RECOVERY OF ANTIBIOTIC RESISTANCE GENES FROM AGRICULTURAL RUNOFF

Kyle B. Jacobs

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Leigh-Anne H. Krometis

William C. Hession

Amy J. Pruden

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ABSTRACT

The reduced capacity of antibiotics to treat infections is one of the greatest health concerns that society faces. There is substantial evidence that links this reduced capacity with the widespread use of antibiotics in livestock production. Livestock can act as reservoirs of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria, which can pass resistance on in the livestock's manure. It is important to understand the fate of antibiotic resistance genes and resistant bacteria in the environment after land-application of manure-based amendments. The goal of this field-scale study was to identify the effects of soil amendments (inorganic fertilizer, compost, or raw manure) and crop cover (lettuce or radish) on sediment transfer, fecal indicator bacteria (FIB), and release of ARGs in runoff over six storm events. Two FIB (*Escherichia coli* and enterococci) and two ARGs (*sulI* and *ermB*) were quantified in runoff from each of the constructed plots throughout the growing season. FIB and ARGs were recovered from all plots, including control plots indicating a background level within the soil. Additionally, only the effects of variability among individual storms had an impact on the concentration of FIB in runoff. Vegetative cover and storm variability affected sediment release. A trend of higher *sul1* and *ermB* in runoff from compost and raw manure-amended plots for at least 2 months after planting crops was observed. Only one of these ARGs (*ermB*) is associated with the class of drugs given to the dairy cows used for the manure and compost, indicating inherent carriage of some ARGs independent of the type of antibiotic administered, and such genes can persist in the environment. These results suggest that there is a risk of ARGs being carried into areas downgradient from agricultural plots that have been amended with compost or manure.

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GENERAL AUDIENCE ABSTRACT

Millions of kilograms of antibiotics are used in livestock production each year in the United States, causing concern that such widespread antibiotic use could be contributing to a decrease in effectiveness of antibiotics for treating illness in humans. The purpose of this study is to understand how antibiotic resistance might be transferred from livestock to manure into the environment and ultimately to people. This field-scale study tested the effect of soil amendment (chemical fertilizer, compost, or manure) and crop cover (lettuce or radish) on the release of fecal indicator bacteria (*Escherichia coli* and enterococci), sediment, and antibiotic resistance genes (*sul1* and *ermB*) in runoff coming from agricultural plots. In part, this study helped evaluate recent US Food and Drug Administration, Food Safety Modernization Act (FSMA) criteria for composting to reduce pathogenic bacteria when using manure-derived soil amendment to grow food for human consumption. This study found that fecal indicator bacteria and antibiotic resistance genes were recovered in runoff from all soil amendment and vegetable types. However, there were higher levels of antibiotic resistance genes recovered in runoff from compost and manure amended soils than from fertilizer control or unamended plots during the growing season. This suggests that composting may not be effective for reducing or removing the genes that encode antibiotic resistance in runoff.

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1. INTRODUCTION

Antibiotic resistant infections pose an enormous threat to public health (Auerbach, 2007; Chang et al., 2015; USCDC, 2016). Globally each year, millions of individuals become ill with bacterial infections that cannot be treated with simple antibiotic regimens (WHO, 2015; USCDC, 2016). Increasing resources are required to determine the sources of these infections and/or to develop strategies to prevent further infection at the individual and community scale. One of the largest consumers of antibiotics is livestock agriculture, as antibiotics are commonly included as feed supplements to promote herd health and weight gain, however, the Veterinary Feed Directive implemented in 2015 prohibits the use of antibiotics as feed supplements (Davis et al., 2006; Chang et al., 2015; USFDA, 2015; Sura et al., 2015). More than 13 million kg of antibiotics are used annually by the agricultural industry, many of which hold critical human importance (Yang and Carlson, 2003; Sura et al., 2015). Therefore, agriculture is a specific area of focus in designing strategies to reduce potential environmental pressures which encourage the dissemination of antibiotic resistance (Chang et al., 2015).

Many antibiotics are not completely broken down or metabolized when they pass through animal digestive systems (Ray et al., 2017). This is critical in environmental management of resistance, as manure is applied to more than 8.9 million ha (22 million acres) of land annually as a soil conditioner and fertilizer (NASS, 2014). Antibiotics, antibiotic resistant organisms, and antibiotic resistance genes found within this land-applied animal waste can be washed away in runoff during storm events (Frank, 2005; Davis et al., 2006; Ribeiro et al., 2012; Joy et al., 2013; Sun et al., 2013; Fahrenfeld et al., 2014; Sura et al., 2015). Guidelines for reducing pathogens in manure via composting were included within the recent US Food and Drug Administration Food Safety and Modernization Act of 2015 in an effort to control potential crop exposure to pathogens within land-applied manure (USFDA, 2015). These guidelines state that following composting at a standard temperature (>55 °C), pathogen and pathogens indicators in manure should be sufficiently inactivated for safe land application to croplands. However, this standard does not address antibiotic resistance genes, the ultimate fate of which may be governed by different processes. While there are studies that measure the change in antibiotic and antibiotic resistance gene concentrations in manure during composting (Storteboom et al., 2007; Sharma et

al., 2009; Wang et al., 2012; Ray et al., 2017), there are few field studies evaluating the effects of composting on the subsequent release of manure-derived antibiotic resistance genes via runoff.

The goal of this thesis was to identify potential differences in ARG concentrations in runoff from vegetable plots treated with different types of field amendments, including chemical fertilizer, composted dairy manure, and raw manure. Fecal indicator bacteria and sediment levels in runoff were characterized in parallel to understand broader transport mechanisms that may be at play. Specific research questions included:

- Do levels of antibiotic resistance genes (ARGs), fecal indicator bacteria (FIB), and sediment in runoff vary depending on soil amendments (chemical fertilizer, composted manure, raw manure)?

- Do levels of ARGs, FIB, and sediment in runoff differ between root crops (radishes) vs leafy vegetables (lettuce)?

- Does inter-storm variability affect the levels of ARG, FIB, and sediment concentrations in runoff?

2. LITERATURE REVIEW

2.1 Antibiotic Resistance as a Global Concern

Antibiotic resistance presents one the most serious public health concerns of our time (Auerbach, 2007; Chang et al., 2015; USCDC, 2016). According to the US Centers for Disease Control and Prevention (CDC), more than 2 million individuals become ill and more than 20,000 people die annually from infections caused by antibiotic resistant bacteria (ARBs) in the United States (USCDC, 2016). Similarly, according to the World Health Organization (WHO), antibiotic resistance causes more than 25,000 deaths in the European Union each year, with associated health care costs of more than 1.5 billion US Dollars annually (WHO, 2015). Increasing incidence of ARB infections is attributed to a variety of sources, though there traditionally has been a preventative focus on reducing clinical overuse and ensuring patient adherence to prescribed regimens. More recently, environmental sources of antibiotic resistance have received increased scrutiny, particularly with respect to antibiotic use in agriculture. Understanding environmental spread of resistance due to agricultural practices is necessary to identify effective means of control (Auerbach et al., 2007; Joy et al., 2013; Pruden et al., 2013; Fahrenfeld et al., 2014; Chang et al., 2015; Sura et al., 2015).

Antibiotics are incorporated into livestock feed to ensure herds are healthier and gain more weight than those without dietary supplements of antibiotics (Davis et al., 2006; Joy et al., 2013; Sura et al., 2015; Ray et al., 2017). Each year, livestock are dosed with more than 13 million kg of antibiotics, generally at sub-therapeutic levels to promote weight gain (Davis et al., 2006; Chang et al., 2015; Sura et al., 2015; Ray et al., 2017). According to the US Department of Agriculture (USDA), more than 75% of feedlots with at least 1,000 beef cattle provided antibiotics in feed or water with the purpose of promoting growth between 1994 and 2011 (USDA, 2016). This practice is different from the dairy industry, where approximately 90% of dairy cows received antibiotics in 2007 specifically to prevent disease (most commonly, mastitis). It is worth noting this is quite an increase from 2006 when only 20% of dairy cows received antibiotics to prevent disease (Sneeringer et al., 2015).

Given that several of the drugs used to promote herd health and weight gain are also critical to the treatment of human disease, there is concern that regular use in livestock agriculture may promote the spread of antibiotic resistant microorganisms (Yang and Carlson, 2003; Sura et al., 2015; Ray et al., 2017). Livestock do not assimilate or fully break down all of the antibiotics in feed, and those that are excreted are disseminated into the broader environment through the application of manure as a fertilizer and soil amendment (Frank, 2005; Davis et al., 2006; Joy et al., 2013; Chang et al., 2015; Sura et al., 2015; Ray et al., 2017). Since animal manure contains millions of bacteria, there is the potential that these microorganisms may become resistant or carry resistance elements via several different pathways (Haug et al., 2011; Wellington et al., 2013; Ray et al., 2017).

2.2 Antibiotic Resistance Genes

The ability to resist specific antibiotics is encoded within the genetic sequences of a bacterium's chromosome. Expression of these genes, hereafter referred to as antibiotic resistance genes (ARGs), provide various mechanisms that render antibiotics ineffective including efflux pumps, inhibitors, and enzymes (Bennett, 1999; Kazimierczak et al., 2007; Wellington et al., 2013, Lin et al., 2015). A bacterium with a functional ARG (e.g., the bacterium is demonstrably resistant to some antibiotics) is referred to as an ARB. However, it is important to note that ARGs can be recovered in the absence of ARBs (i.e., this genetic material may persist in the environment following the lysis of a parent cell), which still has important implications for resistance transfer via transformation (Sykes, 2010; Wellington et al., 2013; Fahrenfeld et al., 2014). Determining the genes that are associated with antibiotic resistance is an important part of all genomic analyses identifying ARBs (Auerbach et al., 2007; Zhang et al., 2015).

Bacteria can become resistant to antibiotics through several pathways. Vertical pathways included random mutation or natural selection following introduction to an antibiotic, with resistant bacteria undergoing binary fission to create a colony of ARBs that are better able to survive than their original parent cells, a process of natural selection that can lead to populations of ARBs (Amábile-Cuevas, 1993; Sykes, 2010). For example, in manure from an animal that has received antibiotics, colonies of ARBs can flourish after the antibiotics kill competing

susceptible bacteria (Kazimierczak et al., 2007; Huag et al., 2011; Finley et al., 2013; Fahrenfeld et al., 2014).

Bacteria can also become resistant through horizontal gene transfer by three mechanisms: conjugation, transduction, and transformation (Amábile-Cuevas, 1993; Bennett, 1999; Kazimierczak et al., 2007; Sykes, 2010; Haug et al., 2011; Finley et al., 2013; Chang et al., 2015). ARGs can be present within the primary bacterial chromosome or on an extrachromosomal circular strand of DNA called a plasmid. These plasmids can encode antibiotic resistance and other genes that allow the bacteria to survive in unfavorable conditions, and can replicate independently from chromosomal DNA (Amábile-Cuevas, 1993; Sykes, 2010; Finley et al., 2013). Conjugation occurs when an ARB directly transfers copies of its genetic material via a pilus to a non-resistant bacterium, imparting resistance (Kazimierczak et al., 2007; Sykes, 2010; Finley et al., 2013). One of the early tests to determine if conjugation had taken place is to expose an antibiotic-sensitive bacterium to an ARB and then expose both to the antibiotic; if conjugation had taken place, both bacteria could demonstrate resistance (Amábile-Cuevas, 1993). Transduction occurs when a bacteriophage, or a virus that infects bacteria, infects an ARB that then incorporates ARGs with the injected viral genome. After the bacteriophage multiplies within the infected ARB, the ARB cell is lysed, or killed, and the ARG-infected bacteriophages can transfer the ARGs to new, non-resistant bacteria (Kazimierczak et al., 2007; Sykes, 2010; Finley et al., 2013). Finally, transformation is the mechanism of one largest concern for environmental sources and pathways of antibiotic resistance, as it involves the absorption of free-floating DNA, including ARGs, by environmental non-resistant bacteria. These bacteria can then incorporate the ARGs into their chromosomes and become antibiotic resistant (Amábile-Cuevas, 1993; Kazimierczak et al., 2007; Sykes, 2010, Finley et al., 2013).

The transfer of ARGs is a common process in nature and horizontal gene transfer can occur across multiple different species. For example, wastewater treatment, drug manufacturing, and agricultural waste all release large quantities of antibiotic residues into the environment (Finley et al., 2013). Trung et al. (2005) demonstrated that a non-pathogenic strain of *Escherichia coli* could transfer its antibiotic resistance genes to a strain of *Shigella* from isolates collected in a

clinical setting. This poses a serious health issue as *Shigella* is an etiological agent of dysentery, a life-threatening diarrhea typically successfully treated with antibiotics (Amábile-Cuevas, 1993; Wellington et al., 2013). Some organisms can obtain resistance from other naturally resistant organisms such as *Pseudomonas aeruginosa* (Mah et al., 2003). Additionally, a majority of antibiotics used in livestock production interact with the natural microflora found in the mammalian gut, which can affect common fecal bacteria such as *E. coli* and enterococci, some of which may develop antibiotic resistance through either natural selection or horizontal gene transfer (Haug et al., 2011). Fahrenfeld et al. (2014) discussed how methicillin-resistant *Staphylococcus aureus* (MRSA) has been associated with nearby swine manure application sites. The resistance is transferred from the resistant bacteria found in the swine manure and is transferred to *S. aureus*.

2.3 Transfer of Antibiotic Resistance Genes from Animals to Humans

In addition to serving as ARG vectors, zoonotic pathogens (i.e., pathogens that can be transferred from animals to humans) can vector antibiotic resistance to the microbes in the natural flora across macro-species including vertebrates and invertebrates. Humans often act as important reservoirs for ARGs due to frequent antibiotic use. This exposure of pathogenic bacteria to antibiotics can lead to the expression or development of ARGs (Amábile-Cuevas, 1993; Haug et al., 2011; Finley et al., 2013; Chang et al., 2015). Grasselli et al. (2008) and Chang et al. (2015) compared the genomes of several strains of isolated *E. coli* from animals (including felines, canines, bovines, and swine) to those of humans to illustrate that the *E. coli* from these hosts were capable of horizontal gene transfer. This is evidence that antibiotic resistant bacteria from animals could potentially be harmful to humans. They concluded that the ability of *E. coli* to survive in a wide variety of environments and the fact that they are found within the digestive systems of multiple species makes them an ideal bacterial model for further study. The species *E. coli* includes multiple pathogenic strains, so understanding how interactions between strains occurs may be important in understanding how ARGs can be transferred from host to host (Grasselli et al.*,* 2008; Chang et al., 2015). Grasselli et al. (2008) isolated *E. coli* from five different species including humans, cows, and pigs. The DNA of these isolates were then extracted and hybridized to determine which sequences were more prone to horizontal movement. After genomic analysis, *E. coli* collected from different species were

found to have a common "backbone" of genes though each individual strain was better adapted to survive in its primarily host organism. The genome most prone to horizontal gene transfer were those related to defense mechanisms, cell motility, and intracellular trafficking. Each of these categories can serve as a target for antibiotics, and if a strain is resistant to a certain antibiotic that targets a specific cellular feature, that strain could potentially exchange its genetic information with a non-resistant organism. This exchange can expose humans to antibiotic resistant *E. coli* if ARGs from agricultural runoff contaminate water supplies or food sources (Haug et al., 2011).

Industrialized antibiotic production and widespread use has drastically increased the quantity of resistance genes in the environment in general (Finley et al., 2013). For example, ARGs in soils were approximately 2-15 times more abundant in 2008 than in the 1970s and there are multiple lines of evidence that pathogens that can infect humans have obtained ARGs from environmental bacteria (Finley et al., 2013). Finley et al. (2013) cite gene sequencing analyses showing the history of the resistant genes collected directly from areas with poor sanitation; they observe a method in which a pathogen can naturally obtain quinolone resistance on its chromosome, and then transfer that resistance to other bacteria.

2.4 Antibiotics in Agriculture

Livestock manure has been land-applied as a soil conditioner and fertilizer for centuries. Although manure nutrients can stimulate plant growth, they can also be adsorbed to the surface of sediments or washed directly into receiving waters following storm events if applied at an inappropriate rate or time (Yang and Carlson, 2003; Davis et al., 2006; Joy et al., 2013). Similarly, if antibiotic residuals within manure are sediment bound, these sediments can then be eroded by surface runoff, and travel into receiving waterbodies such as streams, rivers, and lakes (Davis et al., 2006; Joy et al., 2013). Despite the widespread acknowledgement that manure application and subsequent nutrient loss to waterways is a major source of waterbody impairment (Ribeiro et al., 2012), several researchers note that there are few studies that quantify the loss of antibiotics and ARGs to the environment (Frank, 2005; Ribeiro et al., 2012; Joy et al., 2013). Given the use of multiple types of antibiotics in agriculture (Table 2-1), there is potential for the occurrence of multiple types of antibiotic resistance.

Table 2-1. Common antibiotics used in agriculture.

Antibiotic	Abbreviation	Associated Livestock	Associated ARG	Source
Tetracycline	TC	Beef Cattle	*tet(W)*, *tet(X)*, *tet(Q)*	Joy et al., 2013
Sulfamethoxazole	SUL	Beef Cattle	*sul1*, *sul2*	Heueur et al., 2011
Erythromycin	ERY	Dairy Cattle	*ermF*, *ermB*	Joy et al., 2013
Clindamycin	CL	Dairy Cattle	*msr(A)*	Fiebelkorn et al., 2003

Following antibiotic treatment, livestock excrement contains undigested antibiotics, which has the potential to exert selective pressures on present microorganisms. Manures are often stored in manure pits that can release antibiotics, ARBs, and ARGs to receiving water bodies if they fail. Davis et al. (2006) sprayed soils with antibiotic solutions (Tetracycline, sulfamethoxazole, erythromycin as well as the specific antibiotics tylosin, chlortetracycline, and sulfamethazine) 1-hr prior to a simulated rainfall to evaluate a worst-case scenario. After the simulated rainfall, samples were processed using high-performance liquid chromatography in addition to mass spectrometry to detect the presence of antibiotics in runoff. Results indicated that Tetracycline, erythromycin, and tylosin were more likely than other antibiotics to be adsorbed to sediments rather than suspended in runoff; however, these antibiotics can still be affected by runoff if the rainfall intensity is enough to cause the sediments themselves to be eroded away (Davis et al., 2006). One major limitation of their study was that the manures were applied well above typical agronomic rates or were simply antibiotics being directly sprayed on to the soils (Yang and Carlson, 2003; Davis et al., 2006).

Sura et al. (2015) examined the transport of antibiotics from cow manure excreted in feedlots as opposed to transported from applied manures on an agricultural plot, with a specific focus on how bedding affects antibiotic transport. They found that with the addition of straw bedding in the feedlot, the antibiotics were partially trapped so that the majority of antibiotics by weight remained on site. However, there were still antibiotics found within the runoff directed from the bedded area and the non-bedded area (Sura et al., 2015).

2.5 Antibiotic Resistance Genes in Runoff

There have been multiple investigations as to how actual ARBs and ARGs can migrate into waterways via runoff from similar agricultural areas (Frank, 2005; Ribeiro et al., 2012; Joy et al., 2013; Fahrenfeld et al. 2014). Two studies observed how ARBs and ARGs moved through a karst environment (Frank, 2005; and Ribeiro et al., 2012). Both of these studies focused on large primarily agricultural land areas, and measured the *E. coli* concentrations of springs fed by runoff during both wet and dry periods of the year and after heavy rainfall. Both studies observed a constant background level of antibiotic resistance present in each spring and increased *E. coli* concentrations in springs following rainfall. Ribeiro et al. (2012) observed springs during rainfall events*,* measured *E. coli* concentrations and tested them for antibiotic resistance, where they determined that groundwater sources within a karst system were sensitive to ARBs following a period of heavy rain. They found that there were higher ARB and ARGs detected from springs close to agriculture. Because the targeted groundwater systems were a significant source of drinking water this presented a significant human health concern. This contradicted the findings from Frank (2005), which indicated there might not be a relationship between areas with residential or agricultural runoff and ARG concentrations; however, they failed to account for any best management practices that are already in place to prevent runoff from directly entering the streams. Additionally, the type of agriculture would likely affect the amount of ARGs washed away by runoff (Frank, 2005). The Ribeiro et al. (2012) study had the strength of using actual rainfall to conduct their study in an area where the largest land use was agriculture; however, the area was so large there is no known way of saying if runoff was coming from individual farms.

Two studies used a mass-balance approach to quantify ARG concentrations in runoff or downhill soils (Joy et al., 2013; Fahrenfeld et al. 2014). Both applied manure from livestock that had been given antibiotics to varying spots within a cornfield near a stream, but the Fahrenfeld et al. (2014) study also observed how the type of manure used (dry-stack vs slurry) affected ARG concentrations. Fahrenfeld looked at soil that was downhill from the manure-applied spots. Meanwhile, Joy et al. (2013) focused on the potential effects of differences in application method (broadcasting vs tilling) on ARG concentrations. Both studies found ARG

concentrations from plots amended with manure to be higher than background ARG concentrations, as expected. Joy et al. (2013) found that incorporating the manure into the soil significantly reduced the concentration of ARGs in runoff when compared to simply broadcasting without tilling. Fahrenfeld et al. (2014) determined that slurry manure had a significantly higher concentration of ARGs in soils than dry stack manure. Both studies observed that following manure application, ARG concentrations from runoff or in downhill soils coming from the manure treated plots increased significantly for up to two months post application. Therefore, Fahrenfeld et al. (2014) concluded that efforts to reduce the exposure of water bodies to ARGs following manure application should be focus on maximizing degradation during this initial two-month period. The Fahrenfeld et al. (2014) study showed the effects of manure on ARG contamination, but it did not account for the effect of composting on ARG contamination.

2.6 Reducing Antibiotics and ARGs via Composting

There have been numerous investigations of potential prior to land application (manure treatments) to reduce risk (USFDA, 2015; Ray et al., 2017). Composting is generally recommended as a means to reduce the quantities of fecal indicator bacteria in manure prior to land application (Sharma et al., 2009; Ray et al., 2017). The general idea behind composting is that a majority of the bacteria in manure (including *E. coli*) are mesophiles, which cannot survive at high temperatures during the composting process. In addition to reductions in overall pathogen numbers, recent work has investigated the ability of composting specifically with respect to degradation or inactivation of antibiotics, ARBs, and ARGs (USFDA, 2015). A synthesis of available literature by Pruden et al. (2013) documented reductions of antibiotics by 50-70% in composted agricultural wastes, though it does not directly report on reductions in ARGs. Work by Wang et al. (2012; 2015) reported that composting can decrease TC and ERY resistant bacteria and their respective genes in manure by up to seven logs (99.99999% removal) following small-scale composting at 55 °C. Wang et al. (2015) identified multiple mechanisms driving reductions in resistant bacteria, including high temperature, low moisture, reduced horizontal gene transfer, and high enzymatic rates. Sharma et al. (2009) demonstrated that while composting can reduce fecal indicator bacteria concentrations, ARGs encoding tetracycline and erythromycin resistance can still be detected following composting.

Animal waste management practices proposed in 2013 and implemented in 2015 through the Food Safety Modernization Act (FSMA) directly aim to prevent the incursion of human pathogens (e.g. *Salmonella*, *Listeria*) into farms producing crops for human consumption. (USFDA, 2015). However, it is unclear whether these practices are sufficient to prevent the proliferation of ARGs as well. The current standard proposed by the FSMA for reducing pathogens in manure is to compost wastes at 55 ℃ for several days with the goal of reducing fecal coliforms to less than 1,000 MPN per gram of total solids (USFDA, 2015). While the effect of composting practices on common fecal pathogens has been studied, the ability of these practices to inactivate ARGs is less well established, and there is little to no information on the impact of application of composted manures on ARG presence in agricultural runoff.

3. GOALS AND OBJECTIVES

Based on the available literature regarding the FSMA composting standards, there are two primary objectives that will aid in determining the effectiveness of the standard at reducing ARGs in runoff that flows from plots amended with compost. This study will compare the release of FIB, sediment, and ARGs from plots amended with composted manure, raw manure, and fertilizer into runoff in a field setting. The study will also compare the release of FIB, sediment, and ARGs from plots planted with root vegetables and leafy vegetables into runoff in a field setting.

3.1 Experimental Questions

The field experiment was designed to test the following questions via an unbalanced block design:

- Do differing soil amendments (e.g. chemical fertilizer, compost without antibiotics given to cattle, compost with antibiotics given to cattle, or raw manure) affect the loss of antibiotic resistant genes (ARGs), fecal indicator bacteria (FIB), and sediments to runoff?
- Does vegetation cover (e.g. radishes, lettuce, or barren plots) influence the loss of ARGs and sediments to runoff?
- Does inter-storm variability affect the levels of ARG, FIB, and sediment concentrations in runoff?

4. METHODS

4.1 Experimental Design

Twenty-seven plots were constructed (Table 4-1) in an unbalanced block design due to space constraints at the field site. Three plots were left barren without any soil amendments to act as a control, i.e. these plots neither received additional soil amendment nor were they planted with any radishes or lettuce. The rest of the plots were divided into groups of six plots each across four treatments: chemical fertilizer, compost without antibiotics given to the dairy cattle, compost with antibiotics given to cattle, and raw manure with antibiotics given to the cattle as a "worst case scenario" in terms of potential exposure to ARGs (raw manure is not actually applied to commercial crops). For each treatment group, three plots were planted with lettuce and three plots planted with radish to test for the effects of crop type and ground cover.

Table 4-1. The method in which the treatments were distributed for the plots of the field study.

	Vegetable Treatment		
Soil Treatment	None	Lettuce	Radish
Control	3 plots		
Chemical Fertilizer		3 plots	3 plots
Compost no ABX		3 plots	3 plots
Compost with ABX		3 plots	3 plots
Raw Manure with ABX		3 plots	3 plots

4.2 Study Site and Plot Construction

Prior to the installation, the field area was treated with glyphosate herbicide and tilled per usual farming practices in the fall of 2015. Soil amendments were applied to all plots one month prior to planting the vegetables (February 2016). Amendments were mixed into the soil using rakes that were only used for one type of soil amendment to prevent cross-contamination.

Study plots were installed at the Virginia Tech Urban Horticulture Center (UHC) in Blacksburg, Virginia. This facility is home to dozens of agricultural research projects, though the portion used for this specific project has remained fallow for at least a decade. The primary soils on site are a Berks-Groseclose complex with a 7% slope. These soils are associated with low infiltration rates and high runoff potential. At the beginning of the study soil cores were taken from various areas of the field, which identified a clay layer present throughout the entire field approximately 0.15 m below the surface. Soil testing conducted at the Virginia Tech Soils Lab indicated the soil had a pH of 5.5, 84 kg/ha phosphorus and 322 kg/ha potassium, nitrogen was not specifically tested as nitrogen species are very complex to test. As per the Mid-Atlantic Commercial Vegetable Production Recommendations of 2016, the area had 3.4 MT/ha of lime added to increase the pH to recommended levels.

The twenty-seven 9 m^2 plots were delineated (Fig. 4-1) using three lengths of 16 gauge galvanized 3 m x 0.4 m steel sheets inserted approximately 0.2 m into the ground (Fig. 4-2).
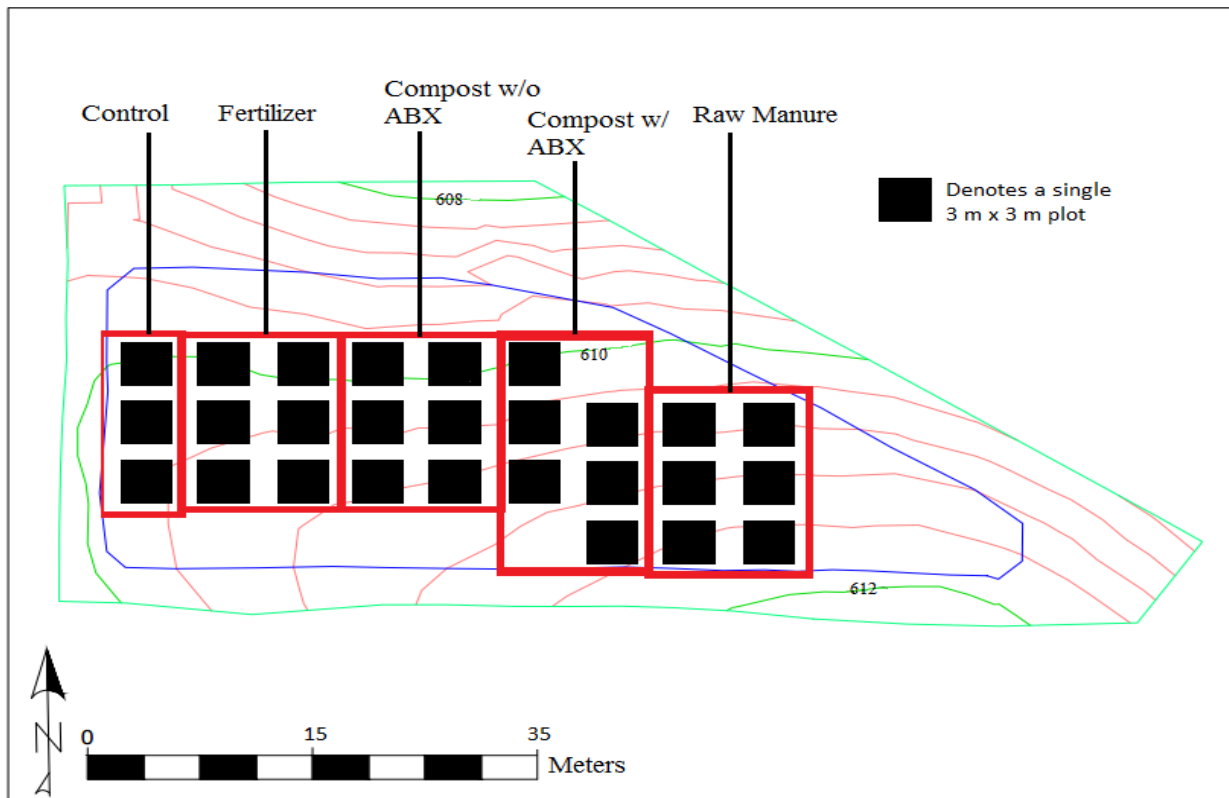
Fig. 4-1. Contour map of the field site showing plot placement with treatment type. The control plots were positioned in such a way to prevent cross-contamination from wind-driven sedimentation.

