

ABSTRACT

Title of Document: FATE OF BACTERIAL AND VIRAL
INDICATORS IN AN ADVANCED
WASTEWATER TREATMENT PLANT

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Wastewater treatment plants (WWTP) are natural aggregators of pathogenic organisms due to the waste they treat. This study examined the fate of two bacterial indicators, fecal coliforms (FC) and *Salmonella*, and one viral indicator, Male-specific coliphages (MSCs), throughout an advanced WWTP. Samples were collected from various points in the WWTP from August 2011 to October 2012. Results show both bacteria and viruses preferentially partition into solids and significant reductions in both bacteria and viruses occur prior to final disposal. The total log removals of FC, *Salmonella*, and MSCs were 4.51, 5.17, and 6.19, respectively for the solids; and the total log removal of FC, *Salmonella*, and MSCs in liquids was 4.47, 5.16, and 3.62, respectively. This study provides the first holistic survey of bacteria and virus indicator fate in a WWTP. Furthermore, results herein demonstrate that current biosolids liming regulations may underestimate the level of viruses in Class B biosolids.

FATE OF BACTERIAL AND VIRAL INDICATORS IN AN ADVANCED
WASTEWATER TREATMENT PLANT

By

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Dedication

To my family for their unwavering support as I pursue my passions, to my friends who were there through good times and bad, and to my mentors whose endless patience made this possible.

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Chapter 1: Introduction

1.1 Viruses

Viruses are a group of infectious agents that are capable of infecting various microorganisms such as animals, plants, and bacteria. These agents are inert in the environment and incapable of growth without a living host.

1.1.1 Background of Waterborne Viruses

Viruses have unique characteristics that make them distinct from other microorganisms such as bacteria and helminthes. One key characteristic is their ability to quickly mutate. This allows them to adapt to harsh environments and vaccines. Their small nanometer-scale size allows them to pass through traditional micron-sized filters. The structural breakdown of a virus is comprised of two basic parts: the genetic information (Ribonucleic Acid, RNA or deoxyribonucleic acid, DNA) and a protein coat that encapsulates the nucleic acids. In some viruses, a third part called a lipid envelope may be found outside of the capsid. These structures allow the virus to infect and replicate within their host cell through the hijacking of their host's biomolecular machinery.

1.1.2 Enteric Viruses

Among the various groups, only certain viruses are capable of infecting human cells and cause disease; we classify these biological agents as pathogens. Enteric viruses are a group of pathogenic viruses whose route of infection are through the fecal-oral route and are spread through waterborne exposure. A major source of these enteric viruses comes from infected individuals who shed large quantities of enteric viruses in their fecal

material (Charles P. Gerba, 2000) which ends up in the sewage and wastewater supply. A survey of sewage contaminated water revealed over 100 different virus species that cause a wide variety of diseases such as hepatitis, gastroenteritis, meningitis, fever, rash, conjunctivitis, and respiratory illness (Bosch, 1998). Several families of viruses that result in illnesses are listed in Table 1.1 (Loret, 2010).

Table 1.1 Common Waterborne Virus Characteristics

Virus Family	Size (nm)	Appearance	Nucleic Acid	Infectious Dose (virus particles)	Significant Notes
Adenovirus	90-100	Non-enveloped, icosahedral	dsDNA	10 -100	<ul style="list-style-type: none"> - Causes respiratory and gastrointestinal diseases - Worldwide distribution
Caliciviruses	27-40	Non-enveloped, cuplike	ss(+)RNA	10-100	<ul style="list-style-type: none"> - Major cause of nonbacterial gastroenteritis - Highest probability of infection from single particle to date (0.5) - Worldwide distribution
Enteroviruses	20-30	Non-enveloped, icosahedral	ssRNA	1-10	<ul style="list-style-type: none"> - Responsible for "Summer Flu" - Worldwide distribution
Hepatitis A	27-28	Non-enveloped, spherical	ss(+)RNA	10-100	<ul style="list-style-type: none"> - Infects the liver - Worldwide distribution but outbreaks only in developing countries
Hepatitis E	27-34	Non-enveloped, spherical	ss(+)RNA	Unknown	<ul style="list-style-type: none"> - Longer incubation period than Hepatitis A - High mortality with pregnant women (30%) - Confined to tropical areas
Rotaviruses	70	Non-enveloped, wheel-like	dsRNA	1	<ul style="list-style-type: none"> - Causes gastroenteritis - Most common cause of diarrhea worldwide - Worldwide distribution

1.1.3 History of Enteric Viruses

Waterborne viral diseases have been prevalent throughout the world and history. One of the first identifiable large waterborne outbreaks that was attributed to an enteric virus was an outbreak of Hepatitis E in New Delhi between December 1955 and January 1956 that affected 290,000 people when raw sewage contaminated the drinking water supply (Gupta & Smetana, 1957). All the enteric viruses that are listed in Table 1.1 have been linked to outbreaks around the world. Noroviruses, for example, are responsible for 50% to 80% of gastroenteritis outbreaks in the United States, Japan, and parts of Europe (Loret, 2010). Enterovirus infections lead to an outbreak of poliomyelitis in Taiwan in 1982 affecting over 1,031 people (Kim-Farley et al., 1984). One Hepatitis A infection in Shanghai in 1988 involving 250,000 people was traced to the consumption of raw clams (Halliday et al., 1991). Hepatitis E struck India again in 1991 in the city of Delhi with an estimated total of over 79,000 cases of disease (Naik, Aggarwal, Salunke, & Mehrotra, 1992). Meanwhile, rotaviruses are estimated to be responsible for the death of approximately 500,000 children a year, primarily in developing countries (Parashar, Hummelman, Bresee, Miller, & Glass, 2003). These global cases of waterborne viral disease demonstrate a need for better surveillance and research of waterborne viruses in the environment as the mechanisms for their survival and persistence are not well understood. This lack of understanding may prove to be responsible for future outbreaks of waterborne illnesses; point sources such as inadequate treated wastewater and solids generated from wastewater treatment plants serve as source material for enteric viruses are released into the environment.

1.2 Fate of Viruses at Wastewater Treatment Plants

Due to the large numbers of enteric viruses that are shed from infected individuals, it should not come as a surprise that wastewater treatment plants (WWTPs) are natural collection points for enteric viruses that may unintentionally lead to waterborne illnesses. In fact, various studies have shown that some waterborne outbreaks around the world have traced the source of their contamination to sewage or otherwise improperly treated wastewater seeping into the drinking water supply (Anderson et al., 2003; Boccia et al., 2002; Okoh, Sibanda, & Gusha, 2010; Symonds, Griffin, & Breitbart, 2009). These outbreaks could be reduced and prevented by better monitoring and tracking of enteric viruses from when they enter to when they leave wastewater treatment plants.

1.2.1 Wastewater Treatment Plant (WWTP) Overview

Within a wastewater treatment plant, viruses have been shown to partition strongly to the solids that are contained, produced, and removed during the wastewater treatment process (Schwartzbrod & Mathieu, 1986). These solids are collected from three treatment processes: primary, secondary, and tertiary treatments. The primary, secondary, and tertiary treatment is comprised of sedimentation, activated sludge/ biological oxygen demand (BOD) removal, and nitrification/denitrification, respectively. The solids are then combined and treated for pathogen reduction to a final product called biosolids and shipped offsite for disposal.

1.2.2 Virus Detection Methods

Historically, widespread enteric virus monitoring in wastewater samples has been hindered by the high cost and complexity of tissue culture-based virus detection methods that are necessary for monitoring human viruses. With the advent of molecular microbiology technology, however, the detection of viruses has been faster and less expensive due to polymerase chain reaction (PCR). PCR has been utilized to detect enteric viruses such as Hepatitis E (Jothikumar et al., 1993), Noroviruses (da Silva et al., 2007), Poliovirus (Karim, Glenn, & Gerba, 2008), and virus surrogates such as MS2 (Wen, Tutuka, Keegan, & Jin, 2009).

1.2.3 Historical Data on Virus Inactivation

A great deal of literature has been published regarding the fate and removal of enteric viruses through various types of wastewater treatment processes using both cell culture and PCR techniques. From the studies in Table 1.2, enteric viruses are found to be present worldwide in significant concentrations in wastewater influent and low levels in final effluent.

These literature values are tabulated in Table 1.3. It should be noted that pathogen removal and inactivation is typically reported in \log_{10} , with 1 log corresponding to 90% removal, 2 log corresponding to 99% removal, etc.

Table 1.2 Influent Concentrations in (PFU or Gene Copies per 100 mL)

Study	HAV*	MS2*	EV*		Polio virus	HAdV*		NoV*	BGMC*
	qPCR	Cul*	Cul*	qPCR	Cul*	Cul*	qPCR	qPCR	Cul*
(Buras 1976)			12778						
(Wen, Tutuka et al. 2009)		3633333			720000				
(Simmons and Xagorarakis 2011)				21000			7300000	43000	1.259
(Ottoson, Hansen et al. 2006)				1000				30	
(Simmons, Kuo et al. 2011)				63096			316228	5011872	
(Hewitt, Leonard et al. 2011)			41	19952		41	190546	18	
(Dryden, Chen et al. 1979)					13000				
(Petrinca, Donia et al. 2009)		751667	26						
(La Rosa, Pourshaban et al. 2010)				251189			199526	1.122E+09	
(Jebri, Jofre et al. 2012)	891250								
Average:	891250	2192500	4282	71247	366500	41	2001575	225414675	1

*HAV = Hepatitis A Virus, MS2 = MS2 Coliphage, EV = Enteroviruses, HAdV = Human Adenoviruses, NoV = Noroviruses, BGMC = Blue Green Monkey Cells, Cul = Samples analyzed for infective viruses via cell culturing, qPCR = Samples analyzed the presence of viral genes via quantitative polymerase chain reaction

Table 1.3 Log Removal (Influent to Effluent) of Viruses at WWTP Worldwide

Study	WWTP	HAV*	MS2*	EV*		Polio virus	HAdV*		NoV*	BGMC*
		qPCR	Cul*	Cul*	qPCR	Cul*	Cul*	qPCR	qPCR	Cul*
(Buras, 1976)	2°			0.33						
(Wen, et al., 2009)	2°		1.85			2.54				
(Simmons & Xagorarakis, 2011)	2°				3.12		4.30	5.63	3.6	
(Ottoson, Hansen, Bjorlenius, Norder, & Stenstrom, 2006)	2°M				1.67			0.95		
(Simmons, Kuo, & Xagorarakis, 2011)	2°M				3.9		3.1	5.7		
(Hewitt, Leonard, Greening, & Lewis, 2011)	2° & 3°			0.67	2.23		2.37	1.33	-2.03	
(Dryden, Chen, & Selna, 1979)	2°					5.2				
(Petrinca et al., 2009)	2°		1.49	0.80						
(La Rosa, Pourshaban, Iaconelli, & Muscillo, 2010)	2°				1.31		0.64	0.54		
(Jebri, Jofre, Barkallah, Saidi, & Hmaied, 2012b)	2°	1.43								
Average:		1.43	1.67	0.60	2.45	3.87	2.37	2.34	2.16	3.6

*WWTP: 2° = Secondary, 2°M = Secondary with Membrane 3° = Tertiary, HAV = Hepatitis A Virus, MS2 = MS2 Coliphage, EV = Enteroviruses, HAdV = Human Adenoviruses, NoV = Noroviruses, BGMC = Blue Green Monkey Cells, Cul = Samples analyzed for infective viruses via cell culturing, qPCR = Samples analyzed the presence of viral genes via quantitative polymerase chain reaction.

