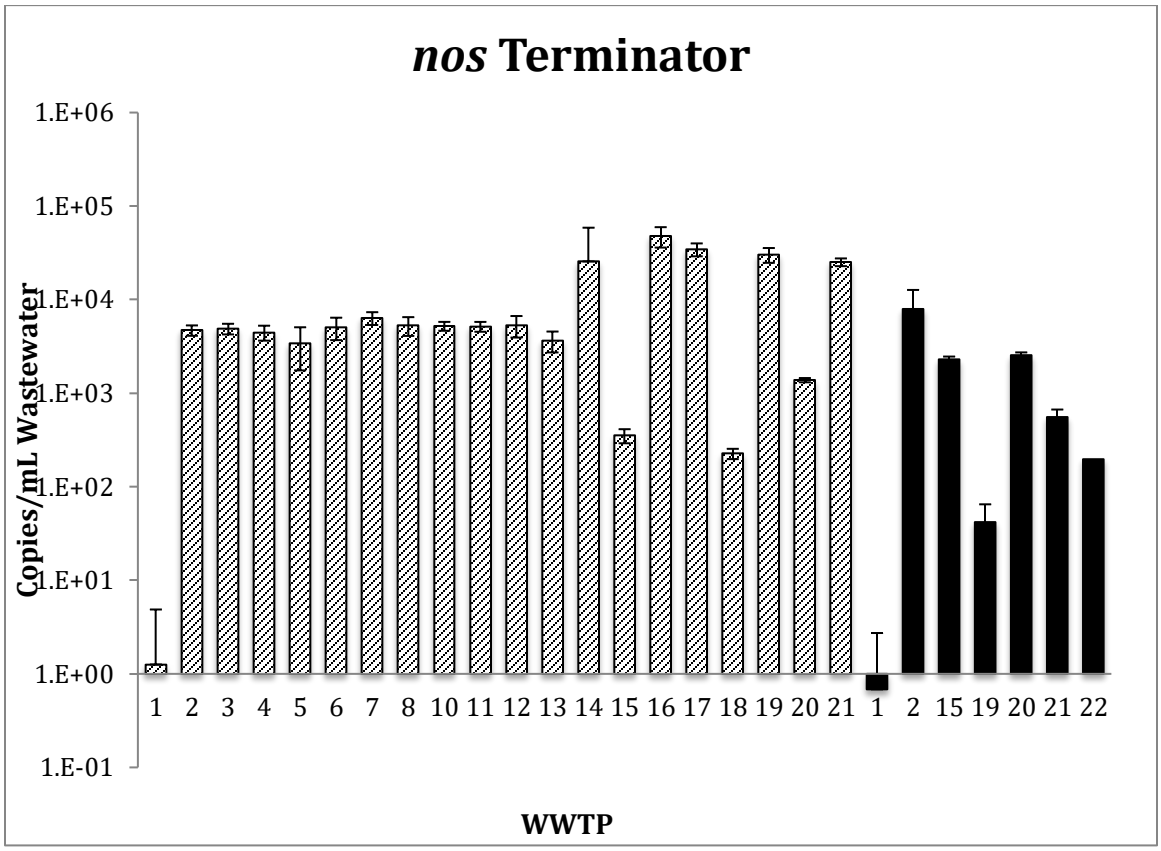

Figure 28: Quantitative detection of terminator gene *nos* in wastewater activated sludge and digester sludge. The observed copy number of *nos* for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

Copy numbers of *nptII* ARGs in domestic activated sludge wastewater varied widely among sampling locations (Figure 37). *NptII* concentrations ranged from 2.46 $x10^2$ to 1.58 $x10^5$ copies per mL of wastewater, with an average of 2.80 $x10^4$ copies per mL. Unlike *p35* and *nos* genes, *nptII* ARG abundance in activated sludge did not appear to be linked to the location of WWTPs (p=0.091). The highest concentrations of *nptII* among

activated sludge samples were found in WWTPs 3, 9, 19, and 21, and were significantly different from other WWTPs (p=0.013). Overall copy numbers among wastewater digesters were similar to those observed in activated sludge samples (p=0.086). *NptII* abundance in US digesters ranged from 8.04 x10^2 to 2.20 x10^4 copies per mL of wastewater, with an average of 5.29 x10^3 copies per mL. The concentration of *nptII* ARGs in control activated and digester sludge (WWTP 1) was 6.30 x10^1 and 3.20 x10^1, respectively, and was significantly lower compared to that of domestic wastewaters (p=0.041).

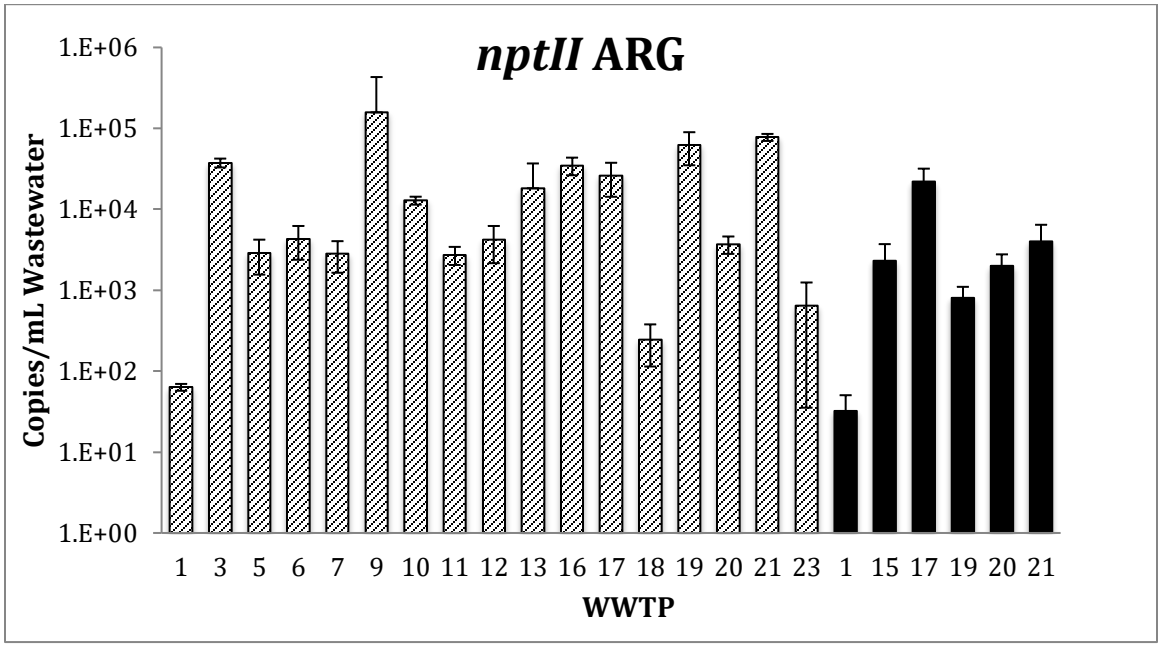

Figure 29: Quantitative detection of antibiotic resistance gene *nptII* in wastewater activated sludge and digester sludge. The observed copy number of *nptII* for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

The detected copy numbers of the *bla* ARGs in domestic wastewater samples were similar to those of *nptII* in that they varied greatly among WWTPs (Figure 38). In activated sludge, *bla* ARGs copy numbers ranged from 7.18 to 1.31 $x10^5$ copies per mL of wastewater, with an average of 5.25 $x10^4$ copies per mL. The lowest concentrations of *bla* among activated sludge samples were found in WWTPs 15, 18, 20, 22, and 23 and were significantly different from other WWTPs (p=0.000080). Overall copy numbers among digester sludge samples was also highly variable. Among these samples *bla* ARG abundance ranged from 2.29 $x10^2$ to 9.71 $x10^3$ copies per mL of wastewater, with an average of 3.67 $x10^3$ copies per mL. The concentration of *bla* genes in control activated sludge was not significantly different from that of domestic wastewaters (p=0.24).

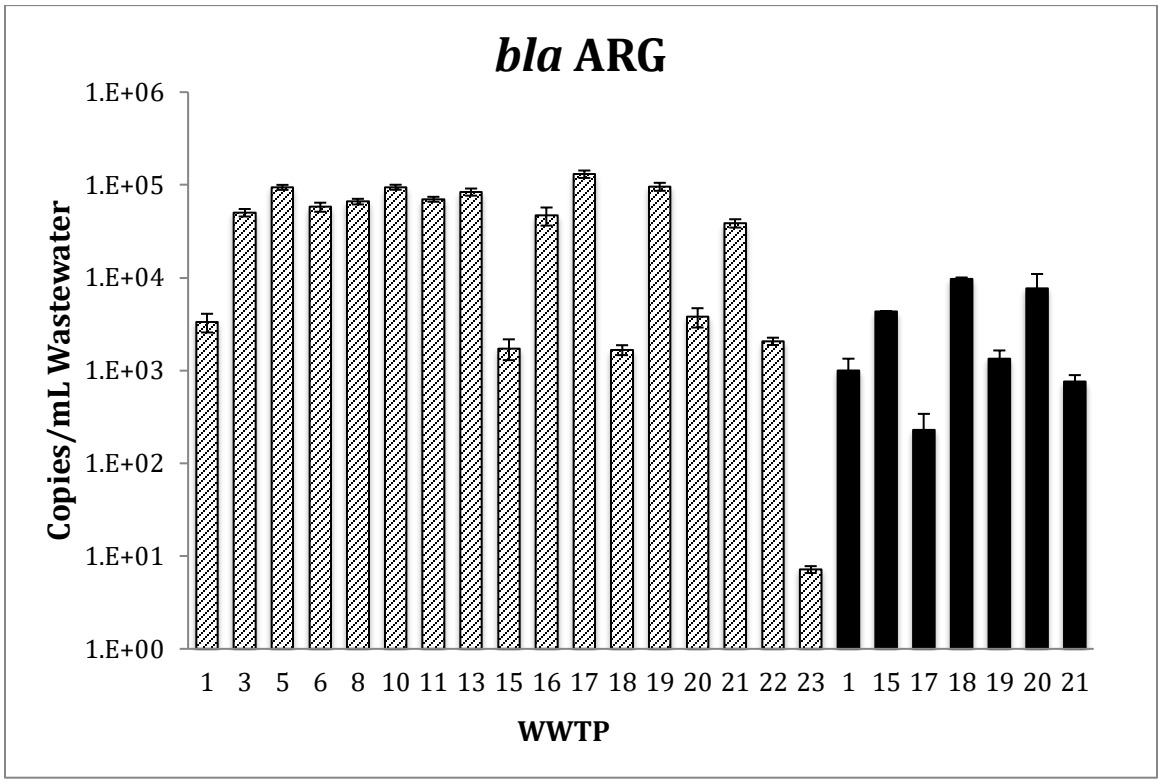

Figure 30: Quantitative detection of antibiotic resistance gene *bla* in wastewater activated sludge and digester sludge. The observed copy number of *bla* for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

5.3.4 Comparison of Transgene Abundance to *16S*

The relative abundance of each gene was also compared to the relative abundance of bacteria in each activated sludge and digester sludge sample, determined through the quantification of the *16S* gene. The concentration of *16S* in wastewater samples was found to be consistent among WWTPs for both activated sludge and digester sludge samples. Activated sludge was found to have a maximum of 1.13 $x10^{10}$ copies/mL and an average of 3.84 $x10^{9}$ copies/mL. Lower copy numbers were observed in digester sludges, where

there was an upper limit of 1.52 $x10^9$ copies of *16S* per mL wastewater and an average of 4.27 $x10^8$ copies/mL. This range is comparable to that estimated by other studies [127, 132].

When comparing the ratio of *p35* promoter genes to *16S* genes in wastewater samples, the same trends were observed as described above (Figure 39). Sixteen WWTPs had similar *p35/16S* ratios, ranging from 6.76 $x10^{-4}$ to 1.05 $x10^{-8}$, with an average of 3.32 $x10^{-5}$. No significant differences existed between the ratios associated with activated sludge and digester sludge. The lowest *p35/16S* ratios were observed in control activated and digester sludge samples (p=0.0090).

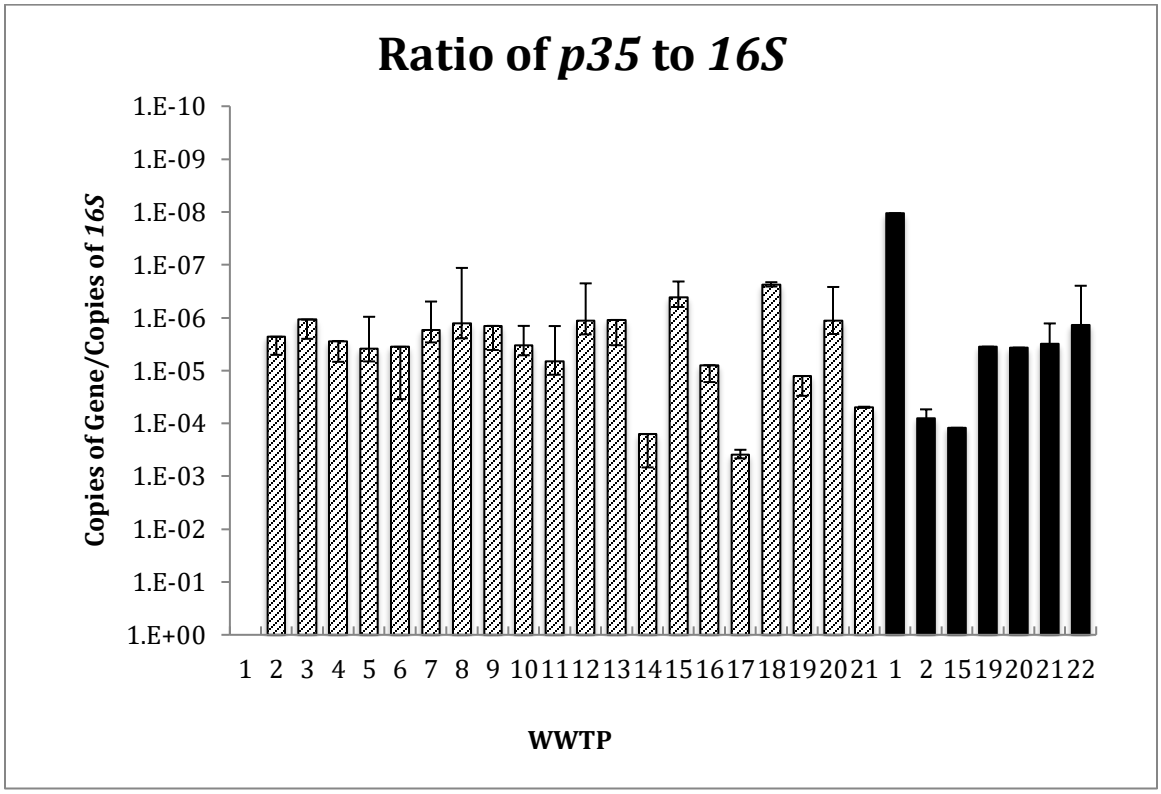

Figure 31: Ratio of detected promoter gene p35 to 16S in wastewater activated sludge and digester sludge. The copy number ratio (p35/16S) for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted in black hatch patterns and digester sludge samples are represented in solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

Similar trends were observed among the ratios of the *nos* terminator and *16S* genes in activated and digester sludges (Figure 40). Similar to the *p35/16S* ratios, fourteen activated sludge samples had similar *nos/16S* ratios, ranging from 1.41 $x10^{-5}$ to 9.83 $x10^{7}$, with an average of 5.44 $x10^{-5}$. WWTPs 14, 17, 19, and 21 had the highest ratios of *nos* to *16S* in activated sludge among all sampled WWTPs, and these were significantly different than the ratios found in other WWTPs (p=0.0050). Digester sludges had high *nos/16S* ratios overall with an average of 1.59 $x10^{-4}$, though these differences were not significant (p=0.12). As before, control wastewater samples were found to have significantly lower *nos/16S* ratios (p=0.038).