Fig. 4-2. The twenty-seven 9 m2 plots installed at UHC.

The downhill side of each plot was fitted with a gutter and bucket for collection of runoff during storm events. The plots were held together by bending the steel into 0.01 m corners and were riveted to prevent runoff from entering the plots from the exterior. The corners were also caulked together to create a water tight seal. A rolling trencher was used to create the 0.2 m deep trench to insert the plots into the ground. The gutters were placed below the open, down-gradient side of the plots to collect runoff, and 0.3 m of aluminum steel flashing was used to cover the lip of the gutter to prevent direct precipitation into the gutter. The gutter was angled such that it would always flow toward a 11-liter collection bucket. A food-grade safety hose was used to connect the gutter to the collection bucket below the surface (Fig. 4-3).

Fig. 4-3. The gutter, hose and bucket runoff collection section. The gutter was then covered by the steel plate on the right to prevent rainfall from directly entering the gutter.

4.3 Soil Treatments

Manure for application to fill plots was obtained from lactating dairy cows that were raised for milk production with or without antibiotics depending on the type of treatment to be applied. The cows that received antibiotics were given Pirlimycin (Lincosamide) as two intramammary doses of 50 mg each therapeutically at peak lactation, to treat disease. Although the cows in this study were healthy, a typical dose of Pirlimycin was given as if they were not healthy. Cephapirin (Macrolide) was given as a single intramammary dose of 300mg as a prophylactic at the end of lactation, to prevent disease. Manure was mixed as ratios of alfalfa hay (4:1) and sawdust (4:3) with the end C:N ratio of 25-30%, and moisture content of 55-65%. Manure was composted using an aerated static process at Kentland Farm, in Blacksburg, VA as described in Ray *et al.* (2017). In brief, compost piles reached a temperature of >55ºC on the 2nd day of composting, and maintained at this temperature for more than five days. Each plot receiving compost or manure received 6.72 Mg of treatment/ha. As the compost/raw manure was not nutritionally sufficient per the Mid-Atlantic Commercial Vegetable Production Recommendations, chemical fertilizer was added to these plots to ensure minimum nutrient requirements. Lettuce and radish plots were amended with a 100-100-75 nutrient ratio added to lettuce plots and a 50-50-20 ratio added to radish plots (VCE, 2016).

4.4 Vegetable Planting and Harvest

In early April 2016 (approximately, one month after soil amendment application), radishes and lettuce were planted separately in three plots per treatment type excluding the control. Radish seeds were planted by hand with 0.305 m (1 ft) spaced rows every 2.5 cm (1 in) for the entire width of the plot resulting in 1000 seeds/plot (VCE, 2016). Lettuce seedlings grown in vermiculite and amended with inorganic fertilizer were secured from a local commercial grower. These seedlings were transplanted to the field plots using a 0.61 meter (2 ft) row spacing with a seedling inserted every 23 cm (9 in) along the width of each plot resulting in 54 plants/plot (VCE, 2016).

Upon harvest, in total, the plots yielded 593 kg of radishes and 323 kg of lettuce. Plots amended with compost with antibiotics yielded the greatest quantity of radishes (66.3±3.1 kg), while plots amended with compost without antibiotics yielded the greatest quantity of lettuce (26.3±2.2 kg). Plots amended with inorganic fertilizer yielded the least lettuce and radishes (14.8±3.1 kg; 30.0±3.6 kg, respectively).

4.5 Runoff Collection

Runoff was collected during a total of 6 storm events: four storms during the growing season, one storm collected after harvesting, and one storm post-harvest following the plot treatment with glyphosate. A storm was defined as sufficient rainfall to produce at least 11 l (approximately 3 gal) of runoff per plot.

Runoff samples were collected using sterilized 11 l buckets attached to the gutters of each plot via food-grade hoses. Each bucket was sterilized with a 10% bleach solution immediately before each storm collection with a thorough rinsing to reduce the chance of the bleach solution affecting bacterial results. These buckets collected the first 11 l of runoff (i.e., "first flush"), and then were sealed to prevent any additional runoff from entering the bucket. A sub-sample of 2 l was collected from each bucket following using sterile pipette tips to stir the contents of the buckets to ensure a homogenous sample of runoff and sediment. The runoff that was not in the first flush entering the buckets was ignored.

4.6 Laboratory Analyses

4.6.1 Fecal Indicator Bacteria Quantification

FIB were quantified in runoff samples using IDEXX Colilert® and Enterolert® defined
Substrate Technology®. This IDEXX Quanti-Tray®/2000 system quantifies the total coliforms,
E. coli, and enterococci using a Most Probable Number (MPN) model (IDEXX Laboratories
Inc., 2017). If the correct dilution was not captured, then a re-test was performed within 24 hr of
collection.

4.6.2. DNA Extraction and qPCR

DNA was filter captured via Isopore® Membrane Filters 0.4 μm HTTP poly-carbonate filters
(MilliporeSigma, 2017) within 72 hours of collection. At least 10 ml of runoff was concentrated
with vacuum filtration. The actual filtered volume varied based on the ease of passage and filter
clogging due to sediment and was recorded on each cryotube. Filters were stored in 1.5 ml
cryotubes at -80°C prior to DNA extraction and qPCR analysis (approximately 6 months).

The filtered samples were processed for DNA extraction and qPCR using FastDNA® SPIN Kit
for Soil by MP Biomedicals following the manufacturer's instructions (MP Biomedicals, LLC,
2017). During the DNA extraction process, the filters were placed into a bead beater to
homogenize and lyse the cells. Inhibition was addressed using the OneStep™ PCR Inhibitor
Removal Kit from Zymo Research (2017) following the manufacturer's instructions.

All extracted samples were analyzed for *sul1*, *ermB*, *16SrRNA*. The genes *sul1* and *ermB* are
genes that encode resistance for sulfonamide drugs and erythromycin respectively. The gene
16SrRNA is present in nearly all bacteria and does not change rapidly over time (Janda et al.,
2007). These genes were chosen for analysis based on the results of a microcosm study, a
greenhouse study, and a field study that followed an identical experimental design and field
condition as those found in Wind (2017). Sulfonamide-class drugs were not given to the dairy
cows, however, previous metagenomic work examining the impact of manure composting
indicated that there is often an increase in *sul1* resistance genes following composting (Pankow,
2017; Williams, 2017). A macrolide-class drug was used in the study (cephapirin) which is the

same class of drug as erythromycin. Since these are of the same class, there is a hypothesis that the resistance for erythromycin caused by *ermB* will increase in manure coming from the dairy cows.

Table 4-2. List of targeted genes and their forward and reverse primers for qPCR.

Target Gene	Primer Sequences	Reference
ermB- 91F	GAT ACC GTT TAC GAA ATT GG	Chen et al., 2017
ermB- 454R	GAA TCG AGA CTT GAG TGT GC	
16S-1369F	CGG TGA ATA CGT TCY CGG	Gaze et al., 2011
16S-1492R	GGW TAC CTT GTT ACG ACT T	
sul1- F	CGC ACC GGA AAC ATC GCT GCA C	Pei et al., 2006
sul1- R	TGA AGT TCC GCC GCA AGG CTC G	

All qPCR analyses used the same formula for the Master Mix: 480 µL EvaGreen® Dye; 76.8 µL Forward Primer (Table 4-2; for *sul1, ermB,* or *16SRNA*); 76.8 µL Reverse Primer (Table 4-2; for *sul1, ermB,* or *16SRNA*); and 240 µL molecular-grade, DNAase-free water. Each well in the qPCR received 10 µL of the Master Mix. Negative controls were included in each qPCR iteration, and standard curves for each gene can be found in Appendix 9.5.

4.6.3 Sediment Analysis

Sediment concentrations in runoff were determined via a Total Suspended Solids (TSS) analysis (Bartram and Ballance, 1996). TSS was used instead of SSC in analyzing sediment concentration due to the high clay content of the soil. In brief, a 100 ml aliquot of sample was filtered through 0.45-µm Glass-Fiber Filters (Thermo Fisher Scientific, 2017). Samples were dried at 105°C for 24 hr and then stored in a desiccator for another 24 hr prior to weighing to determine the sediment mass.

4.7 Statistical Approach

4.7.1 Transforming the Data

All statistical analyses were conducted in R studio version 3.3.2. Normality/Non-Normality of the data were determined by observing the normal Q-Q plot of the residuals from the two-way ANOVA. The entire statistical process (Fig. 4-4) determined the effect of treatments across all storms and within individual storms.
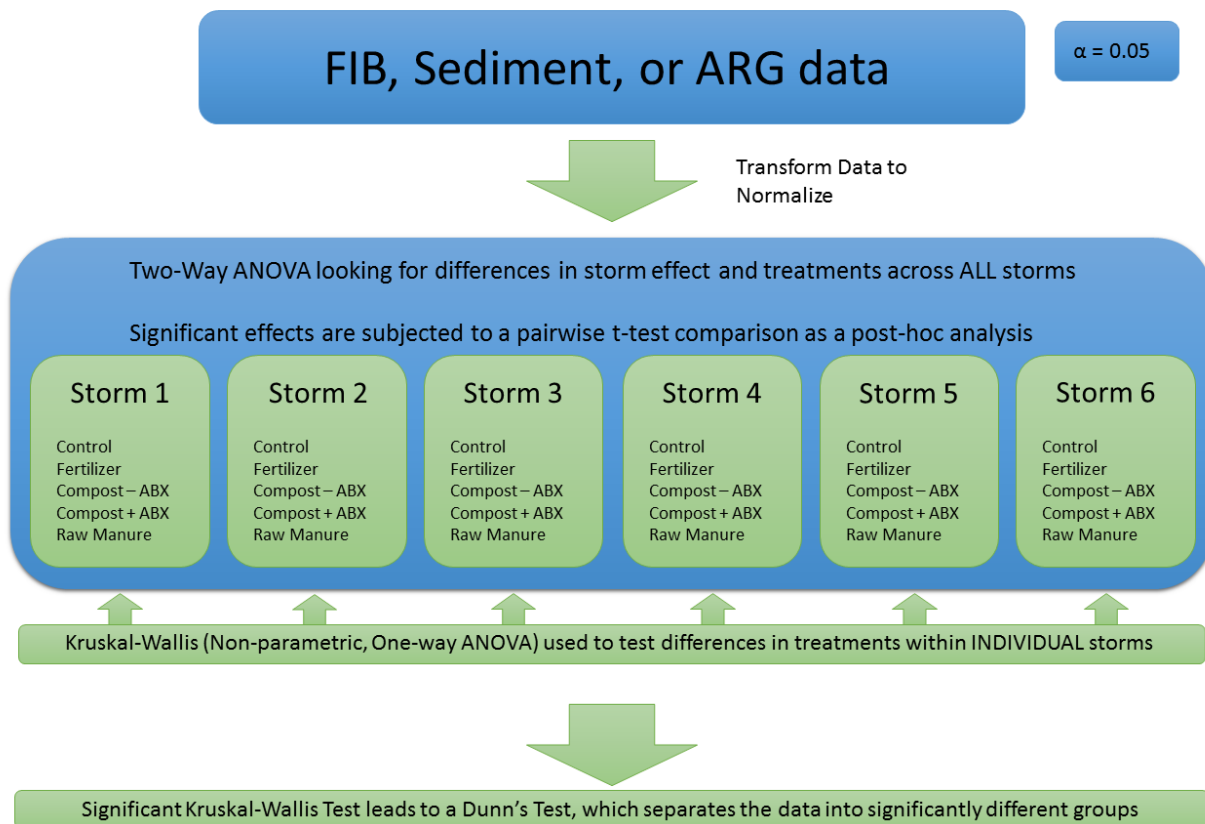
Fig. 4-4. An illustration showing the statistical steps used to interpret the FIB, sediment, and ARG data including parametric and non-parametric ANOVAs as well as post-hoc analysis.

Before conducting the two-way ANOVA for the data across all storms, the FIB and ARGs were normalized to a log10 scale, the 16SrRNA data were normalized by taking the square root, and the sediment data were normalized to a natural log scale. These were sufficient in normalizing the data to use the two-way ANOVA to compare treatments and storm effects across all storms. However, to compare treatments within individual storms, these transformations were not sufficient for normality. For example, the Q-Q plot of the *E. coli* data for the two-way ANOVA

residuals were normal following the log10 transformation. However, the Q-Q plot for individual Storm 1 *E. coli* data that was also transformed to a log10 scale, the data was not normally distributed, and each storm was found to have a different distribution. A non-parametric test was used due to the normality assumption not being met.

4.7.2 Two-way ANOVA for Overall Effects & Post-Hoc Analysis

The two-way ANOVA was performed for measured FIB concentrations (enterococci and *E. coli*), sediment concentrations, and ARG concentrations (*sul1, 16SRNA, and ermB*). Combining the treatments permitted the use of a two-way ANOVA to observe the interaction between storm effect and treatment. An alpha value of 0.05 was used to determine significance. Due to the unbalanced block design used by the experiment, a three-way Analysis of Variance (ANOVA) testing the interaction between soil amendment, storm effect, and planted vegetable type could not be completed. Instead, the individual plot treatments were combined into one variable (e. g., a plot containing chemical fertilizer and lettuce was labeled as an individual treatment: Fertilizer + Lettuce). The two-way ANOVA allowed for a better understanding of how soil treatment and storm effect impacted the measurements of FIB, ARGs, and sediment. If the ANOVA test indicated that there was some significance for the soil treatment or storm effect, then a pair-wise t-test was performed to indicate which groups of treatments or storm effects were significantly different from each other. For example, if the two-way ANOVA showed a significant effect ($p<0.05$) of the treatments on *E. coli* concentrations, then two-sample t-tests would compare these *E. coli* concentrations between each combination of treatment. These combinations are then used to separate the treatments into groups.

4.7.3 Kruskal-Wallis and Post-Hoc Analysis

Two-way ANOVA analyses indicated that differences between storms (i.e. storm effect) consistently influenced concentrations of FIB, sediment, and ARGs in runoff as compared to the soil amendments (treatments). To determine the effectiveness of the treatments within individual storms, a Kruskal-Wallis test was performed (Hollander et al., 1973). This non-parametric one-way ANOVA was selected since the data within individual storms were not always normally distributed even after the transformations used for the two-way ANOVA. The Kruskal-Wallis is used when the normality assumptions of a one-way ANOVA are not met, and it works by

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assigning a rank to each of the values instead of using the data values themselves. An alpha value of 0.05 was used to determine significance. Following a significant Kruskal-Wallis test, a post-hoc analysis was performed.

The post-hoc analysis following a significant Kruskal-Wallis test was a Dunn's test which separates data from independent variables into groups. If the Kruskal-Wallis test is not significant ($p > 0.05$), then the Dunn test cannot separate the independent variable into groups. The Dunn's test was obtained from R package FSA (Ogle, 2017).

<u>4.7.4 Limits of Detection</u>

Although the majority of the collected data were within the upper and lower limits of detection, there were some data points that extended beyond these limits. For the FIB data, if all wells in the IDEXX Quanti-Tray®/2000 were positive, then a value of two times the upper limit of detection was used. Samples from multiple plots were over the limit of detection of enterococci during Storm 5 (11.7% of all enterococci data); all measured *E. coli* values were within the detection limits. A portion of samples fell below the detection limits for ARG targets: *sul1* (3.8% below detection limit) and *ermB* (6.1% below detection limit). The US Environmental Protection Agency (2002) recommends if the number of samples below the limit is below 15% then non-detected data can be substituted with half the lower limit of detection. If a higher percentage of data fell below the lower limit of detection, then models such as a Zero-Inflated Poisson distribution may be used. Given non-detect levels below 15%, more advanced models were not required.

5. RESULTS AND DISCUSSION

5.1 Storm intensity, duration, and timing

The six storms collected varied considerably in terms of total rainfall, rainfall duration, and intensity (Table 5-1). In addition, characteristics of each storm occurred during different times within the growing season (Fig. 5-1). The storm effect therefore accounts for multiple factors including temperature, soil moisture, UV exposure prior to rainfall, ground cover, and overall seasonality. Therefore, storms were considered as distinct events in describing the potential

impacts of storms on the release of sediment, FIB, and ARGs. Storms 1-4 were collected prior to vegetable harvest, and Storm 4 had the largest total rainfall depth. Storm 5 and Storm 6 had the highest average first hour intensities, and both were collected after the vegetable harvest. This higher intensity post-harvest might have impacted the collection system since only the first flush of runoff was collected. High first hour intensities would fill the collection buckets quickly and the buckets would fill completely before the first hour of runoff was complete.
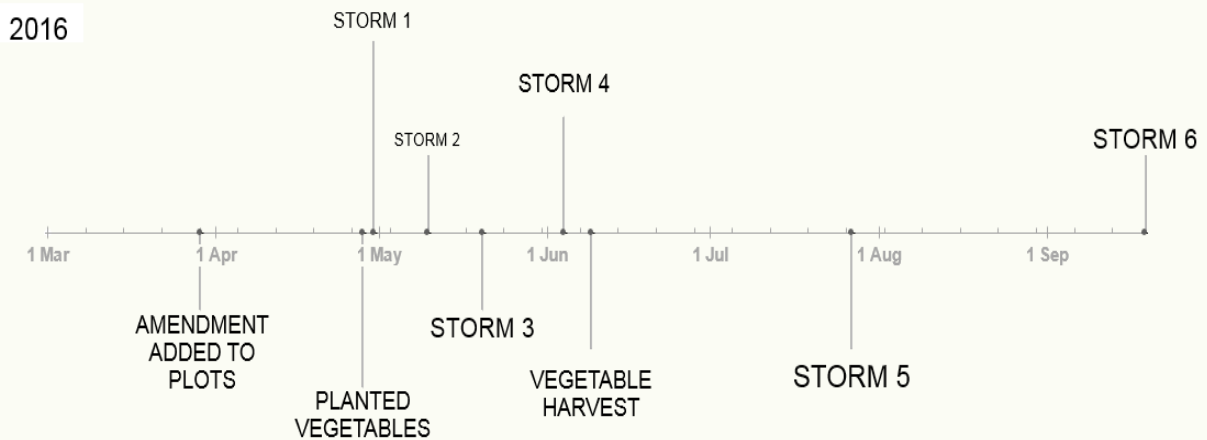
Fig. 5-1. A timeline showing the storm collection in relation to the growing season of the planted vegetables. The larger font size indicates a higher storm intensity.

Table 5-1. Data showing the date, total volume, maximum intensity and duration of each storm collected. Bold indicated the highest first-hour storm intensity.

Date	Storm Number	Total Rainfall Depth (mm)	Average First hour of intensity (mm/hr)	Storm Duration (hr)	Total Theoretical Volume per Plot (l)
4/30/2016	Storm 1	25.5	3.3	18.0	229
5/10/2016	Storm 2	12.9	2.7	16.5	116
5/20/2016	Storm 3	23.2	5.9	5.8	209
6/4/2016	Storm 4	28.4	2.6	20.3	255
7/27/2016	Storm 5	16.8	16.7	1.3	151
9/19/2016	Storm 6	15.1	9.5	24.3	136

5.2 Fecal Indicator Bacteria

Both *E. coli* and enterococci were detected at high levels in runoff, with maximum observations of 10^6 MPN/100 ml for *E. coli* and 10^7 MPN/100 ml for enterococci (Fig 5-2 and 5-3). This is consistent with past literature demonstrating the link between land-application of manure to agricultural lands and contamination of adjacent receiving waters following storm events (Frank,

2005; Ribeiro et al., 2012; Joy et al., 2013; Fahrenfeld et al. 2014). However, it is interesting to note that substantial quantities of both indicators were detected in runoff from plots that did not receive any manure. For example, from plots that received only chemical fertilizer and no manure, enterococci concentrations were as high as $1.4 * 10^7$ MPN/100 ml. Similarly, concentrations of *E. coli* in runoff from control plots (no chemical or manure amendment) were as high as $2.4 * 10^5$ MPN/100 ml.
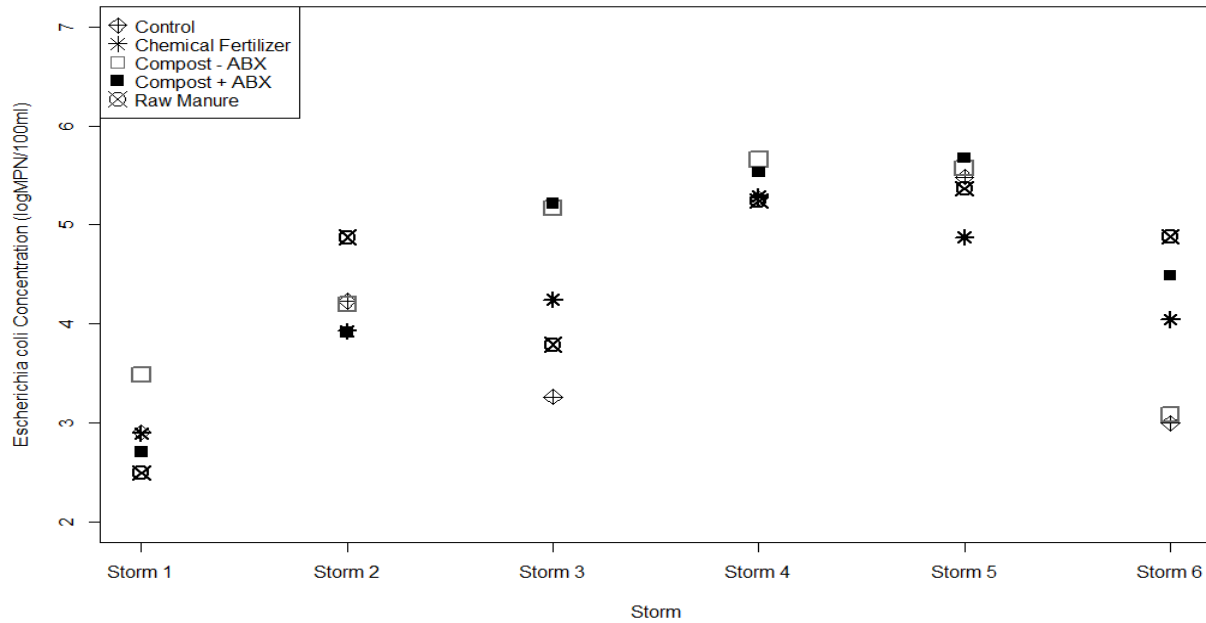
Fig. 5-2. The average *E. coli* concentrations in runoff for each treatment for each storm.
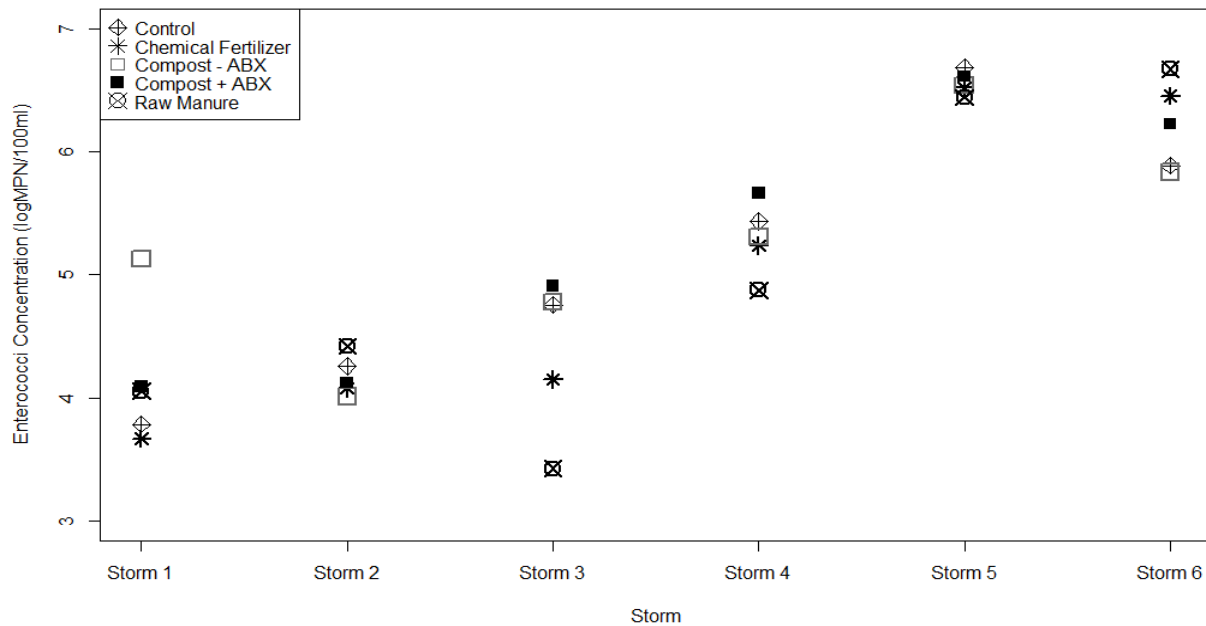
Fig. 5-3. The average enterococci concentrations in runoff for each treatment for each storm.