The average log removal for PCR related studies were 2.37 with the highest being 2.45 and the lowest being 1.67 due to the differences in treatment and the characteristics of the viruses. The average log removal for culture related studies were 2.69 but had a greater variation with the highest log removal at 3.87 and lowest log removal at 0.60. We can also see from the Table 1.3 that Enteroviruses culture results suggest lower removal rates than PCR results. This may be due to the large variation with the Enterovirus family, the low recovery rates of viruses, and issues with PCR detection methods (e.g., genes detected in influent samples are not in infective viruses). The higher values of NoV (mostly genogroup II) recorded by Hewitt in the effluent than the influent was attributed to its sporadic occurrence but all other studies indicate that NoV removal is comparable to other enteric viruses. Three selected studies are broken down in the proceeding paragraphs to provide a snapshot of how these values were obtained.

1.2.3.1 Detailed Wastewater and Biosolids Study

A study by Simmons and Xagorarakis (Simmons & Xagorarakis, 2011) analyzed 30 wastewater and six biosolids samples from five WWTPs for Adenovirus, Enterovirus, and Norovirus genogroup II. Human Adenoviruses (HAdV) were present in wastewater influent at an average log concentration per liter equal to 7.86 with PCR. In pre- and post-disinfection steps at the same wastewater treatment plant, the log adenovirus concentrations had decreased to 3.94 and 3.57, respectively. Enterovirus (EV) were found to have an average log concentration per liter of 5.32, 2.46, and 2.20 in the influent, pre-, and post-disinfection, respectively with pre- and post-disinfection below detection limit; Norovirus genotype II (NoV II) was found at a log concentration per liter of 5.63 in the influent with none detected in the pre- or post-disinfection samples. In the biosolids,

average human adenovirus and Enterovirus concentrations were at log concentrations per gram of 4.1 and 2.9; Noroviruses type I (NoV I) were detected at an average log concentration per gram of 4.3 and type II at an average log concentration per gram of 5.2; lastly, Hepatitis A virus (HAV) was not found in any of the samples tested. The BGM (African green monkey kidney) cell lines were utilized to measure the infectivity states of those viruses that grow in BGM cells, including Enteroviruses, Polioviruses type 1-3, Coxsackievirus A types 7,9,14,16, Coxsackievirus B types 1-6, Echovirus types 1-27, Rhinovirus 2060, measles, Reovirus, and Herpes virus to name a few (Barron, Olshevsky, & Cohen, 1970). Note that the enteric viruses species detected with BGM cells cannot be differentiated; therefore BGM results are aggregate virus values. Based on the combined PCR and BGM culture results, the study suggests that conventional secondary treatment removes approximately 4 logs of enteric viruses from the wastewater. The study points out that a greater solids removal of Noroviruses than adenoviruses or Enteroviruses was seen which was attributed to more adsorptive properties of NoV. In the following Simmons *et. al* (2011) study also included in Table 1.3, adenoviruses were seen to be more removable in sedimentation than Enteroviruses or Noroviruses possibly due to the larger size (2×) of the adenovirus when compared with EV and NoV.

The six treated biosolids measured in this study were slightly higher when compared to historical 0.4-1.6 log units of infectious particles per gram after composting and liming (Guzman, Jofre, Montemayor, & Lucena, 2007; Monpoeho et al., 2004). The large variations were hypothesized to be due to the differences in detention time of each WWTP. The PCR results showed adenoviruses in 100% of the treated biosolids samples followed by Enterovirus (67%) then Noroviruses (10%).

1.2.3.2 Culture versus qPCR Quantification Study

A different study (Hewitt, et al., 2011) analyzed viruses in samples of influent and effluent from wastewater treatment plants with various treatment processes and different population sizes. The plants serving small populations utilized waste stabilization ponds and plants serving medium and large sized population utilized activated sludge or moving bed biofilm reactors. The viruses studied showed minor variances in concentration and prevalence in large WWTPs. This was attributed to the better mixing and extended infrastructure as the wastewater is transported as well as the possibility of continuous infections in a large population community. In contrast, small WWTPs are more sensitive to localized outbreaks –which may cause a spike in the virus concentration due to the smaller inflow when compared to a large WWTP. Enteroviruses and Adenoviruses were able to be cultured in both the influent and effluent of all wastewater treatment processes despite the fact that culture methods resulted in lower values than those measured with qPCR. Overall, greater adenovirus log reductions were observed with culture methods compared to qPCR methods, but the trend was opposite for Enteroviruses. Some adenovirus and enterovirus samples from both influent and effluent were found to be positive by culture but repeatedly negative by PCR. The authors postulated that this was due to the difference in sampling volume (200 mL for culture and 1 mL for PCR). Noroviruses infectivity could not be assessed due to the lack of viable cell lines. Nonetheless, norovirus infectivity losses were postulated based on the correlation between adenovirus culture and PCR concentrations since noroviruses have similar characteristics.

1.2.3.3 Coliphage Partitioning in a WWTP

Lastly, a study by (Tanji et al., 2002) tracked the partitioning of coliphages (i.e. coliform bacterial viruses) of a large wastewater treatment plant (population 200,000) in Japan. Influent coliphage concentrations via culture methods varied between 10^3 to 10^4 PFU/ml throughout the year with slightly higher values found in the summer, between June and August, and late fall, between October and December. The WWTP utilizes primary settling followed by an anaerobic tank followed by an aerobic tank with secondary settling then disinfection. Samples were collected from the influent, primary supernatant, anaerobic tank, aerobic tank, returned sludge, secondary supernatant, and effluent. The samples were then inoculated into three different *E. Coli* strains: K12 (W3110) for F- coliphages, K12 HfrH for F+ coliphages, and C for somatic coliphages. The results indicate that most of the coliphages detected in the influent are suspended in the liquid matrix with a tiny amount in the solids. In the primary effluent, the K12 (W3110) strain showed a decrease in the liquid matrix, and no reductions in the number of F+ coliphages, and a slight decrease in the concentration of somatic phages were observed. In the anaerobic tank, coliphages significantly decreased and began to partition into solids with solid partitioning taking approximately 25%, 65%, and 30%, for F-, F+, and somatic, respectively. In the aerobic tank, all of the coliphages are partitioned into solids. The return sludge had higher concentrations of coliphages. Lastly, no F- and F+ coliphages were present in the secondary effluent and effluent while a tiny amount of somatic coliphage was present in the secondary effluent but none present in the final effluent. This study indicates that partitioning into solids during anaerobic/aerobic tanks are the dominant pathways of coliphage movement through the WWTP. These results

were conducted utilizing various bacterial hosts that were susceptible to viruses that were host compatible. Therefore this study may underestimate viruses that were present in a non-culturable state.

1.2.4 Culture Versus PCR Assays

The studies discussed above utilized two different methods to quantify viruses: quantitative-PCR assays and/or culture infectivity assays. Here we discuss the basics of these methods along with their inherent advantages and disadvantages.

1.2.4.1 PCR Assay

PCR-based methods are powerful in that they are able to detect small amounts of genetic material with relatively high precision and speed. These advantages have made PCR-based methods a prevalent tool in the field of microbiology but their use has a few drawbacks. Polymerase chain reaction requires polymerase enzymes, primers, and genetic material to be in contact with one another to replicate. However, in order to free the genetic material from contaminants so that it may associate with the polymerase, the sample must be lysed, effectively killing any viable organisms that are present in the sample. The drawback comes into play while interpreting the results. Although the genetic material is present and was replicated, the viability of the source of the genetic material is unknown. This drawback has been noted in various studies (Ottoson, et al., 2006; Rodriguez, Pepper, & Gerba, 2009; Toze, 1999), where free floating genetic material or even whole non-infective viruses give false positives when no infective viruses are present (Limsawat & Ohgaki, 1997). Culture assays, on the other hand, avoid this problem by mixing the sample with host cells and then plating the cells. If viruses are present in the sample, holes or “plaques” will form in the cell layers. The presence and

quantification of these plaques allows for a count of the infectious viruses present in the original sample. A study by Baertsch, *et. al* (Baertsch, Paez-Rubio, Viau, & Peccia, 2007) found that although Class A biosolids have lower concentration of pathogen genomes, the calculated reductions were greater with qPCR results compared to culture results; this suggested an overestimation of inactivation. Another drawback that PCR assays face is the need for a known primer sequence for the interested microorganism. Culture Assays can sometimes measure bacterial and viral strains that were not targeted in the analyses; however, culture techniques also face major limitations such as the lack of cellular hosts for a number of important human viruses.

1.2.4.2 Culture Assay

Culture assays requires that a virus has a cultural host that can be grown *in vitro*; unfortunately, there are currently no cell lines that are available for noroviruses (da Silva, et al., 2007; Hewitt, et al., 2011). Virus culture methods also experienced low recovery rates with reported rates around 10% to 25% (C. Gerba, A. Ross, K. Takizawa, & I. Pepper, 2011; Hewitt, et al., 2011). Yet another drawback is the time and cost associated with maintaining cell lines. Despite the associated disadvantages of culture methods, these technique are necessary for assessing the success of wastewater treatment since PCR-based methods often result in inaccurate log removal values.(Sobsey, Battigelli, Shin, & Newland, 1998)

1.2.5 Need for Population Balance Inside WWTP

All of the aforementioned studies focused on the removal and persistence of viruses within the various wastewater treatment processes. However, no one has yet conducted a holistic population balance of viruses through a wastewater treatment plant

to track where exactly viruses are removed and inactivated. A population balance would also aid in determining the effectiveness of solids treatment and allow for more targeted methods of pathogen destruction.

1.3 Biosolid Regulation

1.3.1 Biosolid Overview

Biosolids are solids that are collected and concentrated from various wastewater treatment processes. Due to their high levels of organic carbon and nutrients, they are often used as soil additives or even fertilizer in agricultural settings. In the United States, there are approximately 16,583 WWTPs (as of 2007) of which 20% are responsible for generating 92% of the total quantity of biosolids (Jenkins, et al., 2007). These biosolids are often land-applied to help provide nutrients and improve soil properties. Noncomposted biosolids are highly enriched and decompose quickly in soils. Biosolids ensure pH stability, improve water holding capacity, aeration, and structural stability of the soil (U. EPA, 1999).

If not treated adequately, biosolids can harbor pathogenic bacteria and viruses that have the potential to impact human health when released into the environment. In order to limit and prevent outbreaks from occurring, US Congress and the Environmental Protection Agency (EPA) established federal regulations in 1993 that monitor the concentration of possible pathogens under Title 40 Part 503 of the Code of Federal Regulations. This is commonly called Sludge Rule 503. Under Sludge Rule 503, the presence of pathogens is estimated based on the presence of two different bacterial fecal indicators: fecal coliforms and *Salmonella*.

Fecal coliforms (FC) are a broad family of microbes that are facultative anaerobic (can survive in both aerobic and anaerobic environments), rod-shaped, gram-negative, and non-sporulating bacteria. One well-known species that belongs to the family classified as fecal coliform is *Escherichia coli*. FC are coliforms found in the waste of warm blooded animals and are defined by their ability to ferment lactose at 44.5 °C (G. Berg, 1978). 96.4% of the total coliforms found in human waste can be classified as FC. An estimated 10^6 to 10^9 fecal coliform are present per gram of human stool--lower concentrations are observed in other warm-blooded animals such as rabbits and cats.