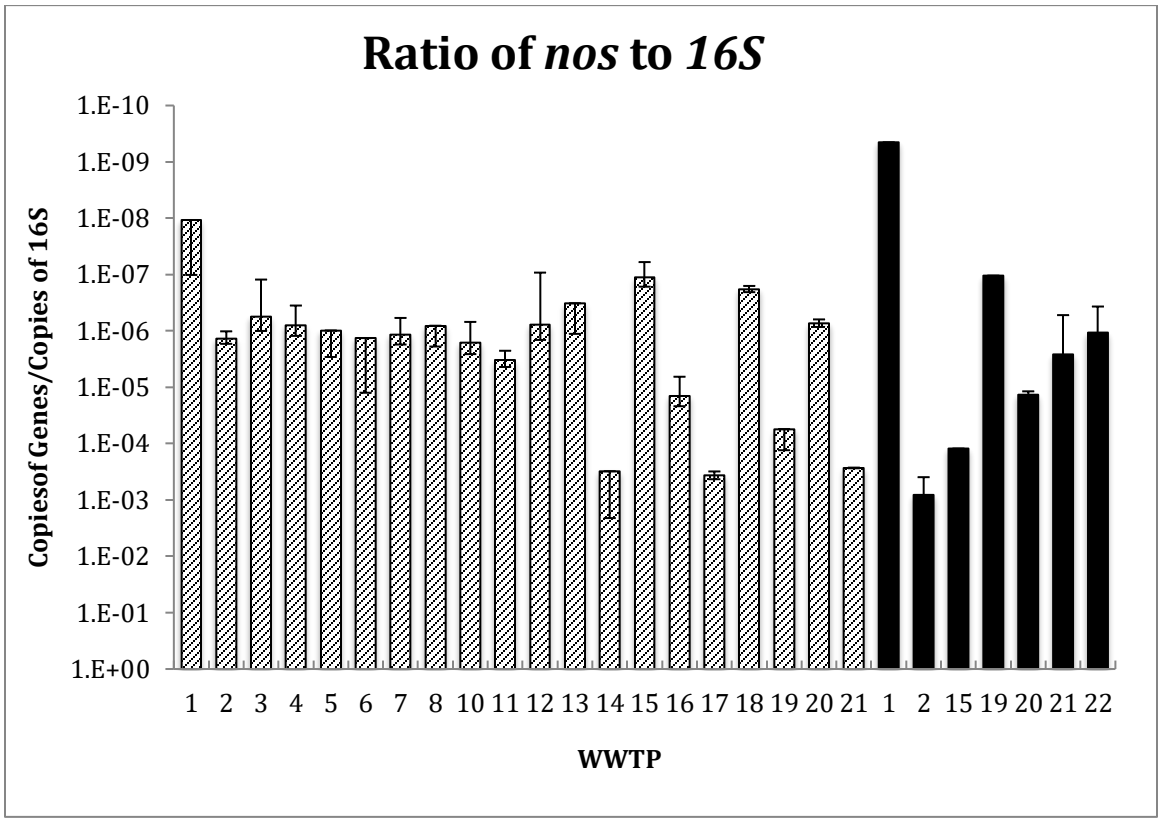

Figure 32: Ratio of detected terminator gene nos to 16S in wastewater activated sludge and digester sludge. The copy number ratio (nos/16S) for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted in black hatch patterns and digester sludge samples are represented in solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

The ratio of *nptII* ARGs to *16S* genes varied widely among both activated sludge and digester sludge samples (Figure 41). Among activated sludge samples, *nptII*/*16S* ratios varied from 1.14 x10^{-4} to 8.31 x10^{-7} with an average of 4.96 x10^{-5}. Digester sludge ratios were equally variable overall with an upper and lower limit of 1.17 x10^{-3} to 4.99 x10^{-6}, respectively, and an average of 2.40 x10^{-4}. The differences in *nptII*/*16S* ratios between

activated and digester sludges were slightly different (p=0.062). Control activated sludge and digester sludge samples were also significantly different from domestic sludge samples (p=0.028).

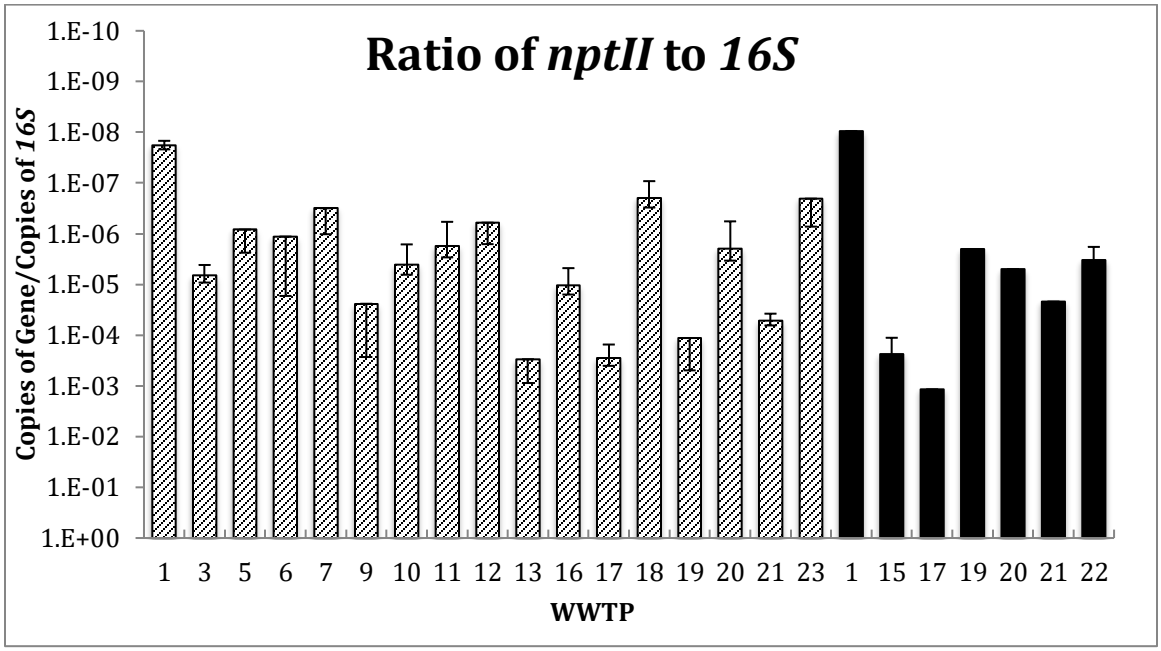

Figure 33: Ratio of detected antibiotic resistance gene nptII to 16S in wastewater activated sludge and digester sludge. The copy number ratio (nptII/16S) for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted in black hatch patterns and digester sludge samples are represented in solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9)

Unlike the ratios of *nptII* to *16S*, the ratios of *bla* to *16S* among WWTPs were consistent and did not vary significantly (Figure 42). Among activated sludge samples, *bla*/*16S* ratios varied from 1.10E x10^{4} to 6.95 x10^{-6} with an average of 2.60 x10^{-5}. Digester sludge ratios were similar overall with an upper and lower limit of 1.04 x10^{-4} and 3.21 x10^{-7}, respectively, and an average of 2.09 x10^{-5}. The differences in ratios between activated

and digester sludges were also only marginally different (p=0.087). Moreover, control sludge samples were also not significantly different from domestic wastewaters (p=0.44).

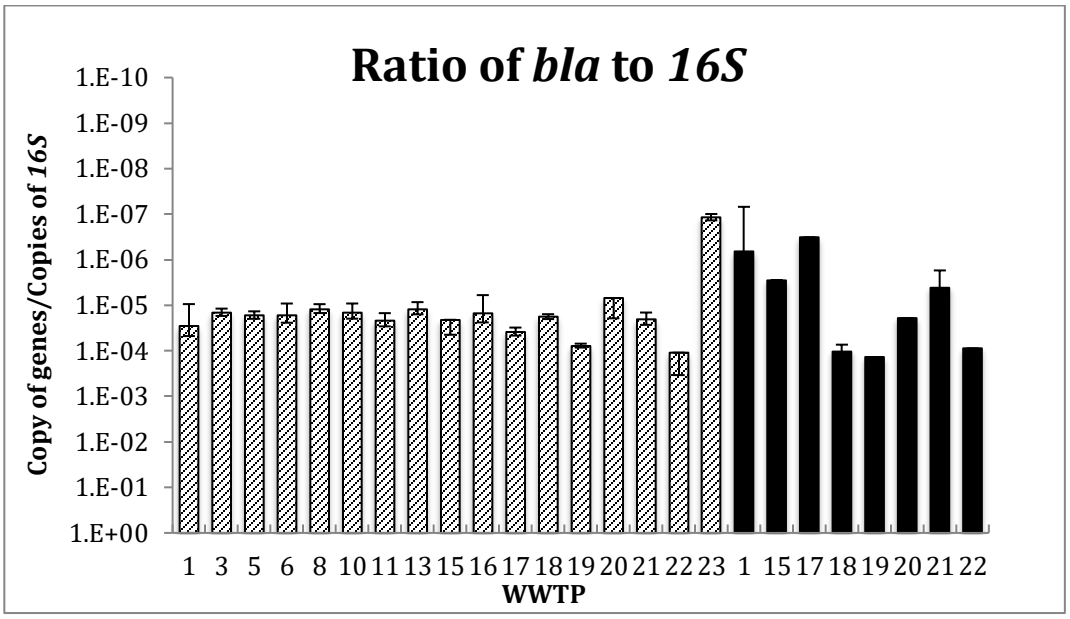

Figure 34: Ratio of detected antibiotic resistance gene *bla* to *16S* in wastewater activated sludge and digester sludge. The copy number ratio (*bla*/*16S*) for each sample has been normalized to copies per mL of wastewater. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. Wastewater treatment plants have been reported anonymously. Error is reported as standard deviation (n=9).

5.3.5 Correlation Analyses

Overall, *p35*, *nos*, *nptII*, and *bla* genes varied widely in concentration among sampled wastewater treatment plants. *P35* promoter and *nos* terminator genes were less abundant than *nptII* and *bla* ARGs on average. However, additional statistical analysis indicated that some transgenes were positively correlated with wastewater treatment plant location and size. The concentrations of all potential transgenes—*p35*, *nos*, *nptII*, and *bla*—among individual wastewater treatment plants was found to be correlated to WWTP

state locations (p=0.029), determined by an ANOVA and multiple linear regression regression. *P35* promoter gene copy numbers were most strongly correlated with WWTP location (p=0.029), and *nptII* and *bla* ARGs were moderately correlated with WWTP location (p=0.051 and p=0.099, respectively). *Nos* terminator and *16S* gene copy numbers were not strongly correlated with WWTP location (p=0.38, p=0.27 respectively). Correlations with WWTP locations appeared to be linked only to individual states and not entire geographic regions. For example, transgene abundance was linked to the US states in which they were located, as noted above. However, the correlations between entire geographic regions were not significant (e.g., Midwestern regions vs. Northeastern regions). This appears counterintuitive, given the large quantities of transgenic crops cultivated in Midwestern states. Rather, these results suggest that the consumption of GMO crops is a more important factor in assessing the prevalence and quantity of transgenes in WWTP sludge.

Notably, the abundance of the *p35, nos,* and *nptII* genes was markedly lower in control sludge samples as compared to domestic sludge samples. Control sludge samples were obtained from France, where transgenic crops are neither cultivated nor consumed by humans. The lack of these transgenes further supports the idea that transgene presence in WWTPs is linked to human consumption and excretion of transgenic crops or their byproducts. The levels of *p35, nos,* and *nptII* genes in these control wastewater samples are therefore a likely indication of the abundance of these genes occurring naturally in

WWTP bacterial populations. As the abundance of the *bla* ARG was not found to differ significantly between control and domestic wastewater samples, it is logical to conclude that the *bla* genes detected in domestic wastewater are not derived from transgenic crops, but are instead a reflection of naturally occurring antibiotic resistance.