Storm effect was the most significant factor influencing runoff concentrations (Fig. 8 and 9), i.e., different storms resulted in significantly different concentrations of both types of FIB recovered in runoff samples (Two-way ANOVA, p<2e-16). No other factor (vegetable cover or treatment type) was significant for *E. coli* (Two-way ANOVA, p>0.05). Interestingly, a post-hoc analysis for *E. coli* concentrations (Table 5-2) indicated that the two lower intensity storms (Storm 2 and 3) were statistically different than the two higher intensity storms (Storm 4 and 5), as well as the two storms that were collected at the beginning and end of the study (Storm 1 and 6). Storm 4 and Storm 5 had the highest *E. coli* concentration in runoff, while Storm 1 had the lowest *E. coli* concentration.
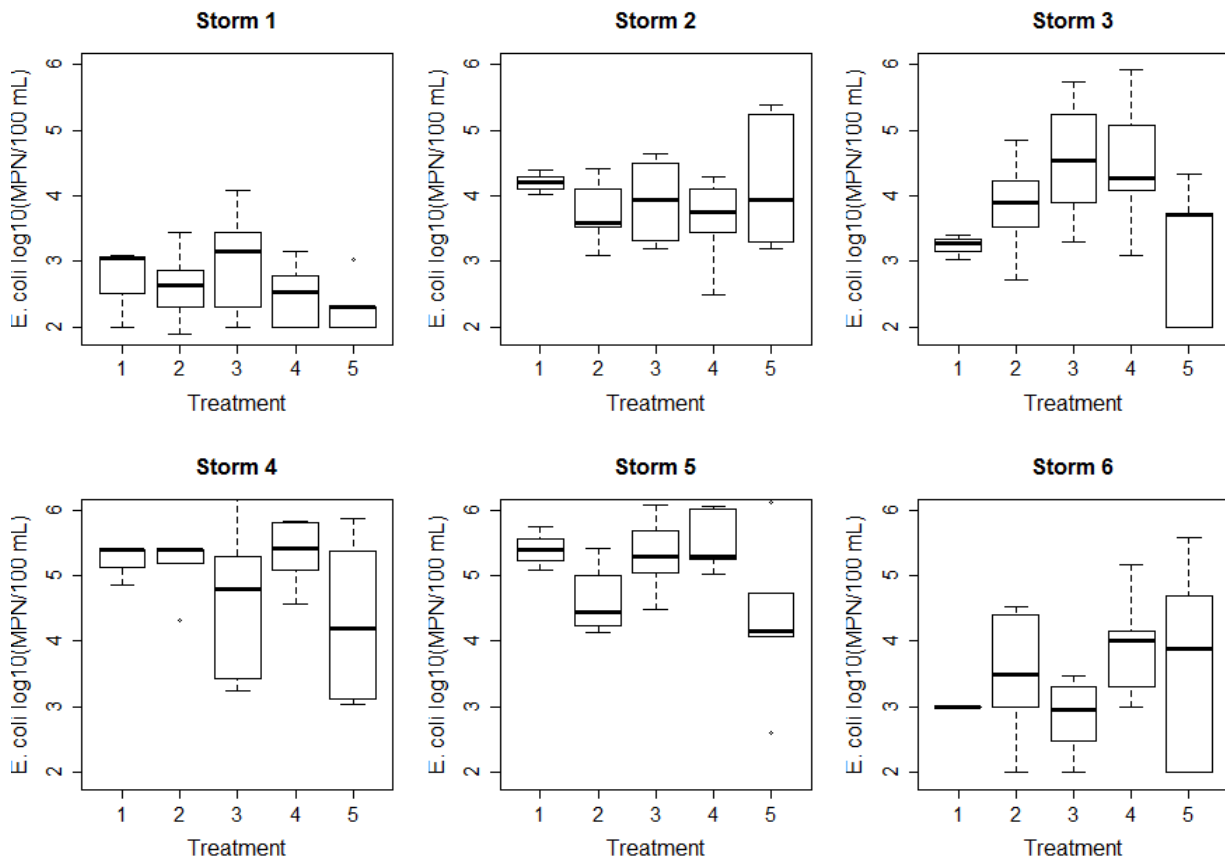
Fig. 5-4. *E. coli* concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length.
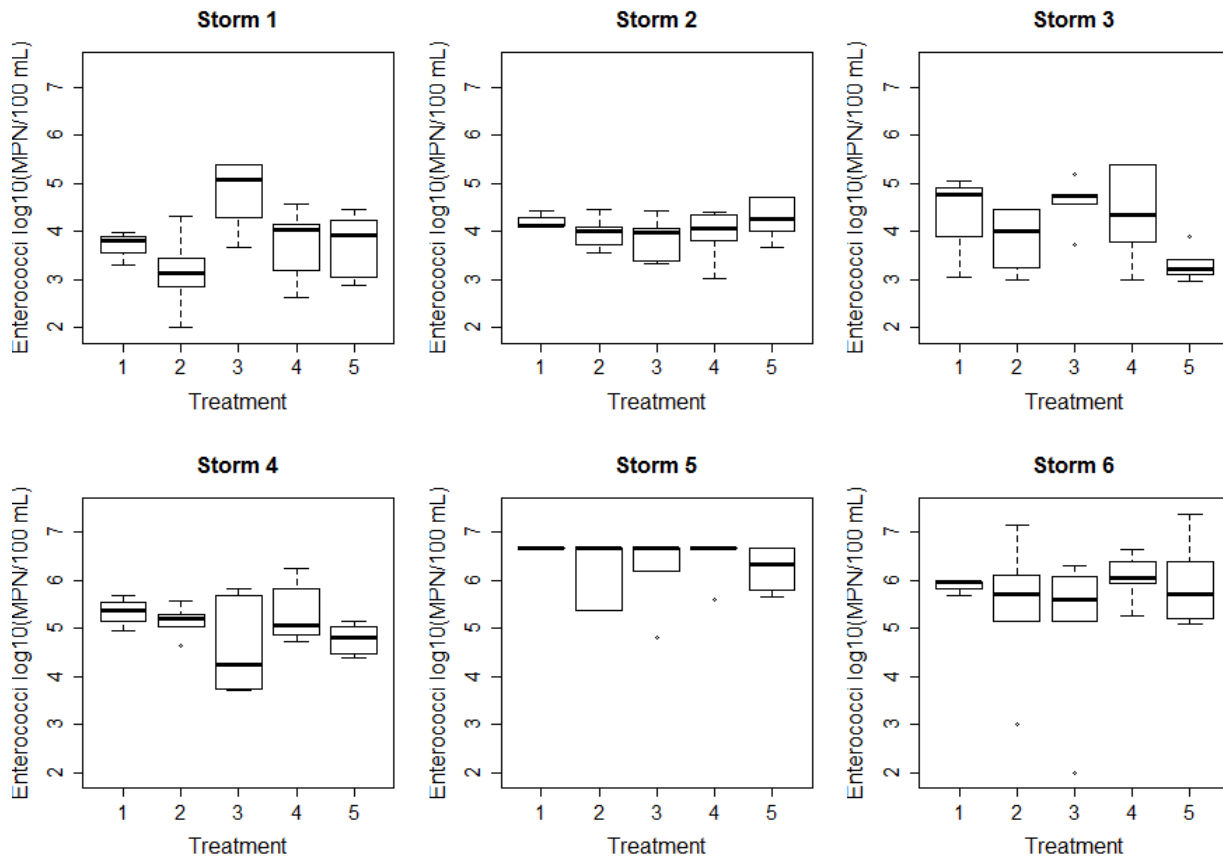
Fig. 5-5. Enterococci concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length.

Similarly to *E. coli*, enterococci demonstrated storm effect was an important factor overall in enterococci release in runoff (Two-way ANOVA, p<2e-16). In this case, more enterococci were released in later storms (Fig. 5-3). A post-hoc analysis indicated Storm 1, 2, and 3 were all statistically similar and Storm 4, 5, and 6 were unique storms. Storm 1, 2, and 3 released significantly less enterococci than the other storms, and Storm 5 led to the largest release of enterococci in runoff. Unlike *E. coli*, for enterococci, one specific treatment combination was significantly different across all storms due to interaction effects (Two-way ANOVA, p=0.04). During Storm 3, the chemical fertilizer and radish combination released a significantly lower concentration of enterococci than all other treatments and vegetable combinations.

Table 1-2. Grouping of effect of different storms based on significant two-way ANOVAs for FIB, where each letter represents a separate group of average FIB concentration in runoff. If two or more Storms have the same number, then they are considered to be statistically similar.

Storm Number	*E. coli* Group	Enterococci Group
1	A	A
2	B	A
3	B	A
4	C	B
5	C	C
6	D	D

Kruskal-Wallis test results examining differences within storms for *E. coli* supported results from the two-way ANOVA across all storms. None of the individual treatments caused significant differences in *E. coli* concentrations in the runoff (Kruskal-Wallis, p>0.05). Tests for individual storms told a slightly different story for enterococci: for Storm 1, enterococci concentrations from plots amended with compost without antibiotics were significantly higher than those recovered from the other plots (Kruskal-Wallis, p=0.03). All other treatments within the other storms were statistically similar (Kruskal-Wallis, p>0.05).

Storm effect was the most significant variable affecting the release of both *E. coli* and enterococci in runoff. Enterococci additionally had one treatment that led to significantly lower enterococci release in runoff: chemical fertilizer + radishes. When observing individual storms, none of the *E. coli* concentrations varied based on soil amendment type. Individual storms for enterococci indicated that plots containing compost without antibiotics had significantly higher concentrations in runoff from Storm 1.

5.3 Sediment

Sediment quantities recovered by storm ranged from 0.001 to 0.484 g/100 ml. Values did not seem connected to storm size; for example, Storm 5, which had the highest intensity (Table 6, Fig. 5-6) produced only slightly higher TSS values than Storm 3, which was the smallest storm. Since only the first flush of runoff was collected, these similar TSS values were expected. If the entirety of each storm was collected, then larger storms would be expected to release more sediment. Weeds overtook many of the plots post-harvest and became overgrown. The plots with lettuce and radishes were not as overgrown as the barren control plots.
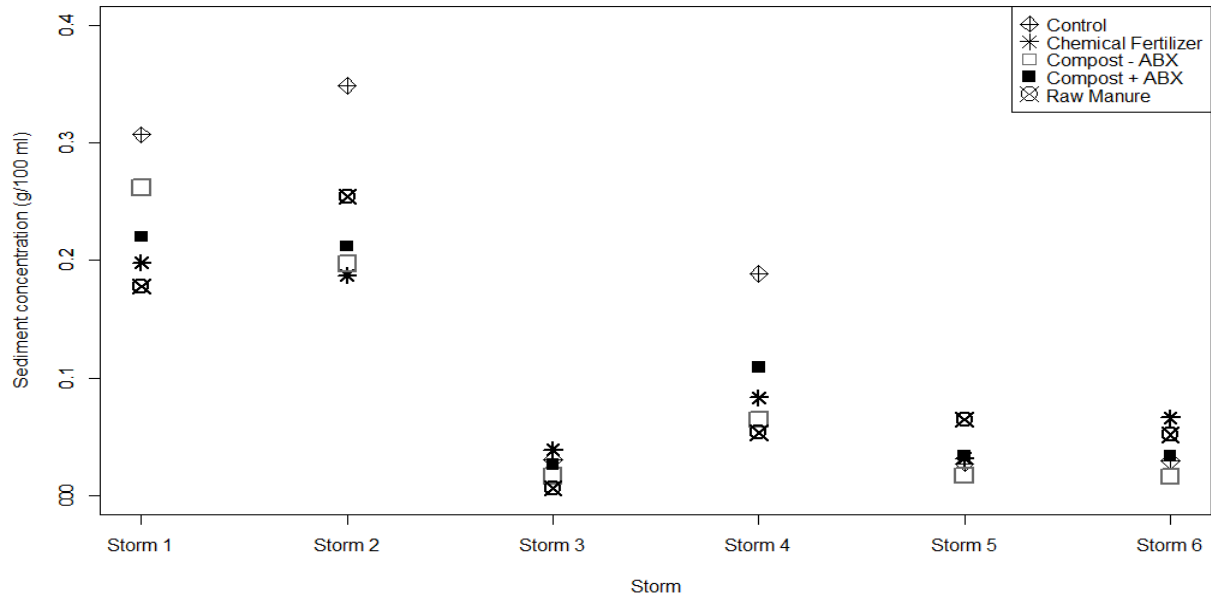
Fig. 5-6. Average concentration of sediment in runoff.

Similarly to the FIB results, the two-way ANOVA indicated that the quantity of sediment recovered differed significantly between storms (Two-way ANOVA, $p < 2e\text{-}16$). A post-hoc analysis (Table 5-3) grouped Storm 1 and 2 into the same group and grouped Storm 5 and 6 into a single group. Storm 3 and 4 were considered unique storms. Storm 1 and 2 led to the highest sediment release in runoff, while Storm 3 led to the lowest.

Table 5-3. The effects of each storm grouped together based on similar sediment release, where each letter represents a separate group of average sediment concentration in runoff. If two or more Storms have the same number, then they are considered to be statistically similar.

Storm Number	Storm Effect on Sediment Group
1	A
2	A
3	B
4	C
5	D
6	D

However, for sediment, treatment type was significant as well, where the barren control plots had significantly higher concentrations of sediment lost to runoff than raw manure plots (Two-way ANOVA, $p = 0.039$). A post-hoc analysis on treatment types indicated that only the control plots were statistically different from other treatment types for sediment.

However, the Kruskal-Wallis tests indicated that within individual storms there were no significant differences in sediment concentrations due to vegetable type or soil amendment (Kruskal-Wallis, p>0.05). This was unexpected, as the barren control plots were anticipated to yield more sediment lost to runoff than those that were planted with lettuce and radishes. However, the treatments within the individual storms look very similar (Fig. 5-7).

Fig. 5-7. Sediment concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length.

Across all storms, barren control plots led to significantly higher concentrations of sediment in runoff before harvest. Within individual storms however, there was no significant difference in sediment release based on treatment or vegetable type.

5.4 Antibiotic Resistance Genes

5.4.1 16SrRNA

The *16SrRNA* concentrations recovered by storm ranged from 10^4 to 10^{10} *16SrRNA* copies/100 ml. There was an upward trend in 16SrRNA concentration with each subsequent storm collection (i.e., more 16SrRNA copies were recovered in samples from later storms; Fig. 5-8). This may be due to increasing soil temperatures, increased plant activity as the growing season progresses, or increased soil microbial community activity.
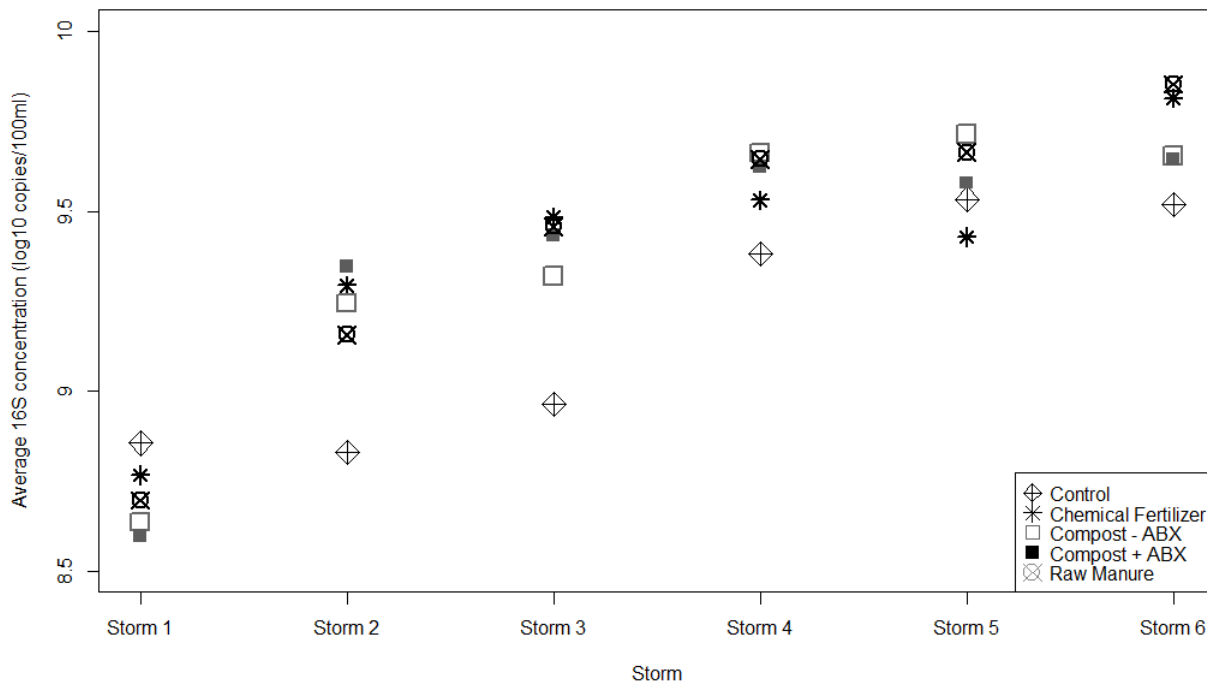
Fig. 5-8. Average concentration of 16SrRNA gene copies in runoff.

Similar to the FIB and sediment, the quantity of 16SrRNA was significantly different between storms (Two-way ANOVA, $p<2e-16$). The post-hoc analysis with the pairwise t-test created groups that indicated that Storm 2 and Storm 3 had similar effects on 16SrRNA release in runoff (Table 5-4). Storm 4, Storm 5, and Storm 6 were similarly grouped together, and Storm 1 was considered a unique storm. Storm 4, Storm 5, and Storm 6 led to statistically higher 16SrRNA release in runoff, while Storm 1 had the lowest 16SrRNA release in runoff.

Table 5-4. The effects of each storm grouped together based on similar 16SrRNA release.

Storm Number	Storm Effect on 16SrRNA group
1	A
2	B
3	B
4	C
5	C
6	C

Kruskal-Wallis tests yielded no significant effect of treatments on 16SrRNA concentration in runoff within individual storms (Kruskal-Wallis, P>0.05). The boxplots demonstrated overlap amongst the treatments within individual storms (Fig. 13).

Figure. 2. 16SrRNA concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length.

The effect of the storms caused a greater influence on differing 16SrRNA release in the first flush of runoff than the individual treatments themselves (Kruskal-Wallis, p>0.05). As will be

discussed in subsequent sections, the increase in 16SrRNA during the study heavily influenced the relative abundance of the two ARGs quantified i.e., higher 16SrRNA concentrations decreased the relative abundance of *sul1* and *ermB*.

5.4.2 Absolute *sul1*

The absolute quantities of *sul1* recovered by storm ranged from 10^3 to 10^6 *sul1* copies/100 ml. Recovery from the first flush of stormwater from the control and chemical fertilizer plots from the first two storms was generally lower than in samples from the other plots (Fig. 5-10).
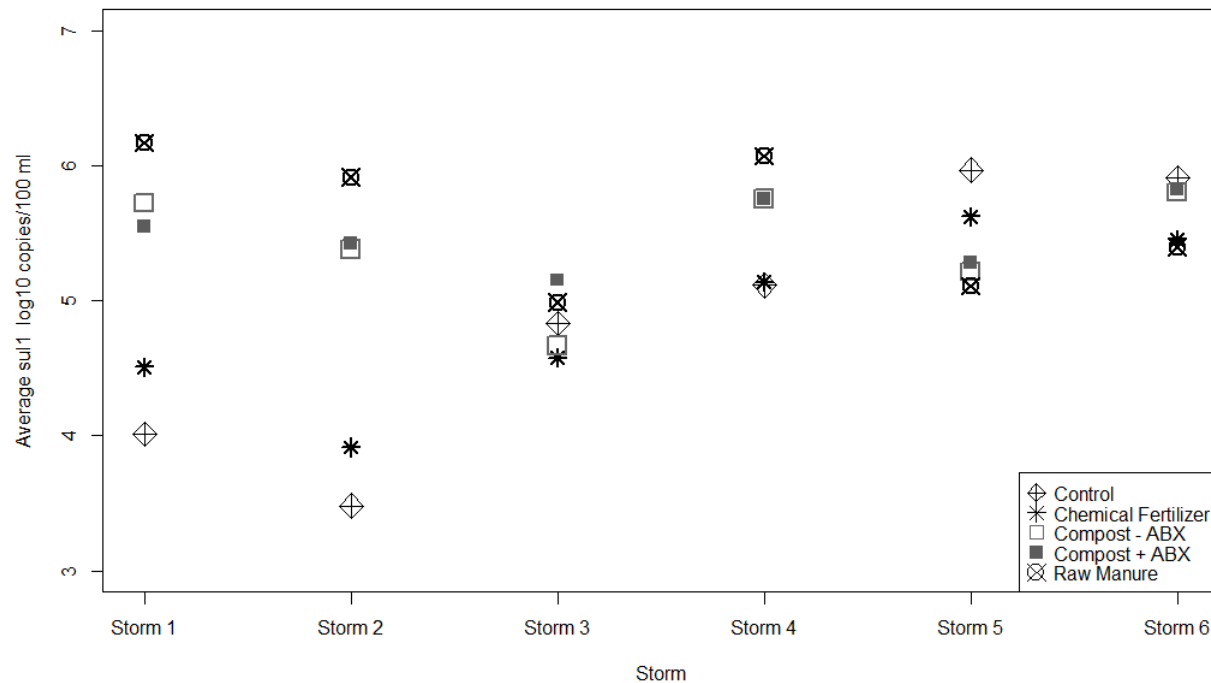
Fig. 5-10. Average concentration of sul1 gene copies in runoff.

The quantity of absolute *sul1* gene copies recovered was significantly different between storms (Two-way ANOVA, p=5.31e-07). Storm 3 had the lowest *sul1* concentrations released to runoff, and Storm 1, 4, 5, and 6 had the highest and were grouped together according to the post-hoc analysis (Table 5-5). There was also a significant effect of the soil treatments on the *sul1* concentrations in runoff (Two-way ANOVA, p=2.74e-06). The groups for the post-hoc analysis indicated that the control and the chemical fertilizer for both lettuce and radishes had the lower *sul1* concentrations released to runoff than the manure and compost treatments (Table 5-5).

Table 5-5. The effects of each storm grouped together based on similar sul1 release. Multiple letters indicate the presence in multiple groups (e.g. Storm 1 is statistically similar to all storms except for Storm 2 and Storm 3).

Storm Number	Storm Effect on *sul1* groups	Treatment	Treatment Effect on *sul1* group
1	A	Control	A
2	B	Fertilizer	A
3	B	Compost-ABX	B
4	A	Compost+ABX	B
5	A	Raw Manure	B
6	A		

Kruskal-Wallis analysis indicated that soil treatments yielded significantly different *sul1* concentrations in runoff in all storms collected prior to harvest (Storm 1, 2, 3, and 4), but that concentrations were statistically equivalent in runoff from all plots for the two post-harvest storms (Storms 5 and 6). There was a gradual decrease in the release of *sul1* genes as the growing season progresses within the compost and raw manure plots (Fig. 5-11). Additionally, there was an increase in *sul1* genes detected in the control and chemical fertilizer plots as the growing season progresses. Although it was unlikely for runoff to move between plots, there are other potential sources that may lead to ARGs contaminating these plots including wildlife and wind-driven sedimentation. The plots were installed in a pattern that was designed to minimize wind-driven sedimentation by placing the control and chemical fertilizer plots up-wind from the compost and raw manure plots.

Interestingly, although no sulfonamide-class drugs were given to the dairy cows producing manure for this study, higher concentrations of *sul1* were released in runoff in both compost types and raw manure plots as compared to the controls and chemical fertilizer plots pre-harvest. These results were not anticipated, as it was expected that there would be more ARGs resisting the classes of drugs given to the dairy cows. Udikovic-Kolic (2014) found similar results in that there was an increase in resistance genes for drug classes in manure that were not given to cattle. It was possible that the high temperatures achieved during composting (>55°C) forced bacteria into expressing genes that enable survival at these high temperatures. Therefore, these bacteria who were capable of surviving these high temperatures also had *sul1* genes present. Whether the *sul1* genes were being expressed was unknown.
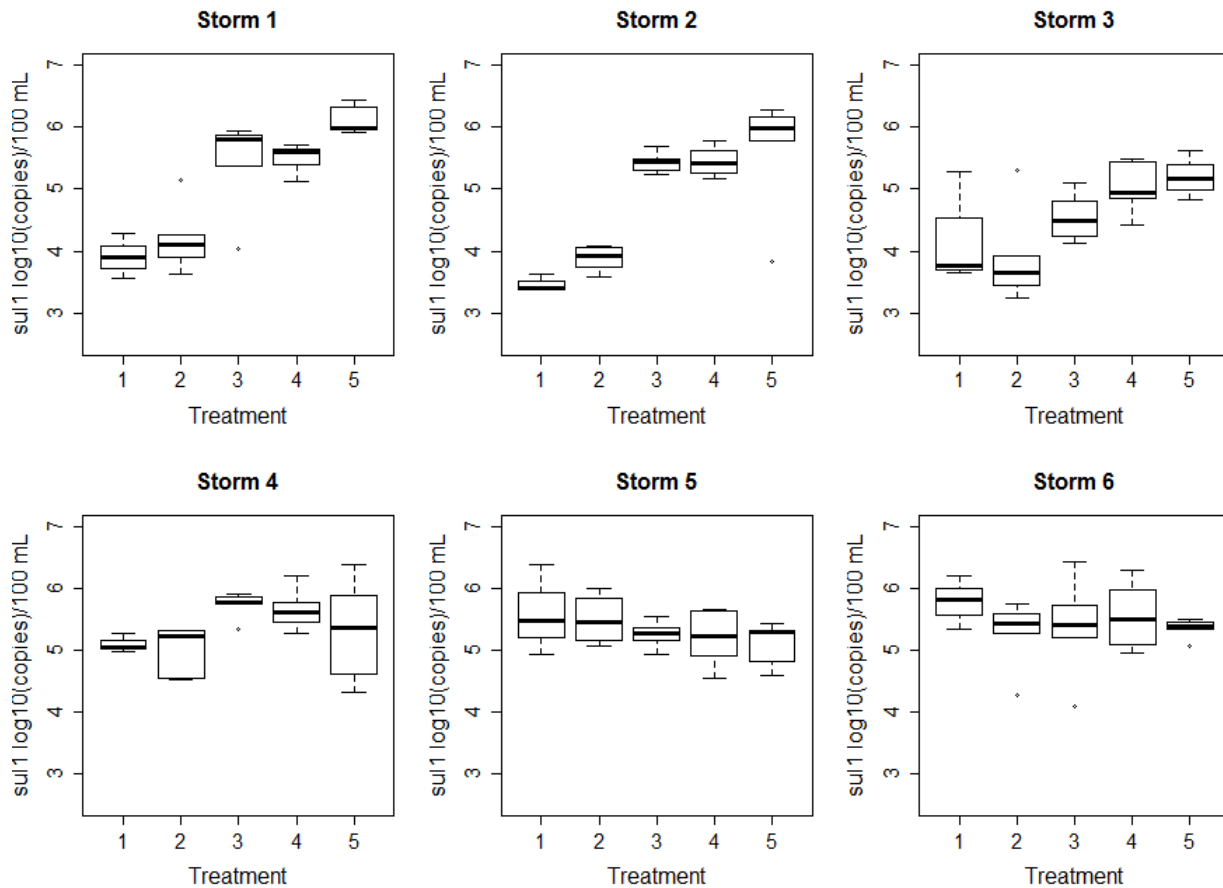
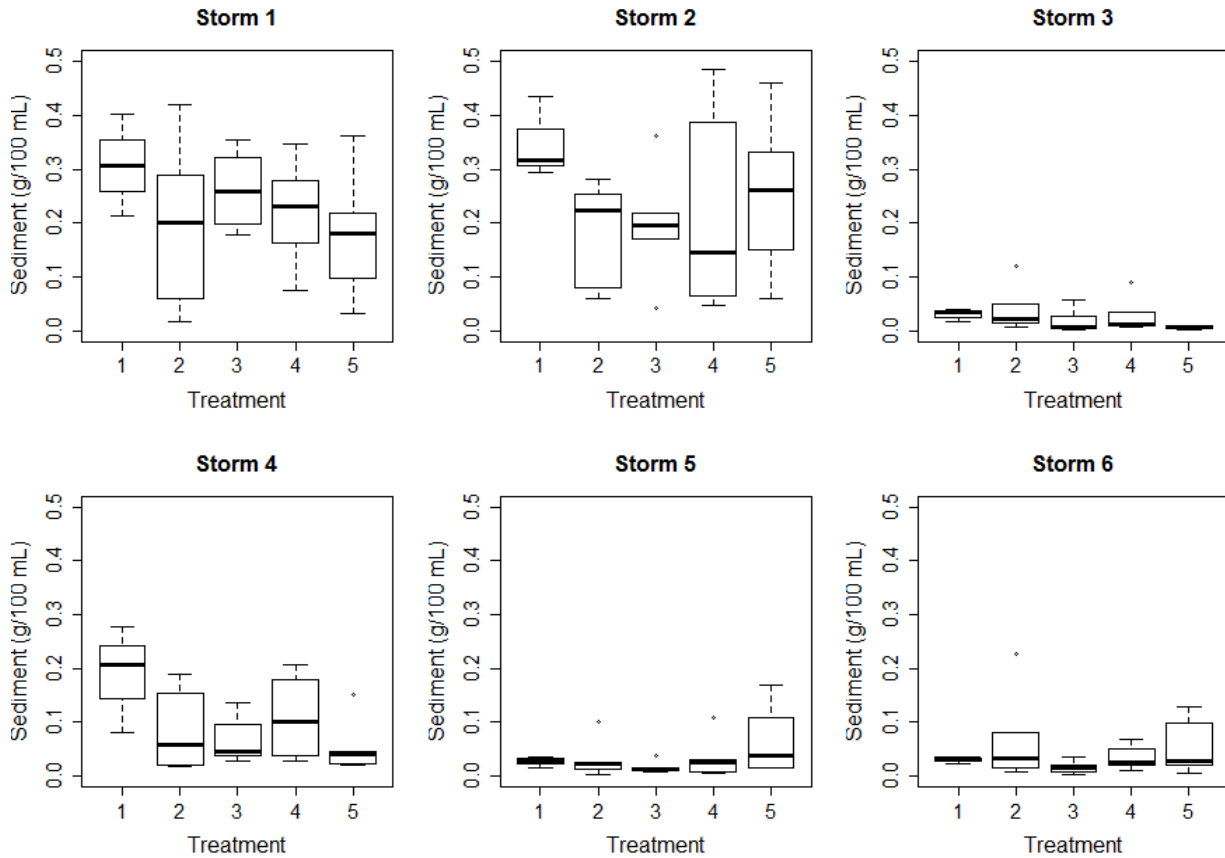Fig. 5-11. Sul1 concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length. Observe how both compost types and raw manure have higher concentrations of sul1 than the control and chemical fertilizer treatments prior to harvest.

A Dunn's test was conducted for Storm 1, 2, 3, and 4 to determine directionality of these differences. Storm 1 had significantly lower concentrations of *sul1* in runoff in both the control and the chemical fertilizer treatments as compared to both compost treatments and the raw manure treatment. Raw manure amended plots yielded significantly higher *sul1* concentrations in runoff as compared to all other plots. Storm 2 results were similar with the significantly lower concentrations of *sul1* in runoff from both the control and the chemical fertilizer treatments than the compost and raw manure treatments. However, in this case the compost and raw manure plot values were statistically equivalent. Storm 3 had significantly lower concentrations of *sul1* in runoff in the chemical fertilizer treatment compared to the control, compost, and raw manure treatments. The compost from cows that received antibiotics and raw manure treatments had significantly higher concentrations of *sul1* gene copies in the runoff than all other treatments.