Salmonella are a genus of rod-shaped, gram-negative, non-sporulating bacteria. *Salmonella* is responsible for 1.3 billion cases of diseases worldwide (Coburn, Grassl, & Finlay, 2007). The *Salmonella* family is comprised of multiple serovars, some of which causes disease in humans: Typhi, Paratyphi, Sendai, Dublin, Typhimurium, and Choleraesuis. The first three serovars named are responsible for enteric fevers and causes what is more popular known as typhoid fever, made famous by Typhoid Mary. The other serovars causes enterocolitis or inflammation of the digestive track. Due to some of its pathogenic serovars and its presence in the digestive track, *Salmonella* has been selected as an indicator for fecal contamination. Therefore it is important to determine how *Salmonella* behaves through the wastewater treatment processes.

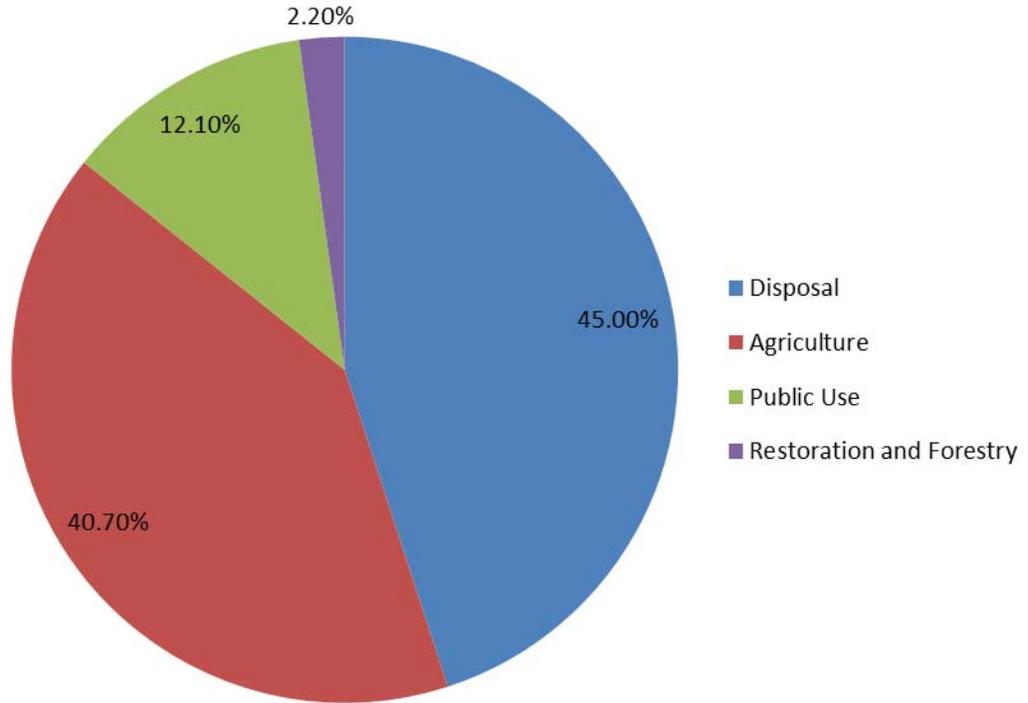
Most species of fecal coliforms and *Salmonella*, however are not pathogenic (U. EPA, 2012). The reason Sludge Rule 503 utilizes bacterial indicators is due to the vast array of pathogens present in the environment. It is therefore not economically feasible to regulate biosolids based on the presence of all pathogens (i.e. norovirus, *Cryptosporidium*

etc.). Bacterial indicators are intended to be conservative surrogates that provide an assessment of the effectiveness of biosolids treatment procedures at removing pathogens.

1.3.2 Class A Versus Class B Biosolid Standards

Under Sludge Rule 503, standards are set depending on the final application of the biosolids (Table 1.4). The utilization of biosolids as fertilizer falls under Subpart D of Sludge Rule 503 which recognizes two classifications of biosolids, Class A and Class B, depending on the extent of treatment. Their classification is measured by using the most probable number (MPN). The classification of biosolids into Class A and Class B represents the degree of pathogenicity with Class A typically considered to be pathogen free and Class B considered to have significantly reduced levels of pathogenicity. Class A biosolids, sometimes referred to as exceptional quality biosolids, are used by the public and have no usage restrictions. Class B biosolids have detectable levels of pathogen surrogates, but have undergone processes that have significantly reduced the pathogen levels. Site restrictions are placed on lands that receive Class B biosolids, such as a minimum distance to public areas.

Figure 1.1 2004 Nationwide Biosolid Breakdown



Source: (Jenkins, Armstrong, & Monti, 2007)

In 2004, 7,189,000 dry tons of biosolids were produced of which 55% (3,953,950 dry tons) were land applied for agronomic, forest, or land restoration; the remaining 45% (3,235,050 dry tons) were disposed in landfills, surface disposal units, or incineration facilities (Figure 1.1). Of the land applied biosolids, 74% (2,925,923 dry tons) were used for agricultural purposes due to their Class B status, 22% (869,869 dry tons) were treated and tested for public use as Class A biosolids, and the remaining 4% (118,619 dry tons) were used for land restoration and forestry (Jenkins, et al., 2007). The values used to determine this classification are shown in Table 1.4 below.

Table 1.4 Current Regulation Limits on Biosolids

Classification	Fecal Coliforms	<i>Salmonella</i>	Coliphage
Class A	< 1,000 MPN/gram*	< 3 MPN/ gram*	1 PFU/ 4 gram*
Class B	< 2,000,000 MPN/gram*		

*grams in grams of dry solids, MPN = Most Probable Number, PFU = Plaque forming Units

1.3.3 Shortcomings of Current Regulations

The biosolids classification system has been criticized due to the fact that indicator bacteria do not accurately reflect the pathogenicity and biological risks posed by viruses (C. P. Gerba, Goyal, LaBelle, Cech, & Bodgan, 1979; Jebri, Jofre, Barkallah, Saidi, & Hmaied, 2012a; Karim, et al., 2008; Limsawat & Ohgaki, 1997; WHO, 2006). Fecal coliforms and *Salmonella* indicators have been unreliable indicators of viruses and thereby misrepresent the pathogen risks that biosolids may present. For instance, water that was consistently free of indicator bacteria was deemed responsible for a hepatitis outbreak and contained hepatitis A virus, rotaviruses, and Enteroviruses (Bosch et al., 1991). In another study, bacterial indicators were found to be considerably more susceptible to chlorine disinfection than viruses (See 1.3.4) (Tree, Adams, & Lees, 2003). The reasons given for these differences have been attributable to the difference in size between bacteria and viruses and the greater physical and biological variations in enteric viruses compared to bacteria (Sidhu & Toze, 2009). The size difference between bacteria and viruses is of great importance in the land application of biosolids due to the possibility that aerosols are generated during and after application. Improved indicators, such as Male-specific coliphages, have been recommended for indicating human health risks posed by biosolids. Further research is needed to survey the presence of viruses in

biosolids and their inactivation through treatments. Due to the complex nature of the constituents in biosolids, an improved understanding of the interactions between biosolids and their environment are also necessary to safeguard the health of the general public and prevent the spread of enteric diseases.

1.3.4 Sludge Rule 503

Biosolids classification rules are outlined in Section 32 of Sludge Rule 503. Solids are classified as Class A if they have if they meet one of the six pathogen reduction requirements, listed below, and either the density of fecal coliforms must be less than 1,000 MPN per total dry gram or less than 3 MPN per 4 grams of total solids. (USEPA – Plain English Guide to EPA Part 503 Biosolids Rule)

1. Time-Temperature Regimes – Fulfills one of four time-temperature regimes based on whether the biosolids is has a solids content less than 7%
2. High pH-High Temperature – Elevating the pH to greater than 12 while maintaining 25 °C for 72 hours or maintaining at least 52 °C for at least 12 hours at a pH of 12 or air drying to over 50% solids after 72 hours at 12 pH
3. Other Known Processes – Density of enteric viruses after virus treatment to be less than 1 plaque forming unit per 4 grams of total dry solids
4. Unknown Processes – Density of viruses less than 1 PFU per 4 grams of total dry solids and the density of helminth ova less than 1 per 4 total dry grams
5. Processes to Further Reduce Pathogens (PFRPs) – utilizing composting, heat drying, heat treatment, thermophilic anaerobic digestion, beta ray irradiation, gamma ray irradiation, or pasteurization outlined

6. Processes Equivalent to PFRPs – treated to any process equivalent to PFRP as determined by the permitting authority

Solids are classified as Class B if they have been treated with Processes to Significantly Reduce Pathogens (PSRP) or process equivalent to one of the PSRPs, as determined by the permitting authority (USEPA – Plain English Guide to EPA Part 503 Biosolids Rule). The PSRPs are listed in Appendix B of Sludge Rule 503 and allows for the five following processes:

1. Aerobic Digestion – where the mean cell residence time and temperature is maintained at 40 days at 20 °C and 60 days at 15 °C.
2. Air Drying – drying on sand beds or on paved or unpaved basins for a minimum of three months during which two of the three months have an ambient average daily temperature above 0 °C.
3. Anaerobic Digestion – maintained in anaerobic conditions for a mean cell residence time and temperature between 15 days at 35 °C to 55 °C and 60 days at 20 °C.
4. Composting – Using within-vessel, static aerated pile, or windrow composting methods to raise the temperature of biosolids to 40 °C or higher and maintained for five days, during which for four hours the temperature in the pile exceeds 55 °C.
5. Lime Stabilization – Sufficient lime is added to raise the pH of the biosolids to 12 after 2 hours of contact.

1.3.7 The Search for a Viral Indicator

Due to the health and method issues associated with human enteric virus measurements, surrogate viruses (e.g. bacterial viruses) are often used to assess the effectiveness of various wastewater treatments including biosolids stabilization. The surrogate viruses need to satisfy the following key requirements: (1) Follow similar behavior to that of enteric viruses, (2) Provide a quick and easy way to determine the concentration in a given sample (3) Do not replicate in the environment or indicate the presence of pathogens when none are present (4) Have limited pathogenic and infectious effects.

In pursuit of a good surrogate for enteric viruses, three different indicators have been previously explored: somatic coliphages, *B. fragilis* bacteriophages, and Male-Specific Coliphage (MSCs) or F-RNA bacteriophage (Sidhu & Toze, 2009). Somatic coliphages are advantageous due to their specificity to *E. coli* and abundance in wastewater but their broad host range and their potential to multiply in the environment makes them severely limited as an indicator of enteric viruses. *B. fragilis* bacteriophages showed high correlation in their behavior with Enteroviruses, but their concentrations tend to be two to three logs lower than the somatic coliphages (Gantzer, Maul, Audic, & Schwartzbrod, 1998). MSCs are the second most common bacteriophage in wastewater and do not replicate in the environment. MSCs appear to be more resistant to thermal inactivation than either *B. fragilis* bacteriophages or somatic coliphages; they are therefore a more conservative indicator of enteric virus presence and tend to undergo the same inactivation rates as enteric viruses during wastewater treatment processes (Lasobras, Dellunde, Jofre, & Lucena, 1999; Moce-Llivina, Muniesa, Pimenta-Vale,

Lucena, & Jofre, 2003). The method used to MSCs are direct, precise, rapid, facile, and cost-effective (Calci, Burkhardt, Watkins, & Rippey, 1998). MSCs were more accurate and stringent indicators of pathogen inactivation in biosolids than traditional fecal coliforms in a recent study (Viau & Peccia, 2009). Ultimately, MSCs seem to be the most ideal surrogates of enteric viruses at this time. The relative behavior of MSCs and indicator bacteria (i.e. FC and *Salmonella*) through wastewater treatment is not well understood. A comparison of MSCs, Fecal Coliforms, and *Salmonella* through wastewater treatment may assist in improving regulations and thus improve the safety of land-applied biosolids.