The relationships among WWTP size, in terms of typical daily flow rates in millions of gallons per day (MGD), and transgene prevalence were also investigated. Daily flow rates in sampled WWTPs varied greatly, from 1.8 to 155 MGD (Table 9). *P35* gene copy numbers were strongly correlated with WWTP size (p=0.0020). *Nos* gene concentrations were slightly correlated (p=0.14), but *bla* and *nptII* ARGs showed little correlation with WWTP size (p=0.33 and p=0.67, respectively). This correlation could be explained by the argument that transgene abundance is a function of human consumption and excretion of transgenic foods, as noted above. Larger WWTPs are more likely to serve larger populations, with larger volumes of volumes of transgenic crops and byproducts being consumed. The prevalence of *16S* genes was most strongly correlated with WWTP size (p=0.00070). This is to be expected, as influent rate and sludge retention time are known to be strongly correlated with the carrying capacities, and therefore bacterial abundance, of WWTP sludges [133, 134].

Overall, the ratios of *p35, nos, nptII*, and *bla* genes to bacterial abundance (i.e., *16S* copy numbers) were unrelated to WWTP geographic location. *p35*/*16S*, *bla*/*16S*, and *nos*/*16S* ratios were not strongly correlated with the geographic locations of sampled

WWTPs—p=0.33, p=0.18, and p=0.27, respectively. *NptII/16S* ratios were only marginally linked to WWTP location (p=0.076). Overall, normalized gene copy numbers were more strongly correlated to WWTP size. As calculated *p35/16S, nos/16S,* and *nptII/16S* ratios were strongly correlated with WWTP size (p=0.011, p=0.019, and p=0.0090, respectively). However, the ratio of *bla* to 16S abundance showed little correlation with WWTP size (p=0.23). This is to be expected as *16S* concentration was strongly correlated with WWTP size, as noted above. Furthermore, it is reasonable to assume that the correlation strength *of p35/16S, nos/16S,* and *nptII/16S* ratios is primarily a reflection of the correlation between *16S* and WWTP size.

In conclusion, it is possible that many of the detected copies of the *p35* promoter, *nos* terminator, and *nptII* AR genes are derived from digested transgenic crops or foods containing their byproducts. This is supported by the strong correlations between gene abundance and WWTP location as well as the large discrepancies in gene abundance between US and control WWTPs, where transgenic crops were not grown or consumed [135]. Additional screenings for CaMV genes in WWTP sludges were negative, suggesting that the detected *p35* genes were not viral in origin. Though without in depth metagenomic analysis, these results do not prove that transgenic crops are the sources of these transgenes they do suggest it, which warrants further investigation into the source and condition of detected transgenes. Horizontal gene transfer (HGT) has been documented to occur within WWTPs at elevated rates, possibly as a function of the high

concentration and mobility of aquatic bacteria as well as the elevated availability of dissolved Ca^{2+} and Mg^{2+} ions [23, 136-138]. Therefore, it is important to verify the sources of potential transgenes in WWTPs, as documented herein, and to evaluate their ability to be taken up by waterborn bacteria.

6. Examining the Behavior of Transgenic Crop-Derived Free DNA in WWTP Anaerobic Digesters

The following chapter outlines the work completed to address Objective IV of this dissertation. The presented work is in preparation and will be submitted for review to the journal of *Environmental Science & Technology* in June 2017.

6.1 Introduction

Consumption of transgenic crops and crop byproducts is extremely common in

the United States; the widespread cultivation and incorporation into foodstuffs makes this almost inevitable. As noted in Chapter 5, DNA encapsulated within food matrices appears to be significantly protected from robust digestive processes, and several studies have indicated that there is evidence of gene transfer from ingested transgenic crop biomass [118]. The level of protection may vary among different foods. For example, foods that are only partially digested by the human body to digest (e.g., corn and some grains) may offer increased protection to internal DNA [139]. Sizeable fragments of ingested transgenes have been detected in the gut of animal models [118], fetuses and newborns [120], and immune-compromised adults [46]. For example, bacterial uptake of conventional and transgenic plant genes has also been observed in the small bowels of individuals with ileostomies. However, uncertainty remains regarding the behavior of this protected DNA—derived from conventional and transgenic foods alike—once it survives the digestive process and enters into other environments within solid waste (i.e., WWTPs). Because several lines of commercially available transgenic crops have been known to contain one or more ARGs, the consequences associated with uptake of transgenic crop DNA within the gut microbiome may have significant consequences with regards to antibiotic resistance.

WWTPs have become increasingly common in the Unites States over the past several decades to treat both industrial and municipal wastewaters. More than 16,000 municipal WWTPs are currently in operation, and they are responsible for treating the

waste produced by more than 75% of US municipal wastewater. The remaining 25% of municipal waste is treated though an alternative method, such as via septic tanks or other onsite treatment systems [121]. Briefly, most municipal waste in the US is subject to primary treatment (grit removal, chemical coagulation, and sedimentation), secondary treatment (activated sludge digestion, BOD and nutrient removal), additional sludge treatment (anaerobic or aerobic digestion), and occasionally tertiary treatment (disinfection by ozone, UV light, or chlorination). The overall wastewater treatment process produces two main waste products: 1) reclaimed water, which may be channeled directly into receiving streams or groundwater, and 2) biosolids, which are either incinerated or incorporated into fertilizers for land application. As two of these core treatment processes are biologically based (i.e., activated sludge and anaerobic digestion), there exists the possibility that WWTP bacteria may come into contact with the free DNA remaining in partially or undigested food material in human waste [140].

Although the activated sludge and anaerobic digestion treatment processes both occur within WWTPs, they should be evaluated independently of each other. Bacterial community composition and structures have been noted as varying widely between activated and digester sludge, due to the different nature of these processes [127]. Activated sludge is maintained as an aerobic mixed culture, while digester sludge is often maintained anaerobically [141, 142]. Targeting how free DNA behaves in anaerobic digesters would ameliorate our understanding of how this DNA may persist or be taken

up by bacteria in digester sludge (a.k.a. biosolids). This area of research is particularly important as more than 50% of WWTP generated biosolids are land applied and reintroduced into the environment as fertilizer [121, 143]. Recent studies have indicated that genetic material contained within land applied biosolids may be escaping into surrounding environments. For example, ARGs found along a human-impacted river in Colorado possessed a molecular signature that was more closely related to those of WWTPs than to nearby concentrated animal feeding operations (CAFOs) [144]. Similarly, relative to control soils, elevated levels of *tet(O), tet(W)* and *sulI* ARG were observed in soils amended with WWTP-derived biosolids [145]. If no significant ARG elimination takes place in biosolids, these ARGs may be introduced elsewhere via land application. Furthermore, elevated ARG concentrations in WWTP wastewater may also increase HGT rates for these genes among competent bacterial populations [146-148].

Just as anaerobic digesters vary in their nutrient removal efficiencies, individual anaerobic digester conditions (e.g., solids retention time and operating temperature) can have a dramatic effect on the removal of free DNA, notably ARGs. Solid retention times (SRTs) of anaerobic sludge for standard municipal domestic WWTP digesters are approximately 20-30 days. Shorter digester SRTs result in decreased ARG removal efficiency, while longer digester SRTs have the opposite effect [127]. The operating temperature of anaerobic digesters has also been found to affect ARG concentrations. Most WWTPs in the US process digester solids under mesophilic conditions (30°-38°C) to

conform to the regulations associated with generating Class B biosolids, which are only approved for land application with restrictions. As a result of increased demand for higher quality biosolids, thermophilic anaerobic digestion (>40°C) is becoming a more popular treatment method, since it produces Class A biosolids that are sanctioned for land application without restriction [149]. Increasing anaerobic digester operating temperatures from mesophilic to thermophilic conditions has been shown to effectively reduce ARG concentrations by several orders of magnitude [132]. However, the degree to which ARGs may be removed is dependent upon how ARGs exist within the WWTPs, namely whether ARGs exist as free extracellular DNA or as functional intracellular DNA.

The nature and location of ARGs within WWTP digesters is also not well understood. Many ARGs are integrated into mobile genetic elements such as plasmids, integrons, and transposons, through which they can be disseminated among bacterial populations via HGT. When incorporated into bacteria as intracellular DNA (iDNA) these mobile elements are relatively stable and may not be transcriptionally active, depending upon the external stressors present in a given environment. ARGs located on iDNA are transferred to other bacteria through conjugation, requiring the formation of a pilus structure to physically connect the two bacteria through which the iDNA is transferred [76, 150]. Conversely, extracellular DNA (eDNA) is located outside the bacterial cell and may be unattached or bound to clays or organic matter compounds. EDNA is integrated into bacteria through the process of transformation, which requires natural competence

in the environment. More than 90 bacterial species have been shown to exhibit natural competence, including *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* [75, 151], and there are likely more in the environment. Transformation events have been detected in a variety of natural environments including, soil [88, 152], marine sediments [153], freshwater biofilms [154], and river epilithon [155]. The rates associated with these HGT events vary widely, ranging from 10^{-9} to 10^{-3} events per bacterium for certain species [88]. Several studies have also indicated that eDNA located in soil and sediment act as a reservoir for free DNA, including a variety of ARGs. These free DNA fragments are often bound to colloid particles and are protected from degradation by DNAse enzymes, and therefore may facilitate HGT in these environments [156]. It stands to reason that WWTP digesters may also harbor these extracellular genes, warranting further investigation.

The ability of bacteria to take up transgenes in a WWTP is a factor of: 1) the observed rates of HGT in anaerobic environments, 2) the abundance of genes of interest within WWTP anaerobic digesters, and 3) the presence of a selective pressure. Observing and quantifying HGT events in anaerobic environments has proved to be a difficult task. There have been many instances of HGT being observed *in vitro*, though this often occurred under highly controlled conditions and does not reflect environmentally relevant scenarios [147]. Past studies have looked instead to the nucleotide sequences specific to detected ARGs to ascertain the occurrence of HGT. A sequence similarity of 95% or higher among separate gene copies has been used to imply that these genes were

transferred though HGT events rather than convergent evolution, which would result in a sequence dissimilarity of more than 90% [157].

Antibiotic resistance has increased among some anaerobic species in recent decades, most notably *Bacteroides*, implying that HGT events are becoming more common among anaerobes [148]. Most work conducted on HGT in anaerobic environments has focused on human health: specifically, how antibiotic resistance is propagating among bacteria located in the human digestive system. Researchers have recently discovered a network of more than 10,000 newly transferred genes among 2,235 bacterial genomes in the human gut and estimated HGT rates to be 25-fold lower among ecologically diverse communities. In other words, bacteria that fill the same ecological niche (e.g., the same WWTP sludge tank) are more likely to take part in HGT events than bacterial communities who do not share a common ecology [158]. Extensive conjugal gene transfer among genera has also been observed in the human colon [159]. HGT events within WWTPs have proved more difficult to detect and track, though many believe that biologically based treatment tanks act as HGT hot spots [160]. Some studies suggest HGT may take place at a rate of 3.79×10^{-5} to 4.76×10^{-5} events per recipient cell in anaerobic environments [137]. Several studies have also noted that antibiotic resistance levels present in WWTP influent significantly increase by anaerobic digester and effluent stages of treatment, suggesting the occurrence of HGT events [138].

The abundance of potential transgenes in WWTP digester sludge has been investigated for this project and is presented in Chapter 5. In brief, screened transgenes included the *p35* promoter gene, *nos* terminator gene, *nptII* ARG, and *bla* ARG. *P35* promoter genes were detected in 20 WWTPs with an average concentration of 1.13 $x10^3$ copies/mL digester sludge. *Nos* terminator genes were detected in 19 WWTPs with an average concentration of 2.77 $x10^3$ copies/mL of sludge. *Bla* and *nptII* ARGs were found in 15 and 17 of the 26 screened WWTPs respectively, with an average concentration of 3.67 $x10^3$ and 5.29 $x10^3$ copies/mL of wastewater. Arguably, the most significant of these genes are the *nptII* and *bla* ARGs, as they have the potential to contribute to antibiotic resistance in wastewater bacteria. However, all ARGs, regardless of source, can contribute to antibiotic resistance so it is equally important to understand how other free ARGs behave in WWTPs. Furthermore, it has been widely noted that antibiotics have become ubiquitous constituents of municipal wastewater across the US. Traditional municipal wastewater treatment plants have been shown to contain both antibiotic resistant bacteria and antibiotic chemicals. The levels of resistance and ARGs found in these systems are the highest outside of hospital wastewater settings [23, 42-44]. Resistance to vancomycin, cefazolin, and penicillin was found to be 11%, 39%, and 30% among total bacteria in WWTP activated sludge basins, respectively. The effluent of the same WWTPs, which is commonly discharged into nearby rivers, still showed significant resistance rates of 15%, 39%, and 20%, respectively. When taking into account the number of CFUs per mL, these

levels of resistance are significantly higher than those found in either drinking water or surface water sources (Table 1). Additional studies have characterized the class and concentration of contaminating antibiotics, which are often detected at a scale of μg per liter of wastewater [161] (Table 11).

Table 11: Summary of average concentration and average percent recovery of antibiotics from WWTP influent and effluent samples. Adapted from Karthikeyan et al. [161].

Compound	Influent				Effluent			
	Average Concentration (μg/L)	Standard Deviation (μg/L)	Average Recovery	Standard Deviation	Average Concentration (μg/L)	Standard Deviation (μg/L)	Average Recovery	Standard Deviation
Sulfamethizole	0.28	0.03	100	11	0.34	0.03	90	23
Sulfathiazole	0.30	0.04	99	18	0.35	0.04	88	11
Sulfamerazine	0.29	0.04	97	8.2	0.33	0.03	130	7.6
Sulfamethazine	0.30	0.03	100	24	0.36	0.01	78	6.2
Sulfachloropyrizadine	0.33	0.02	71	4.6	0.32	0.01	88	3.4
Sulfamethoxazole	0.31	0.03	108	17	0.27	0.04	98	6.0
Lincomycin	0.36	0.05	121	11	0.51	0.11	81	14
Carbadox	0.34	0.05	112	13	0.36	0.03	97	2.7
Trimethroprim	0.44	0.04	138	19	0.39	0.01	106	8.4
Sulfadimethoxine	0.29	0.15	111	5.4	0.35	0.04	82	8.2
Tylosin	1.5	0.02	95	15	0.72	0.09	105	15
Erythromycin-H_2O	3.9	0.31	113	12	1.1	0.23	93	13
Roxithromycin	1.5	0.15	84	5.7	0.87	0.05	96	4.4
Tetracycline	48	3.21	94	9.3	3.6	0.31	96	10
Chlortetracycline	0.31	0.02	98	13	0.42	0.06	114	22

Oxytetracycline	47	3.61	92	17	4.2	0.40	110	13
Doxycycline	10	0.76	89	22	10.9	2.76	73	6.2
Norfloxacin	0.25	0.03	91	7.8	0.33	0.04	97	3.3
Ciprofloxacin	0.31	0.04	102	9.3	0.31	0.02	87	9.2
Enrofloxacine	0.25	0.04	97	11	0.27	0.03	92	7.1
Sarafloxacine	0.25	0.03	108	7.2	0.25	0.01	98	10

Because municipal WWTPs are not designed to eliminate these pharmaceutical compounds, they are present and persist within every stage of the wastewater treatment system. As indicated in Table 11, antibiotic concentrations in the effluent are largely similar to those in the influent, suggesting that little if any degradation takes place. Despite this, the observed levels are not elevated enough to be toxic to the majority of bacteria that reside within activated sludge and digester tanks. However the constant low levels of these antibiotics likely act as a selective pressure to these bacteria, thereby inducing them to maintain ARGs already present in their genetic constructs or take up ARGs from their environments (e.g., through transformation or conjugation events).