Storm 4 also had significantly lower concentrations of *sul1* in runoff from the chemical fertilizer treatment as compared to the control, compost, and raw manure treatments. In this case, only the compost from cows without antibiotics was significantly higher than the other treatments.

The analysis across all storms indicated that both treatment type and storm effect was significant for absolute *sul1* abundance. For individual storms, compost without antibiotics, compost with antibiotics, and raw manure plots led to significantly higher absolute *sul1* release in runoff in storms collected before vegetable harvest.

5.4.3 Relative *sul1*

In addition to examining potential differences in absolute values of *sul1*, quantities relative to total 16SrRNA were examined. Targeting relative *sul1*/16SrRNA indicates potential changes in the relative quantity of resistance over treatments and storms; resultant relative data cover a wide range (Fig. 5-12).
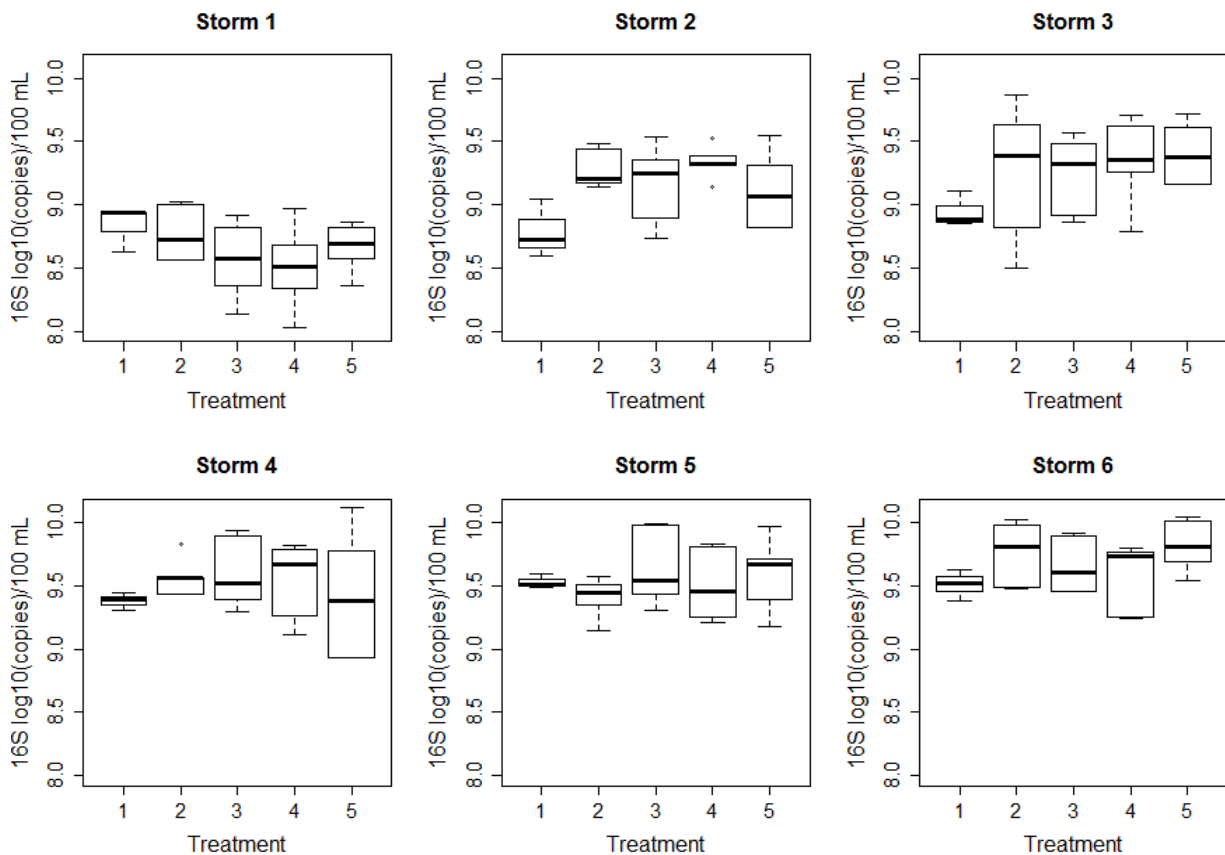

Fig. 5-12. Relative sul1 concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note different y-axes.

The lowest relative *sul1* value was 10^{-6} *sul1* copies/16SrRNA copies, and the highest relative *sul1* value was 10^{-1} *sul1* copies/16SrRNA copies. Storm 1 had the most interesting transformation which mimicked the shape of the absolute *sul1* gene box and whisker plot (Fig 5-11). This means that during Storm 1, the compost treatments and the manure treatment had higher relative numbers than the fertilizer and control. Storm 2 had slightly elevated relative *sul1* gene copies in runoff from compost and raw manure plots and then quickly returned to uniform levels for the remaining storms. Although there was continuous detection of *sul1* in the runoff, with the increasing 16SrRNA concentrations, the proportion of *sul1* genes decreased over time. This is important as it illustrated how the population of microbes within the plots are behaving. Due to the decrease in proportion of *sul1* genes, this suggests that there is competition between microbes that do not contain *sul1* genes and those that do.

The two-way ANOVA (p>0.05) across all storms indicated that there was no significant effect on relative *sul1* abundance from either soil amendment or storm effect. However, the Kruskal-Wallis tests for individual storms suggested that soil treatment was important in the release of relative *sul1* genes in runoff. Storm 1 indicated that compost without antibiotics and raw manure both contributed to higher relative *sul1* genes in runoff. Storm 2 started illustrating the gradual decline in relative *sul1* abundance and only the raw manure treatment led to significantly higher relative *sul1* genes than the other treatments. Storm 3 indicated how chemical fertilizer released significantly lower relative *sul1* than other treatments, but it is important to note that all treatments relative abundance had decreased significantly. Storm 4 and Storm 5 showed no significant effect of treatment on relative *sul1* release in runoff. Finally, Storm 6 revealed that at the final storm collection, the control plots had a higher relative *sul1* abundance than all other treatments.

A higher proportion of *sul1* genes to 16SrRNA genes in the earlier storms (Storm 1, 2) demonstrated how the microbes with *sul1* genes were competing with the microbes that did not. Additionally, for Storm 1 and Storm 2, the raw manure treatment led to significantly higher relative *sul1* abundance than other treatments.

<u>5.4.4 Absolute *ermB*</u>

The range of absolute *ermB* gene copies detected ranged was from 10^1 to 10^3 *ermB* copies/100 ml of runoff; not all samples were quantifiable (6.1% of samples were below detectable limits). Any values that were below 10^1 *ermB* copies/100 ml were given a value of $10^{0.698}$ which is approximately half the lower limit of detection. There was little variation in the level of *ermB* found in the runoff coming from the plots relative to observations of FIB, sediment, 16SrRNA, or *sul1* (Fig. 5-13).
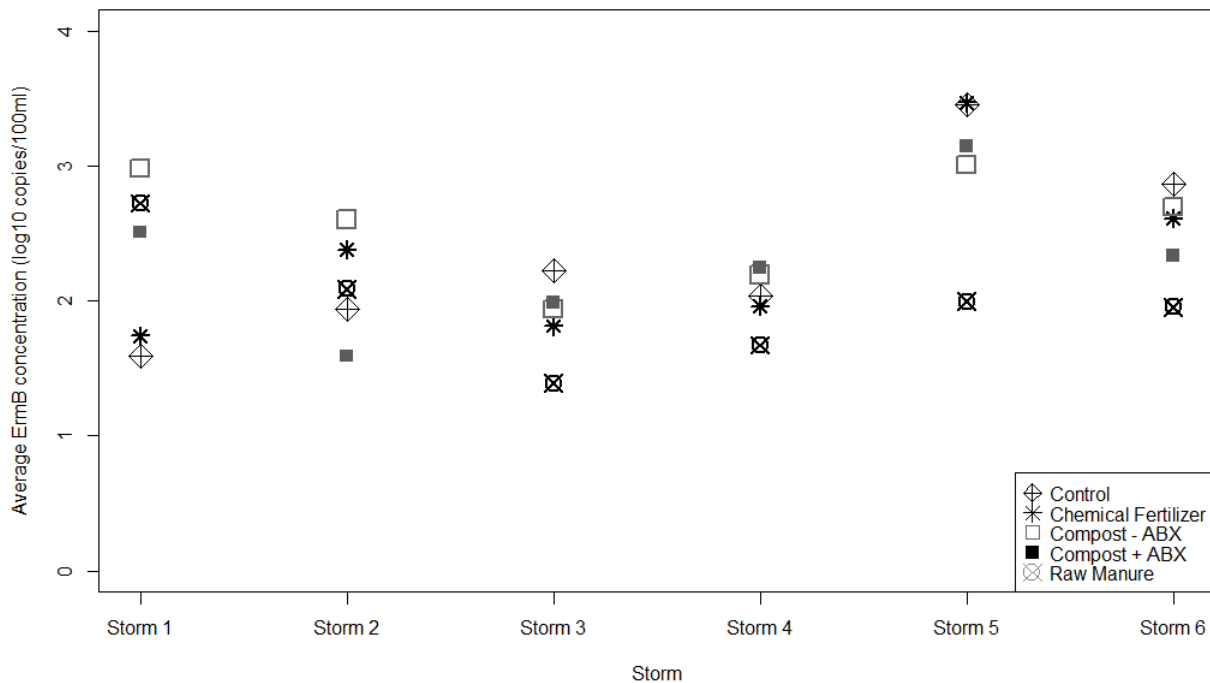
Fig. 5-13. Average concentration of ermB gene copies in runoff.

Concentrations of absolute *ermB* in runoff were significantly different between storms (p=2.51e-08) and treatments (p=2.74e-04) for the two-way ANOVA. The post-hoc analysis indicated that values in runoff following Storm 5 (the largest storm) were statistically higher than all other storms on the release of *ermB* in runoff (Table 5-6). The release of *ermB* from treatments were slightly different across all storms; interestingly, the compost without antibiotics had higher *ermB* concentrations on average than all other treatments. Unexpectedly, the raw manure treatments led to lower average *ermB* concentrations in runoff than other treatments across all storms.

Table 5-6. The effects of each storm grouped together based on similar ermB release. If a treatment has more than one letter, then it is not significantly different from either group.

Storm Number	Storm Effect on *ermB* groups	Treatment	Treatment Effect on *ermB* group
1	A	Control	B
2	A	Fertilizer	BC
3	A	Compost-ABX	A
4	A	Compost+ABX	BC
5	B	Raw Manure	C
6	A		

The Kruskal-Wallis tests indicated that within individual storms, only Storm 2 and Storm 5 indicated that there was a significant difference between the treatments (Kruskal-Wallis, $p<0.05$). The *ermB* concentrations look largely uniform within individual storms (Fig. 5-14).
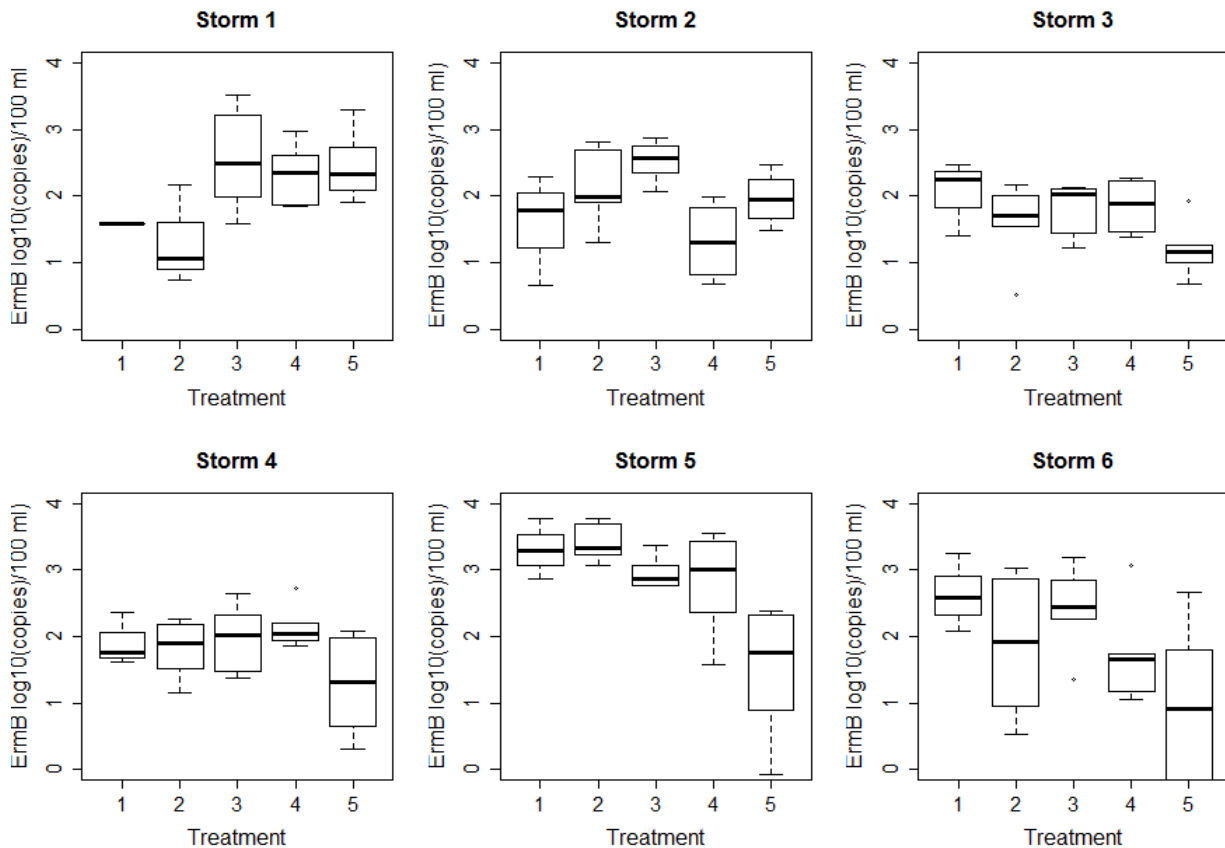
Fig. 5-14. ErmB concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note that all Y-axes are the same length. After Storm 2, the raw manure begins to have visually lower concentrations of *ermB* in the runoff.

The Dunn's Test for Storm 2 indicated that only the compost without any antibiotics given to the cows yielded significantly higher concentrations of *ermB* than other treatments within that storm. The Dunn's Test for Storm 5 indicated that only the raw manure treatment yielded significantly lower concentrations of *ermB* in runoff than other treatments within that individual storm.

5.4.5 Relative *ermB*

Similarly to *sul1*, in addition to examining potential differences in absolute values of *ermB*, quantities relative to total 16SrRNA were examined. The two-way ANOVA (p>0.05) for relative *ermB* concentrations in runoff yielded no significant effect of soil treatment or storm effect. There are lower relative abundances of *ermB* in the runoff than *sul1* (Fig. 5-15).
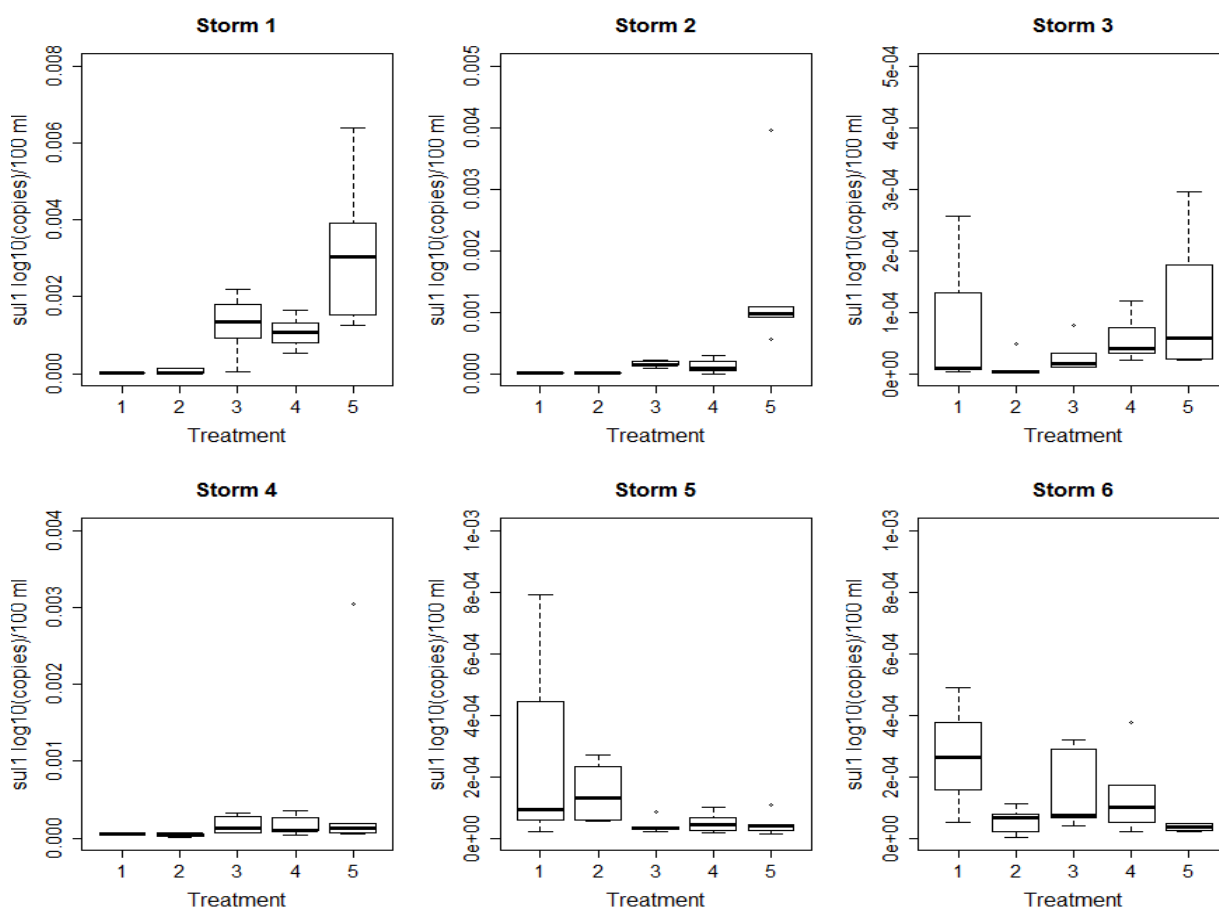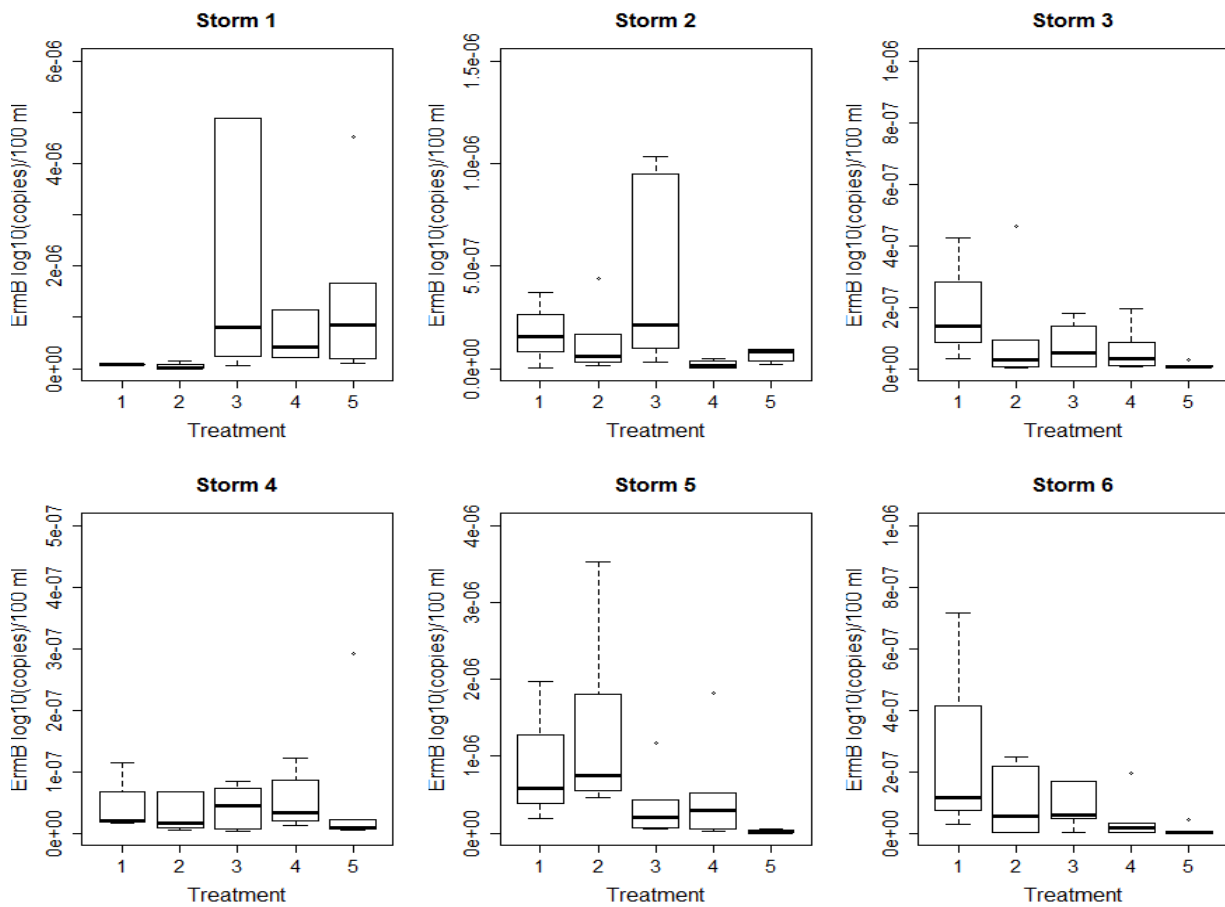

Fig. 5-15. Relative ermB concentrations in runoff for each treatment for each storm. Where the treatments are: 1. Control; 2. Chemical Fertilizer; 3. Compost without antibiotics; 4. Compost with antibiotics; and 5. Raw Manure. Note different y-axes.

The Kruskal-Wallis tests indicated Storm 1, Storm 2, and Storm 5 had treatments with significant differences in relative *ermB* release in runoff. Storm 1 demonstrated relative *ermB* concentrations were higher in plots containing both types of compost and raw manure than

control and chemical fertilizer plots. Storm 2 interestingly indicated that compost without antibiotics led to higher relative abundances of *ermB* than compost with antibiotics and the control, chemical fertilizer, and raw manure plots led to higher relative abundances of *ermB* than compost with antibiotics. For Storm 5, only chemical fertilizer was significantly lower than other soil treatments, and all other soil treatments were statistically similar.

It was hypothesized that since the dairy cow feed was supplemented with Pirlimycin, a lincosamide, which has the same mode of action for antibiotic resistance as erythromycin (macrolide), there would be a higher concentration of *ermB* in the runoff coming from the plots with compost with antibiotics and raw manure treatments. Both lincosamides and macrolides are protein synthesis inhibitors (Tenson et al., 2003). The erythromycin resistance gene, *ermB,* was chosen because erythromycin is critical for human health. The results however, indicate the opposite is true. There were lower concentrations of *ermB* in the runoff coming from these plots than the control and chemical fertilizer plots, indicating that there is likely a background reservoir of *ermB* found naturally in the soil. One potential confounding factor is the concentrations of *ermB* are so low in the runoff, the conclusions drawn from the statistical analysis are not actually meaningful. When normalized to 16SrRNA concentrations, the detected ermB concentrations had lower relative abundances than *sul1* from the same plots and were as low as 10^{-10} *ermB* copies/ 16SrRNA copies. There were elevated levels of *ermB* early in the storm collection followed by a decline, and then a resurgence during the storm with the highest intensity (Storm 5).

Joy et al. (2013) confirms that following the amendment of swine manure, *ermB* can be detectable in soils within plots and in runoff leaving the plot during a simulated storm. One key difference worth noting is that these plots were amended with swine manure treated with chlortetracycline, tylosin, and bacitracin which were different from the antibiotics the dairy cows in this study. Interestingly, the Joy et al. experiment saw the same or higher concentrations (10^1-10^4 gene copies/ml) of *ermB* genes in runoff as this study. One of the antibiotics fed to the swine in the Joy et al. (2013) study, tylosin, was a macrolide-class drug. This is the same class of drug of the cephapirin given to the dairy cows in this study, and the type of drug targeted by the resistance gene *ermB*. Given that there are comparable numbers despite the two types of

manures, the combined results of this study and the Joy et al. (2013) study suggests that *ermB* may not be usually found in high abundance in runoff.

6. FUTURE RESEARCH RECOMMENDATIONS

6.1 Methodological Improvements

Multiple methodological refinements would likely increase the ability of similar field studies to answer questions related to ARG persistence in agricultural runoff. Increasing the number of plots would increase the statistical power of the experiment. Adding on-site rain gages immediately adjacent to the plots would provide a more accurate volume of rainfall during storms. Collecting samples across the entire storm duration instead of the first flush would lead to further analyses about how FIB, sediment, and ARGs behave in runoff. Perhaps of greatest usefulness, intra-storm sampling would permit the calculation of FIB, sediment, and ARG loadings within individual storms. Measuring the flow velocities in the collection system yields the data necessary to create hydrographs of each plot. These hydrographs could then be used to compare loadings of FIB, sediment, or ARG data across individual plots within the same storm for the entire duration the runoff is produced. If piezometers were added to the plots, one could observe how FIB and ARGs may infiltrate into the groundwater. Although there are some data which show that ARGs can move through groundwater systems (Ribiero et al, 2012), many of these studies observed large areas that are fed by hundreds of acres of groundwater. Groundwater monitoring for FIB and ARGs within the on-site locations observed in this study could help identify which areas contributed more to FIB and ARG contamination.

6.2 Linking ARGs and Live Bacteria

The presence of ARGs does not necessarily mean there is a risk of antibiotic resistant bacterial infection. ARGs are ubiquitous in the environment, and these genes can be taken up by many bacteria (Amábile-Cuevas, 1993). There is always a risk of pathogenic bacteria absorbing free-floating ARGs, though the presence of a pathogenic bacteria is not necessarily a public health threat if it is present at levels below the infectious dose. Culture-based analyses of live bacteria are needed to specifically look at pathogens in runoff to determine if there are any actual risks of infection, however, many environmental bacteria are not capable of growing in a laboratory setting.

Wind (2017) quantified the antibiotic resistant fecal coliform bacteria using MacConkey agar from the plots used in this study during the same storm collection period used for this project. This study found a spike in sulfamethoxazole-resistant bacteria initially following the manure and compost application followed by a return to background-levels within seven-days. However, *sul1* genes were still detected following the decline in detectable sulfamethoxazole-resistant bacteria in the soil, i.e., although ARGs were still detectable, the resistance that they convey was not being expressed phenotypically by the fecal coliforms that were cultured on MacConkey agar. An obvious next step would be to statistically examine potential links between soil concentrations of ARBs and he ARGs observed in this study.

Additionally, the ultimate goal of this study was to determine if the soil amendment had an effect on the quantity of ARBs and ARGs on vegetables for human consumption harvested from plots containing compost or raw manure. The study wanted to determine if vegetables from these plots had higher counts of bacteria or ARGs than plots without compost or raw manure. The plants in this study were harvested and tested for live aerobic bacteria as well as *sul1* genes (Fogler et al., 2017). Fogler et al. (2017) found no significant differences in bacteria counts on non-selective R2A plates from the surfaces of lettuce or radishes harvested from this study. However, lettuce that were grown in plots containing raw manure had significantly higher copies of *sul1* resistance genes, which agrees with the findings of the higher concentrations of *sul1* in runoff from manure plots. This study found no significant difference in *sul1* on vegetable surfaces between compost without antibiotics and compost with antibiotics (Fogler et al., 2017).