1.4 Lime Treatment for Stabilizing Biosolids

Alkaline or lime is often added to biosolids as a chemical treatment to stabilize the solids in lieu of other stabilization methods such as anaerobic digestion, aerobic digestion, or composting (Williford, Chen, Shamas, & Wang, 2007). Advantages of lime stabilization over other methods include low associated costs and the simplicity of operation. Liming processes aim to stabilize solids by increasing the pH to 12.0 for at least 2 hours—heightened pH levels inactivate pathogens and reduce odor.

1.4.1 Requirements for Class A/B Biosolids Utilizing Lime Treatment

Alkaline treatment of biosolids in the wastewater industry is conducted in one of two ways: pre-lime stabilization or post-lime treatment (Federation., 2011). Pre-lime stabilization can include two processes: 1) The process of adding lime slurry to liquid biosolids to satisfy Class B requirements and 2) Conditioning biosolids with lime before dewatering. The first method requires a greater volume of lime per dry ton of biosolids

limiting it to smaller wastewater treatment plants or those with short distances to land application sites. In the second method, lime is added in addition to other conditioners such as aluminum or iron salts to enhance dewatering and the amount of lime added typically exceeds those required for pathogen reduction.

Post-lime treatment is the process of adding lime to a dewatered cake to meet Class A or Class B requirements. In this case, lime is critically mixed into the dewatered cake using a screw conveyer, paddle mixer, or a similar mixing device. When the lime is then hydrolyzed by the cake, the pH increases and the solids are heated. Class B standards for lime stabilization requires a pH of 12 after 2 hours of contact. Class A standards requires a pH of 12 for at least 72 hours with temperatures of at least 52 °C maintained for 12 hours or 70 °C maintained for at least 30 minutes. After fulfilling the relevant pH, temperature, and time requirements for the desired class of biosolids, the solids are tested for fecal coliforms or *Salmonella* to determine their classification prior to their ultimate disposal.

1.4.2 Advantages and Disadvantages of Using Lime in Biosolids

Biosolids can be limed utilizing a variety of materials such as hydrated lime, quicklime, fly ash, lime, cement kiln dust, or carbide lime. Fly ash, lime kiln dust, and cement kiln dust are commonly utilized due to their availability and relatively low cost; quicklime is also commonly utilized due to its high heat of hydrolysis that can enhance pathogen destruction. Lime stabilized biosolids (LSB) can improve soils by remediating the pH, texture, and water capacity of the soil to which they are applied. LSB helps remediate the soil by lowering the acidity of acid soils due to acid mine drainage,

improve the nutrient value of the soil, and reduce elevated levels of heavy metals. LSB are also sometimes applied as the daily covers in landfill management.

Lime is the most commonly used alkaline material in the wastewater industry (Turovski*i* & Mathai, 2006). Lime application is advantageous compared to other stabilization processes due to its low cost, ease of management, equivalent pathogen reduction, associated odor reduction, improved dewaterability, and decreased mobility of certain metallic ions. However, compared to other stabilization techniques, lime stabilization does not reduce the amount of solids; LSB also has lower nitrogen and phosphorous concentrations than digested solids and may produce ammonia and other gasses.

1.4.3 Lime's Effectiveness in Pathogen Reduction

In terms of pathogen reduction, LSB reduces fecal coliforms, total coliforms, and fecal streptococci concentrations by more than 99.9%. *Salmonella* and *Pseudomonas aeruginosa* are typically reduced below detection limits (U. S. EPA, 2000). Virus destruction on the other hand, is poorly understood. A few studies have examined the effects of lime flocculation on virus removal from wastewater. Poliovirus at pHs greater than 11 were rapidly inactivated in one of these studies (Gerald Berg, Dean, & Dahling, 1968). Another study (Abu-Orf et al., 2004), looked at the reduction of viruses utilizing a combination of lime stabilization and anoxic conditions that reduce the amount of fecal coliforms, reoviruses, and helminth to Class A levels in 12 days at 100 g of lime per kg of biosolids. Bacterial indicators were found to be inactivated much more quickly than viruses and runoff from biosolid amended soils might lead to surface water contamination

and movement through the soil column to pollute underground reservoirs due to rain events that reduce the cation binding of the virus to the soil (Bean et al., 2007). MS2, a model virus for MSCs, were found to be a conservative indicator under alkaline treatment when compared to other viral pathogens such as adenovirus and rotavirus (Hansen, Warden, & Margolin, 2007). Although these studies demonstrate alkaline treatment is effective at inactivating pathogenic microorganisms, they also point out a need for better understanding of the fate and mechanisms behind the inactivation of bacterial indicator and viruses.

1.4.4 Homogeneity of Limed Biosolid Sample and Sampling Size

Due to the heterogeneity of the way lime is distributed in limed biosolids, the sampling procedure likely impacts the ultimate level of pathogens and indicators detected in the grab samples. Of particular importance, is the sample size. Due to the presence of zones void of lime chips, pathogen indicators that are still viable in the biosolids may persist within the biosolid matrix, in localized zones with pH values below 12. The extent of biosolids mixing after lime application and the moisture level can influence the concentration of microorganisms due to the distribution of lime within the biosolid matrix and the movement of these zones to other parts of the biosolids. Therefore the effect of sampling size and the homogenous distribution of lime within the matrix are important factors in determining the concentration of viruses and therefore the safety of the biosolids.

1.4.5 Aerosol Risk Associated with the Land Application of Biosolids

In addition to presence/absence of pathogens in the biosolids, further consideration must be given to possible environmental transmission routes (i.e. wind, water runoff) for infectious agents during and after land-application. One study (Viau & Peccia, 2009) found that pathogenic genomes survive aerosolization and are transported through aerosols; they may therefore pose a risk to surrounding communities where biosolids are being applied. In another study (Baertsch, et al., 2007), DNA-based microbial source tracking was performed on aerosols released from biosolids during high wind events (classified as greater than 5 m/s). This study showed that biosolids were found to be present in 56% of the downwind samples compared with 3% of upwind samples. Another study, however, concluded that the annual risk of infection by bioaerosols was only 0.04%. Few studies have examined the risks for workers who apply the biosolids, with one study finding a 34% chance of coxsackievirus A21 infection and a 2% chance of *Salmonella* infection (Brooks et al., 2005). These results indicate that viruses are sometimes present in biosolids and may lead to occupational and residential exposure.

1.5 Study Objectives

In summary, knowledge gaps continue to exist in our collective knowledge on virus fate in biosolids—more information is therefore needed to optimize resource recovery from wastewater treatment while maintaining public safety. First, although the removal of viruses at various steps and cumulatively over the wastewater treatment process has been analyzed, but a holistic population balance and survey of viruses across an entire WWTP has not yet been conducted. Second, the risks of viral exposure from

biosolids through handling or aerosolization after their transport from the wastewater remain an important area of inquiry. Last, due to the widespread use of liming methods in biosolids stabilization, a better understanding of sampling size and representative samples when determining biosolids quality is needed to insure public safety.

In this study, we attempt to address these issues by 1) providing a population balance of Male-specific coliphages through an advanced wastewater treatment plant utilizing nitrification/denitrification, multi-media filtration, and disinfection, 2) assessing the effectiveness of liming and alkalinity on Male-specific coliphage infectivity, and 3) investigating virus homogeneity in biosolids produced by an advanced wastewater treatment plant utilizing lime stabilization.

Chapter 2: Materials and Methods

2.1 Materials

The following paragraphs list the chemicals, supplies, and equipment that were used through the course of this study.

2.1.1 Chemicals

All chemicals were used as received without further purification. The following items were purchased from BD Difco (Sparks, MD): Bacto Agar (214010), Bacto Tryptic Soy Broth (211822), Bacto Yeast Extract (288620), Beef Extract Powder (212303), mFC Broth Base (288320), Rappaport-Vassiliadis (MSRV) Medium Semisolid Modification (218681), Novobiocin Antibiotic (231971), Rosolic Acid (232281), and Tryptone (211705). Additional items were purchased from Acros Organics (Fair Lawn, NJ): Aluminum Chloride (195785000), Calcium Chloride (349615000), and Chloroform (383760010). Other items were purchased from Fisher Scientific (Pittsburg, PA): Hydrochloric acid (A142-212), Sodium Phosphate Dibasic (S373-500), Sodium Chloride (BP358-1), and Sodium Hydroxide (S320-1). Citric Acid (0529-500G) and Glucose (0188-1KG) were purchased from Amresco (Framingham, MA). Ethyl Alcohol (DSP-KY-417) was purchased from Pharmco-AAPER (Brookfield, CT). Lastly, Polyethylene Glycol-MW: 6,000 (A17541) was purchased from Alfa Aesar (Ward Hil, MA).

2.1.2 Supplies

All the supplies were used as received without modification: The following items were purchased from Millipore (Billerica, MA): 0.22 μm filter (GSWP047S0), 0.45 μm

filter (HAWP047S0), Swinnex 47 mm filter holder (SX0004700), 0.1 μm syringe filters (SLVV033RS), and 0.45 μm syringe filters (SLHA033SBA). Additional supplies were purchased from Fisher Scientific (Pittsburg, PA): 16 oz Specimen Storage Container (14955117A), 100-1000 μL pipet (HH34076), 10-100 μL pipet (HH16525), 1-10 mL pipet (HH16851), 48 well 5 mL blocks (14-222-874), Centrifuge Nalgene Bottles (05-564-1), Culture tubes (14-959-25E), Falcon Tubes (352070), Petri Dishes (875713), and Pipette Boy (111253). Supplies purchased from Monoject (Mansfield, MA) were: 12 mL leur-lock syringe (8881-512878) and 12 mL Syringe (888-1112059). Lastly, a 100-1200 μL multi-channel pipet (L-1200 XLS) was purchased from Ranin (Columbus, OH).

2.1.3 Instruments

Instruments used in this study include an Isotemp Incubator (6845) from Fisher, UV-Vis Spectrophotometer (Cary 60 UV-Vis) from Agilent Technologies (Santa Clara, CA), pH Meter (Accumet Basic AB15) from Fisher, Stir plate (SP131325) from Thermo (Pittsburg, PA), Microbalance (PI-114) from Denver Instruments, Balance (P-8001) from Denver Instruments (Bohemia, NY), Tabletop Centrifuge (Sorvall Legend Micro 21R) from Thermo, High-Speed Centrifuge (Sorvall RC 6+) from Thermo, Autoclave (Systec VE-150) from Microbiology International (Frederick, MD), and Water Baths (2345) from Thermo.

2.2 Wastewater Treatment Plant and Sampling Protocol

2.2.1 Overview of the Wastewater Treatment Processes

Samples from a Mid-Atlantic Wastewater Treatment Plant were collected on a weekly basis for analysis. The WWTP treats municipal wastewater for 1.6 million people

and processes 300 million gallons of water per day (MGD). The treatment process includes five major steps: preliminary, primary, secondary, tertiary, and disinfection. The preliminary step at this plant includes an addition of ferric chloride for coagulation. The plant includes a tertiary treatment processes with nitrification and denitrification tanks to reduce nitrogen levels and tertiary clarifiers to retain flocs generated during treatment. Disinfection processes at the plant are comprised of filtration through a multi-media filter that contains sand and carbon, disinfection with sodium hypochlorite, and then dechlorination prior to discharge. The solids generated from primary and secondary treatment are collected and blended together with tertiary solids. The blended solids are put through gravity thickeners, dissolved air floatation, doped with polymer, and then centrifuged to form biosolids, which are then treated with quicklime (Calcium Oxide) to reduce odor and pathogens before they are shipped offsite for final disposal. In 2009, for example, 419,368 tons of biosolids were generated on site with 376,806 tons applied to various farmlands throughout Maryland and Virginia, and landfilling 4,893 tons (Water, 2009).