The goal of this study was to determine the fate of transgenic maize-derived ARGs during anaerobic digester treatment. To this end, **t**hermophilic anaerobic digesters were constructed and inoculated with various known concentrations of GM maize transgenes. The abundance of these transgenes and corn marker genes were monitored over a period of 60 days.

6.2 Materials and Methods

6.2.1 Transgenic BT Maize DNA Isolation

Genomic DNA from transgenic BT maize was isolated using the following CTAB isolation protocol outlined by Delobel et al. [162]. First, 500 μL CTAB Buffer (100mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone, 2% hexadecyl trimethyl-amonium bromide (CTAB) (w/v), and 0.5% β-Mercaptoethanol (v/v)) was added to 100 mg maize biomass and incubated at 60°C for two h. Samples were removed from heat and 1.0 μL RNAse A (10 mg/mL) was added to the mixture and incubated at 37°C for 30 min to remove RNA and increase the accuracy of spectrophotometric measurements. Then 700 μL phenol:chloroform:IAA was added, tubes were vortexed briefly and centrifuged at 13,000x g for 60s. This step was repeated until no debris could be seen at the interface. Next, 700 μL of chloroform was added to the aqueous phase and tubes were centrifuged at 13,000x g for 2 min. The aqueous phase was transferred to a new tube and 0.08 volumes of cold 5 M ammonium acetate and 0.54 volumes of isopropanol were added, mixed by inversion, and incubated on ice for 45 min. DNA was then purified by ethanol precipitation and the resulting pellet was resuspended in 50 μL TE Buffer. Resuspended DNA was further purified with the DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) according to manufacturer instructions and quality was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA). Only

extracted DNA with a 260/280 ratio of 1.8-2.0 and a 260/230 ratio of 2.0-2.2 were used for transgene isolation and digester construction.

6.2.2 Transgene Isolation and Quantification

6.2.2.1 Isolation of *bla*, *nptII*, and *LUG* Genes from Transgenic BT Maize

Whole *bla*, *nptII* genes and partial *LUG* genes were then amplified from isolated DNA using the primers found in Table 12. End-Point PCR conditions for each gene were performed using a BioRad T100 Thermocycler (BioRad, Hercules, CA) as follows: Initial denaturation at 95°C for 3min, followed by 35 cycles of 95°C for 45s, 55°C for 60s, and 72°C for 30s. Negative and positive controls were incorporated into each PCR run. PCR products were purified using the DNA Clean & Concentrator kit according to manufacturer instructions (Zymo Research, Irvine, CA).

Table 12: End-point PCR primer sequences used for *bla*, *nptII*, and *LUG* gene isolation from transgenic BT maize.

Target Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Amplicon Size (bp)	Source

bla	GAAAGGGCCTCGTG ATACGC	CGGGGTCTGACGCTCA GTGG	1205	[130]
nptII	AAATCGGCTCCGTCG ATACT	CCCAATCAGGCTTGAT CCCC	929	This Study
LUG	TCCAGTGCTACAGGG AAGGT	GTTAGTTCTTGAGCCC ACGC	178	[163]

Nested PCRs with *bla* and *nptII* whole-gene PCR products were performed with the qPCR primers outlined in Table 8 to ensure the presence of smaller qPCR gene targets within the whole gene amplicons. Nested PCRs were performed as described above. The resulting nested PCR products were run on a gel to test qPCR primer specificity.

6.2.2.2 Quantification of Whole-Gene PCR Products

The concentration of purified *bla*, *nptII*, and *LUG* PCR products was determined with qPCR using methods described in Section 6.2.2.1. *Bla* genes were amplified using SYBR Green chemistry under the following conditions: Initial denaturation at 95°C for 3min, followed by 40 cycles of 95°C for 45s, 55°C for 60s, and 72°C for 30s. *LUG* genes were also amplified using SYBR Green chemistry under the following conditions: Initial denaturation at 95°C for 3min, followed by 40 cycles of 95°C for 45s, 55°C for 30s, and 72°C for 30s. Fluorescence measurements for SYBR Green chemistry assays were taken during elongation steps. The dissociation curve was assessed at the end of the run under the following conditions: 95°C for 60s, 55°C for 30s, and 95°C for 30s. QPCR amplification

using TaqMan®™ Probe chemistry was performed for *nptII* genes using the following conditions: Initial denaturation at 95°C for 60s, followed by 40 cycles of 95°C for 30s and 60°C for 30s. Fluorescence measurements for each TaqMan®™ assay were taken during the annealing steps. All qPCR assays were performed using a Stratagene Mx3000P thermocycler (Agilent Technologies, Santa Clara, CA).

Gene copy number per μL was determined for each transgene and PCR products were then diluted to three different stock concentrations: 10^3 copies/mL, 10^5 copies/mL, and 10^7 copies/mL. Stocks were stored at -20°C until anaerobic digesters could be constructed.

6.2.3 Anaerobic Digester Set Up

Anaerobic digester sludge was obtained from the North Durham Wastewater Treatment Facility (Durham, NC). Digester sludge was collected in sterile 1L containers and transported in a warm water bath to Duke University, where it was immediately placed in an incubator set to 47°C. All digester reactors were constructed in quadruplicate under sterile anaerobic conditions. First, 50 mL of anaerobic digester sludge was added to sterile 100mL serum bottles. Anaerobic digesters were then set up under five treatment conditions (Table 13).

Table 13: Digester setup components for anaerobic digester treatments with Low, Medium, and High concentrations of added transgenic crop transgenes.

Treatment	Sludge Volume (mL)	Penicillin-Kanamycin Addition	*bla* Concentration (copies/mL)	*nptII* Concentration (copies/mL)	*LUG* Concentration (copies/mL)

Control A	50	0	0	0	0
Control B	50	5 μg/mL	0	0	0
Low (10^2 copies/mL)	50	5 μg/mL	10^2	10^2	10^7
Medium (10^4 copies/mL)	50	5 μg/mL	10^4	10^4	10^7
High (10^6 copies/mL)	50	5 μg/mL	10^6	10^6	10^7

Serum bottles were immediately sealed following set up and placed into a 47°C incubator. Feed sludge was used for time zero measurements. Serum bottles for each treatment were sacrificed in quadruplicate after 1, 7, 30, and 60 days of incubation.

6.2.4 Intracellular and Extracellular DNA Isolation

Gaining a deeper understanding of how free DNA behaves in complex anaerobic sludge matrices was accomplished by extracting and analyzing both iDNA and eDNA. Anaerobic batch reactors were sacrificed in quadruplicate for each time point. Two mL of digester sludge were centrifuged at 11,000x g for one minute to obtain solids and supernatants for iDNA and eDNA extraction, respectively. Solids were washed thrice with 0.1 M PBS solution (pH 8.0) and centrifuged at 11,000x g for one minute and the resulting supernatant was decanted. IDNA was extracted from washed digester solids using the PowerLyzer PowerSoil kit according to kit manufacturer instructions (MoBio Laboratories Inc., Vancouver, CA). DNA was eluted in 50μL of elution buffer and stored at -20°C for downstream analysis.

Resulting supernatants from the initial centrifugation were transferred to a sterile 1.5mL centrifuge tube for extracellular DNA extraction. EDNA was extracted according to the protocol outlined by Mao et al. [156] and Corinaldesi et al. [164]. Briefly, 4 mL of 0.12M NaH_2PO_4 (pH 8.0) and 0.2 g of polyvinyl polypyrrolidone (PVPP) was added to supernatants and shaken at 250 rpm and 25°C for 10 min. The mixture was centrifuged at 10,000x g and 4°C for 10 min and supernatants were transferred to a fresh sterile 15mL tube and stored on ice. This was performed in triplicate. The resulting supernatants were pooled and filtered through sterile 0.2 μm PVDF membrane filters (Whatman, Maidstone, UK) and stored on ice. Filtered supernatants were then centrifuged at 10,000x g and 4°C for 20 min and stored on ice. After centrifugation, the supernatants were filtered through 0.02 μm Anotop 25 membrane filters (Whatman, Maidstone, UK) to remove potential contaminating viral or bacterial cells. EDNA was precipitated by adding 1 volume of a cetyltrimethylammonium bromide (CTAB) solution, consisting of 1% CTAB in 50 mM Tris and 10 mM EDTA (pH 8.0). Samples were incubated at 65°C for 30 min and centrifuged at 5,000x g and 4°C for 10 min. Supernatants were decanted and pellets were resuspended in TE buffer solution (10 mM Tris-HCl, 0.1 mM EDTA, and 1 M NaCl; pH 8.0). Then 0.6 volumes of ice-cold isopropanol was added to each sample and samples were incubated on ice for one hour. Tubes were then centrifuged at 10,000x g and 4°C for 15 min. Supernatants were decanted and pellets were resuspended in Tris-EDTA solution (10 mM Tris-HCl and 0.1 mM EDTA; pH 8.0). An equal volume of phenol:chloroform:isoamyl

alcohol (25:24:1) was added to each sample, vortexed briefly to mix, and centrifuged at 10,000x g and room temperature for 5 min. The aqueous phase was transferred to a sterile 1.5 mL centrifuge tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added to each sample. Tubes were centrifuged again at 10,000x g and room temperature for 5 min. The supernatant was then precipitated with cold ethanol (final concentration, 70%) and NaCl (final concentration, 0.2 M), and incubated at −20°C for one hour. Tubes were centrifuged at 10,000x g and room temperature for 15 min. The pellet was then precipitated again with 70% and 95% ice-cold ethanol and the resulting pellet was dried gently at 37°C. Finally, the pellet was resuspended in 50μL of TE buffer (pH 8.0) and stored at -20°C for downstream analysis.

6.2.5 Detection of Transgenes via qPCR

Extracted iDNA and eDNA were then screened for the presence of *bla*, *nptII*, and *LUG* transgenes. QPCR assay conditions for *bla*, *nptII*, and *LUG* genes were performed as outlined above in Section 6.2.2.2. The abundance of *16S* genes was also quantified in each sample and served as a proxy for bacterial abundance. The following conditions were used for *16S* TaqMan®™ assays: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30s and 60°C for 60s. Fluorescence was measured during elongation steps. Results were normalized to gene copies per mL of digester sludge.

6.2.6 Measuring Digester Biogas Production

Biogas production was measured in each anaerobic digester every seven days and on every digester sampling day. Serum bottles were removed from the 47°C incubator and placed in a fume hood. Biogas was collected from each digester and measured with sterile 30mL syringes fitted with sterile 1″ 25 gauge needles. Needles were changed between serum bottles and were not permitted to contact digester sludge. Average biogas production rates and standard deviations were calculated for each time point.

6.2.7 Statistical Analysis

The data were normalized to gene copies per mL of wastewater. The copy number of each transgene was also compared the copy number of *16S* genes per mL of wastewater (number of bacteria per mL). Multiple regression analyses and correlation analyses were performed to investigate the relationships among gene concentration, digester treatment, and incubation time. Averages and standard deviations of all data were calculated using Microsoft Excel 2007. All other statistical tests-including t-tests and ANOVAs-were conducted in RStudio 1.0.136.

6.3 Results and Discussion

6.3.1 Anaerobic Digester Biogas Production

Biogas production was measured in each anaerobic digester to assess the metabolic activity of microbial communities throughout the incubation period. Biogas

production rates were highest just after anaerobic digesters were set up, with an average of 212.2 mL of biogas produced per week across all treatments (Figure 43). Biogas production steadily decreased over time, decreasing to 60.3 mL of biogas/week by day 7 and 11.65 mL of biogas/week by day 30. Production rates remained steady between days 30 and 49, before decreasing to 2.76 mL biogas/week by day 60.

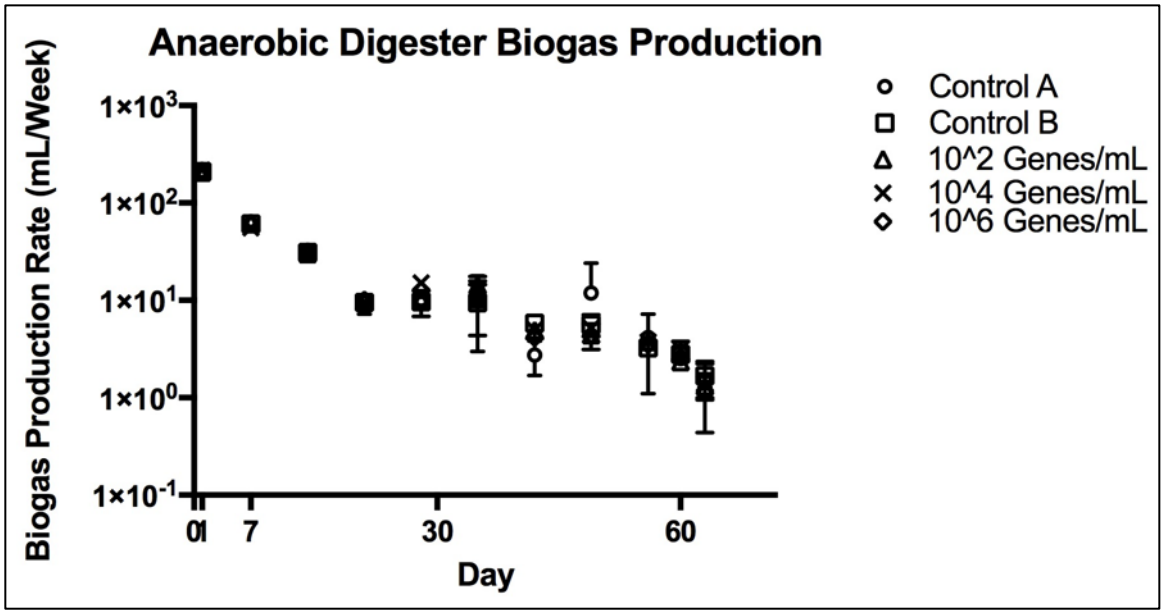

Figure 35: Average biogas production rates (mL biogas/week) of all anaerobic digester treatments. Error is reported as standard deviation (n=20).