6.3 Informing Producers and Risk
Currently, there is not enough information to inform a producer about the potential increase in risk to farm workers and/or consumers following the detection of ARGs and/or ARBs in runoff. Background levels of ARGs were observed in these plots and are likely not uncommon in agricultural soils. Ashbolt et al. (2013) suggest that to properly perform a human health risk assessment of ARGs and ARB in the environment, several research gaps needed to be addressed. These research gaps include measuring the minimum selective concentration of antibiotic needed to select for resistance, developing dose-response data for ARGs and ARBs, and identifying

environmental hot spots where antibiotic resistance can develop such as sewage discharges and agricultural fields with applied animal manure (Ashbolt et al., 2013). To properly assess risk with ARG and ARB exposure, a microbial risk assessment (MRA) should be performed. An MRA is a systematic approach to quantify risk associated with microbial infections. This requires describing the problem (ARGs and ARBs), assessing the exposure of the problem, and quantifying the relationship between dose of ARGs or ARBs and the rate ARB infections in humans (Ashbolt et al., 2013). Currently, however, a there are too many data gaps to create a proper MRA associated with manure-based soil amendments in agriculture. The chemical and physical properties of soil, background soil microorganisms, and the antibiotic resistance environments can differ significantly between farms. Most importantly, if there are ARGs detected in background concentrations, the detection of ARGs does not indicate whether the genes are capable of being expressed by pathogenic bacteria. Therefore, a risk assessment of potential of horizontal gene transfer must also be performed. The runoff from these fields would have to be further analyzed for culturable ARBs in order to determine the relationship between ARG and ARB occurrence.

In addition to risk assessment, another important step would be determining how long it takes for ARGs, ARBs, and antibiotics to decay over time. Properly implementing best management practices to control erosion from manure-applied land would be difficult because a producer would not know how long it takes for the ARGs, ARBs, and antibiotics that may be in runoff to degrade or die off. A future study that quantifies the decay of more ARGs, ARBs, and antibiotics in runoff from full-scale fields that have been treated with compost following the FSMA guidelines would help inform what steps to make with best management practices. If ARGs, ARBs, and antibiotics naturally decay or die off while in agricultural best management practices such as vegetative buffer zones or cover crops, implementing best management practices for producers would be relatively cheap. However, if they do not degrade or die off while in a storage system, other steps would need to be taken to prevent runoff from entering rivers or entering groundwater.

Additionally, this study observed greater vegetable yields from plots treated with compost or manure as compared to yields from plots that were treated with chemical fertilizer. Despite the

same nutritional content applied to each plot, compost or raw manure yielded up to twice as much lettuce (per kg) and up to nearly six times as much radishes (per kg) than chemical fertilizer alone. This suggests that the nutrients supplied by the compost or raw manure are more easily available to plants than inorganic chemical fertilizer.

7. CONCLUSIONS

One of the most important observations obtained from the results is that there are FIB and ARGs found in the runoff coming from the control and chemical fertilizer plots. These results indicate there are naturally occurring fecal indicator bacteria in each of the treatment plots despite there being no manure applications occurring for more than a decade. The presence of background fecal indicator bacteria and antibiotic resistance genes is supported by the literature (Leclerc et al., 2001; Wellington et al., 2013; Fahrenfeld et al., 2014). If there are higher than background levels of FIB and ARGs in runoff, producers would likely have to implement best management practices to capture sediment in runoff. For producers, it is important to maintain these best management practices such as contour farming, adding cover crops, creating a grassed waterway, adding buffer strips in between crop rows, or planting in areas to minimize erosion caused by runoff. This runoff could go to other parts of a field or waterway where livestock or wildlife could be exposed to ARGs, ARBs, or antibiotics that are adsorbed onto sediment. If a producer has runoff coming from fields that have been treated with compost, the runoff may enter a neighbor's property or could enter a river that may lead to a public health risk

Recovery of FIB and ARGs in runoff from agricultural plots treated with manure was anticipated, as this has been observed at multiple scales in previous studies (Finley et al., 2013; Joy et al., 2013; Wellington et al., 2013; Fahrenfeld et al., 2014). Finley et al. (2013) suggested that water is a key vector for the storage and dispersal of antibiotic resistance. Although the specific genes discussed in the Finley et al. (2013) study were not identical to those found in this study, they still confirm that ARGs can survive in bodies of water that are fed by agriculture runoff.

Comparing compost from cows that did receive antibiotics and compost from cows that did not receive antibiotics prior to field application indicated that whether the cow received or did not

receive antibiotics did not seem to have an impact on reducing ARGs in runoff. Some studies suggest that the stress of composting on the bacteria may increase the expression of antibiotic resistance (Sharma et al., 2009; Pruden et al., 2013). However, the absolute and relative *sul1* data demonstrated that the raw manure had higher gene copy numbers than either of the compost types in early storms. This suggested that *sul1* genes may be affected by composting.

The recovery of higher *sul1* genes in runoff from plots containing compost and raw manure is especially alarming since the dairy cows were not dosed with sulfonamide-class drugs. For absolute *sul1* gene copies, raw manure and both types of compost had higher concentrations than those of the control and the chemical fertilizer for the storms collected pre-harvest. The *sul1* relative abundance showed similar results to the absolute *sul1* abundance: the compost and manure treatments both had higher relative *sul1* gene copy abundance than the control and fertilizer treatments at the beginning of the study, but not after harvest.

It was expected that there would be higher concentrations of *ermB* genes in the compost with antibiotics and raw manure plots due to erythromycin resistance having the same mode of action as pirlimycin which was given to the cows. The expectation was the exposure to the pirlimycin would kill non-resistant bacteria and allow naturally resistant bacteria to grow. Then because pirlimycin and erythromycin have the same resistance type, erythromycin resistance would be a good analog for pirlimycin resistance. However, this did not occur; the absolute *ermB* data did not demonstrate the same pattern as the absolute *sul1* data, which suggests that different ARGs behave differently during composting and when being excreted from the dairy cow. Compost without antibiotics showed the most significant difference from other treatments for absolute *ermB*, which was further confirmed by the relative *ermB* concentrations in the runoff. Additionally, raw manure had the lowest *ermB* concentrations in both absolute and relative abundances in the final storms collected.

In summary, plot runoff from six natural storms was collected during the growing season of planted radishes and lettuce to analyze the effect of soil amendment and vegetable type on the release of fecal indicator bacteria, sediment, and antibiotic resistance genes to runoff. The effects

of differing soil amendments were dependent on the variable tested. Runoff concentrations of *E. coli* and enterococci were statistically equivalent regardless of the soil amendment of the respective plot. Level of released sediment also did not differ across soil amendments. Antibiotic resistance genes did display higher concentrations of absolute *sul1* genes in the manure-based plots (including compost with and compost without antibiotics given to the dairy cows) relative to the control/fertilizer plots. The relative *sul1* abundance was significantly higher in compost and manure treatments for the first and second collected storm, and then all treatments led to statistically similar *sul1* concentrations in runoff following Storm 2. *ErmB* resistance genes were elevated in manure treated plots after application, however the manure treatment had the lowest final *ermB* concentrations in runoff in the final two collected storms. Vegetable type was not an important factor in the release of FIB or ARGs to runoff. More sediment was released from barren plots than those planted with vegetables. However, there was no significant difference between the sediment released from plots containing lettuce or radishes.

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APPENDIX A

A.1 Raw Excel Data

Storm
1

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	1220	9590	0.3063	4.27E+08	3524	39
B	1100	6630	0.2122	8.61E+08	7715	*
C	100	2020	0.403	8.62E+08	19559	*
D	200	100	0.2884	1.15E+04	9111	*
E	630	1710	0.0168	1.06E+09	17153	11
F	300	1090	0.4199	3.62E+08	4253	*
G	80	21420	0.2332	5.95E+08	18141	*
H	740	710	0.0594	1.01E+09	141784	149
I	2790	2810	0.1702	4.78E+08	8119	5
J	1870	241960	0.2846	2.29E+08	10685	200
K	1080	241960	0.1988	6.66E+08	861117	500
L	200	61310	0.3537	4.05E+08	564996	99
M	2810	241960	0.1786	3.39E+08	746336	1656
N	100	19180	0.235	1.35E+08	241291	3308
O	12360	4590	0.3218	8.20E+08	754689	39
P	600	1520	0.1621	9.27E+08	503927	402
Q	600	12810	0.0756	1.07E+08	135401	959
R	100	410	0.2091	3.37E+08	444080	75
S	1450	8730	0.3457	3.07E+08	249843	69
T	200	13850	0.2505	2.18E+08	362101	252
U	100	36230	0.2783	4.84E+08	440483	193
V	200	750	0.2176	2.29E+08	897648	380
W	1090	17270	0.144	3.71E+08	829877	549
X	100	29090	0.3615	4.32E+08	2759193	1951
Y	200	4350	0.0972	6.62E+08	1006205	122
Z	100	1090	0.2156	7.24E+08	913986	81
Θ	200	15290	0.0327	5.55E+08	2139810	121

*Denotes measurements that were below the limit of detection

Storm
2

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	10500	13010	0.316	1.12E+09	4216	5
B	15760	13540	0.295	3.95E+08	2349	62
C	24890	27550	0.4349	5.22E+08	2512	194
D	12460	12740	0.2039	1.70E+09	8578	114
E	3410	12740	0.0802	1.47E+09	5568	641
F	3930	3590	0.2436	2.74E+09	3843	79
G	3730	8130	0.253	3.00E+09	11382	501
H	1220	5280	0.2821	1.38E+09	11984	20
I	26130	29090	0.0603	1.52E+09	7914	85
J	43520	9590	0.1693	7.80E+08	169919	740
K	8160	2100	0.0425	3.40E+09	493193	116
L	30760	11460	0.199	5.41E+08	*	559
M	2130	9590	0.2176	1.55E+09	307944	312
N	9320	26130	0.3625	2.25E+09	196138	229
O	1600	2480	0.1925	2.00E+09	279759	455
P	19560	24810	0.3861	3.34E+09	176891	*
Q	12910	22470	0.2221	2.08E+09	147915	5
R	11260	6270	0.0708	2.07E+09	598662	44
S	2790	1080	0.484	2.44E+09	259479	*
T	2810	17890	0.046	1.39E+09	*	9
U	310	7060	0.0638	2.02E+09	412609	99
V	241960	10140	0.2837	1.74E+06	6870	175
W	27230	51720	0.4608	2.06E+09	*	47
X	2010	11120	0.3314	3.50E+09	1938237	294
Y	1600	4650	0.0599	8.87E+08	955721	31
Z	2790	51720	0.2407	6.53E+08	597388	62
Θ	173290	29090	0.1499	1.50E+09	1447374	129

*Denotes measurements that were below the limit of detection

Storm 3

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	1090	57940	0.04	1.28E+09	4479	176
B	1870	111990	0.01585	7.62E+08	195234	26
C	2560	1100	0.0355	7.11E+08	5727	303
D	16640	28510	0.0206	3.17E+08	1718	146
E	68670	29090	0.00615	4.34E+09	4576	3
F	520	950	0.01315	1.39E+09	4197	44
G	5630	5830	0.1192	7.37E+09	8247	35
H	3410	1710	0.0505	4.21E+09	206226	104
I	10760	18500	0.022	6.56E+08	2743	60
J	7710	36540	0.02605	2.56E+09	33027	17
K	2010	57940	0.00465	8.16E+08	63712	114
L	8570	5350	0.0039	7.32E+08	13206	131
M	141360	57940	0.001	1.76E+09	17495	103
N	547500	155310	0.0566	3.00E+09	30084	134
O	173290	48840	0.00695	3.68E+09	123088	27
P	13340	6160	0.0335	5.15E+09	107861	170
Q	816400	241960	0.089	2.19E+09	72666	189
R	120330	241960	0.0105	4.20E+09	310255	29
S	24810	68670	0.01135	1.79E+09	70292	52
T	12110	6830	0.00885	2.30E+09	270711	24
U	1220	970	0.00625	6.10E+08	26633	120
V	21430	2590	0.00625	5.24E+09	129933	5
W	5460	1990	0.0088	4.13E+09	94943	18
X	5120	920	0.0019	3.16E+09	67903	85
Y	100	1390	0.0045	1.79E+09	162034	14
Z	100	1270	0.0072	1.43E+09	252448	10
Θ	4950	7760	0.00935	1.45E+09	426229	15

Storm 4

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	241960	241960	0.2065	2.78E+09	96455	58
B	72700	86640	0.2776	2.44E+09	182529	41
C	241960	488400	0.082	2.00E+09	112387	228
D	155310	173290	0.0181	1.80E+05	34098	151
E	241960	141360	0.0652	3.66E+09	206747	32
F	241960	43520	0.0205	2.74E+09	35355	183
G	241960	368100	0.1541	6.77E+09	195914	116
H	261300	198630	0.1904	3.56E+09	209052	15
I	20460	111990	0.0505	3.66E+09	147087	55
J	72700	41060	0.0374	2.44E+09	217174	206
K	1730	5200	0.0428	2.44E+09	782372	177
L	51720	5580	0.0279	1.96E+09	562224	60
M	198630	472100	0.047	8.57E+09	603414	23
N	2419600	686700	0.1372	7.77E+09	563032	446
O	2720	7760	0.0956	4.40E+09	704407	29
P	629400	1732900	0.2063	6.63E+09	286073	87
Q	387300	73800	0.1423	1.83E+09	185254	73
R	173290	648800	0.0588	4.51E+09	1585462	118
S	120330	54750	0.1796	4.90E+09	454901	101
T	37300	77010	0.0387	1.30E+09	353734	160
U	686700	173290	0.0277	6.12E+09	579245	531
V	727000	111990	0.1507	1.30E+10	2417044	95
W	81640	141360	0.0392	5.96E+09	391603	54
X	235900	43500	0.0461	5.59E+09	761140	121
Y	1320	24810	0.0235	8.48E+08	40128	4*
Z	3010	98040	0.0196	1.04E+09	134254	8
Θ	1100	29090	0.0418	6.81E+06	20692	2*

Storm
5

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	241960	4839200[1]	0.0361	3.25E+09	301722	1901
B	120330	4839200[1]	0.0281	3.06E+09	2418770	6003
C	547500	4839200[1]	0.0168	3.95E+09	82940	728
D	24810	241960	0.0258	2.56E+09	139767	1186
E	13540	4839200[1]	0.0133	1.39E+09	114047	4896
F	261300	4839200[1]	0.0244	3.21E+09	189650	5804
G	32550	4839200[1]	0.023	2.98E+09	692607	2244
H	17230	241960	0.0034	3.73E+09	1000592	2071
I	98040	4839200[1]	0.1001	2.24E+09	402489	1662
J	155310	4839200[1]	0.0145	2.73E+09	231650	1185
K	1203300	64880	0.038	2.87E+09	84554	585
L	30760	4839200[1]	0.009	2.04E+09	*	2381
M	111990	4839200[1]	0.0115	9.77E+09	188546	632
N	241960	4839200[1]	0.0159	4.20E+09	139794	821
O	488400	1553100	0.014	9.45E+09	343448	577
P	198630	4839200[1]	0.0277	1.95E+09	35744	3555
Q	1119900	4839200[1]	0.0297	6.41E+09	166444	2640
R	173290	4839200[1]	0.0246	4.24E+09	420745	232
S	198630	4839200[1]	0.0062	1.79E+09	78655	38
T	104620	396800	0.1077	1.63E+09	*	858
U	1046200	4839200[1]	0.0083	6.75E+09	454065	1211
V	410	4839200[1]	0.1076	4.81E+09	64922	210
W	1299700	4839200[1]	0.0242	9.22E+09	*	116
X	54750	4839200[1]	0.0533	4.53E+09	204200	236
Y	11530	456900	0.0157	2.46E+09	264587	28
Z	11870	613100	0.1689	1.50E+09	38628	8
Θ	17890	980400	0.0165	5.17E+09	197750	1*

* Denotes measurements that were below the limit of detection
[1] Denotes measurements that were above the limit of detection

Storm
6

Plot	E. COLI	ENTEROCOCCI	SEDIMENT	16S	SUL1	ErmB
A	1000	913900	0.0321	2.42E+09	635879	1728
B	1000	488400	0.0231	3.31E+09	1613710	384
C	1000	920800	0.0333	4.20E+09	215045	118
D	100	1000	0.2266	1.05E+10	18517	3*
E	33600	14136000	0.0302	3.02E+09	183702	652
F	2430	214100	0.0802	9.45E+09	212645	1045
G	1000	139600	0.0349	7.40E+09	567526	11
H	25300	1299700	0.0076	5.71E+09	390640	9
I	4100	1299700	0.016	3.04E+09	337441	747
J	100	100	0.0029	4.18E+07	12138	1529
K	1000	435200	0.0214	3.86E+09	157608	183
L	310	1986300	0.0174	4.18E+09	285172	709
M	860	137600	0.013	7.89E+09	541428	373
N	3000	344800	0.0076	2.88E+09	226055	204
O	2000	1203300	0.0345	8.26E+09	2635924	22
P	14500	2419600	0.0206	6.30E+09	750733	11
Q	11000	186000	0.0113	1.77E+09	87482	44
R	2000	1299700	0.0497	5.23E+09	1959322	15
S	1000	1011200	0.0303	1.76E+09	136875	55
T	146700	857000	0.0694	5.80E+09	123122	1138
U	9500	4352000	0.0214	5.59E+09	955236	46
V	100	260300	0.1281	5.36E+09	260224	21
W	9700	24196000	0.0244	1.04E+10	216328	451
X	6300	2419600	0.0992	1.11E+10	275062	64
Y	100	163100	0.0324	4.93E+09	231195	3*
Z	49500	119800	0.0043	3.49E+09	116683	0*
Θ	387300	1046200	0.0215	7.65E+09	320560	1*

* Denotes measurements that were below the limit of detection

A.2 R Codes and Outputs

A.2.1 E. coli Codes and Outputs

Two-way ANOVA
```
> ##2wayECOLIANOVA
> Ecoli2way = read_excel("Quantitray counts.xlsx", sheet="EcoliANOVA")
> attach(Ecoli2way)

> a1=aov(Storms~Treatment*StormNum)
> summary(a1)
                     Df Sum Sq Mean Sq F value Pr(>F)
Treatment             8   6.45   0.806   1.308  0.247
StormNum              5 107.71  21.542  34.976 <2e-16 ***
Treatment:StormNum   40  33.38   0.835   1.355  0.111
Residuals           108  66.52   0.616
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


> #posthoc
> pairwise.t.test(Storms,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  Storms and StormNum

        Storm 1 Storm 2 Storm 3 Storm 4 Storm 5
Storm 2 7.5e-08 -       -       -       -
Storm 3 3.6e-08 0.88123 -       -       -
Storm 4 < 2e-16 1.4e-05 2.6e-05 -       -
Storm 5 < 2e-16 3.4e-06 6.5e-06 0.74035 -
Storm 6 0.00043 0.04177 0.02911 8.3e-10 1.4e-10

P value adjustment method: none
> aggregate(Storms, list(Ecoli2way$StormNum), mean)
   Group.1        x
1 Storm 1 2.630719
2 Storm 2 3.900108
3 Storm 3 3.933736
4 Storm 4 4.908533
5 Storm 5 4.983132
6 Storm 6 3.438836
```

Individual Kruskal-Wallis Tests
```
> ##Individual Storms Kruskal-Wallis Tests
> setwd("D:/Grad school/_WRITING/Data")
> x= read_excel("Quantitray counts.xlsx", sheet="logecoli")
> attach(x)

> #Storm1
> trt=c(x$trt)
> storm1ecolia=x$Storm1
> storm1ecoli=data.frame(trt,storm1ecolia)
> kruskal.test(storm1ecoli$storm1ecolia~storm1ecoli$trt)

        Kruskal-Wallis rank sum test
```

```
data:  storm1ecoli$storm1ecolia by storm1ecoli$trt
Kruskal-Wallis chi-squared = 3.9324, df = 4, p-value = 0.4152

> boxplot(storm1ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
>
> #Storm2
> storm2ecolia=x$Storm2
> storm2ecoli=data.frame(trt,storm2ecolia)
> kruskal.test(storm2ecoli$storm2ecolia~storm2ecoli$trt)

        Kruskal-Wallis rank sum test

data:  storm2ecoli$storm2ecolia by storm2ecoli$trt
Kruskal-Wallis chi-squared = 1.7484, df = 4, p-value = 0.7819

> boxplot(storm2ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
>
> #Storm3
> storm3ecolia=x$Storm3
> storm3ecoli=data.frame(trt,storm3ecolia)
> kruskal.test(storm3ecoli$storm3ecolia~storm3ecoli$trt)

        Kruskal-Wallis rank sum test

data:  storm3ecoli$storm3ecolia by storm3ecoli$trt
Kruskal-Wallis chi-squared = 8.9922, df = 4, p-value = 0.0613

> boxplot(storm3ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
>
> #Storm4
> storm4ecolia=x$Storm4
> storm4ecoli=data.frame(trt,storm4ecolia)
> kruskal.test(storm4ecoli$storm4ecolia~storm4ecoli$trt)

        Kruskal-Wallis rank sum test

data:  storm4ecoli$storm4ecolia by storm4ecoli$trt
Kruskal-Wallis chi-squared = 4.0811, df = 4, p-value = 0.3951

> boxplot(storm4ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
>
> #Storm5
> storm5ecolia=x$Storm5
> storm5ecoli=data.frame(trt,storm5ecolia)
> kruskal.test(storm5ecoli$storm5ecolia~storm5ecoli$trt)

        Kruskal-Wallis rank sum test

data:  storm5ecoli$storm5ecolia by storm5ecoli$trt
Kruskal-Wallis chi-squared = 9.3675, df = 4, p-value = 0.05254

> boxplot(storm5ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
>
> #Storm6
> storm6ecolia=x$Storm6
> storm6ecoli=data.frame(trt,storm6ecolia)
> kruskal.test(storm6ecoli$storm6ecolia~storm6ecoli$trt)

        Kruskal-Wallis rank sum test

data:  storm6ecoli$storm6ecolia by storm6ecoli$trt
```

```
Kruskal-Wallis chi-squared = 6.0526, df = 4, p-value = 0.1953

> boxplot(storm6ecolia ~ trt, ylab="Ecoli conc", xlab="Treatment")
```

A.2.2 Enterococci Codes and Outputs

Two-way ANOVA
```
> ##2wayEnteroANOVA
> Entero2way = read_excel("Quantitray counts.xlsx", sheet="EnteroANOVA")
> attach(Entero2way)
> summary(aov(Storms~Treatment*StormNum))
                    Df Sum Sq Mean Sq F value Pr(>F)
Treatment            8   8.50   1.063   2.106 0.0412 *
StormNum             5 142.04  28.408  56.293 <2e-16 ***
Treatment:StormNum  40  26.68   0.667   1.322 0.1307
Residuals          108  54.50   0.505
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> pairwise.t.test(Storms,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  Storms and StormNum

        Storm 1 Storm 2 Storm 3 Storm 4 Storm 5
Storm 2 0.35955 -       -       -       -
Storm 3 0.29397 0.89352 -       -       -
Storm 4 1.1e-07 7.1e-06 1.2e-05 -       -
Storm 5 < 2e-16 < 2e-16 < 2e-16 5.3e-10 -
Storm 6 2.2e-15 4.7e-13 1.0e-12 0.00142 0.00092

P value adjustment method: none
> aggregate(Storms, list(Entero2way$StormNum), mean)
  Group.1        x
1 Storm 1 3.847165
2 Storm 2 4.036798
3 Storm 3 4.064465
4 Storm 4 4.996235
5 Storm 5 6.363660
6 Storm 6 5.666583
```

Individual Kruskal-Wallis Tests
```
> ##Individual Storms Kruskal-Wallis Tests
> setwd("D:/Grad school/_WRITING/Data")
> x= read_excel("Quantitray counts.xlsx", sheet="logentero")
> attach(x)

> #Storm1
> trt=c(x$trt)
> storm1enteroa=x$Storm1
> storm1entero=data.frame(trt,storm1enteroa)
> kruskal.test(storm1entero$storm1enteroa~storm1entero$trt)

        Kruskal-Wallis rank sum test

data:  storm1entero$storm1enteroa by storm1entero$trt
Kruskal-Wallis chi-squared = 10.215, df = 4, p-value = 0.03695
```

```
> boxplot(storm1enteroa ~ trt, ylab="Enterococi conc", xlab="Treatment")
>
> #Storm2
> storm2enteroa=x$Storm2
> storm2entero=data.frame(trt,storm2enteroa)
> kruskal.test(storm2entero$storm2enteroa~storm2entero$trt)

        Kruskal-Wallis rank sum test

data:  storm2entero$storm2enteroa by storm2entero$trt
Kruskal-Wallis chi-squared = 4.5095, df = 4, p-value = 0.3414

> boxplot(storm2enteroa ~ trt, ylab="Enterococi conc", xlab="Treatment")
>
> #Storm3
> storm3enteroa=x$Storm3
> storm3entero=data.frame(trt,storm3enteroa)
> kruskal.test(storm3entero$storm3enteroa~storm3entero$trt)

        Kruskal-Wallis rank sum test

data:  storm3entero$storm3enteroa by storm3entero$trt
Kruskal-Wallis chi-squared = 8.8362, df = 4, p-value = 0.06533

> boxplot(storm3enteroa ~ trt, ylab="Enterococi conc", xlab="Treatment")
>
> #Storm4
> storm4enteroa=x$Storm4
> storm4entero=data.frame(trt,storm4enteroa)
> kruskal.test(storm4entero$storm4enteroa~storm4entero$trt)

        Kruskal-Wallis rank sum test

data:  storm4entero$storm4enteroa by storm4entero$trt
Kruskal-Wallis chi-squared = 5.4746, df = 4, p-value = 0.242

> boxplot(storm4enteroa ~ trt, ylab="Enterococi conc", xlab="Treatment")
>
> #Storm5
> storm5enteroa=x$Storm5
> storm5entero=data.frame(trt,storm5enteroa)
> kruskal.test(storm5entero$storm5enteroa~storm5entero$trt)

        Kruskal-Wallis rank sum test

data:  storm5entero$storm5enteroa by storm5entero$trt
Kruskal-Wallis chi-squared = 2.395, df = 4, p-value = 0.6635

> boxplot(storm5enteroa ~ trt, ylab="Enterococi conc", xlab="Treatment")
>
> #Storm6
> storm6enteroa=x$Storm6
> storm6entero=data.frame(trt,storm6enteroa)
> kruskal.test(storm6entero$storm6enteroa~storm6entero$trt)

        Kruskal-Wallis rank sum test

data:  storm6entero$storm6enteroa by storm6entero$trt
Kruskal-Wallis chi-squared = 2.007, df = 4, p-value = 0.7345
```

```
> boxplot(storm6enteroa ~ trt, ylab="Enterococci conc", xlab="Treatment")
```

A.2.3 Sediment Codes and Outputs

Two-Way ANOVA
```
> ##2waySedANOVA
> Sediment2way = read_excel("TSS Sample 1.xlsx", sheet="2wayANOVA")
> attach(Sediment2way)
> a3=aov(Storms~Treatment*StormNum)
> summary(a3)
                    Df Sum Sq Mean Sq F value Pr(>F)
Treatment            8  12.16    1.52   2.128 0.0391 *
StormNum             5 172.08   34.42  48.174 <2e-16 ***
Treatment:StormNum  40  38.22    0.96   1.338 0.1208
Residuals          108  77.16    0.71
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


> pairwise.t.test(Storms,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  Storms and StormNum

        Storm 1 Storm 2 Storm 3 Storm 4 Storm 5
Storm 2 0.96545 -       -       -       -
Storm 3 < 2e-16 < 2e-16 -       -       -
Storm 4 3.5e-05 4.1e-05 5.0e-10 -       -
Storm 5 2.6e-14 3.4e-14 0.01331 5.9e-05 -
Storm 6 2.1e-13 2.7e-13 0.00483 0.00023 0.72285

P value adjustment method: none
> aggregate(Storms, list(Sediment2way$StormNum), mean)
  Group.1         x
1 Storm 1 -1.687572
2 Storm 2 -1.698247
3 Storm 3 -4.369879
4 Storm 4 -2.736869
5 Storm 5 -3.753651
6 Storm 6 -3.666217
> pairwise.t.test(Storms,Treatment, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  Storms and Treatment
```

	CompostnoLet	CompostnoRad	CompostyesLet	CompostyesRad	Control	FertilizerLet
CompostnoRad	0.694	-	-	-	-	-
CompostyesLet	0.480	0.754	-	-	-	-
CompostyesRad	0.260	0.462	0.672	-	-	-
Control	0.059	0.133	0.233	0.440	-	-
FertilizerLet	0.456	0.725	0.969	0.701	0.249	-

```
FertilizerRad 0.317        0.542        0.767        0.900        0.369   0
.797
ManureLet     0.908        0.611        0.412        0.215        0.045   0
.390
ManureRad     0.209        0.387        0.581        0.897        0.520   0
.608
              FertilizerRad ManureLet
CompostnoRad  -             -
CompostyesLet -             -
CompostyesRad -             -
Control       -             -
FertilizerLet -             -
FertilizerRad -             -
ManureLet     0.264         -
ManureRad     0.798         0.171

P value adjustment method: none
> aggregate(Storms, list(Sediment2way$Treatment), mean)
        Group.1         x
1  CompostnoLet -3.342511
2  CompostnoRad -3.162595
3 CompostyesLet -3.019282
4 CompostyesRad -2.825731
5       Control -2.472126
6 FertilizerLet -3.001357
7 FertilizerRad -2.883432
8     ManureLet -3.395171
9     ManureRad -2.766449
```