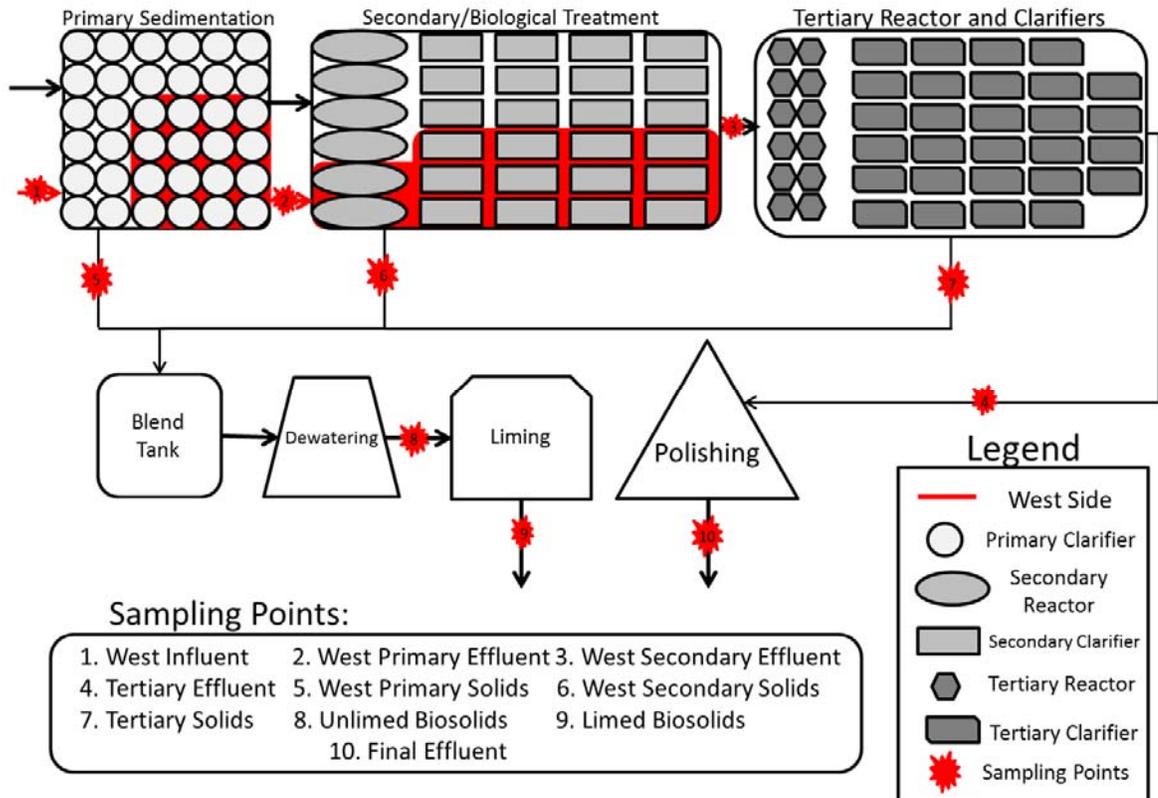
2.2.2 Sampling Points and Transportation Procedure

For the population balance study of bacterial and viral indicators, water samples were collected in 16 oz (473 mL) specimen storage containers weekly for two months at the following points of the wastewater treatment plant: influent, primary effluent, primary solids, secondary effluent, secondary solids, tertiary solids, tertiary effluent, final effluent, unlimed biosolids, and limed biosolids. The samples were put into a 5-gallon bucket filled with ice to inhibit biological growth and samples were processed within 2 hours after collection. For the study on the effects of liming on the infectivity of Male-

specific coliphages, samples were taken weekly for four months from the unlimed and limed biosolids. For the study on the homogeneity of biosolids, samples from the unlimed and limed biosolids were taken weekly from August to October of 2012.

For the study of the population balance of coliphages in the wastewater treatment plant, liquids, solids, and biosolids samples were analyzed to capture the effect of various treatments on Male-specific coliphages. The sampling points (Figure 2.1) were developed in consultation with plant staff. In this particular treatment plant, the influents are separated into two streams for primary and secondary treatment and are later combined prior to nitrification/denitrification. Sampling was therefore done at one of the streams for influent, primary effluent, primary solids, secondary effluent, and secondary solids. The methods for collecting the liquids, solids, and biosolids are outlined below.

Figure 2.1 Wastewater Treatment Sampling Schematic



2.2.3 Liquid and Solids Sampling Procedure

The liquid samples were collected with the use of an orange 2 L plastic bucket tied to a 50 feet rope. For the influent sample, the bucket was tossed into the influent stream and rinsed multiple times prior to obtaining the sample. Primary, secondary, and tertiary effluent were collected by tossing the bucket into basins after the treatment clarifiers and then drawn up to be put into sampling cups. Each sample was collected after rinsing the sampling cups with the liquid in the 2 L bucket to prevent any contamination and reactivity. The solids samples were collected from sampling ports that pump the sludge from the bottom of reactors for total solids analysis. The sampling cups were again rinsed multiple times with the solids solution to prevent contamination. The

biosolids were collected after dewatering and centrifuging at two points: prior and after lime introduction with the use of a T-shaped auger. All of the samples were then put on ice and transported back to the lab at the University of Maryland, College Park and processed within two hours.

2.3 Bacterial and Viral Assays

2.3.1 Bacterial Assay Procedure and MPN Calculation

Bacterial measurements for Fecal Coliforms and *Salmonella* were processed following a modified version of EPA's Method 1680 (U. S. EPA, 2005). All equipment was autoclaved prior to use to prevent contamination. The liquid and solid samples were first vigorously shaken to improve homogeneity. Quadruplicate samples of 0.5 mL were then taken and mixed into 4.5 mL of LTB Broth (Table 2.1) in wells for Most Probable Number (MPN) analysis. The blocks were comprised of 8 by 6 rows and each were 5 ml deep. Each block was filled with 4.5 mL of LTB broth and a serial dilution was performed using a multi-channel pipette at 0.5 mL to perform up to 12-log dilutions. The blocks were incubated overnight at 37 °C and then checked for bacterial growth by increased turbidity. A 50 uL sample aliquot of the sample was then plated onto MSR/V with Novobiocin or mFC Broth with agar (1.5 grams/100 mL) and Rosolic Acid for *Salmonella* and Fecal Coliforms, respectively. The plates were incubated at 42 °C overnight and then checked for the presence of *Salmonella* (white spots) and Fecal Coliforms (blue spots). Biosolids samples were analyzed with the procedure with the inclusion of an initial liquification step (30 grams of biosolids in 270 mL of deionized water). The limed biosolids were then neutralized using diluted hydrochloric acid and sodium hydroxide (40 g/L) until the pH reached 7.0. These liquefied samples were

then inoculated into the MPN blocks as described above. Positive controls were verified with strains generously provided by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) with the following bacterial strains: *E. Coli* with green fluorescent protein (USDA 4321) and *Salmonella* Typhimurium (ATCC 13311) for *Salmonella*.

For MPN analysis, samples are serially diluted and incubated in growth serum to estimate bacterial concentrations. The presence of bacteria was indicated by the turbidity of the solution after overnight incubation. The MPN values are determined by entering the number of positive and negative wells and the equivalent grams into an MPN calculator. This method assumes a few conditions), which estimates the density of microorganisms in a liquid assuming (1) that organisms are randomly distributed and (2) that the culture medium is certain to exhibit growth (Cochran, 1950).

2.3.2 Viral Assay Procedure and Controls

Viruses were extracted from solids using ASTM Method D4994-89 and liquid samples were directly plated for virus enumeration. The virus was extracted from sludges in each treatment by adsorption, with, 100 mL of solids added to a beaker and acidified to pH 3.5. 1 mL of AlCl₃ (0.091 M) was then added to the sample to encourage the coagulation and settling of the solids. Samples were shaken on a rotor for 30 min and then centrifuged at 2500× g for 15 min. The supernatant was decanted and 100 mL of 10% beef extract solution (Table 2.2) was added to the solids, mixed again for 30 minutes, and then centrifuged at 10,000× g for 30 mins at 4 °C. The resultant supernatant was then filtered through a 47 mm, 0.22 µm filter and plated for viruses. Biosolids sampling followed a similar procedure, but due to their dewatered and centrifuged state;

the biosolid virus extraction followed the procedure outlined elsewhere (Viau & Peccia, 2009). In brief, 6 grams of biosolids was first mixed with 25 mL of 10% beef extract (Table 2.2) for 2 hours. After mixing, the mixture was centrifuged for 30 mins at 10,000× g at 4 °C, followed by filtration through a 47 mm, 0.22 µm filter, and then plated.

Positive controls and stocks for male-specific viruses were created using bacteriophage MS-2 (ATCC 15597-B1) with *E. coli* as the host (ATCC 15597) by following the process outlined in (Brennecke, 2009). The host used in the detection of MSC was *E. coli Famp* (ATCC 700891) using the double-layered agar method (American Public Health, American Water Works, & Water Pollution Control, 2005). Briefly, the double-layered plaque assay is performed by first creating a hard base layer of agar containing growth media with streptomycin and ampicillin. A soft agar tube containing the same growth media is inoculated with 100 ul of *E. coli Famp* (*Famp*) and the environmental sample. The mixture is then poured on top of the hard agar layer and allowed to solidify and then incubate overnight at 37 °C. Plaques that form due to the presence of viruses are enumerated the following day. Serial dilution of the environmental samples are plated to determine the concentration of MSCs.

2.3.3 Detection Limit and Daily Load Calculations

The holistic determination of bacteria and viruses at a WWTP is viewed through the daily load that the plant received. This value is calculated by multiplying the known concentration with the daily flow rates measured at each of the processes. This unit gives a comparable look at the total amount of bacteria and viruses present across the entire treatment works. The minimum daily load that can be detected is derived from the lowest concentration detected through the bacterial and viral assays. This detection limit is

calculated by finding the lowest possible detection of bacterial and viral indicators, 1 MPN or PFU per gram or 500 μL multiplied by the daily flow rate. From these values we get the detection limit for the various media are for the liquids (2.1×10^{15} PFU), primary solids (1.1×10^{14} MPN), secondary solids (1.3×10^{14} MPN), denitrification solids (8.6×10^{14} MPN), and biosolids (2.0×10^{10} MPN).

2.3.4 Lime Removal Experiments

To determine the extent of indicator organism inactivation in different zones of the Lime Stabilized Biosolids (LSBs), the solids were spread out in a tray and lime was selectively removed (Figure 2.2). The delimited biosolids were then randomly sampled and assayed following the same biosolid extraction procedures described above.

Figure 2.2 Screening of Picked Out Limed Biosolids



The pile on the left shows the limed biosolids and the pile on the right shows the biosolids after lime has been removed.