The trends observed in these digesters are similar to those of other thermophilic anaerobic digesters (Ferrer et al., 2008). Biogas production rates were significantly affected by sludge age (p=0.016). No significant differences in biogas production were detected

between treatments at each sampling time, suggesting that the treatment reactors were operating similarly to the control reactors.

6.3.2 Detection of Transgenes in Anaerobic Digesters

In order to determine the behavior of free transgenic crop DNA in WWTP digesters, *bla* and *nptII* ARGs were isolated from BT transgenic maize and added to anaerobic digesters in the following concentrations: 10^2 copies/mL, 10^4 copies/mL, and 10^6 copies/mL. *LUG*, a corn-specific marker gene, was also added to all experimental treatment digesters at a concentration of 10^7 copies/mL. Negative control anaerobic digesters—with and without added penicillin and kanamycin antibiotics—without added *bla, nptII,* or *LUG* genes were used as controls. Digesters were incubated at 47°C for the duration of the experiment to simulate the thermophilic digestion conditions necessary to produce Class A biosolids. The concentration of each gene (copies/mL wastewater) in both iDNA and eDNA was monitored using qPCR for a period of 60 days.

Bla genes were detected in eDNA in all digesters at 0, 1, 7, 30, and 60 days of incubation (Figure 44). Feed anaerobic digester sludge was found to have an average of 3.05 $x10^2$ copies of *bla* ARGs per mL wastewater. The *bla* gene copy numbers in both Control A and B anaerobic digesters remained constant for 60 days of incubation, and dropped in the subsequent 30 days. During the first month, Control A had a maximum concentration of 1.67 $x10^3$ copies/mL wastewater (day 7), a minimum of 1.67 $x10^3$ copies/mL (day 30), and an average of 8.66 $x10^2$ copies/mL wastewater. Similar trends

were observed in Control B digesters during this time frame. By 60 days of incubation, both Control A and Control B digesters experienced a significant decrease in *bla* concentration (8.58 $x10^{-1}$ copies/mL and 7.49 $x10^{-1}$ copies/mL, respectively) (p=0.045). The concentrations of *bla* in Low treatment digesters were not statistically different from Control A and B digesters across 60 days of incubation (p=0.36). Given the starting concentration of *bla* genes in wastewater eDNA, this is not surprising; naturally occurring *bla* concentrations were an order of magnitude higher than added free DNA. *Bla* levels in 10^4 copies/mL treatment digesters were also similar to those of Control A and B digesters across all time points (p=0.24). Concentrations in Middle treatments peaked at 7.82 $x10^3$ copies/mL (day 7), with an average of 3.79 $x10^3$ copies/mL during the first 30 days of incubation. *Bla* concentrations in High treatment digesters were significantly elevated relative to Control treatments, with an average of 1.64 $x10^5$ copies/mL wastewater across all time points (p=0.042). *Bla* ARGs increased from an initial concentration of 3.05 $x10^2$ copies/mL to 8.69 $x10^4$ copies/mL by day 1, with statistically similar concentrations detected at day 7 (2.76 $x10^4$ copies/mL). *Bla* levels increased further at day 30 (5.34 $x10^5$ copies/mL), a statistically significant increase relative to all other treatments at this time point (p=0.012). By day 60 *bla* levels in these digesters decreased to 6.42 $x10^3$ copies/mL wastewater but were still significantly elevated compared to *bla* gene copy numbers in other treatments at day 60 (p=0.016). Overall, trends in *bla* abundance within digester eDNA were more influenced by treatment type (p=0.012) than time (p=0.21).

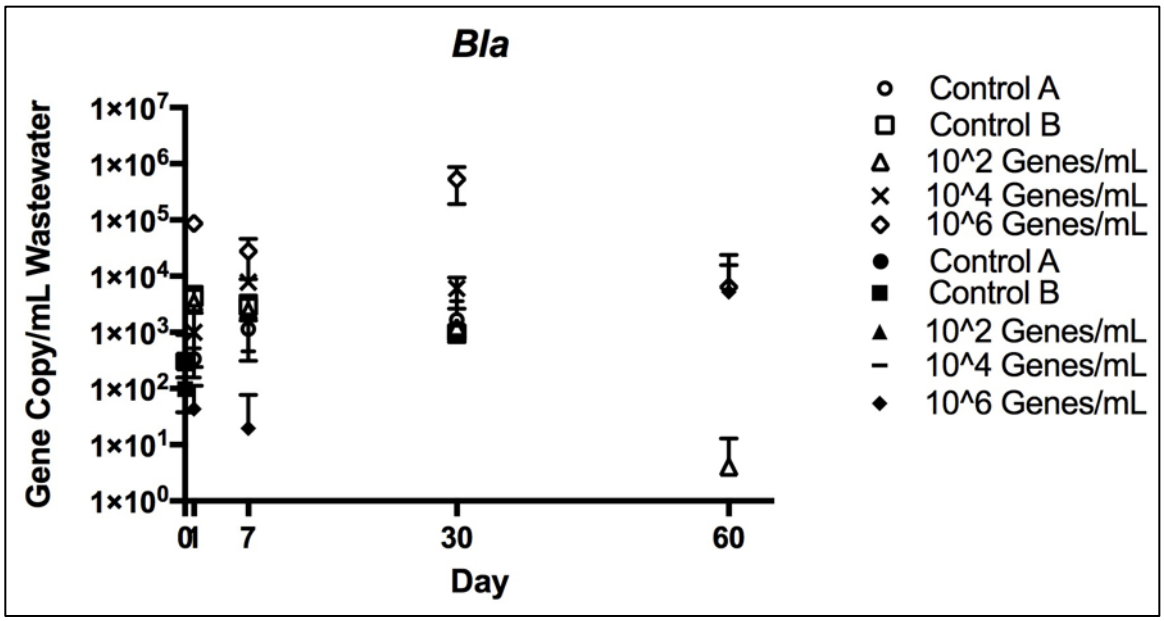

Figure 36: Detection of transgenic maize-derived *bla* ARGs in anaerobic sludge digesters across 60 days of operation. The detected copy number of *Bla* for each has been normalized to copies per mL of wastewater. Hollow data points correspond to extracted intracellular DNA (iDNA) and solid data points correspond to extracted extracellular DNA (eDNA). The following samples were below detection and are not represented on this graph: iDNA in all treatments at 0 days, 1 day, 7 days, 30 days; iDNA in High treatments at 30 days. Error is reported at standard deviation (n=12). *Bla* assay detection ranged from 10^1-10^8 copies/μL.

Bla genes were detected in iDNA from the following samples: 0 days High treatments at 1, 7 days, and 60 days.

Similarly to *bla*, the majority of *nptII* genes in anaerobic digesters were found in eDNA at all time points (Figure 45). The feed digester sludge was found to have 1.04 x10^4 extracellular copies of *nptII* per mL wastewater at day zero. The concentration of *nptII* of both Control A and B anaerobic digesters varied across the duration of the experiment, decreasing to 8.04 x10^2 copies/mL (Control A) and 7.43 x10^2 copies/mL (Control B) by day

30. At 60 days of incubation, both Control A and Control B digesters experienced an increase in *nptII* concentration in eDNA. However, these concentrations at day 60 were not significantly different from starting concentrations at day zero (p=0.11). Similar to *bla* genes, the concentrations of *nptII* in Low treatment digesters were indistinguishable from Control A and B digesters across 60 days of incubation. *NptII* levels in Middle treatment digesters remained consistent across all time points. Concentrations peaked at 6.64 $x10^4$ copies/mL (day 30), with an average of 2.93 $x10^4$ copies/mL across all time points. The difference in trends between Middle treatments and Control treatments was marginally significant (p=0.064). Levels in High treatment digesters were significantly elevated relative to Control treatments, with an average of 9.97 $x10^5$ copies/mL wastewater across all time points (p=0.013). *NptII* concentrations increased to 2.48 $x10^6$ copies/mL by day 1, with similar concentrations detected at day 30 (1.16 $x10^6$ copies/mL). *NptII* levels were lowest at day 60, where 2.25 $x10^4$ copies/mL was detected. The concentration at day 60 in High treatments was not significantly different than Control digesters. Overall, *nptII* concentrations in digester eDNA were significantly affected by neither treatment (p= 0.59) nor time (p=0.24).

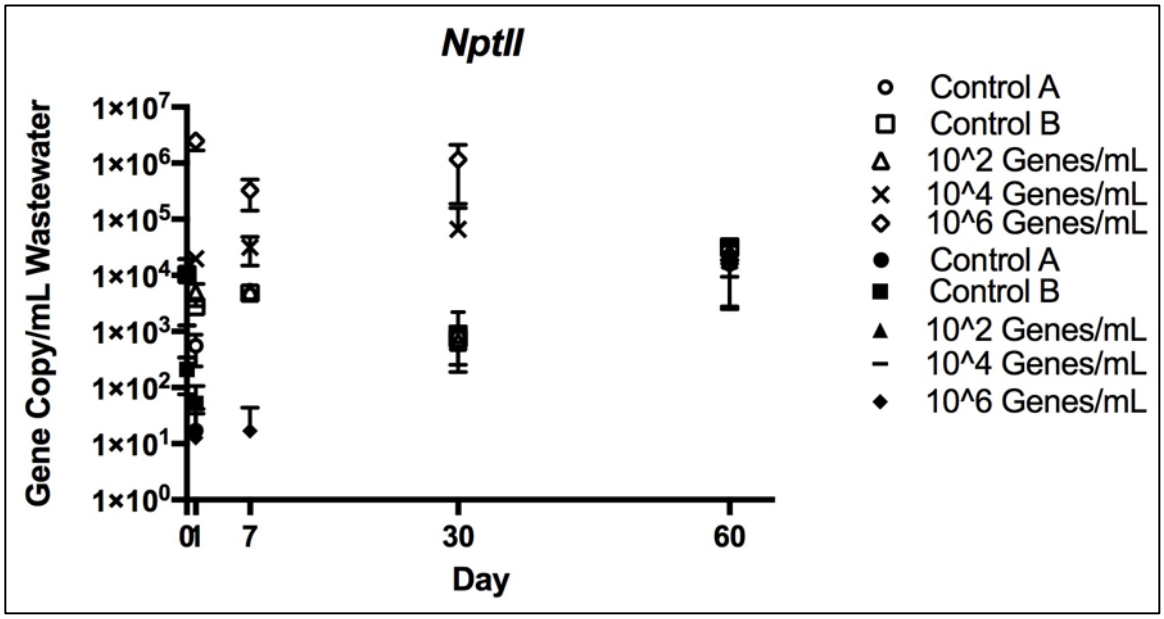

Figure 37: Detection of transgenic maize-derived *nptII* ARG DNA in anaerobic sludge digesters across 60 days of operation. The detected copy number of *NptII* for each has been normalized to copies per mL of wastewater. Hollow data points correspond to extracted intracellular DNA (iDNA) and solid data points correspond to extracted extracellular DNA (eDNA). The following samples were below detection and are not represented on this graph: iDNA in Control B, Low, and Middle treatments at 1 day, 7 days, 30 days; in iDNA in Control A treatments at 7 days, 30 days, and 60 days; and iDNA in High treatments at 30 days and 60 days. Error is reported at standard deviation (n=12). *NptII* assay detection ranged from 10^1-10^8 copies/μL.

The number of *nptII* genes was below detection in the following samples: iDNA in Control B, Low treatments, and Middle treatments at 1, 7, 30 days; in iDNA in Control A treatments at 7, 30, and 60 days; and iDNA in High treatments at 30 and 60 day time points. *NptII* was detected in iDNA associated with Control A, Control B, and High treatments at 0 days and 1 day post-incubation. However, the observed *nptII* concentrations were not significantly different among treatments (p=0.76).

LUG genes were added at a concentration of 10^7 copies/mL the following treatments: 10^2 transgene copies/mL, 10^4 transgene copies/mL, and 10^6 transgene copies/mL. The *LUG* gene produces the Leunig protein and acts as a repressor gene during early stages of floral meristem development in maize and tobacco [165]. It has also been suggested to be involved in plant ovule and pollen development [166]. Current literature indicates this gene is a strong reference gene for maize, with high expression stability and specificity to the maize genome [163]. *LUG* genes were added to treatment digesters at an artificially high concentration to help elucidate the potential mechanism of *bla* or *nptII* HGT, should the concentration of these ARGs increase significantly in anaerobic digesters over time. For example, in High treatments a *LUG*/*ARG* ratio of less than 10 (i.e., 10^7 copies of LUG/mL: 10^6 copies of *bla*/mL) could indicate the presence of conjugation events within digesters. *LUG* concentrations in digesters were not expected to increase across time, as *LUG* genes do not confer any useful traits for bacteria. Therefore, a relative increase in detected ARG DNA would indicate the possibility that bacteria are transferring ARGs and increasing the overall number of detectable ARGs. Similar trends could also be observed in Low and Middle treatment digesters, though the magnitudes of *LUG*/ARG ratios would depend on individual treatments.