Individual Kruskal-Wallis Tests
```
> ##Individual Storms Kruskal-Wallis Tests
> setwd("D:/Grad school/_WRITING/Data")
> x= read_excel("TSS Sample 1.xlsx", sheet="Sheet7")
> attach(x)

>
> trt=c(x$trt)
> storm1seda=x$Storm1
> storm1sed=data.frame(trt,storm1seda)
> kruskal.test(storm1sed$storm1seda~storm1sed$trt)

        Kruskal-Wallis rank sum test

data:  storm1sed$storm1seda by storm1sed$trt
Kruskal-Wallis chi-squared = 3.4683, df = 4, p-value = 0.4827

> boxplot(storm1seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> #Storm2
> storm2seda=x$Storm2
> storm2sed=data.frame(trt,storm2seda)
> kruskal.test(storm2sed$storm2seda~storm2sed$trt)

        Kruskal-Wallis rank sum test

data:  storm2sed$storm2seda by storm2sed$trt
Kruskal-Wallis chi-squared = 4.3862, df = 4, p-value = 0.3562

> boxplot(storm2seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> #Storm3
```

```
> storm3seda=x$Storm3
> storm3sed=data.frame(trt,storm3seda)
> kruskal.test(storm3sed$storm3seda~storm3sed$trt)

        Kruskal-Wallis rank sum test

data:  storm3sed$storm3seda by storm3sed$trt
Kruskal-Wallis chi-squared = 8.9485, df = 4, p-value = 0.0624

> boxplot(storm3seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> #Storm4
> storm4seda=x$Storm4
> storm4sed=data.frame(trt,storm4seda)
> kruskal.test(storm4sed$storm4seda~storm4sed$trt)

        Kruskal-Wallis rank sum test

data:  storm4sed$storm4seda by storm4sed$trt
Kruskal-Wallis chi-squared = 6.6243, df = 4, p-value = 0.1571

> boxplot(storm4seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> #Storm5
> storm5seda=x$Storm5
> storm5sed=data.frame(trt,storm5seda)
> kruskal.test(storm5sed$storm5seda~storm5sed$trt)

        Kruskal-Wallis rank sum test

data:  storm5sed$storm5seda by storm5sed$trt
Kruskal-Wallis chi-squared = 4.4286, df = 4, p-value = 0.3511

> boxplot(storm5seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> #Storm6
> storm6seda=x$Storm6
> storm6sed=data.frame(trt,storm6seda)
> kruskal.test(storm6sed$storm6seda~storm6sed$trt)

        Kruskal-Wallis rank sum test

data:  storm6sed$storm6seda by storm6sed$trt
Kruskal-Wallis chi-squared = 4.4379, df = 4, p-value = 0.35

> boxplot(storm6seda ~ trt, ylab="Sediment conc", xlab="Treatment")
```

A.2.4 16SrRNA Codes and Outputs

Two-way-ANOVA
```
> #Two Way ANOVA
> twoway = read_excel("16sAll.xlsx", sheet="16s Anova")
> attach(twoway)

> a4=aov(`16s`~Treatment*StormNum)
> summary(a4)
                    Df    Sum Sq   Mean Sq F value Pr(>F)
Treatment            8 4.372e+09 5.465e+08   1.801 0.0846 .
StormNum             5 4.132e+10 8.264e+09  27.231 <2e-16 ***
Treatment:StormNum  40 1.491e+10 3.729e+08   1.229 0.2015
Residuals          108 3.277e+10 3.035e+08
```

```
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> pairwise.t.test(`16s`,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  16s and StormNum

       Storm1  Storm2  Storm3  Storm4  Storm5
Storm2 0.00039 -       -       -       -
Storm3 9.0e-07 0.13853 -       -       -
Storm4 1.1e-11 0.00029 0.02787 -       -
Storm5 3.4e-13 2.7e-05 0.00513 0.53656 -
Storm6 < 2e-16 4.8e-09 5.4e-06 0.01378 0.06313

P value adjustment method: none
> aggregate(`16s`, list(twoway$StormNum), mean)
  Group.1       x
1  Storm1 21244.07
2  Storm2 39278.73
3  Storm3 46681.53
4  Storm4 57718.40
5  Storm5 60797.96
6  Storm6 70103.60
```

Individual Kruskal-Wallis Tests

```
> ##Individual Storms Kruskal-Wallis Tests
> setwd("D:/Grad school/_WRITING/Data/16 results")
> x= read_excel("16sAll.xlsx", sheet="Sheet7")
> attach(x)

> #Storm1
> trt=c(x$Treatment)
> storm116sa=x$Storm1
> storm116s=data.frame(trt,storm116sa)
> kruskal.test(storm116s$storm116sa~storm116s$trt)

        Kruskal-Wallis rank sum test

data:  storm116s$storm116sa by storm116s$trt
Kruskal-Wallis chi-squared = 3.9312, df = 4, p-value = 0.4154

> boxplot(storm116sa ~ trt, ylab="16s conc", xlab="Treatment")
>
> #Storm2
> storm216sa=x$Storm2
> storm216s=data.frame(trt,storm216sa)
> kruskal.test(storm216s$storm216sa~storm216s$trt)

        Kruskal-Wallis rank sum test

data:  storm216s$storm216sa by storm216s$trt
Kruskal-Wallis chi-squared = 7.537, df = 4, p-value = 0.1101

> boxplot(storm216sa ~ trt, ylab="16s conc", xlab="Treatment")
>
> #Storm3
> storm316sa=x$Storm3
```

```
> storm316s=data.frame(trt,storm316sa)
> kruskal.test(storm316s$storm316sa~storm316s$trt)

        Kruskal-Wallis rank sum test

data:  storm316s$storm316sa by storm316s$trt
Kruskal-Wallis chi-squared = 4.0794, df = 4, p-value = 0.3954

> boxplot(storm316sa ~ trt, ylab="16s conc", xlab="Treatment")
>
> #Storm4
> storm416sa=x$Storm4
> storm416s=data.frame(trt,storm416sa)
> kruskal.test(storm416s$storm416sa~storm416s$trt)

        Kruskal-Wallis rank sum test

data:  storm416s$storm416sa by storm416s$trt
Kruskal-Wallis chi-squared = 1.3148, df = 4, p-value = 0.8589

> boxplot(storm416sa ~ trt, ylab="16s conc", xlab="Treatment")
>
> #Storm5
> storm516sa=x$Storm5
> storm516s=data.frame(trt,storm516sa)
> kruskal.test(storm516s$storm516sa~storm516s$trt)

        Kruskal-Wallis rank sum test

data:  storm516s$storm516sa by storm516s$trt
Kruskal-Wallis chi-squared = 2.8333, df = 4, p-value = 0.5861

> boxplot(storm516sa ~ trt, ylab="16s conc", xlab="Treatment")
>
> #Storm6
> storm616sa=x$Storm6
> storm616s=data.frame(trt,storm616sa)
> kruskal.test(storm616s$storm616sa~storm616s$trt)

        Kruskal-Wallis rank sum test

data:  storm616s$storm616sa by storm616s$trt
Kruskal-Wallis chi-squared = 4.9418, df = 4, p-value = 0.2933

> boxplot(storm616sa ~ trt, ylab="16s conc", xlab="Treatment")
```

A.2.5 *Sul1* Codes and Outputs

Two-Way ANOVA
```
> #Two-Way ANOVA
> sul12way = read_excel("All storms starting quanties w concentration.xlsx",
sheet="sul1 Anova")
> attach(sul12way)

> a5=aov(sul1~Treatment*StormNum)
> summary(a5)
                   Df Sum Sq Mean Sq F value   Pr(>F)
Treatment           8  21.98  2.7480  11.843 8.31e-12 ***
```

```
StormNum              5   14.47  2.8950   12.476 1.87e-09 ***
Treatment:StormNum   40   29.85  0.7462    3.216 1.14e-06 ***
Residuals           102   23.67  0.2320
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
6 observations deleted due to missingness


> pairwise.t.test(sul1,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  sul1 and StormNum

       Storm1  Storm2  Storm3  Storm4  Storm5
Storm2 0.01780 -       -       -       -
Storm3 0.00857 0.78936 -       -       -
Storm4 0.27117 0.00061 0.00023 -       -
Storm5 0.73969 0.04085 0.02110 0.15268 -
Storm6 0.19360 0.00030 0.00011 0.84068 0.10333

P value adjustment method: none
> aggregate(sul1, list(sul12way$StormNum), FUN=mean, na.action=na.pass, na.rm
=TRUE)
   Group.1        x
1   Storm1 5.163187
2   Storm2 4.663181
3   Storm3 4.607314
4   Storm4 5.393726
5   Storm5 5.093697
6   Storm6 5.435764
> pairwise.t.test(sul1,Treatment, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  sul1 and Treatment

             CompostnoLet CompostnoRad CompostyesLet CompostyesRad Control
CompostnoRad 0.01611      -            -             -             -
CompostyesLet 0.38285     0.12123      -             -             -
CompostyesRad 0.37137     0.12638      0.98299       -             -
Control      0.00160      0.43653      0.02066       0.02183       -
FertilizerLet 0.00129     0.39912      0.01742       0.01842       0.94791
FertilizerRad 0.00045     0.25046      0.00746       0.00793       0.70929
ManureLet    0.31779      0.15442      0.89902       0.91592       0.02850
ManureRad    0.75582      0.03545      0.57381       0.55940       0.00426
             FertilizerLet FertilizerRad ManureLet
CompostnoRad -             -             -
CompostyesLet -            -             -
CompostyesRad -            -             -
Control      -             -             -
FertilizerLet -            -             -
FertilizerRad 0.75845      -             -
ManureLet    0.02419       0.01068       -
ManureRad    0.00349       0.00130       0.49076

P value adjustment method: none
> aggregate(sul1, list(sul12way$Treatment), mean, na.action=na.pass, na.rm=TR
UE)
      Group.1        x
1 CompostnoLet 5.503539
2 CompostnoRad 4.880059
```

```
3 CompostyesLet 5.279308
4 CompostyesRad 5.273835
5        Control 4.680187
6 FertilizerLet 4.663420
7 FertilizerRad 4.584490
8     ManureLet 5.246742
9     ManureRad 5.423723
```

Individual Kruskal-Wallis Tests
```
> ##Individual Storms Kruskal-Wallis Tests
> x= read_excel("All storms starting quanties w concentration.xlsx", sheet="S
heet7")
> attach(x)

> #Storm1
> trt=c(x$Treatment)
> storm1sul1a=x$Storm1
> storm1sul1=data.frame(trt,storm1sul1a)
> kruskal.test(storm1sul1$storm1sul1a~storm1sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm1sul1$storm1sul1a by storm1sul1$trt
Kruskal-Wallis chi-squared = 20.913, df = 4, p-value = 0.0003295

> boxplot(storm1sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
>
> PT=dunnTest(storm1sul1$storm1sul1a~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
         Group Letter MonoLetter
1  Compost+ABX     ab         ab
2 CompostnoABX     ac        a c
3      Control      b          b
4   Fertilizer      b          b
5    RawManure      c          c
>
>
>
> #Storm2
> storm2sul1a=x$Storm2
> storm2sul1=data.frame(trt,storm2sul1a)
> kruskal.test(storm2sul1$storm2sul1a~storm2sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm2sul1$storm2sul1a by storm2sul1$trt
Kruskal-Wallis chi-squared = 15.979, df = 4, p-value = 0.003048

> boxplot(storm2sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
>
> PT=dunnTest(storm2sul1$storm2sul1a~trt, method="none")
Warning messages:
```

```
1: trt was coerced to a factor.
2: Some rows deleted from 'x' and 'g' because missing data.
>
>
> PT = PT$res
>
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
          Group Letter MonoLetter
1  Compost+ABX        a          a
2 CompostnoABX        a          a
3      Control        b           b
4    Fertilizer       b           b
5     RawManure       a          a
>
>
> #Storm3
> storm3sul1a=x$Storm3
> storm3sul1=data.frame(trt,storm3sul1a)
> kruskal.test(storm3sul1$storm3sul1a~storm3sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm3sul1$storm3sul1a by storm3sul1$trt
Kruskal-Wallis chi-squared = 11.614, df = 4, p-value = 0.02047

> boxplot(storm3sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
>
> PT=dunnTest(storm3sul1$storm3sul1a~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
          Group Letter MonoLetter
1  Compost+ABX        a          a
2 CompostnoABX       ab         ab
3      Control       ab         ab
4    Fertilizer       b           b
5     RawManure       a          a
>
>
> #Storm4
> storm4sul1a=x$Storm4
> storm4sul1=data.frame(trt,storm4sul1a)
> kruskal.test(storm4sul1$storm4sul1a~storm4sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm4sul1$storm4sul1a by storm4sul1$trt
Kruskal-Wallis chi-squared = 11.148, df = 4, p-value = 0.02495

> boxplot(storm4sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
>
> PT=dunnTest(storm4sul1$storm4sul1a~trt, method="none")
Warning message:
```

```
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
          Group Letter MonoLetter
1  Compost+ABX     ab         ab
2 CompostnoABX      a          a
3      Control     bc         bc
4   Fertilizer      c          c
5    RawManure    abc        abc
>
>
> #Storm5
> storm5sul1a=x$Storm5
> storm5sul1=data.frame(trt,storm5sul1a)
> kruskal.test(storm5sul1$storm5sul1a~storm5sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm5sul1$storm5sul1a by storm5sul1$trt
Kruskal-Wallis chi-squared = 2.394, df = 4, p-value = 0.6637

> boxplot(storm5sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
>
> #Storm6
> storm6sul1a=x$Storm6
> storm6sul1=data.frame(trt,storm6sul1a)
> kruskal.test(storm6sul1$storm6sul1a~storm6sul1$trt)

        Kruskal-Wallis rank sum test

data:  storm6sul1$storm6sul1a by storm6sul1$trt
Kruskal-Wallis chi-squared = 1.6111, df = 4, p-value = 0.8068

> boxplot(storm6sul1a ~ trt, ylab="sul1 conc", xlab="Treatment")
```

Relative *sul1* codes
```
> a1=aov(Storms~Treatment*StormNum)
> summary(a1)
                    Df   Sum Sq   Mean Sq  F value Pr(>F)
Treatment            8 0.001791 0.0002239   0.999  0.441
StormNum             5 0.001088 0.0002175   0.971  0.439
Treatment:StormNum  40 0.008829 0.0002207   0.985  0.507
Residuals          107 0.023974 0.0002240
1 observation deleted due to missingness

> #Storm1
> trt=c(x$trt)
> storm1seda=x$Storm1
> storm1sed=data.frame(trt,storm1seda)
> kruskal.test(storm1sed$storm1seda~storm1sed$trt)

        Kruskal-Wallis rank sum test

data:  storm1sed$storm1seda by storm1sed$trt
Kruskal-Wallis chi-squared = 19.247, df = 4, p-value = 0.0007027
```

```
> boxplot(storm1seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> library(rcompanion)
> library(FSA)
>
> PT=dunnTest(storm1sed$storm1seda~trt, method="none")
Warning messages:
1: trt was coerced to a factor.
2: Some rows deleted from 'x' and 'g' because missing data.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
        Group Letter MonoLetter
1  Compostno      a          a
2 Compostyes     ab         ab
3    Control      c           c
4 Fertilizer     bc          bc
5     Manure      a          a
>
>
> #Storm2
> storm2seda=x$Storm2
> storm2sed=data.frame(trt,storm2seda)
> kruskal.test(storm2sed$storm2seda~storm2sed$trt)

        Kruskal-Wallis rank sum test

data:  storm2sed$storm2seda by storm2sed$trt
Kruskal-Wallis chi-squared = 11.283, df = 4, p-value = 0.02356

> boxplot(storm2seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> PT=dunnTest(storm2sed$storm2seda~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
        Group Letter MonoLetter
1  Compostno     ab         ab
2 Compostyes     ab         ab
3    Control      a          a
4 Fertilizer      a          a
5     Manure      b          b
>
> #Storm3
> storm3seda=x$Storm3
> storm3sed=data.frame(trt,storm3seda)
> kruskal.test(storm3sed$storm3seda~storm3sed$trt)

        Kruskal-Wallis rank sum test

data:  storm3sed$storm3seda by storm3sed$trt
```

```
Kruskal-Wallis chi-squared = 10.775, df = 4, p-value = 0.02921

> boxplot(storm3seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> PT=dunnTest(storm3sed$storm3seda~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
        Group Letter MonoLetter
1  Compostno     ab         ab
2 Compostyes      a          a
3    Control     ab         ab
4 Fertilizer      b          b
5     Manure      a          a
>
> #Storm4
> storm4seda=x$Storm4
> storm4sed=data.frame(trt,storm4seda)
> kruskal.test(storm4sed$storm4seda~storm4sed$trt)

        Kruskal-Wallis rank sum test

data:  storm4sed$storm4seda by storm4sed$trt
Kruskal-Wallis chi-squared = 7.2037, df = 4, p-value = 0.1255

> boxplot(storm4seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> #Storm5
> storm5seda=x$Storm5
> storm5sed=data.frame(trt,storm5seda)
> kruskal.test(storm5sed$storm5seda~storm5sed$trt)

        Kruskal-Wallis rank sum test

data:  storm5sed$storm5seda by storm5sed$trt
Kruskal-Wallis chi-squared = 8.6905, df = 4, p-value = 0.06932

> boxplot(storm5seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> #Storm6
> storm6seda=x$Storm6
> storm6sed=data.frame(trt,storm6seda)
> kruskal.test(storm6sed$storm6seda~storm6sed$trt)

        Kruskal-Wallis rank sum test

data:  storm6sed$storm6seda by storm6sed$trt
Kruskal-Wallis chi-squared = 9.6878, df = 4, p-value = 0.04603

> boxplot(storm6seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> PT=dunnTest(storm6sed$storm6seda~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
```

```
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
        Group Letter MonoLetter
1  Compostno      a          a
2 Compostyes      a          a
3    Control      a          a
4  Fertilizer    ab         ab
5     Manure      b          b
```

A.2.6 *ErmB* Codes and Outputs

Two-Way ANOVA

```
> twoway = read_excel("ErmB.xlsx", sheet="ErmB Anova")
> attach(twoway)

> a6=aov(`ermB`~Treatment*StormNum)
> summary(a6)
                     Df Sum Sq Mean Sq F value   Pr(>F)
Treatment             8  12.52   1.565   4.091 0.000274 ***
StormNum              5  20.34   4.069  10.638 2.51e-08 ***
Treatment:StormNum   40  37.90   0.948   2.478 0.000111 ***
Residuals           108  41.30   0.382
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


> pairwise.t.test(ermB,StormNum, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  ermB and StormNum

       Storm1  Storm2  Storm3  Storm4  Storm5
Storm2 0.81    -       -       -       -
Storm3 0.22    0.33    -       -       -
Storm4 0.58    0.75    0.50    -       -
Storm5 9.9e-05 3.8e-05 5.6e-07 1.1e-05 -
Storm6 0.89    0.92    0.28    0.68    5.8e-05

P value adjustment method: none
> aggregate(ermB, list(twoway$StormNum), mean)
  Group.1        x
1  Storm1 1.927695
2  Storm2 1.877127
3  Storm3 1.671744
4  Storm4 1.811741
5  Storm5 2.761607
6  Storm6 1.898408
> pairwise.t.test(ermB,Treatment, p.adj="none")

        Pairwise comparisons using t tests with pooled SD

data:  ermB and Treatment

            CompostnoLet CompostnoRad CompostyesLet CompostyesRad Control
```

```
CompostnoRad  0.2247         -             -             -             -
CompostyesLet 0.4389         0.0478        -             -             -
CompostyesRad 0.3635         0.0347        0.8925        -             -
Control       0.5637         0.0742        0.8438        0.7398        -
FertilizerLet 0.4136         0.0432        0.9652        0.9271        0.8098
FertilizerRad 0.2755         0.0220        0.7506        0.8550        0.6067
ManureLet     0.0053         8.2e-05       0.0417        0.0569        0.0258
ManureRad     0.1120         0.0055        0.4121        0.4930        0.3094
              FertilizerLet FertilizerRad ManureLet
CompostnoRad  -             -             -
CompostyesLet -             -             -
CompostyesRad -             -             -
Control       -             -             -
FertilizerLet -             -             -
FertilizerRad 0.7839        -             -
ManureLet     0.0462        0.0847        -
ManureRad     0.4373        0.6149        0.2202

P value adjustment method: none
> aggregate(ermB, list(twoway$Treatment), mean)
        Group.1          x
1  CompostnoLet 2.212158
2  CompostnoRad 2.539907
3 CompostyesLet 2.003491
4 CompostyesRad 1.967100
5       Control 2.056567
6 FertilizerLet 1.991741
7 FertilizerRad 1.917883
8     ManureLet 1.451302
9     ManureRad 1.782333
```

Individual Kruskal-Wallis Tests

```
> #Storm1
> trt=c(x$trt)
> storm1ermba=x$Storm1
> storm1ermb=data.frame(trt,storm1ermba)
> kruskal.test(storm1ermb$storm1ermba~storm1ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm1ermb$storm1ermba by storm1ermb$trt
Kruskal-Wallis chi-squared = 5.9447, df = 4, p-value = 0.2033

> boxplot(storm1ermba ~ trt, ylab="ermb conc", xlab="Treatment")
>
> #Storm2
> storm2ermba=x$Storm2
> storm2ermb=data.frame(trt,storm2ermba)
> kruskal.test(storm2ermb$storm2ermba~storm2ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm2ermb$storm2ermba by storm2ermb$trt
Kruskal-Wallis chi-squared = 10.765, df = 4, p-value = 0.02934

> boxplot(storm2ermba ~ trt, ylab="ermb conc", xlab="Treatment")
>
>
> library(FSA)
```

```
> PT=dunnTest(storm2ermb$storm2ermba~trt, method="none")
Warning messages:
1: trt was coerced to a factor.
2: Some rows deleted from 'x' and 'g' because missing data.
>
>
> PT = PT$res
>
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
           Group Letter MonoLetter
1  CompostnoABX       a          a
2 CompostyesABX       b           b
3        Control       b           b
4     Fertilizer      ab          ab
5      RawManure       b           b
>
> #Storm3
> storm3ermba=x$Storm3
> storm3ermb=data.frame(trt,storm3ermba)
> kruskal.test(storm3ermb$storm3ermba~storm3ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm3ermb$storm3ermba by storm3ermb$trt
Kruskal-Wallis chi-squared = 9.0397, df = 4, p-value = 0.06012

> boxplot(storm3ermba ~ trt, ylab="ermb conc", xlab="Treatment")
>
> #Storm4
> storm4ermba=x$Storm4
> storm4ermb=data.frame(trt,storm4ermba)
> kruskal.test(storm4ermb$storm4ermba~storm4ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm4ermb$storm4ermba by storm4ermb$trt
Kruskal-Wallis chi-squared = 5.2593, df = 4, p-value = 0.2617

> boxplot(storm4ermba ~ trt, ylab="ermb conc", xlab="Treatment")
>
> #Storm5
> storm5ermba=x$Storm5
> storm5ermb=data.frame(trt,storm5ermba)
> kruskal.test(storm5ermb$storm5ermba~storm5ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm5ermb$storm5ermba by storm5ermb$trt
Kruskal-Wallis chi-squared = 15.339, df = 4, p-value = 0.004048

> boxplot(storm5ermba ~ trt, ylab="ermb conc", xlab="Treatment")
>
> PT=dunnTest(storm5ermb$storm5ermba~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
```

```
>
> library(rcompanion)
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
            Group Letter MonoLetter
1  CompostnoABX      a          a
2 CompostyesABX      a          a
3       Control      a          a
4     Fertilizer     a          a
5      RawManure     b           b
>
> #Storm6
> storm6ermba=x$Storm6
> storm6ermb=data.frame(trt,storm6ermba)
> kruskal.test(storm6ermb$storm6ermba~storm6ermb$trt)

        Kruskal-Wallis rank sum test

data:  storm6ermb$storm6ermba by storm6ermb$trt
Kruskal-Wallis chi-squared = 6.9762, df = 4, p-value = 0.1372

> boxplot(storm6ermba ~ trt, ylab="ermb conc", xlab="Treatment")
```

Relative *ermB* codes and outputs

```
> a2=aov(Storms~Treatment*StormNum)
> summary(a1)
                     Df   Sum Sq    Mean Sq F value Pr(>F)
Treatment             8 0.001791 0.0002239   0.999  0.441
StormNum              5 0.001088 0.0002175   0.971  0.439
Treatment:StormNum   40 0.008829 0.0002207   0.985  0.507
Residuals           107 0.023974 0.0002240
1 observation deleted due to missingness
```

Kruskal-Wallis Tests

```
> #Storm1
> trt=c(x$trt)
> storm1seda=x$Storm1
> storm1sed=data.frame(trt,storm1seda)
> kruskal.test(storm1sed$storm1seda~storm1sed$trt)

        Kruskal-Wallis rank sum test

data:  storm1sed$storm1seda by storm1sed$trt
Kruskal-Wallis chi-squared = 14.991, df = 4, p-value = 0.00472

> boxplot(storm1seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> PT=dunnTest(storm1sed$storm1seda~trt, method="none")
Warning messages:
1: trt was coerced to a factor.
2: Some rows deleted from 'x' and 'g' because missing data.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
```

```
         Group Letter MonoLetter
1  Compostno      a          a
2 Compostyes      a          a
3    Control      b           b
4 Fertilizer      b           b
5     Manure      a          a
>
> #Storm2
> storm2seda=x$Storm2
> storm2sed=data.frame(trt,storm2seda)
> kruskal.test(storm2sed$storm2seda~storm2sed$trt)

        Kruskal-Wallis rank sum test

data:  storm2sed$storm2seda by storm2sed$trt
Kruskal-Wallis chi-squared = 12.444, df = 4, p-value = 0.01434

> boxplot(storm2seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> PT=dunnTest(storm2sed$storm2seda~trt, method="none")
Warning messages:
1: trt was coerced to a factor.
2: Some rows deleted from 'x' and 'g' because missing data.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
         Group Letter MonoLetter
1  Compostno      a          a
2 Compostyes      b           b
3    Control     ab          ab
4 Fertilizer      a          a
5     Manure     ab          ab
>
> #Storm3
> storm3seda=x$Storm3
> storm3sed=data.frame(trt,storm3seda)
> kruskal.test(storm3sed$storm3seda~storm3sed$trt)

        Kruskal-Wallis rank sum test

data:  storm3sed$storm3seda by storm3sed$trt
Kruskal-Wallis chi-squared = 7.8862, df = 4, p-value = 0.09584

> boxplot(storm3seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
> #Storm4
> storm4seda=x$Storm4
> storm4sed=data.frame(trt,storm4seda)
> kruskal.test(storm4sed$storm4seda~storm4sed$trt)

        Kruskal-Wallis rank sum test

data:  storm4sed$storm4seda by storm4sed$trt
Kruskal-Wallis chi-squared = 2.5794, df = 4, p-value = 0.6305

> boxplot(storm4seda ~ trt, ylab="Sediment conc", xlab="Treatment")
>
```

```
> #Storm5
> storm5seda=x$Storm5
> storm5sed=data.frame(trt,storm5seda)
> kruskal.test(storm5sed$storm5seda~storm5sed$trt)

        Kruskal-Wallis rank sum test

data:  storm5sed$storm5seda by storm5sed$trt
Kruskal-Wallis chi-squared = 16.81, df = 4, p-value = 0.002105

> boxplot(storm5seda ~ trt, ylab="Sediment conc", xlab="Treatment")
> PT=dunnTest(storm5sed$storm5seda~trt, method="none")
Warning message:
trt was coerced to a factor.
>
>
> PT = PT$res
>
>
>
> cldList(comparison = PT$Comparison, p.value = PT$P.adj, threshold=0.05)
        Group Letter MonoLetter
1  Compostno      a          a
2 Compostyes      a          a
3    Control      a          a
4 Fertilizer      a          a
5     Manure      b          b
>
> #Storm6
> storm6seda=x$Storm6
> storm6sed=data.frame(trt,storm6seda)
> kruskal.test(storm6sed$storm6seda~storm6sed$trt)

        Kruskal-Wallis rank sum test

data:  storm6sed$storm6seda by storm6sed$trt
Kruskal-Wallis chi-squared = 7.5688, df = 4, p-value = 0.1087

> boxplot(storm6seda ~ trt, ylab="Sediment conc", xlab="Treatment")
```