2.4 Recovery Measurements

Male-specific virus recovery measurements were performed on solid samples collected at the wastewater treatment plant by spiking known amounts of MS2 (male specific coliphage) and measuring its concentration. For primary, secondary, and nitrification solids, 100 mL of the sample was added to centrifuge bottles and then spiked and mixed with MS2 (at least one order of magnitude above background MSC levels). For biosolids samples, 12.5 mL of virus dilution buffer was first added to liquefy the biosolids prior to virus spiking and then 12.5 mL of 20% beef extract solution was added; this ratio of beef extract to sample volume was the same ratio used to extract viruses from the unspiked biosolids samples.

2.5 Sampling Variation Experiments

To determine the homogeneity of the biosolids, various sample sizes were randomly taken from biosolids and measured for virus and bacterial indicator concentrations. For the virus analyses the following samples sizes were tested: 2 grams, 3 grams, 6 grams, 30 grams, and 60 grams. For bacteria analyses, the following sizes were tested: 1 gram and 10 grams. The virus extraction procedure followed the biosolid extraction procedure described above and the bacterial measurements were performed using the modified EPA Method 1680.

Table 2.1 Tryptone Media Recipe per 100 mL

Ingredients	Amount	Units	BD Number	Notes
Tryptone	1	gram	211705	
Yeast Extract	0.1	gram	288620	
Glucose	0.1	gram	0188-1KG	
Sodium Chloride	0.8	gram	BP358-1	
Calcium Chloride	0.022	gram	349615000	
Agar (Top)	0.7	gram	214010	
Agar (Bottom)	1.5	gram	214010	
Ampicillin	1	mL	0339-25G	0.15 g/100mL
Streptomycin	1	mL	61224-0500	0.15 g/100mL

Table 2.2 Beef Extract Recipe per 100 mL

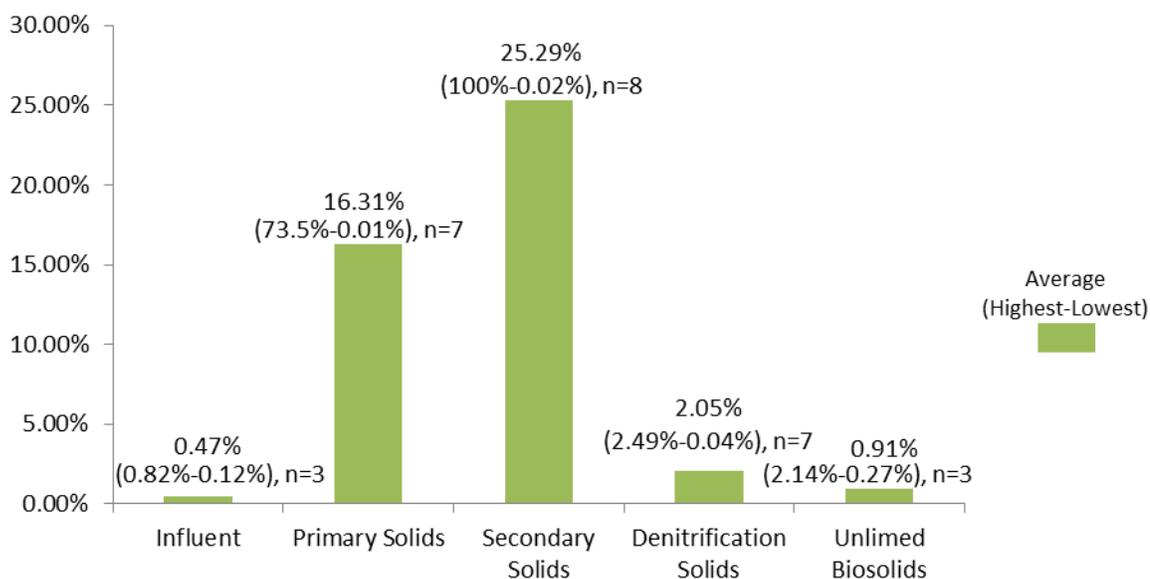
Ingredients	Amount	Units	BD Number
Beef Extract	10	gram	212303
Citric Acid	0.12	gram	0529-500G
Sodium Phosphate Dibasic	1.34	gram	S373-500

Chapter 3: Results and Discussion

3.1 Recoveries

To better estimate the concentration of viruses in the collected samples, the virus extraction recovery was determined for all of the wastewater matrices (Figure 3.1). The highest recovery was seen in secondary solids (n=8) and the lowest recovery was seen in the influent wastewater (n=2). In general, recoveries varied significantly amongst the replicate samples, especially for the primary and secondary solids. Despite the variation from one day to the next (presumably due to differences in the sample characteristics), the relative MSC recoveries from the different solids were constant on each sample day, with the recovery of virus from secondary solid recovery > primary solids recovery > nitrification solids recovery.

Figure 3.1 Virus Recovery Percentages for Various Media



The observed variance in viral recovery rates may be due to several reasons, including the dynamic nature of wastewater samples. The influent samples were spiked with a known concentration of viruses that were then mixed and assayed. Virus were

spiked into the solid mixtures and mixed prior to the addition of acid and aluminum chloride—this was to insure the extraction process mirrored the process of the unspiked samples. Primary solids have the lowest residence time of the three wastewater treatments. Perhaps due to the fact that this process is at the beginning of the wastewater treatment process, variable solid content and therefore virus recoveries are due to the changing nature of the influent. The secondary solids are comprised of both return activated sludge and nitrification/denitrification sludge making it a complex ecosystem with active microbial communities of both bacteria and viruses. The nitrification solids are comprised of nitrifiers and denitrifiers in an anoxic environment that is more selective than aerobic secondary solids. These unique characteristics may cause the lower nitrification solid recoveries. It should be noted that we did not test the recovery of viruses from limed biosolids due to the fact that liquefying limed biosolids lead to a rapid increase in pH, which rapidly inactivated viruses.

Poor recoveries may be due to a number of reasons, including that virus may be inactivated when they interact with the solids or may be bound so strongly to the solid particulates that they are not extractable. A few studies have looked at the recovery rates of viruses from biosolids with maximum recovery rates of 24.6% for Poliovirus I in anaerobically digested Class B biosolids (C. P. Gerba, A. Ross, K. Takizawa, & I. L. Pepper, 2011) and 16% from somatic coliphages in anaerobically digested biosolids (Murthi, Praveen, Jesudhasan, & Pillai, 2012). Both of these recovery rates are much greater than the recovery obtained in this study; suggesting a high variability and extraction rate across biosolid types. The variability in the various solids and influent may limit the measured viral concentrations in each sampling event and it is suggested

that parallel to the viral measurements, recovery measurements also be made to determine the recovery percentage for that particular sample.

These large variations in recovery percentages demonstrate a need to perform recovery studies whenever measuring viruses in environmental samples. They also highlight the degree of confidence in the measured MSC concentrations. It is well-acknowledged that improved virus sampling/extraction methods are needed in the future (Murthi, et al., 2012) as is a better understanding of the mechanisms that underlie extraction processes (C. P. Gerba, et al., 2011).

3.2 Fate of Indicators in the Wastewater Treatment Process

3.2.1 Fecal Coliforms

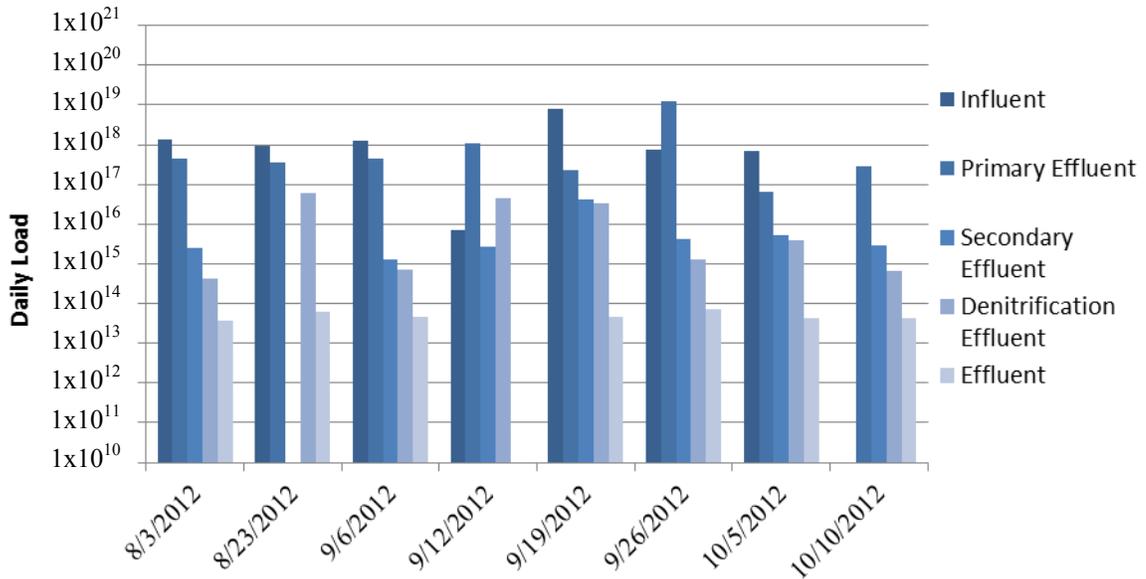
In order to establish how fecal coliforms (FC) behave in a wastewater treatment utility, samples from each treatment step were measured for FC and a population balance was performed across the entire treatment plant. The samples were collected on a weekly basis (when available) during the summer and fall of 2012.

3.2.1.1 Fate of Fecal Coliforms in Wastewater Effluents

Figure 3.2 shows the daily load of fecal coliforms in the liquid stream of the wastewater treatment processes. The daily load of fecal coliforms decreased from influent to effluent, with the influent FC daily loads around 1×10^{17} - 1×10^{18} MPN/day and the final effluent daily loads around 1×10^{13} - 1×10^{14} MPN/day. As such, approximately 4-log fecal coliform removal was achieved through the liquid side of wastewater treatment. The FC daily load minimum detection limit in liquids was approximately 4×10^{11} per day; this was

calculated by multiplying the lowest MPN obtainable (0.25 MPN/gram or mL) by the flow rate.

Figure 3.2 Fecal Coliforms in the Liquid Compartment



The MPN values in liquids were obtained by multiplying the MPN value from samples by the flow rate going into each process. The flow rate was calculated by converting the millions of gallons a day into liters per day. Then multiplying the literes per day by the MPN/L measured to obtain the daily load value. Samples for the secondary effluent from 8/23 and the influent from 10/10 were not included due to contamination and WWTP construction issues, respectively.