LUG genes were exclusively detected in eDNA extracted from anaerobic digesters (Figure 46). LUG genes were below detection in iDNA for all treatments at 0, 1, 7, 30, and 60 day time points. In the eDNA, an average of 2.64 x10^3 copies/mL of wastewater was

detected in feed anaerobic sludge at day 0. Control A and B digesters had similar concentrations of *LUG* genes across 60 days of incubation. Control A digesters contained a maximum of 1.86 $x10^4$ copies/mL (day 60), a minimum of 1.34 $x10^3$ copies/mL (day 1), and an average of 7.99 $x10^3$ copies/mL wastewater. Control B digesters contained a maximum of 4.34 $x10^4$ copies/mL (day 60), a minimum of 1.02 $x10^3$ copies/mL (day 1), and an average of 1.18 $x10^4$ copies/mL wastewater. Detected *LUG* genes in anaerobic sludge eDNA was similar among all treatments until day 30; Low treatments had an average of 2.66 $x10^6$ copies of LUG/mL, Middle treatments had an average of 2.02 $x10^6$ copies of LUG/mL, and High treatments had an average of 2.12 $x10^6$ copies of LUG/mL. The concentration of *LUG* genes significantly decreased in Low, Middle, and High treatments by day 30, with an average of 1.85 $x10^4$ copies/mL, 1.39 $x10^4$ copies/mL, and 8.26 $x10^3$ copies/mL respectively. On days 30 and 60 of incubation, the concentrations of *LUG* genes among all treatments were similar to those of Control digesters (p=0.59). Overall, *LUG* gene concentrations in anaerobic digesters were more influenced by time (p=0.029) than digester treatment type (p=0.18).

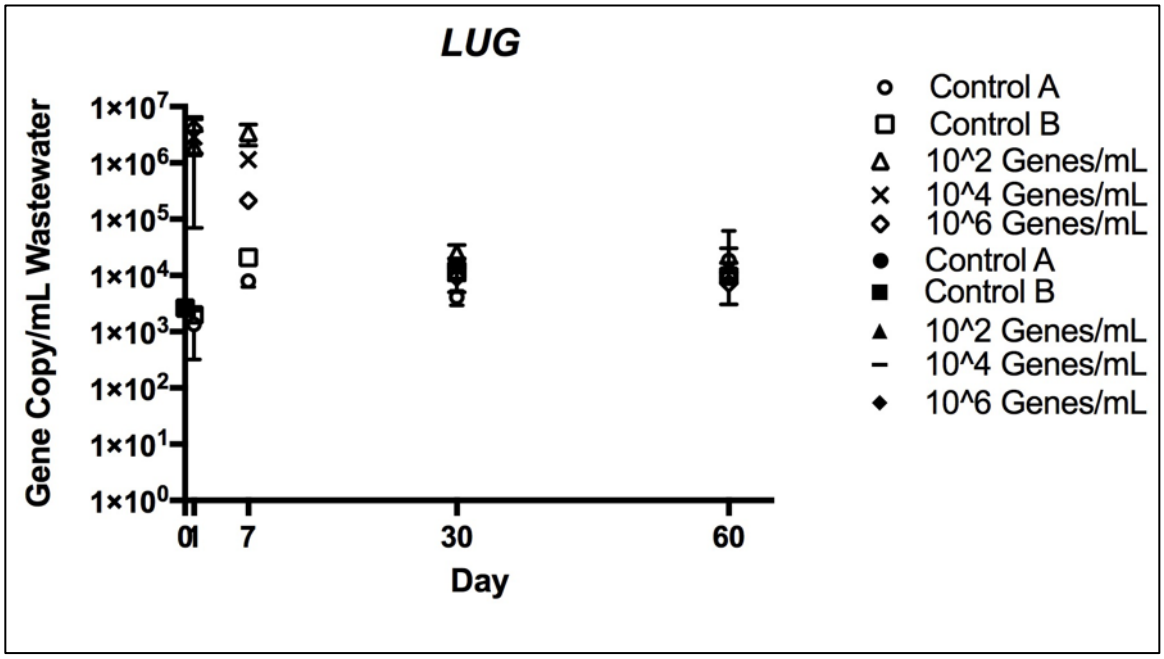

Figure 38: Detection of transgenic maize-derived *LUG* DNA in anaerobic sludge digesters across 60 days of operation. The detected copy number of *LUG* for each has been normalized to copies per mL of wastewater. Hollow data points correspond to extracted intracellular DNA (iDNA) and solid data points correspond to extracted extracellular DNA (eDNA). The following samples were below detection and are not represented on this graph: iDNA in all treatments at 0 days, 1 day, 7 days, 30 days, and 60 days. Error is reported at standard deviation (n=12). *LUG* assay detection ranged from 10^1-10^8 copies/μL.

The number of *16S* genes was also quantified in each sample to serve as a proxy for total bacteria (Figure 47). Detection of *16S* in iDNA remained consistent for all treatments across all sampling times and did not vary significantly with treatment (p=0.91) or sampling time (p=0.82). Average copy numbers of *16S* genes for each treatment were as follows: Control A-3.28 x10^8 copies/mL wastewater; Control B-4.62 x10^8 copies/mL wastewater; Low treatment-1.65 x10^8 copies/mL wastewater; Middle treatment-8.52 x10^8 copies/mL wastewater; and High treatment-5.39 x10^8 copies/mL wastewater. The number

of *16S* genes was below detection in eDNA extractions for all treatments at 0 days, 1 day, 7 days, and 30 days.

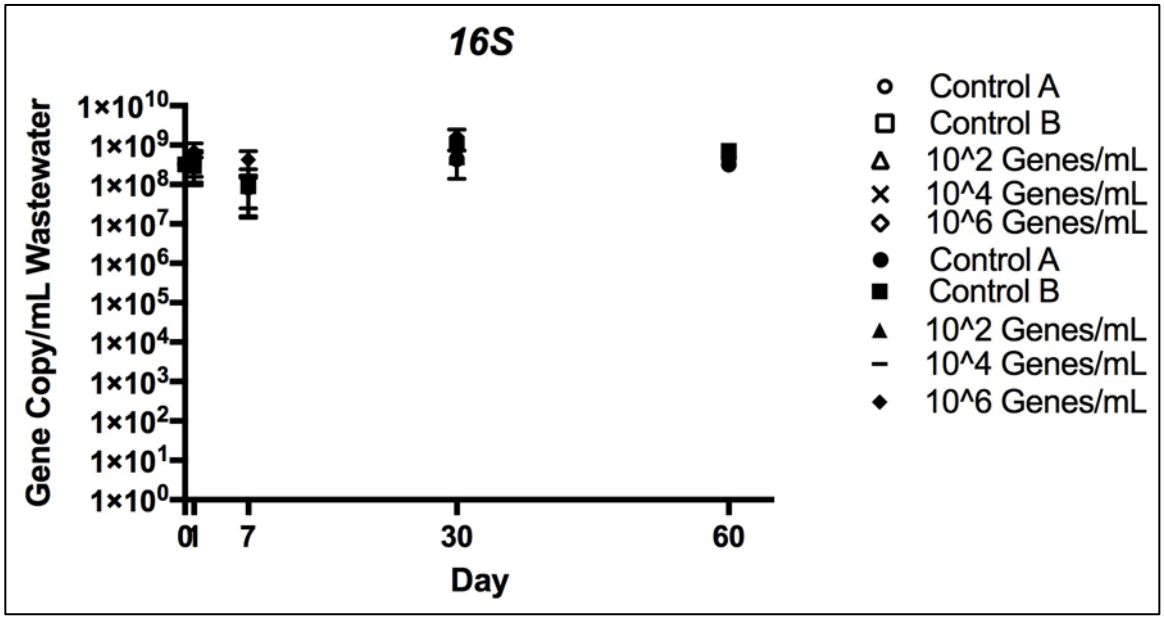

Figure 39: Detection of *16S* DNA in anaerobic sludge digesters across 60 days of operation. The detected copy number of *16S* for each has been normalized to copies per mL of wastewater. Hollow data points correspond to extracted intracellular DNA (iDNA) and solid data points correspond to extracted extracellular DNA (eDNA). The following samples were below detection and are not represented on this graph: eDNA in all treatments at 0 days, 1 day, 7 days, and 30 days. Error is reported at standard deviation (n=9). *16S* assay detection ranged from 10^1-10^8 copies/μL.

Overall, there was no observable difference in detected concentrations of *bla*, *nptII*, or *LUG* genes in eDNA or iDNA associated with Control A and B anaerobic digesters. With regard to *bla* and *nptII* ARGs, this implies that the small amount of penicillin and kanamycin antibiotics added to Control B digesters (5 μg/mL) was not a strong enough

selective pressure to induce high levels of *bla* or *nptII* ARG HGT within these native bacterial communities. Differential HGT rates of these ARGs would have been apparent, since *bla* and *nptII* genes were naturally present in anaerobic sludge at relatively low levels.

LUG genes were exclusively detected in wastewater sludge eDNA. Because LUG genes are not known to be associated with bacterial communities, the presence of these genes in eDNA suggests these are DNA fragments derived from partially digested corn material in WWTP influent. *LUG* genes in eDNA behaved differently than *nptII* and *bla* genes in anaerobic digesters. Although *LUG* genes were added to anaerobic digesters at a much higher concentration than either ARGs, the concentrations of this gene decreased much more rapidly over time. Indeed, the observed LUG levels were indistinguishable from those of Controls A and B by day 30 of thermophilic digestion. The *LUG* DNA fragment added to each treatment was smaller than *nptII* and *bla* genes, 178 bp. Thus, it is possible that this smaller fragment was more susceptible to enzymatic or temperature-based degradation. This is supported by current literature, which suggests that free and undesirable DNA (e.g., ARGs) is more effectively removed in digesters that are operated at higher temperatures with longer SRTs [127, 132, 167].

Both *nptII* and *bla* genes were more often detected in eDNA extracted from digester sludge than extracted iDNA. *NptII* ARGs were more abundant in anaerobic sludge than *bla* ARGs, though this difference may be a characteristic specific to the feed sludge used

for anaerobic digesters. Anaerobic digester sludge generally contains similar levels of both *nptII* and *bla* ARGs, as reported in Chapter 5. Additionally, the general trends for both genes were largely similar across 60 days of digestion. This was expected, as both genes were similar in size: 929 bp (*nptII*) and 1,205 bp (*bla*). Thus it is likely they be similarly protected from degradation from external DNases or other physical-chemical forces. The concentrations of *nptII* and *bla* in 10^2 copies/mL treatment digesters were indistinguishable from Control A and B digesters across 60 days of incubation. However, since the naturally occurring concentration of these ARGs in wastewater were an order of magnitude higher than added free DNA this is to be expected. *NptII* concentrations in Middle and High treatment digesters remained significantly elevated through 30 days of digestion—the average SRT for domestic anaerobic digesters [121]—before decreasing to levels observed in Control reactors. This suggests that the majority of added eDNA had been degraded by day 60 and the remaining ARGs exist as a naturally occurring fraction found in digester eDNA. However, *bla* concentrations in eDNA extracted from High treatment digesters were significantly higher than those of Control digesters. As there was no difference in *bla* levels in Control digesters with and without added antibiotics, this is likely not due to a selective pressure inducing uptake of free *bla* DNA. Instead, the evidence suggests that these *bla* genes are the non-degraded fraction of genes remaining from the DNA added during digesters setup.

A significant increase in *bla* gene concentration was observed in High treatment digesters at 30 days of incubation. It is possible that this increase is a result of HGT among WWTP bacteria, induced by the presence of a selective pressure (i.e., the addition of penicillin to digesters) and high level of free *bla* genes in eDNA. Additionally, *bla* was only detected at significant levels in iDNA at day 60 in these digesters. It is therefore possible that the detected *bla* genes in this iDNA fraction are a result of bacterial uptake of free *bla* ARGs. However, because the concentration of *LUG* marker genes had decreased significantly by day 30, a ratio of remaining *bla*/*LUG* genes could not be obtained to help elucidate the cause of this increase. In lieu of *bla*/*LUG* ratios, plating on selective media could provide additional insights into verifying HGT events. However, even if the observed increase is due to HGT events, this significant increase was only observed in digesters containing the highest augmented concentration of *bla* genes, which are not realistic of actual environmental conditions. It is unlikely that significant HGT will occur in a WWTP, where *bla* genes from transgenic crops are significantly less abundant. *Bla* copy numbers in High treatments far exceed the detected *bla* concentrations in natural WWTPs detailed in this chapter as well as Chapter 5. Nonetheless, this experiment was run on a relatively acute exposure time. In most cases, the growth rates of anaerobes are significantly lower than those of aerobic bacteria. Thus, it is logical to assume that the rates of HGT among these populations would also be lower. Additional studies involving characterization of eDNA persistence and uptake in anaerobic digesters, across several

SRTs would help elucidate trends associated with long-term WWTP operation. Additionally, other ARGs have been detected in wastewater at much higher levels than *bla* (e.g., *tet*, *erm*, and *sul*) and may be larger contributors to HGT rates in wastewater digesters [127, 156].

Perhaps most unexpectedly, the bulk of detected ARGs genes were found in eDNA rather than iDNA at all time points. Though all artificially added, *bla*, *nptII*, and *LUG* DNA essentially disappears after day 30 of anaerobic digestion, with only a fraction of each gene remaining in eDNA at day 60. The remaining eDNA may be the natural fraction adsorbed onto the surface of colloids in wastewater, protected from degradation and only detectable after eDNA extraction. Their continued presence in extracted eDNA at day 60 of thermophilic digestion suggests that this free DNA is bound to colloids, clays, or organic matter in wastewater, which can significantly enhance their ability to persist for a significant period of time in digesters [168]. It is therefore able to remain as a potential source of free DNA for bacterial transformation, though the rates of bacterial uptake of bound DNA are poorly understood [39]. Other studies have noted that eDNA may act as a significant reservoir for ARGs and HGT potential in other similarly complex environments [156]. Transformation of indigenous bacteria with added DNA has also been observed in these environments under a similarly short time scale. Conversely, it is not surprising that *LUG* genes were exclusively detected in extracted eDNA. *LUG* genes are historically associated with maize and tobacco plants rather than bacteria. They are

not known to confer advantageous traits to prokaryotes, and are therefore unlikely to be taken up and maintained in the bacterial genome.