A.3 Q-Q Residual Plots for Normality

E. coli

Normal Q-Q Plot
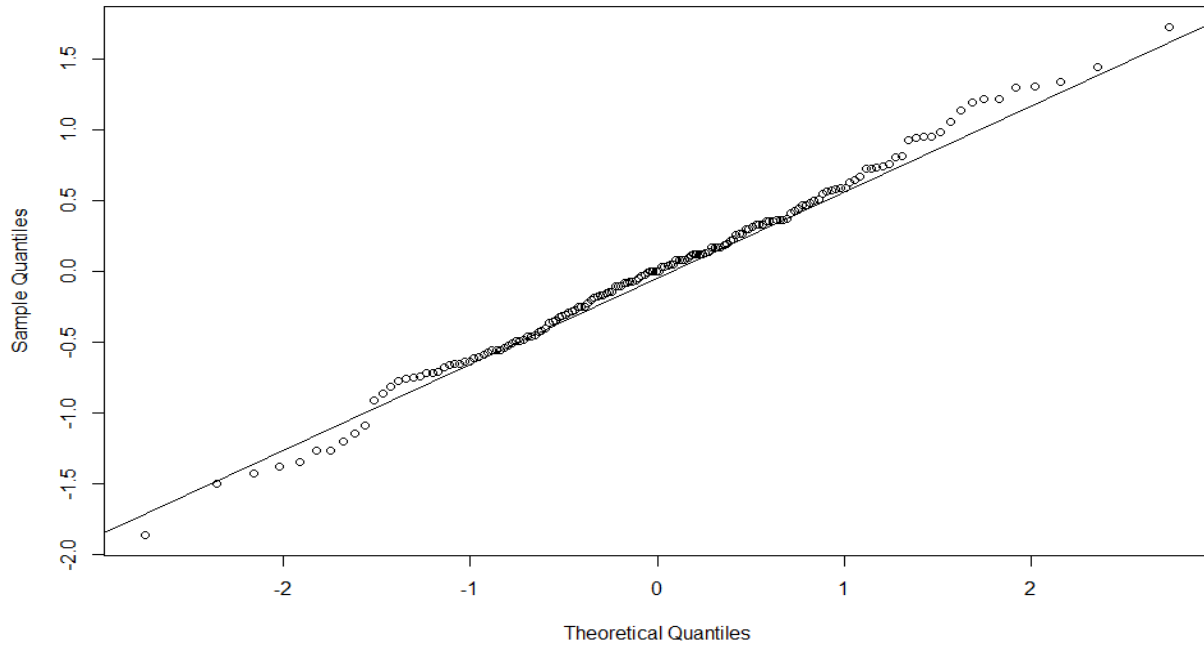
Enterococci

Normal Q-Q Plot

Sediment

Normal Q-Q Plot

16SrRNA

Normal Q-Q Plot

Sul1

Normal Q-Q Plot

ErmB

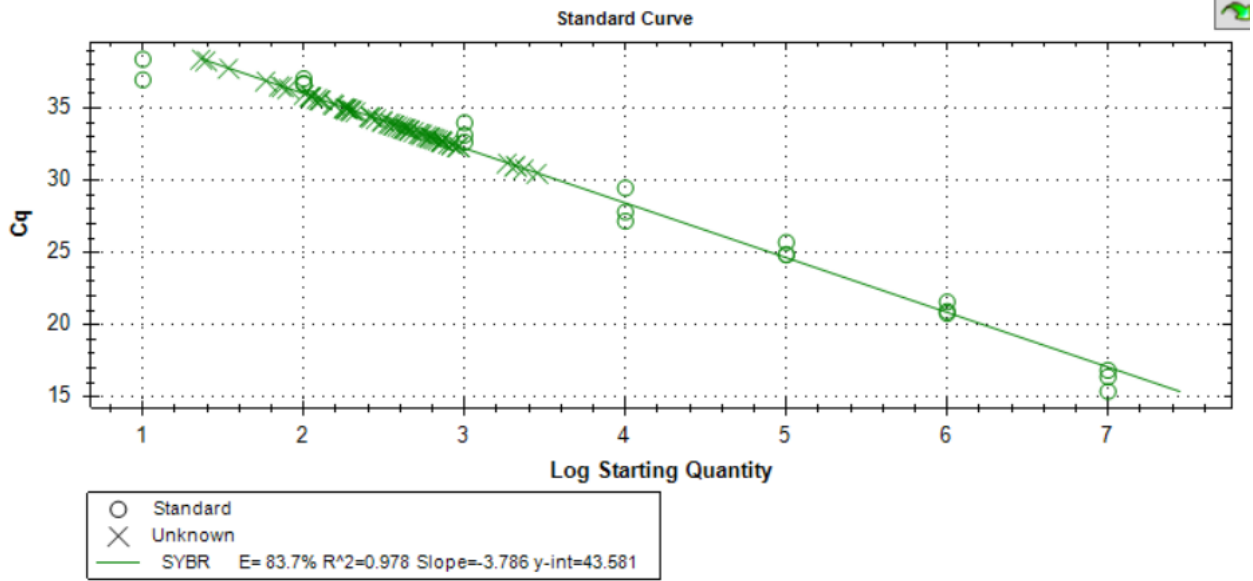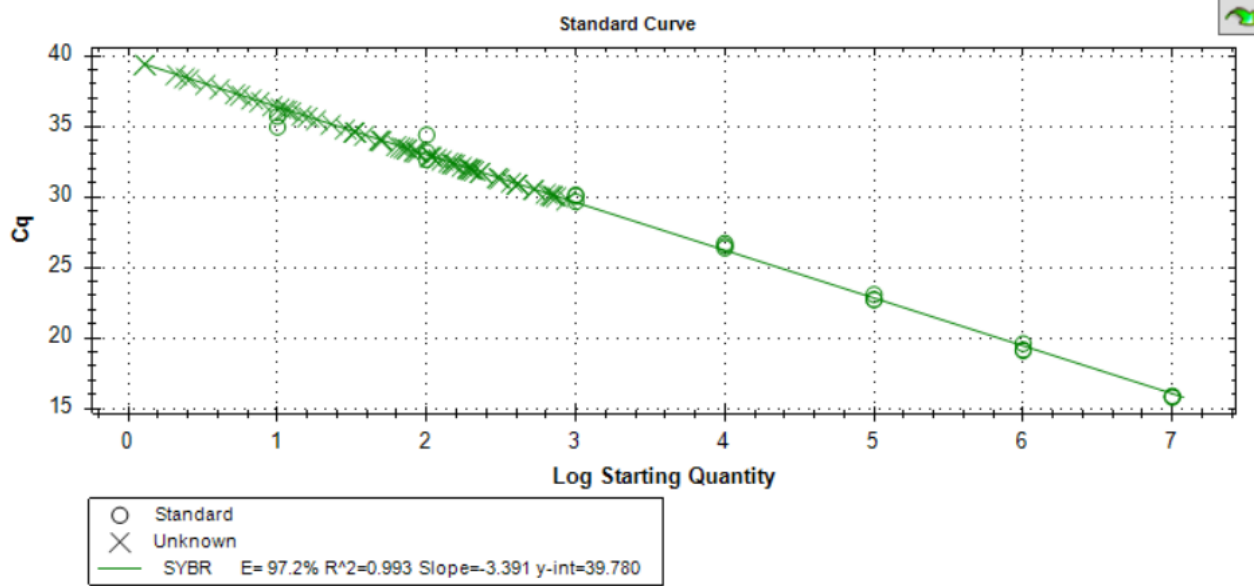**Normal Q-Q Plot**
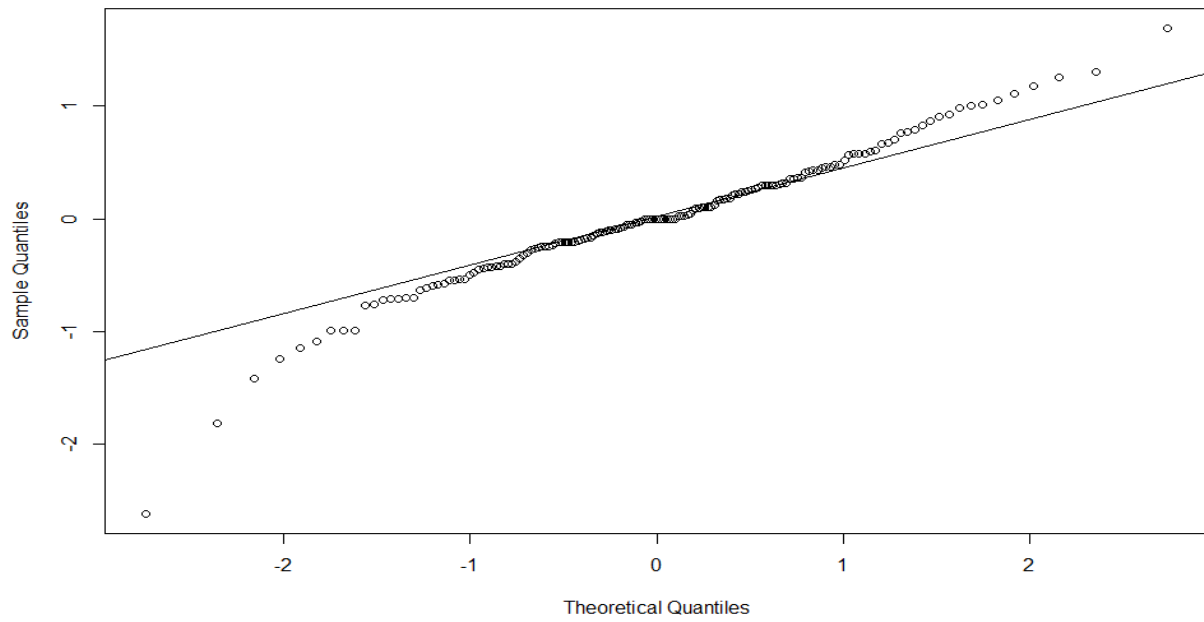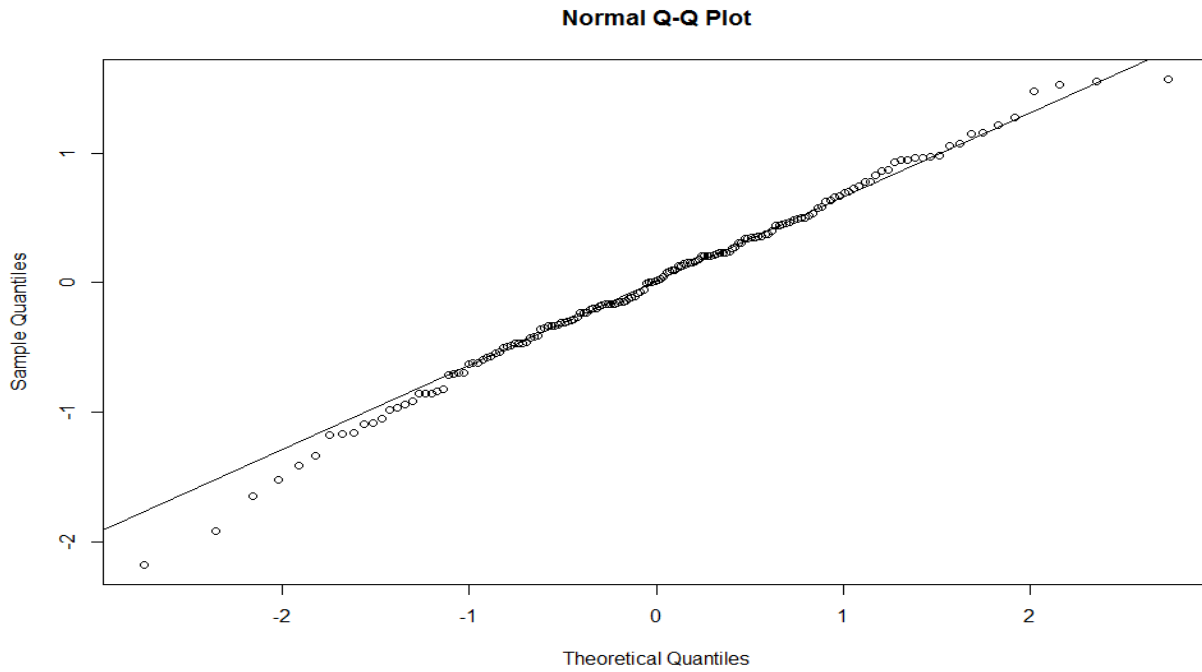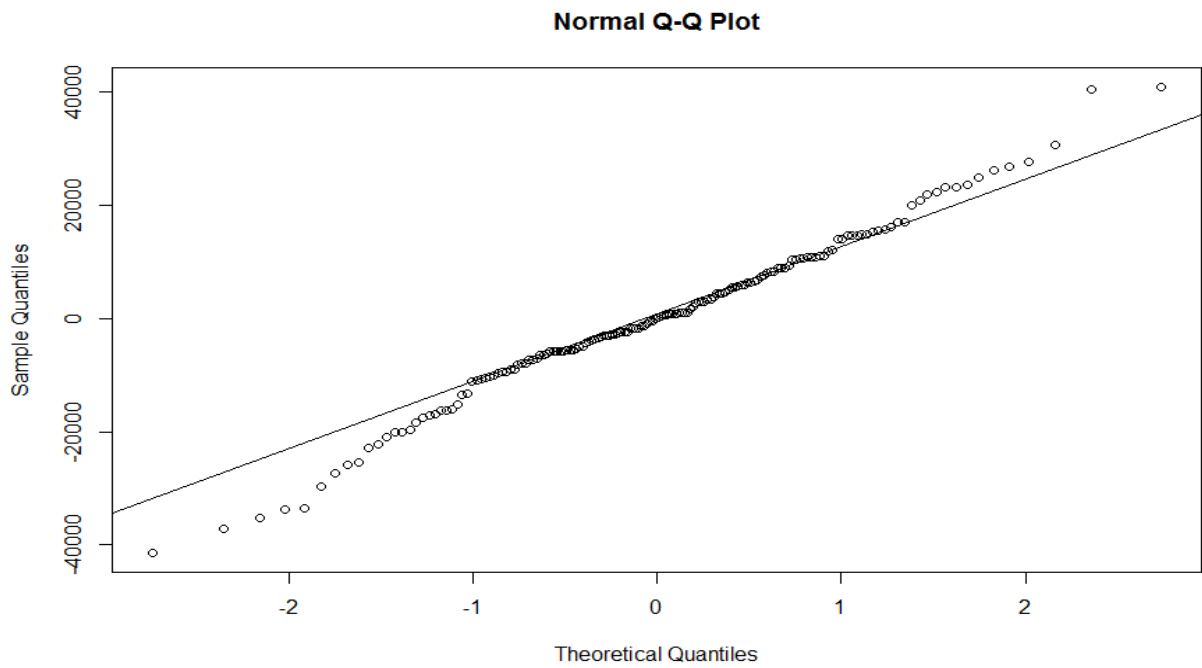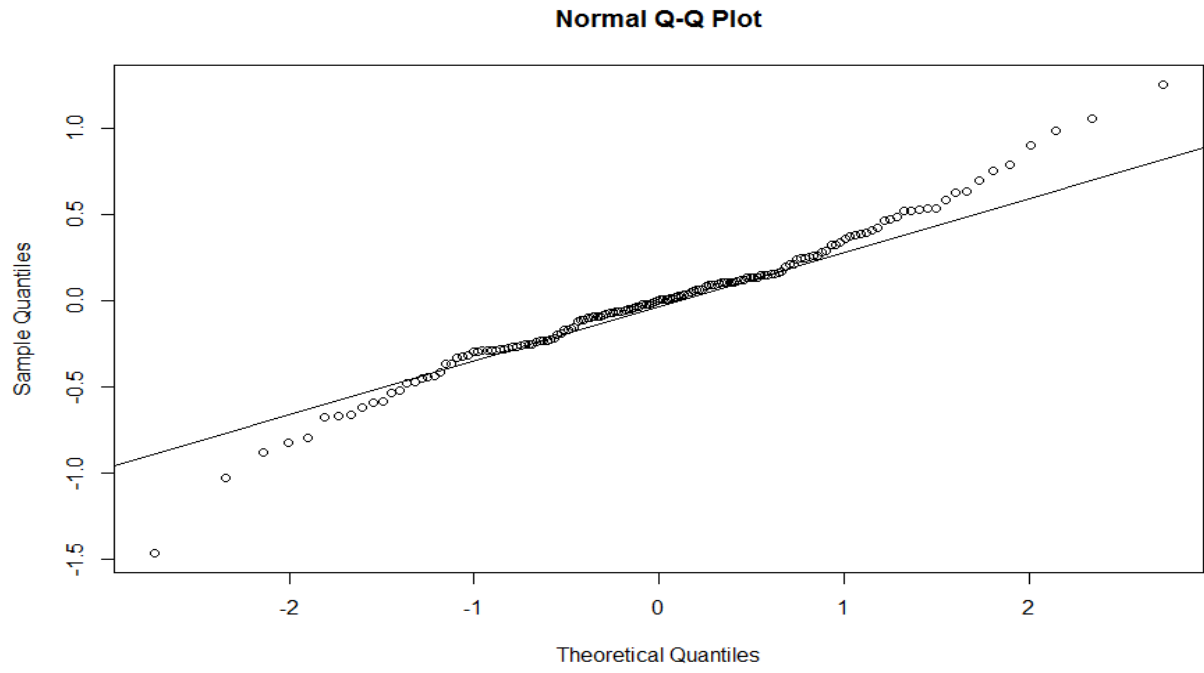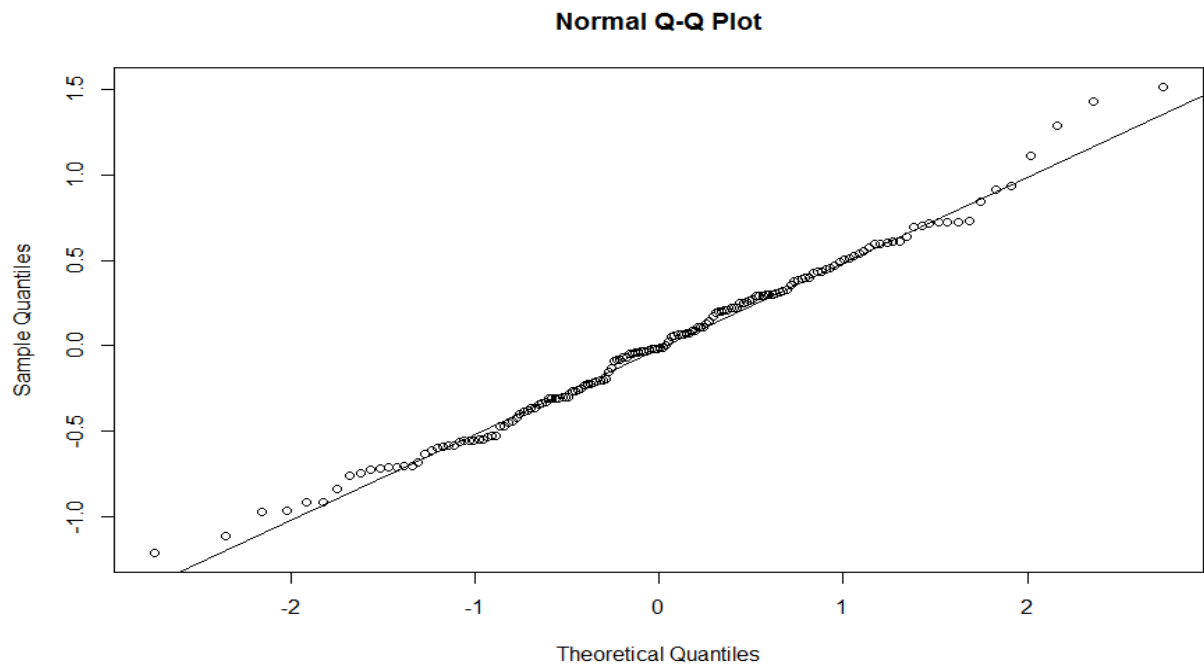
A.4 BioRad qPCR Output

A.4.1 16SrRNA
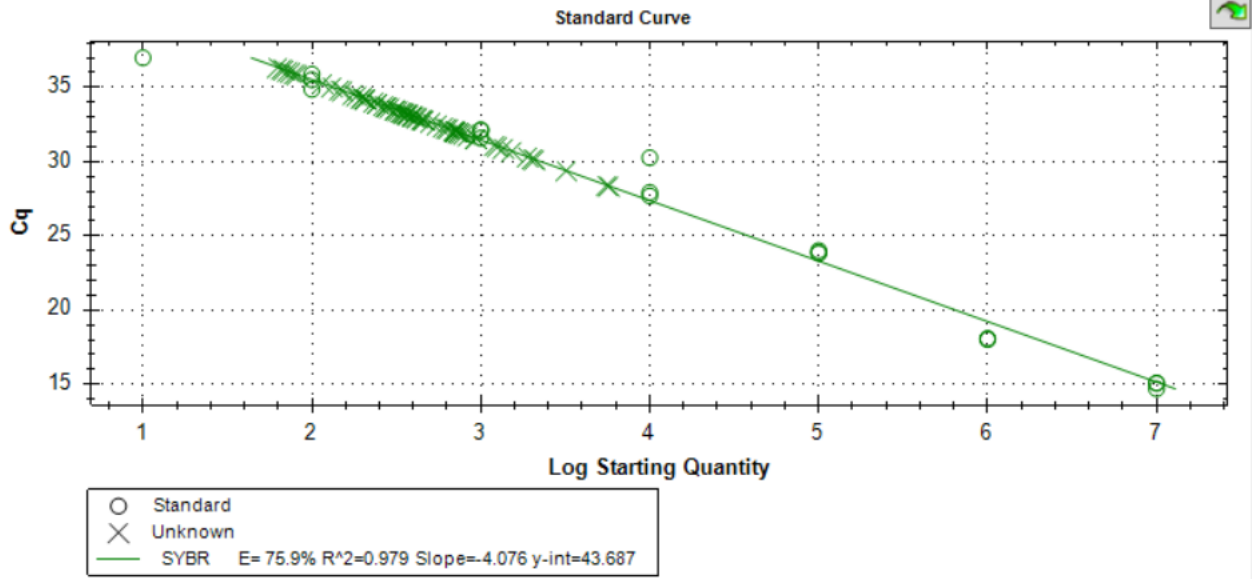
Storm 1 Plots A-W
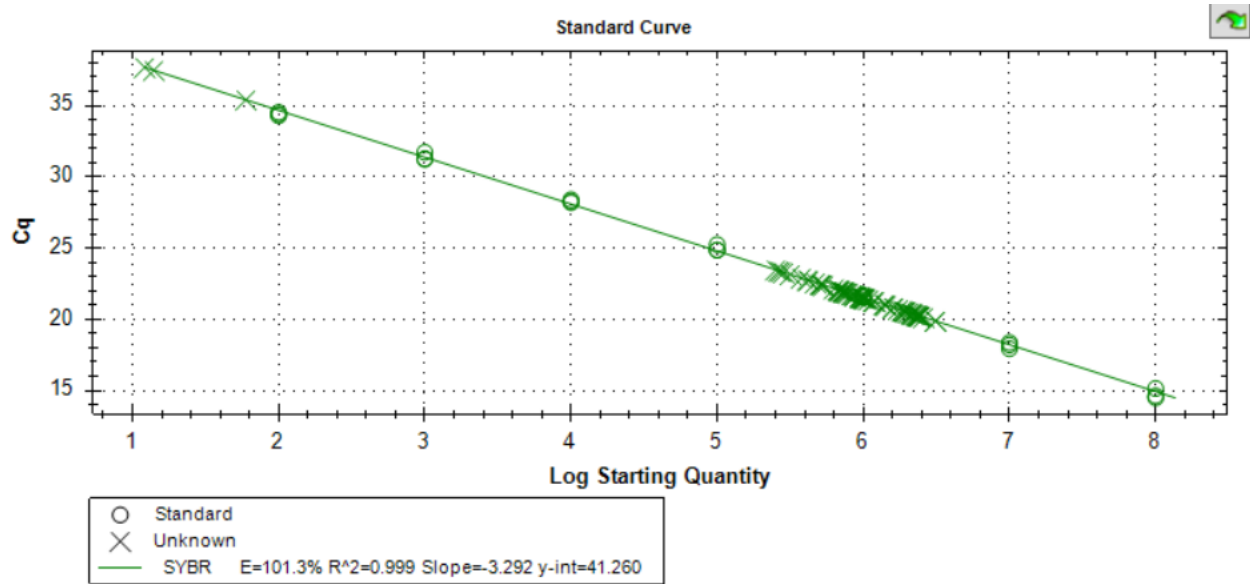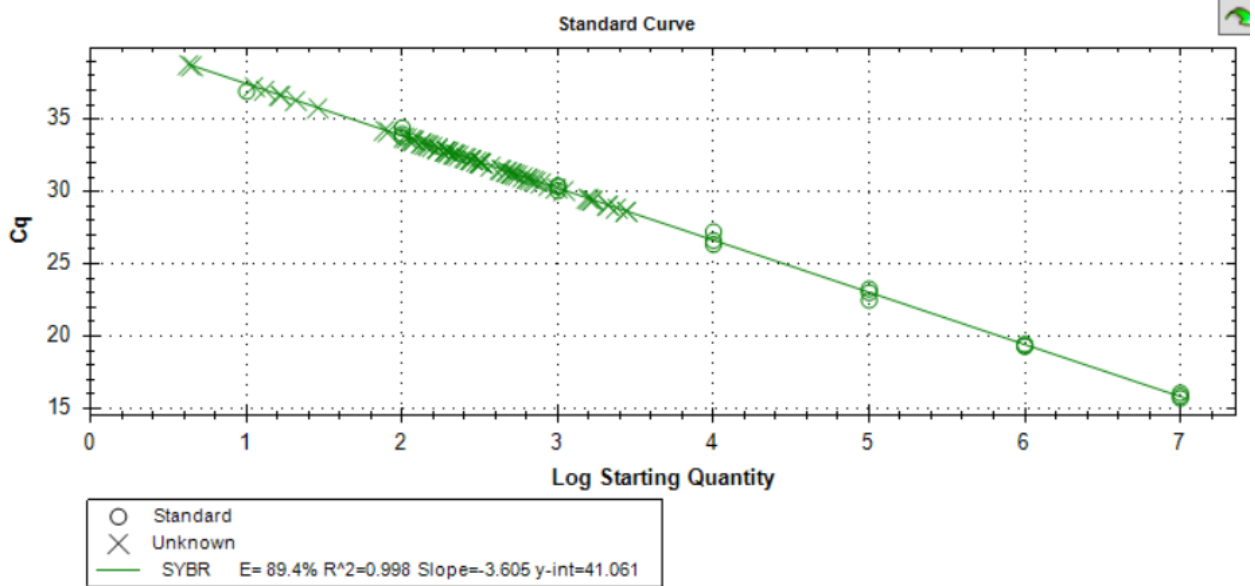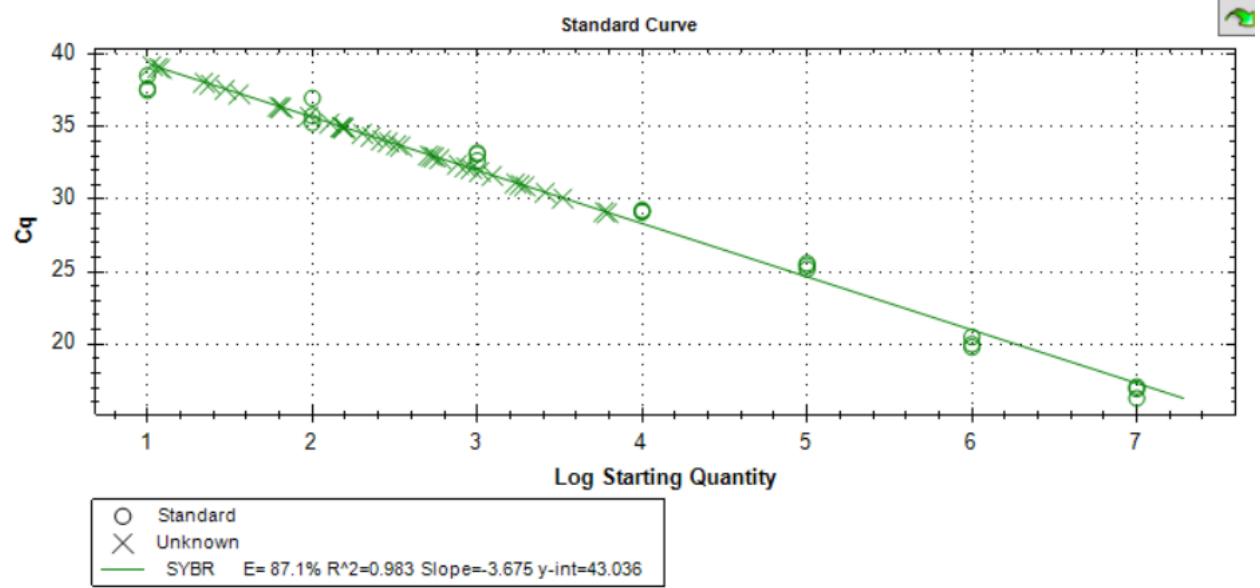