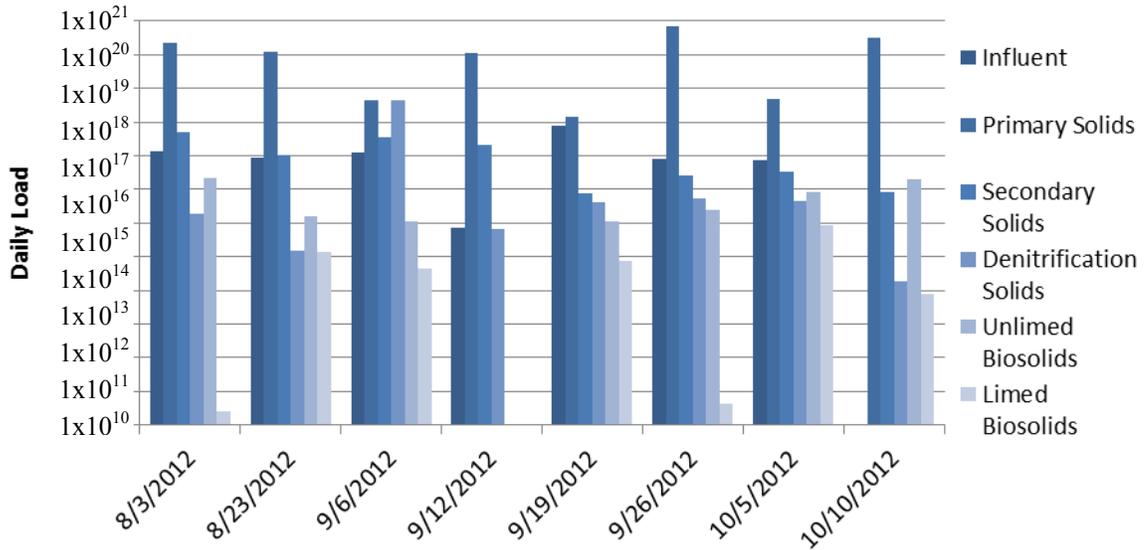
Two atypical events were observed on 9/12 and 9/26 in which the fecal coliform loads increased between the influent and primary treatment. This phenomenon may be explained by sampling errors or by temporary FC population growth in the primary settling tank. Regrowth of FC may occur in water with high nutrient levels and at least one previous study demonstrated fecal coliform growth in non-refrigerated wastewater samples (George, Petit, Theate, & Servais, 2001). The average log reduction between the influent and effluent was 4.47 (99.99%). Sampling conducted in Canada showed similar log removal of 4.3-5.7 logs for FC in an advance wastewater treatment plant (Zhang & Farahbakhsh, 2007). With respect to the individual treatment steps, the greatest log

decrease in the liquid train of FC loads occurred in the secondary treatment with an average decrease of 2.11 log units. Reductions in fecal coliform levels in WWTP processes have been attributed to the competitive advantage that nonpathogenic organisms have over *E. Coli* (Rose, Dickson, Farrah, & Carnahan, 1996). In past studies, FC were mostly removed in secondary treatment and very little removal took place in primary treatment (George, Crop, & Servais, 2002). Another large decrease in FC in the liquid train was observed between the denitrification effluent and the final effluent, with an average log decrease of 1.79. This was expected, as the step includes filtration through a multimedia filter and chlorination.

The National Pollutant Discharge Elimination System (NPDES) permit sets the discharge limits of fecal coliforms to be at 200 MPN per 100 mL; that corresponds to a daily load of approximately 2×10^{12} . All of the daily loads taken of fecal coliforms in Figure 3.2 were higher than expected, in respect to the established limit. However, this cannot be equated to regulatory standards due to the difference in methodology required by EPA. In addition, some studies have shown that fecal coliforms include coliform genera such as *Klebsiella*, which account for less than 3% of fecal flora but show up as positive under elevated fecal coliform incubation (Elmund, Allen, & Rice, 1999).

3.2.1.2 Fate of Fecal Coliforms in Wastewater Solids

Figure 3.3 Fecal Coliform in the Solids Compartment



Note: Sampling date 9/12 lacked biosolids data due to system maintenance

The solids treatment train was also analyzed in order to obtain an overall population balance of fecal coliforms at the plant (Figure 3.3). The highest concentration of FC was always in the primary solids; in fact, primary solids FC levels were often greater by several logs. The greatest reduction in FC load took place in the liming steps on every sampling day (Figure 3.3). Higher load quantities were always present in the solids train (Figure 3.3) compared the liquid train (Figure 3.2).

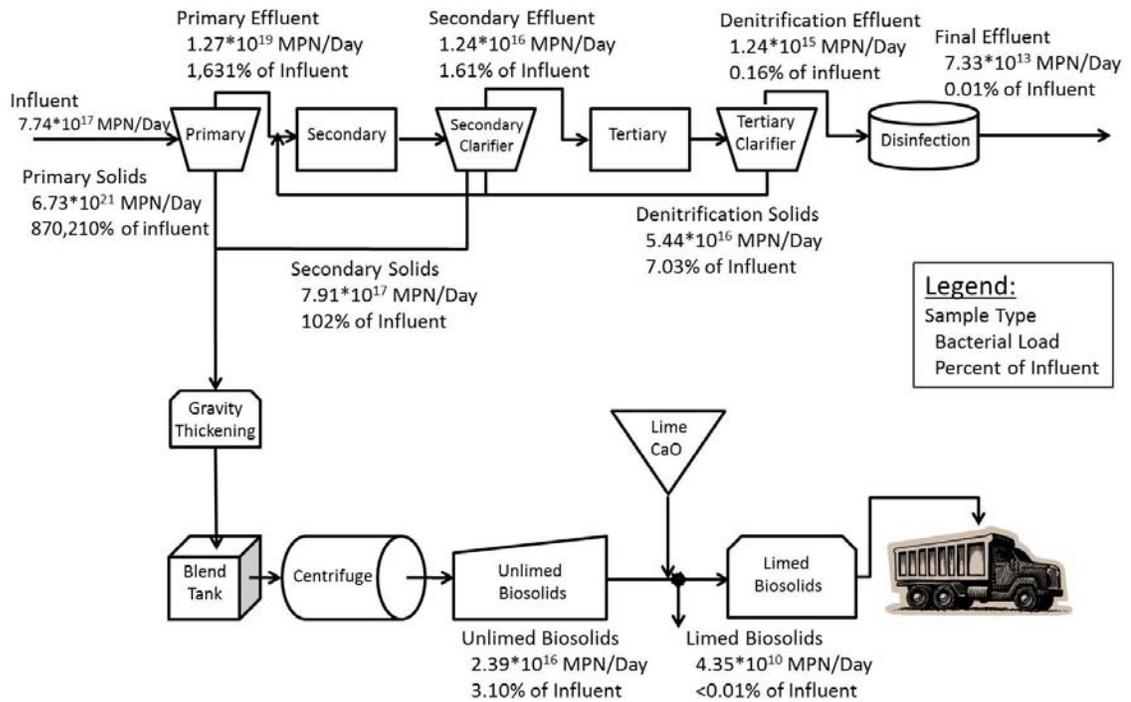
Despite the seemingly erratic behavior of fecal coliforms in solids, a general decreasing trend in fecal coliforms loads occurs through the treatment plant (Figure 3.3). The average FC log reduction through the solids treatment train (e.g. from influent to limed biosolids) was 4.51 (99.99%), although this value varied significantly on the different sampling dates. The solids liming process resulted in the greatest reduction of FC, with an average reduction of 2.95 logs. FC growth was observed in the primary

solids collection process (+2.80 logs). The increase in FC loads in the primary solids suggests fecal coliforms grow in the primary reactor. The increase of fecal coliforms in primary treatment (G. Berg, 1978) and in the centrifugal dewatering of digested biosolids (Qi, Dentel, & Herson, 2007) has been reported previously. A slight increase in FC loads was observed between the denitrification solids and the unlimed biosolids: to the author's knowledge, this has not been reported previously.

3.2.1.3 Plantwide Fecal Coliform Concentrations in One Day

A snapshot of the liquid and solid train fecal coliform data from September 26, 2012 provides a population balance and comparison of the Fecal Coliforms (FC) through the liquid and solid treatment trains of a wastewater treatment plant (Figure 3.4). We report the bacterial loads as both overall MPN/day and as relative percentage of the influent levels.

Figure 3.4 Fecal Coliform Sampling Snapshot 9/26/12



Significant log reduction of fecal coliforms took place through both the liquids and solids treatment processes, with daily log removals of 4.02 and 7.25, respectively. The highest daily load was observed in the primary solids (6.73×10^{21} MPN) and the lowest daily load was observed in the limed biosolids at 4.35×10^{10} MPN. This snapshot data suggests that fecal coliforms have a stronger affinity for solids compared to liquids and that solids treatment is effective at reducing the overall FC levels. Previous studies have demonstrated that sediments tend to have 100 to 1000 fold higher FC concentrations compared to the overlying water column (Ausland, Stevik, Hanssen, Kohler, & Jenssen, 2002; van der Drift, van Seggelen, Stumm, Hol, & Tuinte, 1977).

The higher FC levels in the primary effluent and primary solids compared to the influent indicate FC growth took place in the primary treatment processes. The high BOD levels of the water provide an ideal environment for FC to replicate.

The secondary effluent and secondary solids both have reduced FC levels compared to the primary effluent. Various studies have attempted to describe the mechanisms responsible for FC removal in activated sludge processes. Based on these studies, two key factors have been identified; first, the bacteria are rapidly adsorbed onto sludge flocs during the first hour of the activated treatment processes (van der Drift, et al., 1977). Subsequently, ciliates (the dominant protozoa in activated sludge) predate on the available FC in the activated sludge (Curds, 1973). Ciliates are very effective at reducing FC; *E. coli* had a half-life of 16 hours when activated sludge was void of ciliates, but the half-life plummeted to 1.8 hours (Curds & Fey, 1969) when ciliates were present. In the current study, the combined retention time in the secondary reactor was

around 4.5 hours; both flocculation and predation are therefore expected to take place during secondary treatment.

Tertiary treatment did not lead to significant FC inactivation, although substantial partitioning to the tertiary solids is observed. Chlorine and filtration led to an average log reduction of 1.72. The average 2.31 log reductions from liming were likely due to cellular membrane rupture from high alkaline conditions.

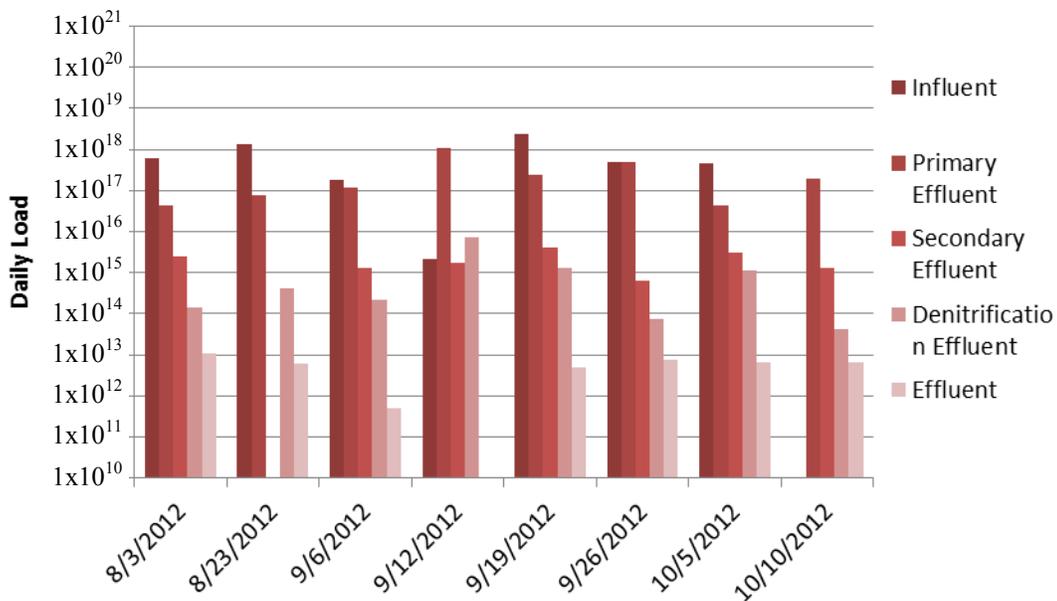
3.2.2 Plantwide *Salmonella* Concentrations in One Day

Similar to the fecal coliform (FC) analysis, liquid and solid samples from each treatment were taken and a population balance was performed for *Salmonella*.