Overall, *bla*, *nptII*, and *LUG* free DNA is significantly removed in thermophilic anaerobic digesters over 60 days of operation at 47°C. However, a significant fraction of free ARG DNA exists naturally as eDNA in wastewater digester sludge, and could act as a persistent reservoir of ARGs. Possible transformation events involving the uptake of *bla* DNA were also detected in some digesters at day 60 of operation, suggesting HGT events may readily occur in digester sludges containing ARGs and a relevant selective pressure. The presence of *LUG* genes in the eDNA fraction of Control digesters also suggests that partially digested DNA, including that of transgenic crops, readily enters WWTPs through human waste. However, more work is needed to fully understand how eDNA from transgenic crops and more conventional sources of ARGs contributes to ARG abundance, persistence, uptake, and transfer in complex WWTP environments.

7. Conclusions and Significance

7.1 Behavior of free DNA in Clays and Soils

7.1.1 Adsorption Capacities of Clay Minerals and Environmental Soils

The overall adsorption capacities of montmorillonite and kaolinite clay minerals are significantly lower than the values cited in previous literature by one to two orders of magnitude. Despite these differences, it appears that a substantial amount of DNA is still able to adsorb onto these matrices (up to 200 mg DNA per gram of clay). Similarly, the amount of transgenic maize DNA that can potentially adhere to soil matrices in the environment is still significant, approximately 70 μg of DNA per dry gram of environmental soil. Kaolinite clay minerals also had a consistently elevated ability to sequester free DNA, suggesting that specific clay structures have an innate adsorption capacity that must be taken in to account when assessing the adsorption capacities of complex environmental soils. These results also suggest that transgenic maize DNA is generally comparable to previous model DNA systems in terms of its adsorption capacity for clay minerals and soils, as there were no significant differences in adsorption among most tested isotherms. Isotherms testing adsorption of DNA sources to kaolinite minerals proved the one exception to this, with calf thymus DNA adsorbing at a higher rate than BT maize DNA. Due to the significantly different G-C contents of each DNA source, this suggests that both clay mineral and DNA structures may be strong predictors of overall adsorption capacity.

7.1.2 Loss of Free DNA to Degradation in Soils

Overall, pH was found to have a more significant impact on the ability of GM crop-derived DNA to persist in soil matrices than soil clay content. Among tested soil pH ranges, pH 5 had the most pronounced ability to sequester free DNA (p=0.052) compared to pH 6 (p=0.085) and pH 7 (p=0.29). This is likely due to increased protonation of soil mineral surfaces because of the abundance of free H^+ ions. The negatively charged phosphate backbone of ssDNA and dsDNA would have an increased ability to bind to soil and clay particles with an overall cationic surface charge, as opposed to an anionic surface charge. In addition, the negative charges associated with these phosphate groups is likely decreased in more acidic environments (i.e., pH 5) as the isoelectric point of DNA is approximated as 5 [41]. This would make adsorption to nearby soil particles easier in acidic soil environments. Transgenic maize and soy, crops that most often contain antibiotic resistance gene (ARG) selection markers, are typically cultivated at a more neutral pH of 6-7 [169, 170]. This suggests that the soils around these major transgenic crops will have a limited ability to sequester free DNA. However, it is worth noting that a significant number of *nptII* genes were still detected in tested soils with a pH of 6 and 7 after 18 weeks.

7.1.3 Study Limitations

It was determined through end-point that a fragment of the *bla* ARG contained within the investigated transgenic maize line was present in the final DNA elution used

to construct the adsorption isotherms, though the fraction adsorbed to clays and soils remains unknown. It should be noted that we did not investigate the integrity of the maize DNA after the DNA extraction protocol to determine if its incorporation into a bacterial genome led to the acquisition of the antibiotic resistant phenotype. We also could not control for the size or GC composition of the extracted DNA fragments in our experiments. Decay rates of *nptII* genes derived from GM maize were also only assessed on an acute time scale of 18 weeks. Longer studies would be needed to assess the long-term ability of these ARGs to persist in different agricultural soils.

7.1.4 Implications for Soils as a Reservoir of ARGs

As described in Chapter 3, DNA adsorbed to clays and soils can persist in soils for a significant period of time, especially in clay matrices of neutral or slightly acidic pH commonly used in agriculture. The degree to which DNA is able to adsorb is substantially lower than past literature estimates, but still remains significant. The acquisition of antibiotic resistance occurs naturally in the environment as a result of the horizontal gene transfer (HGT) of ARGs. Selective pressures in the form of background concentrations of several antibiotics are known to be present in the environment as a result of antibiotic-producing fungi or the disposal of pharmaceuticals, which act as a selective pressure to induce the uptake, transfer, and maintenance of ARGs by sensitive bacterial communities [45, 80]. Based on the ubiquitous presence of these selective pressures and the demonstrated ability of GMO-derived DNA to persist in agricultural soils, the risk of

microbial exposure to ARGs contained within transgenic maize lines, and any uptake thereof, should be investigated more thoroughly.

7.2 Maize Microbiome Characterization and Assessment of DNA Release During Tissue Decomposition

7.2.1 Maize and Rhizosphere Microbiome Characterization

The hypothetical uptake of antibiotic resistance genes contained within genetically modified crops by environmental bacteria has often been cited as a potential risk associated with the widespread cultivation of these plants [130]. However, the microbiomes associated with these crops have not been described in the literature. This dissertation work has fully characterized the community structures of bacterial endophyte and rhizosphere communities associated with conventional maize across 56 days of maize development. Endophyte community structure and composition were heavily influenced by the growth stage of the maize and location within maize pith tissue. Endophyte communities were dominated by *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria* phyla, though the exact percentage varied across time and sampling depth. Rhizosphere communities were only moderately affected by maize growth stage at the phylum level. Rhizosphere soils were dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* phyla. Though no significant differences among phyla were detected, the maize microbiome characterization presented in this dissertation may advance current research in related fields, such as the use of maize for bioethanol production or phytoremediation [171, 172].

7.3 Presence of GMO-Derived Genes in Wastewater

7.3.1 Detection of GMO-Derived Genes in WWTP Activated and Digester Sludge

The findings presented in Chapter 5 detail the detection of *p35, nos, bla*, and *nptII* transgenes in activated sludge and digester sludge. All four genes were detected ubiquitously in 23 of the 26 screened WWTPs in the United States. Based on these findings, it is possible that some of these genes are derived from partially digested transgenic crop material present in human waste. This is supported in part by the significant differences in *p35*, *nos*, and *nptII* concentrations in French WWTP systems relative to domestic WWTP systems. France neither cultivates nor consumes transgenic crops, which provides a possible explanation for these differences. The concentrations of the *bla* ARG were not significantly different among control and domestic wastewaters, so no conclusions can be drawn regarding this specific gene.

Additional PCR screenings support the idea that DNA from transgenic crops can be found in WWTP environments. The *p35* promoter gene found in many transgenic crops is derived from the Cauliflower Mosaic Virus (CaMV), a unique feature among most transgenic crops. WWTP activated and digester sludge was screened for additional CaMV genes, though none were detected by end-point PCR. This suggests that the detected *p35* genes have not been extracted from virus sources and thus exist either as eDNA or within bacterial iDNA. However, the exact source has yet to be identified. The mixed primer screenings described in section 5.2.2 also hint at the possibility of transgenic crop-derived

DNA being present in wastewater sludge. *P35F/nosR* primer screenings resulted in the amplification of multiple large gene fragments in all WWTP samples. Some of these bands were found to contain the *bla* ARG when performing a nested PCR with the *P35F/nosR* PCR products from each WWTP sample. If some of these fragments do originate from transgenic crops, the multiple band sizes are easily explained by the example transformation plasmid outlined in Figure 28. Multiple copies of promoters, terminators, genes of interest, and selection markers are often incorporated into a single transformation plasmid [110], and could therefore generate multiple bands using a single PCR primer set. However, as copyright and intellectual property laws often protect the sequence information for transformation plasmids of transgenic crops, the identity and source of these bands could not be verified.

Overall, it can only be concluded that it is possible for genes contained within transgenic crops and processed foods containing their byproducts to survive the human digestive process and enter into WWTP environments. Though the source of the detected genes could not be definitely confirmed, the results presented in this dissertation warrant further exploration into the possibility that these constructs originate from transgenic crops.

7.4 Behavior of GMO-Derived Genes in Anaerobic Digesters

7.4.1 Behavior of Free DNA in Thermophilic Anaerobic Digesters

Persistence of free, unbound DNA in anaerobic digester wastewater was significantly influenced by DNA fragment length. Longer fragments were protected from degradation for a significantly longer period of time relative to shorter fragments across all tested treatments. In the case of added *LUG* marker genes (178bp) and ARGs (>900bp), *LUG* genes experienced a 4-log decrease in concentration after 7 days of incubation while ARGs remained unchanged after 30 days of incubation. This correlation between DNA sequence length and environmental persistence has been noted in several other environments [59, 70, 174], though this is the first study to confirm this trend in WWTP anaerobic digesters. DNA adsorption to particle surfaces further increases this protection, as evidenced by the elevated persistence of bound eDNA in Control digesters.

The indigenous concentrations of *bla* and *nptII* ARGs in anaerobic sludge are consistent with the findings presented in Chapter 5 of this dissertation. However, the concentration of *LUG* genes in wastewater sludge eDNA was an unexpected finding. This gene is primarily associated with regulatory systems controlling sexual reproduction in maize and tobacco plants, and has no known connection to sequence similarity to known bacterial genes [163, 165, 166]. One possible conclusion is that these genes have entered WWTP environments as partially digested maize DNA in human waste. Maize biomass is known to be difficult to digest by the human body, and DNA contained within food

matrices has been demonstrated to be significantly protected from degradation by both physical and enzymatic forces [139]. If these genes are in fact derived from plant biomass, it also implies that other genes contained within difficult to digest crops (e.g., GMO-derived ARGs) may also partition into WWTP systems, though this has yet to be demonstrably proven.

7.4.2 Extracellular DNA as a Reservoir of ARGs in Wastewater

The naturally occurring concentrations of *bla* and *nptII* ARGs in anaerobic digesters are consistent with those of other wastewater treatment plants. However, the existence of these ARGs as primarily eDNA is novel and presently unreported in the literature. The mobility and transfer of ARGs is likely dependent upon its location; ARGs present in eDNA would be differentially available to bacteria compared to ARGs present in iDNA within a prokaryotic genome or plasmid [152].

In the case of ARGs, the presence of a selective pressure (i.e., relevant antibiotics) may induce competent bacteria to take up ARGs in free eDNA resulting in the increase of an antibiotic resistant phenotype. Antibiotics have been ubiquitously detected at significant levels in wastewater influent, treatment tanks, effluent, and even WWTP-derived agricultural biosolids [145, 175, 176]. Thus, WWTP bacteria are chronically exposed to these selective agents, though bacterial transformation rates are likely dependent upon the concentrations of individual antibiotics and their corresponding ARGs [177]. *Bla* ARGs were detected in iDNA only within 10^6 transgene copies/mL

treatments at day 60 of incubation. It is possible that this increase is due to bacterial uptake of free *bla* in eDNA as a result of an antibiotic-driven selective pressure, inducing the integration of *bla* into bacterial cells. Biogas production rates reached a low of 2.76 mL/week at day 60, suggesting many bacteria were no longer thriving by this time and the remaining may be living in nutrient-limiting conditions. Transformation events have been indicated to occur more frequently under these conditions, perhaps to increase the overall fitness of the remaining bacteria [178, 179]. Assuming the observed increase is due to HGT events, this significant increase was only observed in digesters containing the highest augmented concentration of *bla* genes. It is therefore unlikely that significant HGT of this ARG occurs in normal wastewater, where *bla* genes are significantly less abundant; *bla* copy numbers in 10^6 transgene copies/mL treatments far exceed the detected *bla* concentrations in natural wastewater detailed in this chapter as well as Chapter 5.

7.5 Future Directions

This is the first study to demonstrate that the largest fraction of ARGs in WWTP sludge may be found in eDNA, though the scope of ARG detection was limited to *bla* and *nptII* ARGs. While these genes are most relevant to ARGs found within transgenic crops, they represent only a small fraction of the ARGs commonly found in WWTPs. Additional studies should be conducted to evaluate what fraction of other ARGs exist as eDNA, such as sulfonamide, erythromycin, and tetracycline resistance genes. These genes are significantly more abundant in WWTP environments compared to *bla* and *nptII* genes

[127], increasing the likelihood that HGT events are occurring at detectable levels in WWTP environments. Additional metagenomic analyses can help elucidate whether the ARGs contained within eDNA exist as fragments or part of extracellular plasmids. This information would significantly aid in estimating the potential for HGT of ARGs in WWTP environments.

Appendix – Chapter 4

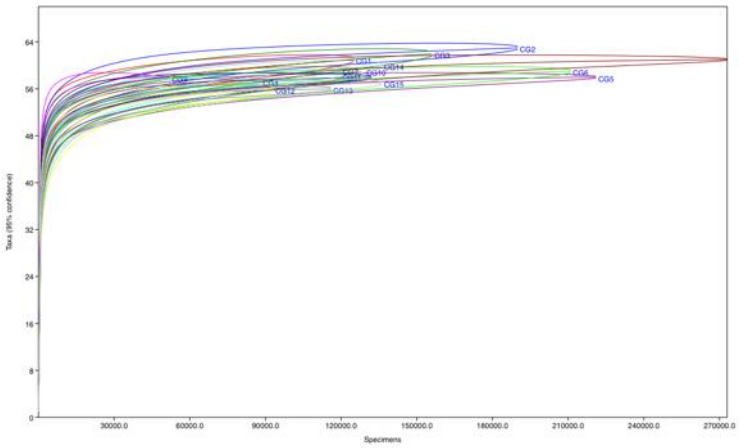

Figure 40: Rarefaction curves calculating species richness as a function of the number of samples (X-axis) and number of species (Y-axis) for example bulk soil and rhizosphere soil samples. All bulk and rhizosphere soil samples possessed satisfactory rarefaction curves.