Storm 2 Plots A-W
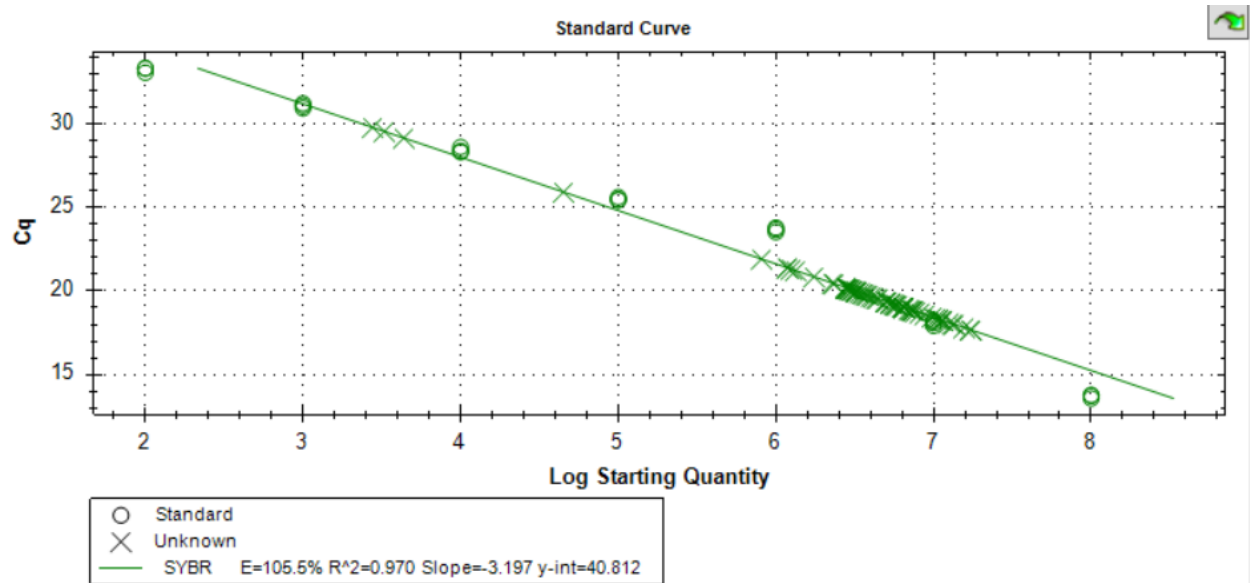
Storm 3 Plots A-W
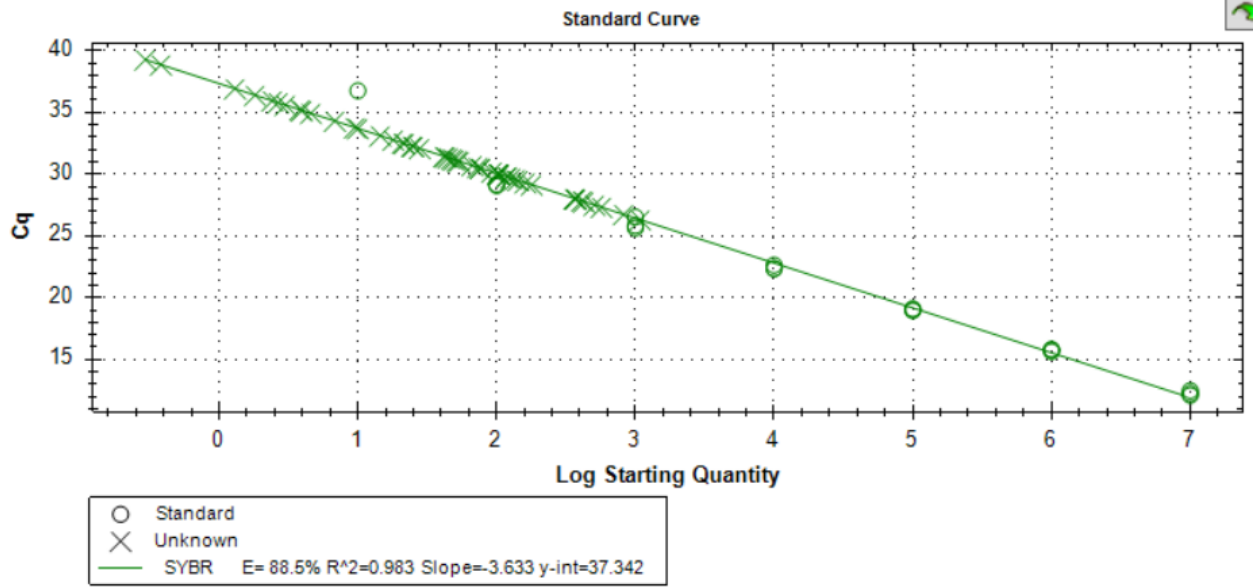

Storm 4 Plots A-W

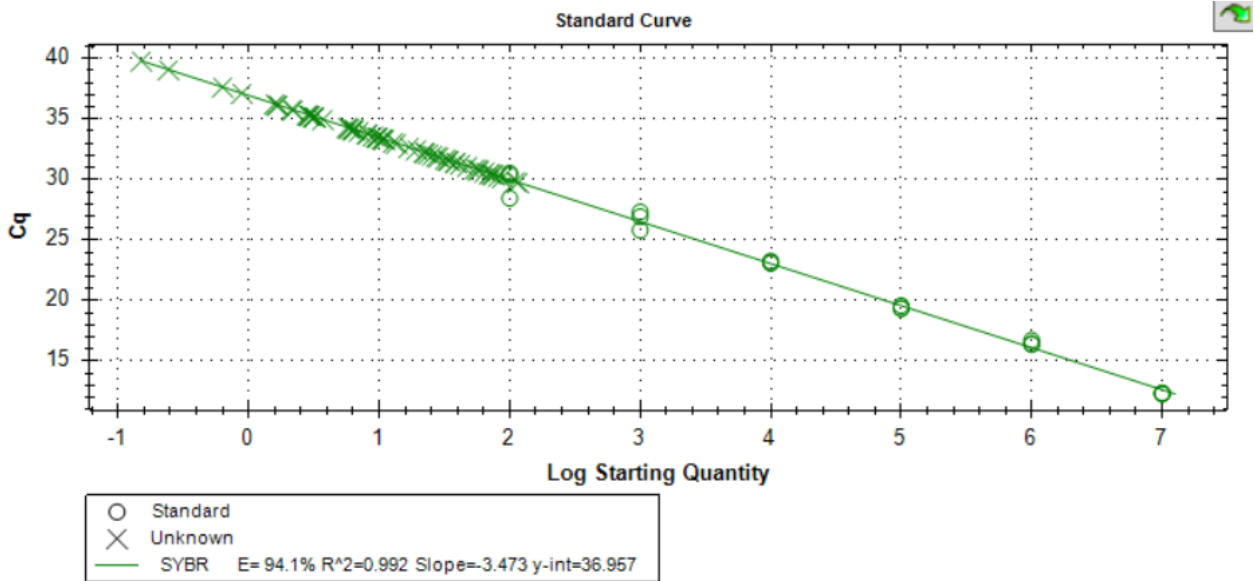Storm 5 Plots A-W

Storm 6 Plots A-W

Storm 1-6 Plot X, Y, Z, and Θ

Standard Curve

Cq vs Log Starting Quantity

- ○ Standard
- ✕ Unknown
- — SYBR   E= 99.9% R^2=0.992 Slope=-3.324 y-int=40.913
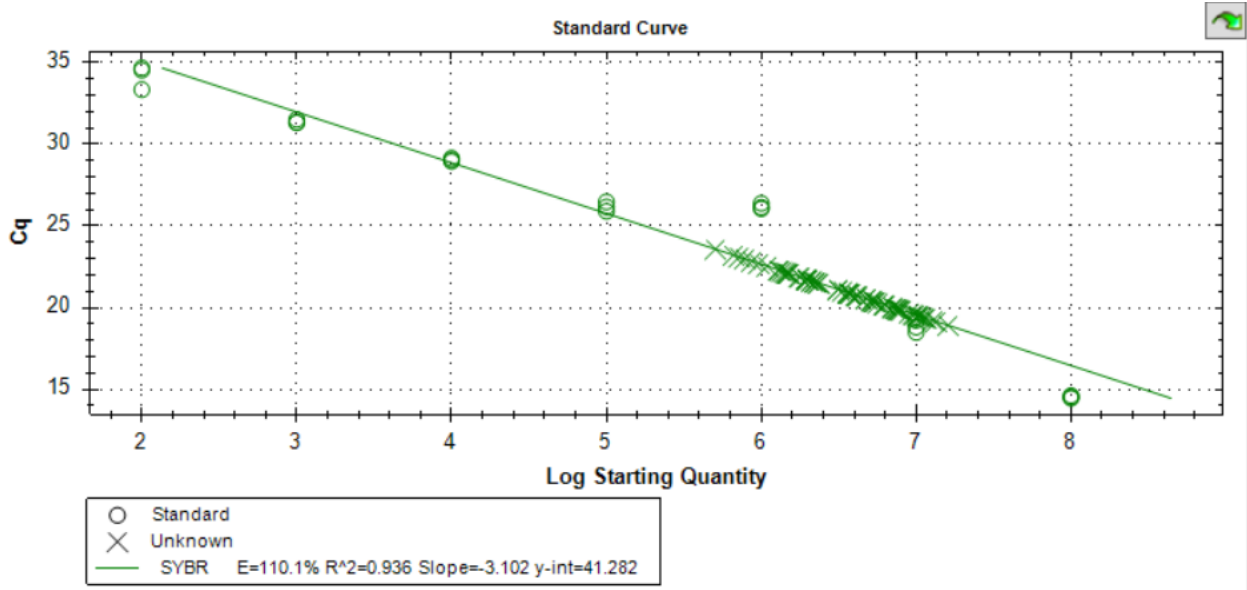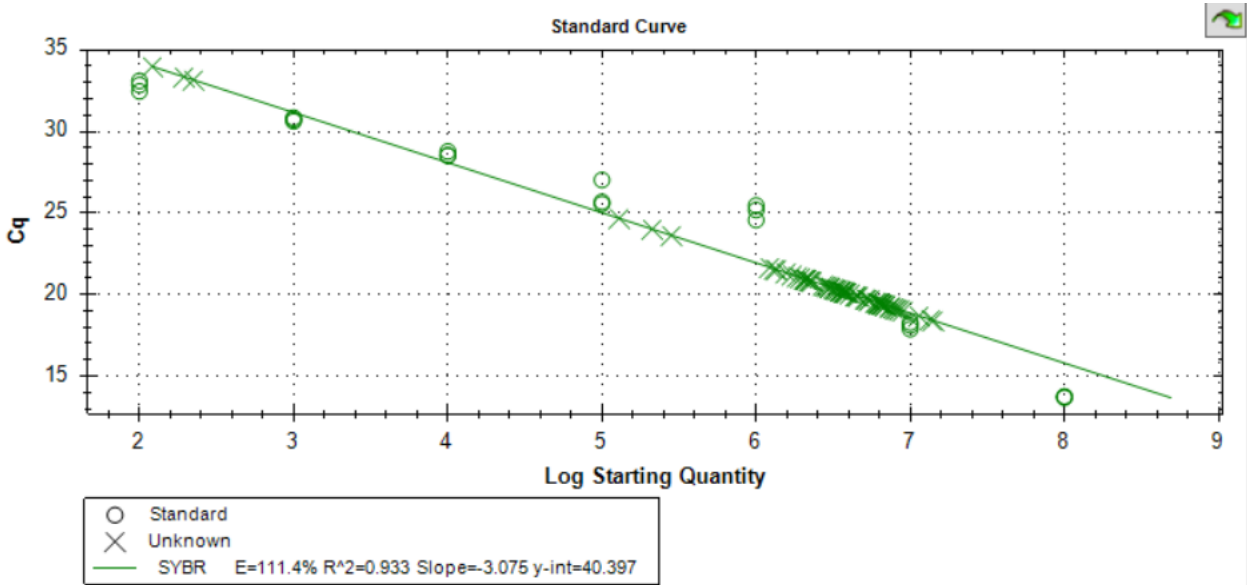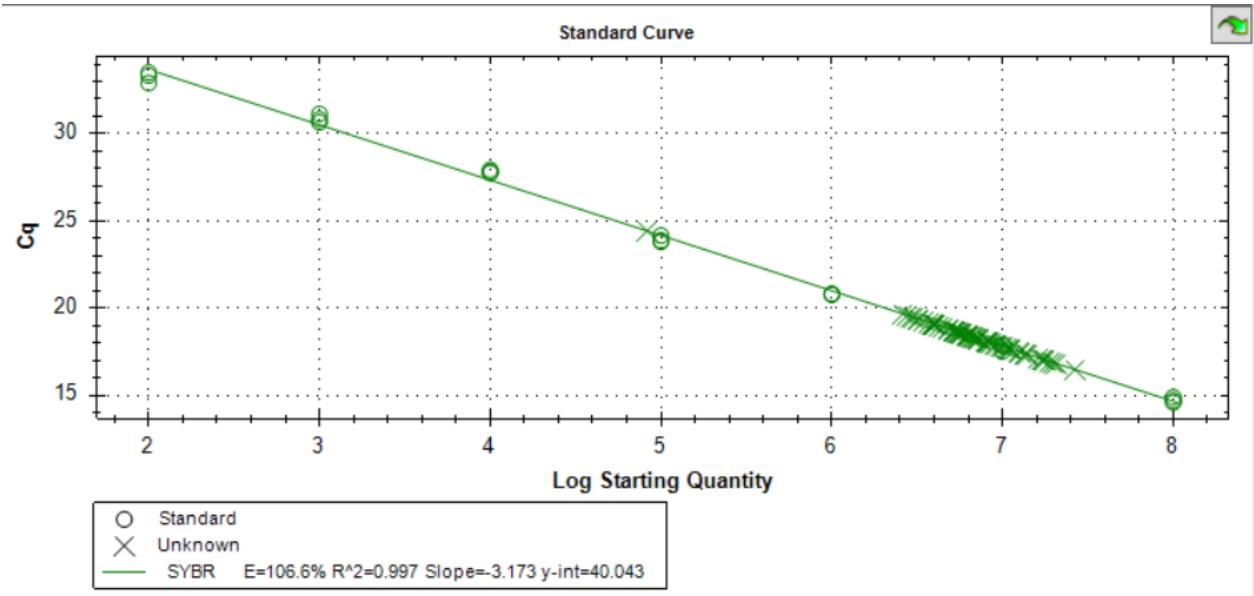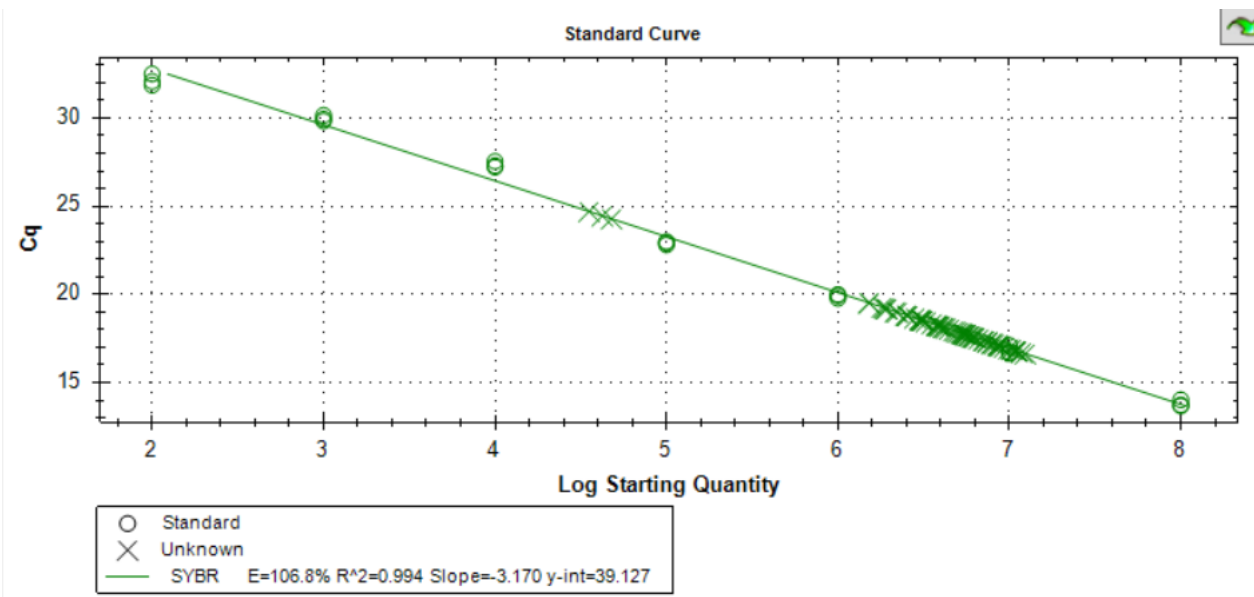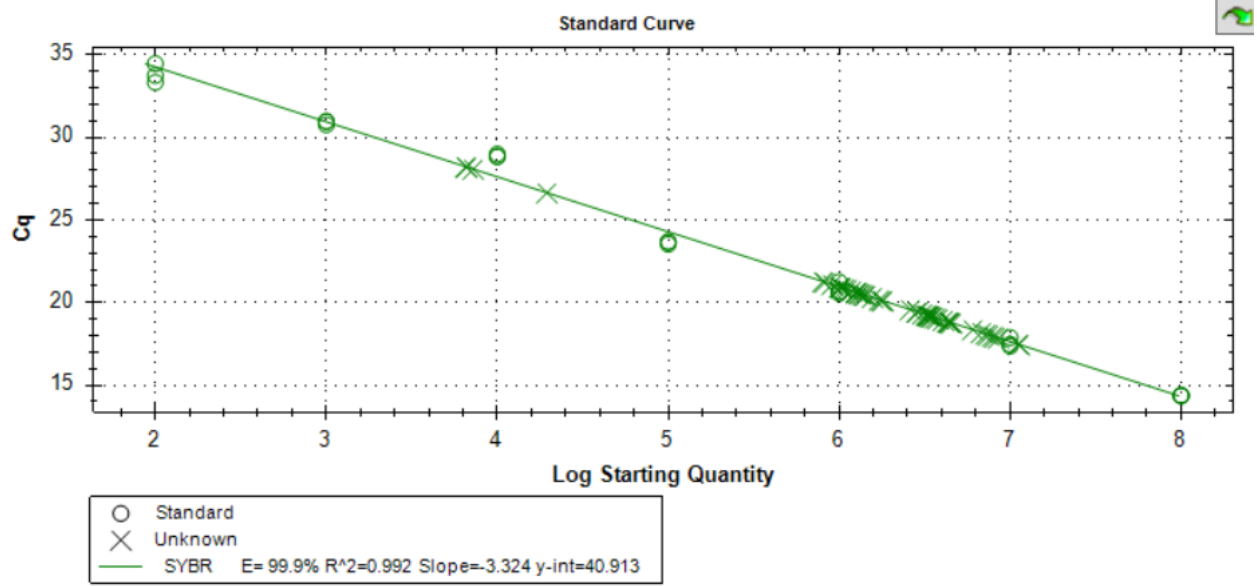
A.4.2 Sul1

Storm 1 Plots A-W
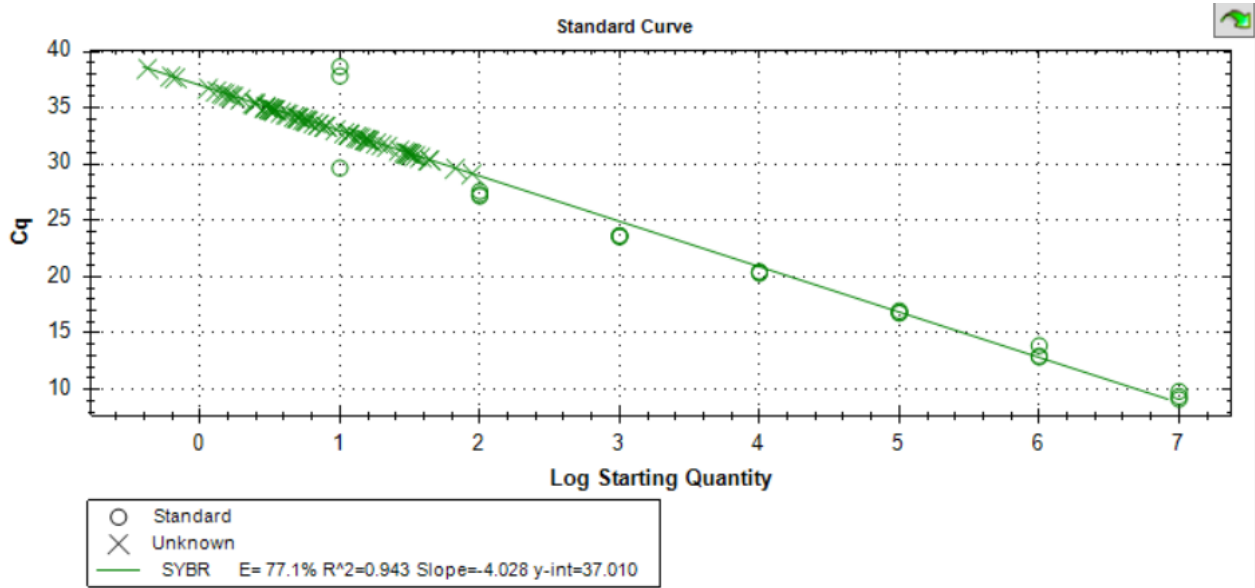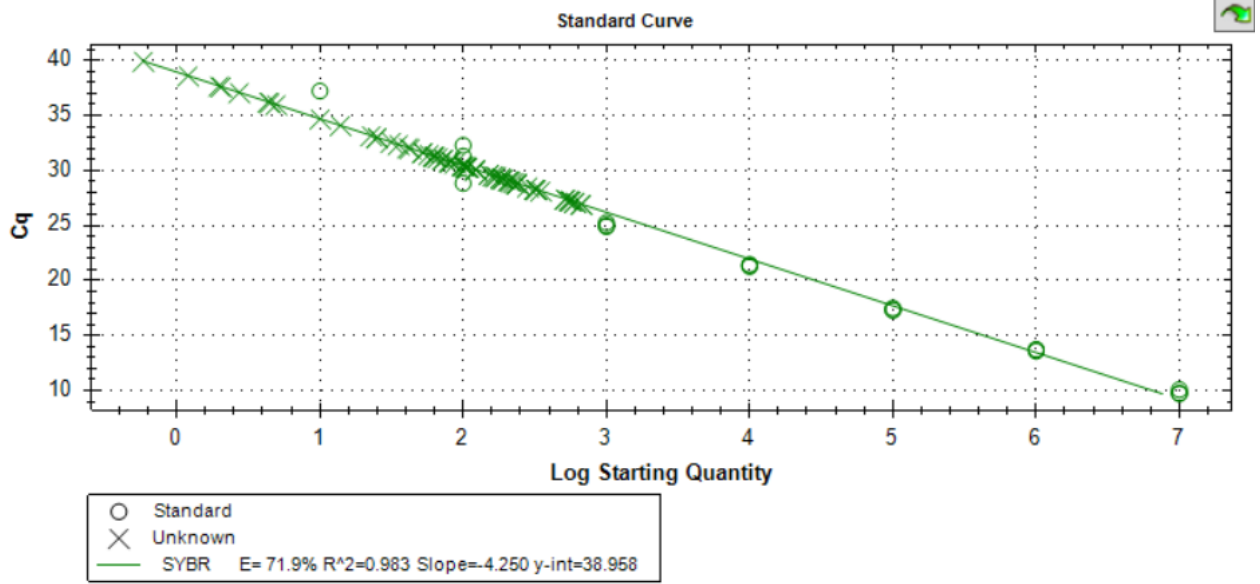

Storm 2 Plots A-W
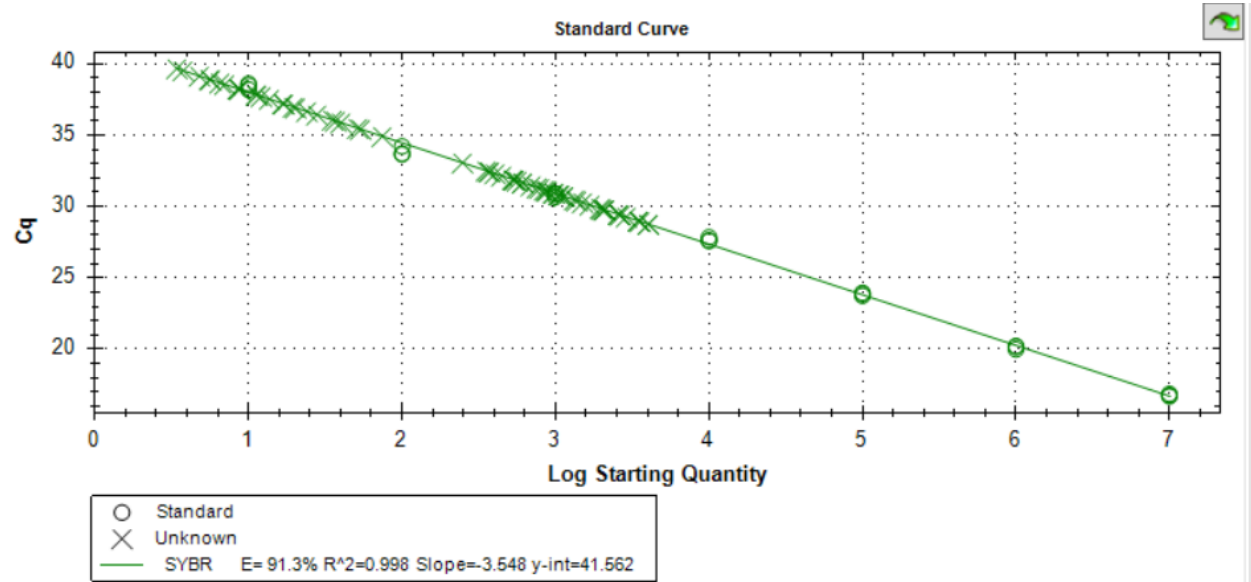
Storm 3 Plots A-W

Storm 4 Plots A-W

Storm 5 Plots A-W

Storm 6 Plots A-W

Storm 1-6 Plot X, Y, Z, and Θ

Standard Curve

Plot with Y-axis labeled "Cq" ranging from 20 to 40, X-axis labeled "Log Starting Quantity" ranging from 1 to 7.

Legend:
- O   Standard
- X   Unknown
- ——   SYBR   E= 87.1% R^2=0.983 Slope=-3.675 y-int=43.036

A.4.3 ErmB

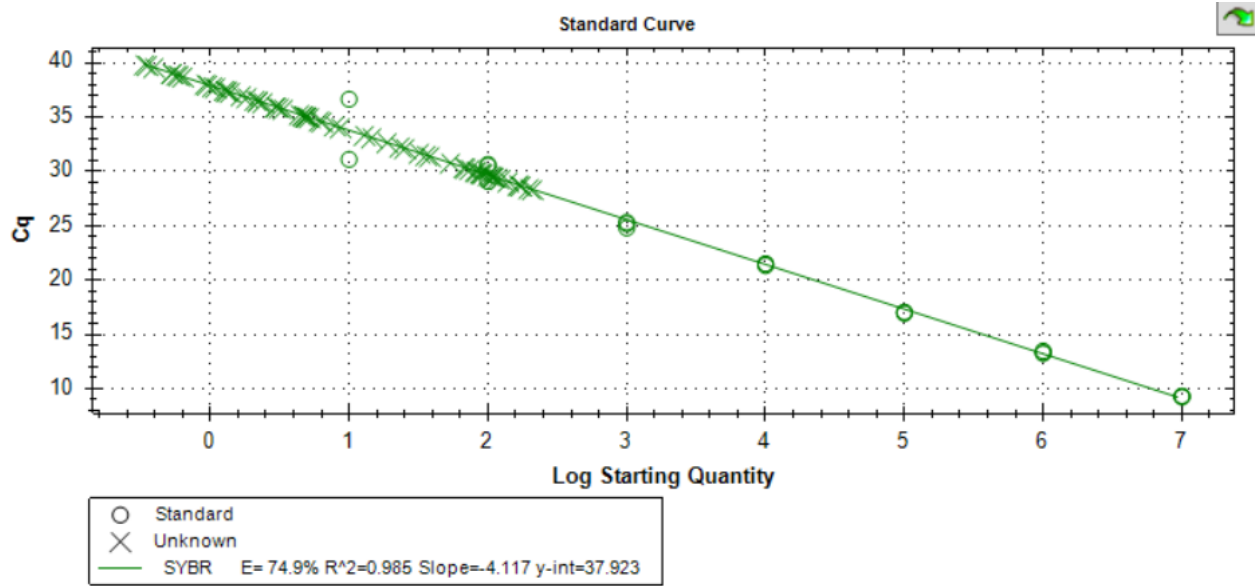Storm 1 Plots A-W

Storm 2 Plots A-W

Storm 3 Plots A-W

Storm 4 Plots A-W

Storm 5 Plots A-W

Storm 6 Plots A-W

Storm 1-6 Plot X, Y, Z, and Θ

Standard Curve

E= 94.3% R^2=0.998 Slope=-3.466 y-int=33.529