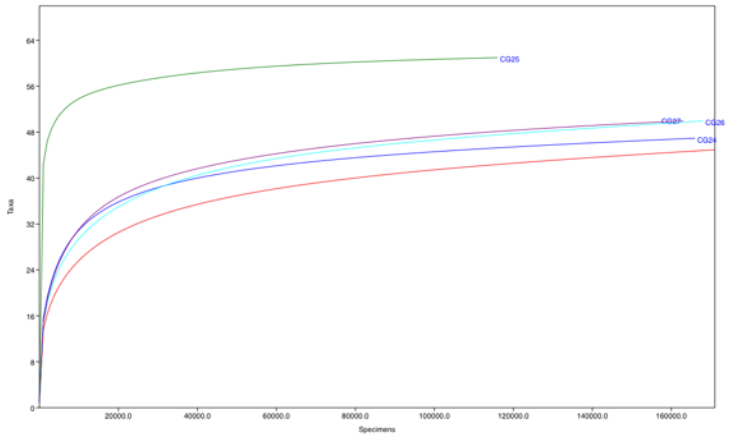

Figure 41: Rarefaction curves calculating species richness as a function of the number of samples (X-axis) and number of species (Y-axis) for example bulk maize endophyte samples. Endophyte samples 78, 84, and 110 did not possess satisfactory rarefaction curves and were excluded from further analysis.

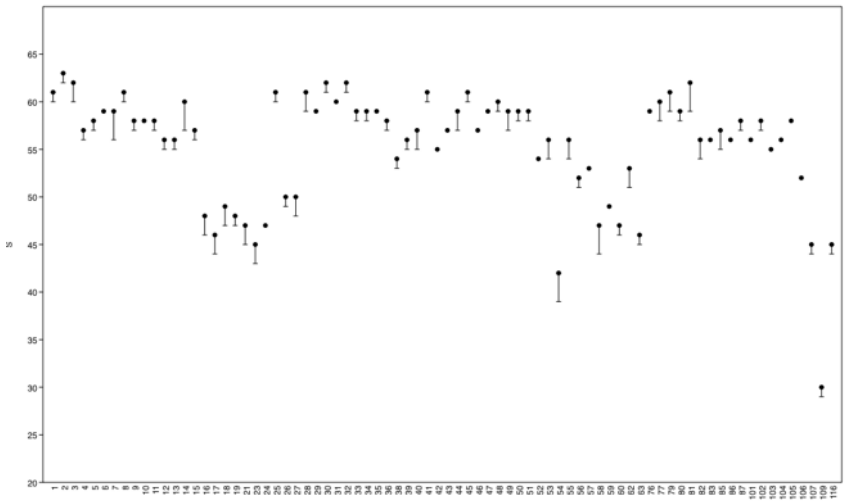

Figure 42: Alpha-diversity plots (mean species diversity within samples) for class-level prokaryotic taxa in bulk soil, rhizosphere soil, and endophyte samples.

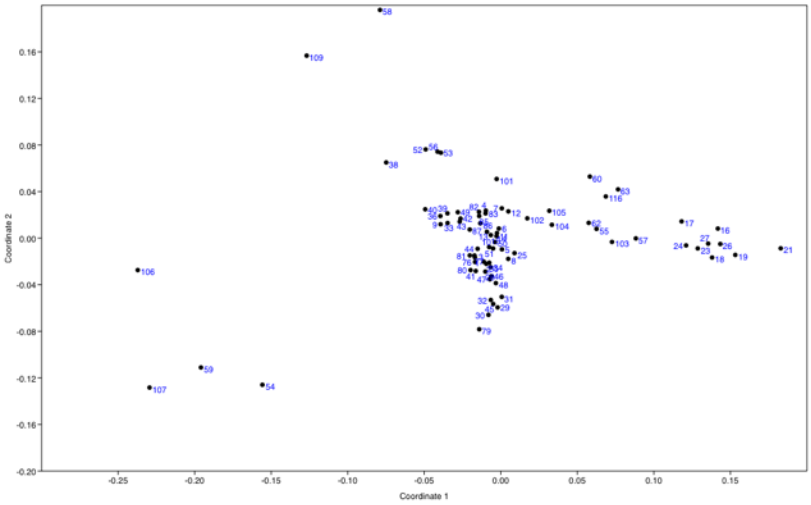

Figure 43: Non-Metric Dimensional Scaling (NMDS) analysis for class-level prokaryotic taxa in bulk soil, rhizosphere soil, and endophyte samples using Bray-Curtis distances. Stress was calculated as 0.046, indicating a good ordination fit.

Table 14: Line of best fit conditions for relative abundance trends of nitrogen cycling bacteria in bulk and rhizosphere soils associated with conventional maize (Figure 22). *Clostridium*, *Cyanobacterium*, *Nitrosospina*, *Nitrosovibrio*, *Nitrosopumilus*, *Nitrosospira*, and *Nitrobacter* genera were excluded from analysis due to poor R^2 values for each sample trend line.

Genera	Soil	R^2	Trendline Slope	Bulk vs Rhizosphere
Azospirillum	Bulk	0.31	-0.016	*p*=0.53
	Rhizosphere	0.28	-0.024	
Rhizobium	Bulk	0.39	0.0014	*p*=0.019
	Rhizosphere	0.85	0.011	
Frankia	Bulk	0.73	0.0075	*p*=0.028
	Rhizosphere	0.83	0.0033	
Nitrospira	Bulk	0.18	-0.015	*p*=0.49
	Rhizosphere	0.46	-0.014	
Denitrobacter	Bulk	0.64	0.0052	*p*=0.78
	Rhizosphere	0.11	0.0013	
Nitrosococcus	Bulk	0.60	-0.0012	*p*=0.0024
	Rhizosphere	0.60	-0.0012	

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Table 15: Standard curve conditions and detection ranges for *p35*, *nos*, *nptII*, and *bla* qPCR assays

Target Gene	Range (copies/µL)	R^2 Range	Efficiency Range (%)
p35	10^1-10^8	0.955-0.982	97.4-99.7
nos	10^1-10^8	0.976-0.978	100.2-100.9

bla	10^1-10^8	0.930-0.985	91.5-103.2
nptII	10^1-10^8	0.978-0.998	97.5-103.5
16S	10^1-10^8	0.942-0.993	93.3-96.2

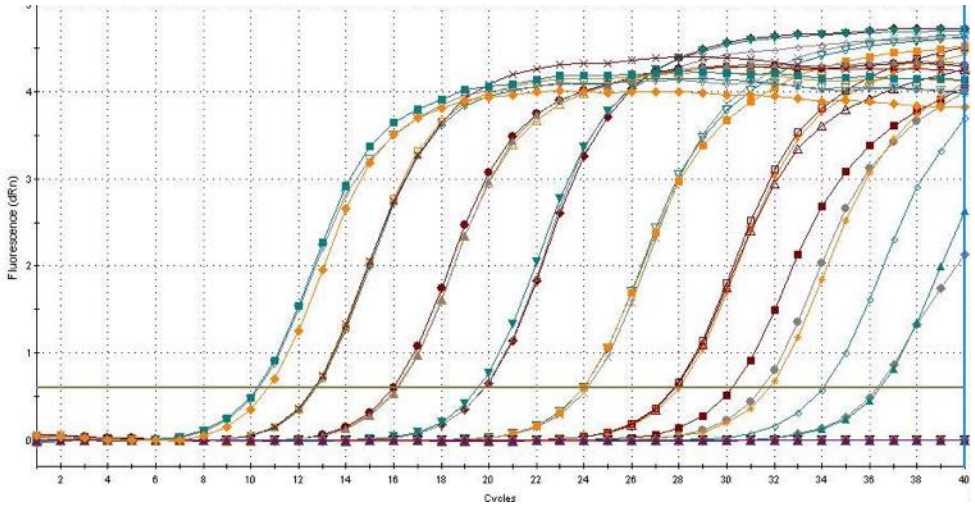

Figure 44: *Bla* qPCR assay amplification plot for DNA extracted from WWTP sludge samples. Assay standard curve ranges from 10^1-10^8 copies/µL. Assays were performed on Stratagene Mx3000P qPCR systems (Agilent, Santa Clara, CA).

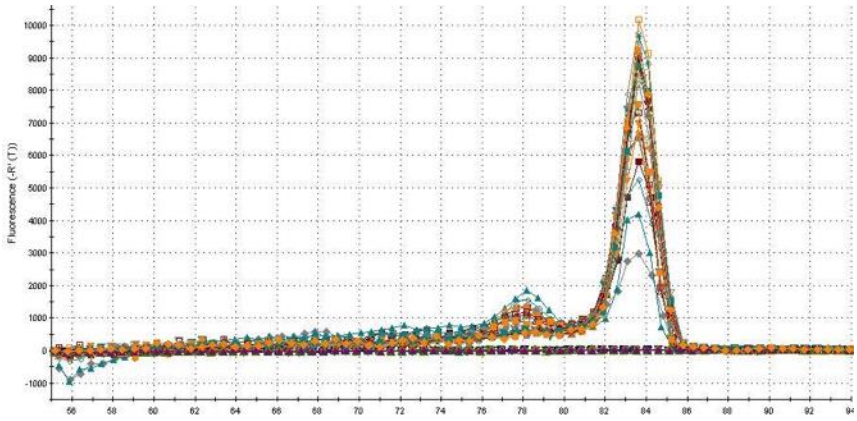

Figure 45: *Bla* qPCR assay dissociation curve for DNA extracted from WWTP sludge samples. Assay standard curve ranges from 10^1-10^8 copies/µL. Assays were performed on Stratagene Mx3000P qPCR systems (Agilent, Santa Clara, CA).

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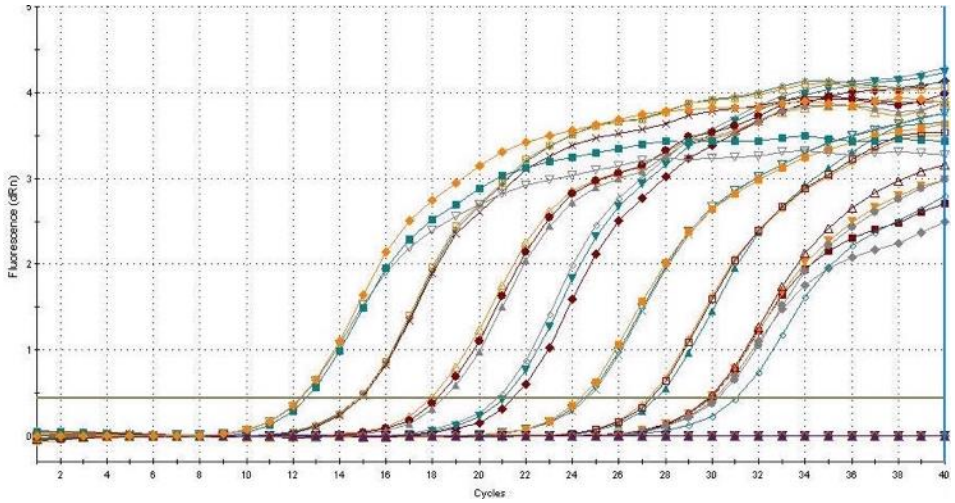

Figure 46: *LUG* qPCR assay amplification plot for DNA extracted from WWTP anaerobic sludge digesters. Assay standard curve ranges from 10^1-10^8 copies/µL. Assays were performed on Stratagene Mx3000P qPCR systems (Agilent, Santa Clara, CA).

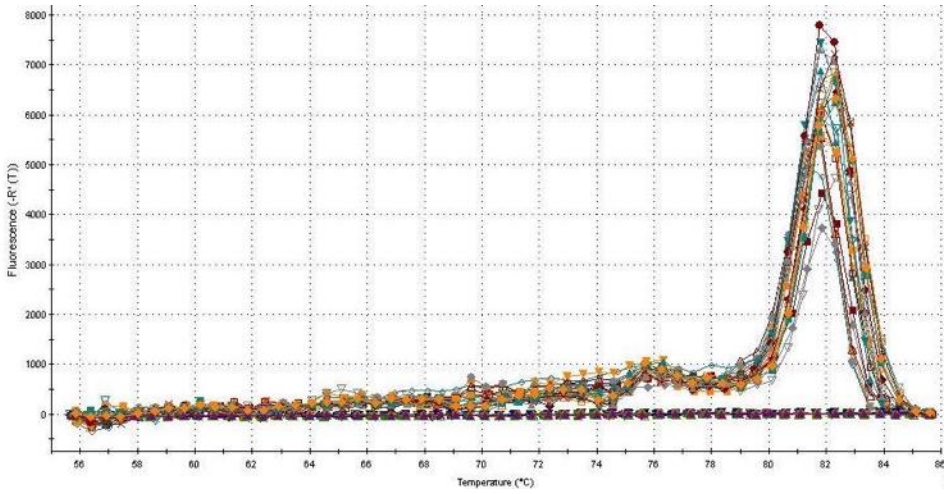

Figure 47: *LUG* qPCR assay dissociation curve for DNA extracted from WWTP anaerobic sludge digesters. Assay standard curve ranges from 10^1-10^8 copies/µL. Assays were performed on Stratagene Mx3000P qPCR systems (Agilent, Santa Clara, CA).

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Biography

Courtney Maye Gardner was born in Tampa, Florida on December 17, 1988 to Denise and Robert Gardner. She graduated Cum Laude from Stetson University (DeLand, FL) with a Bachelor of Science in Biology and a minor in Psychology in May of 2011. She also graduated with High Distinction from Stetson University's Honors Program for her thesis work, "*Determination of Seed Paternity to Evaluate Reproductive Fitness in Passiflora incarnata Using DNA Microsatellites*." She began her doctoral work in 2012 and was awarded a NSF Graduate Research Fellowship in 2013. Mrs. Gardner obtained a Master of Science in Civil and Environmental Engineering from Duke University (Durham, NC) in December of 2015. The articles published by Courtney M. Gardner include: "Part I: Cocopeat for wastewater treatment in the developing world:

comparison to traditional packing media in lab scale biofiltration columns"(*Journal of Environmental Engineering,* 2015) and "Adsorption capacity of multiple DNA sources to clay minerals and environmental soil matrices less than previously estimated" (*Chemosphere,* 2017). Mrs. Gardner has been a member of the following professional societies: ACS, AEESP, AWIS, ASM, and the National Biological Honor Society (inducted 2007).