3.2.2.1 Fate of *Salmonella* in Wastewater Effluents

A decreasing trend in *Salmonella* daily load occurred through the liquid treatment train (Figure 3.5); similar to what was observed with fecal coliform.

Figure 3.5 *Salmonella* in the Liquid Compartment



The average log reduction between the influent and effluent was 5.16 (99.999%). The greatest log removal in the treatment process took place in the secondary treatment with an average removal of 2.00 logs. Past studies on the reduction of *Salmonella* in wastewater treatment plants have reported wide ranges, with a 1 log reduction reported at a secondary treatment plant in a Dutch city (Kampelmacher & Noorle Jansen, 1970) and 6.5 log reduction observed in the summer and 2.6 logs in the winter at the Natural Resources Research Institute's alternative treatment system test facility in northern Minnesota (Pundsack et al., 2001). Here, the average *Salmonella* decrease in primary treatment was 51 %: previous studies of *Salmonella* showed approximately 70% - 80% reduction of *Salmonella* in primary treatment (Yaziz & Lloyd, 1979). The average reduction of *Salmonella* in the secondary treatment was 99%; previously, one to two log reductions of *Salmonella* was observed in activated sludge treatment (Koivunen, Siitonen, & Heinonen-Tanski, 2003; Yaziz & Lloyd, 1982); here we showed similar average of reductions of 51% in primary treatment and 2 log reduction in activated sludge treatment.

It should be noted that the daily load detection limit for the *Salmonella* in liquids is calculated to be at 4×10^{11} per day, calculated by multiplying the lowest MPN obtainable (0.25 MPN/gram or ml) by the flow and recovery percentage.

In the liquid stream, *Salmonella* loads were very similar to FC on all sampling days and in all treatments. The daily load of FC and *Salmonella* follow similar trends in both the liquid and solid treatment trains. The strongest agreement between the two indicators in the liquid stream is between the influent and denitrification steps; the correlation becomes weaker at the effluent. This may be due to possible regrowth of FC

in the final step of the treatment as FCs have undergone regrowth in chlorinated effluents (Shuval, Cohen, & Kolodney, 1973). Historically, FC and *Salmonella* have been well correlated with one another; in one study, for example, when FC was detected at low concentrations [less than 200 per 100 mL] with *Salmonella* was detected sparsely [6.5%-31% of the time], but when FC was detected at higher concentrations [greater than 1000 per 100 mL] *Salmonella* was detected twice as often [55.8%-89.5%] (G. Berg, 1978).

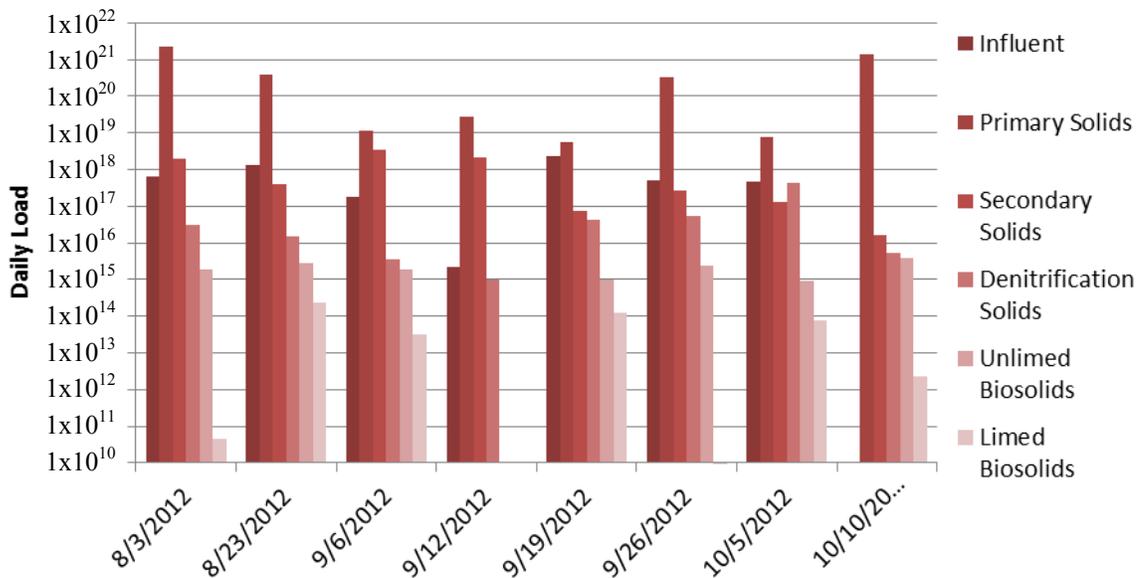
Some analysis error is likely due to the small sample size of five hundred uL—results from these small volumes were extrapolated to the entire plant with hundreds of millions of gallons per day. Attempts were made to minimize error such as sampling during the same time of the day and week.

3.2.2.2 Fate of *Salmonella* in Wastewater Solids

Salmonella daily loads in the solids treatment train showed an increase in the primary solids and then steadily decrease as the treatment train progresses. The average log reduction in *Salmonella* from influent to limed biosolids was 5.17 (99.999%). A high reduction in *Salmonella* was found in the liming process with an average reduction of 2.62 logs.

FC and *Salmonella* are highly correlated in terms of their behavior in the solids treatment processes. As with FC, the *Salmonella* preferentially partition to the solids, with approximately 10× higher loads in the solids steps compared to the liquid steps. Like observed in the FC and *Salmonella* liquid train samples, the solids *Salmonella* average daily loads were approximately 1 log lower than the solids FC daily load.

Figure 3.6 *Salmonella* in the Solids Compartment



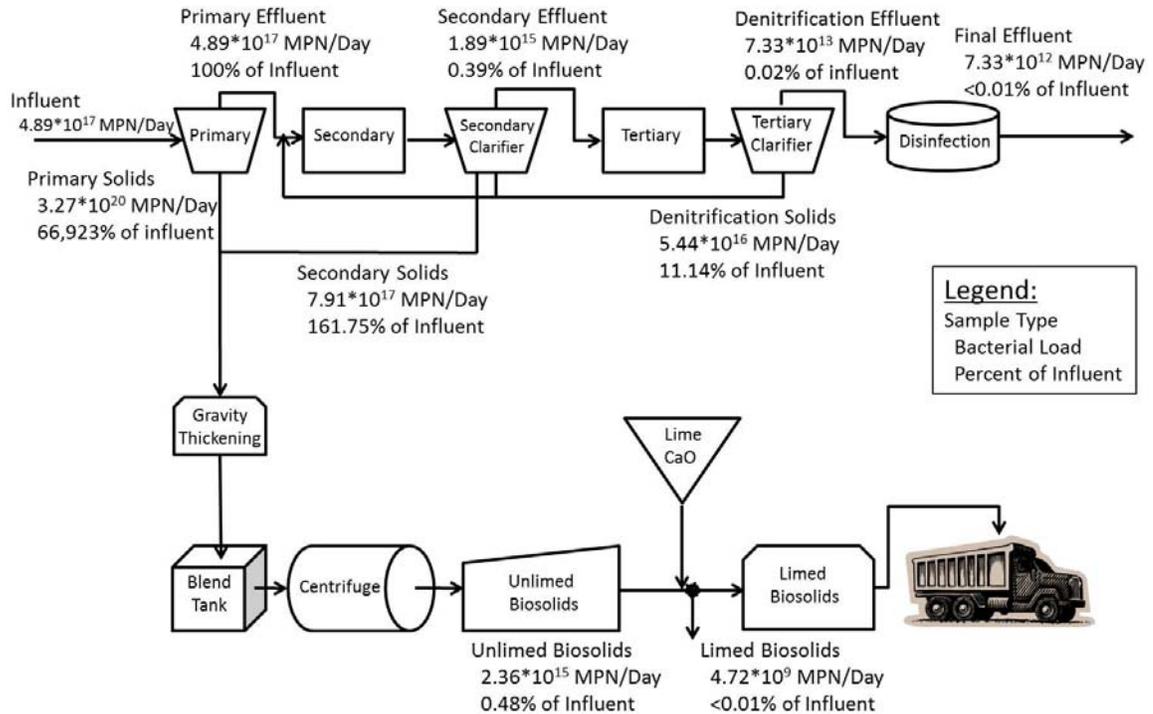
Both FC and *Salmonella* (Figures 3.2 and 3.6) loads increase in primary solids compared to influent levels. These increases indicate that FC and *Salmonella* are growing in the primary treatment process. After the increase of FC and *Salmonella* in the primary solids, secondary solids and denitrification had lower daily loads. In the secondary solids, FC and *Salmonella* loads are in large agreement with each other averaging a daily load of approximately 10^{17} . The denitrification solid shows the only time where the daily loads of *Salmonella* was found to be greater than that of FC by an average of 0.41 logs (the outlier on September 6th was not included in this calculation). In the limed biosolids, the behavioral trend between the FC and *Salmonella* is similar with FC, maintaining a higher daily load than *Salmonella* by an average of 0.99 logs.

3.2.2.3 Plantwide *Salmonella* Population Balance on a Single Day

Figure 3.7 provides a snapshot of *Salmonella* loads at all the sampling points on September 26. The highest daily load levels were detected in the primary solids

(5.70×10^{18} MPN) and the lowest daily load was in the limed biosolids (4.72×10^9 MPN). The liquid treatment train removed approximately 4.5 log and the solids trains removed approximately 7.55 log.

Figure 3.7 *Salmonella* Snapshot: 9/26/12



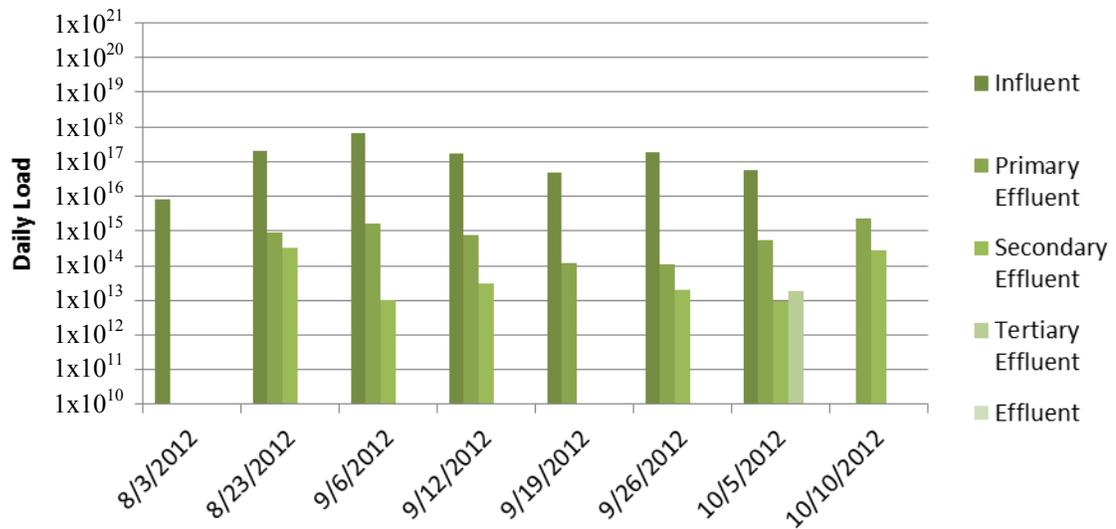
Salmonella generally has a lower daily load in the solids than fecal coliforms while they have approximately the same daily loads for the liquid stream. Lower *Salmonella* numbers have been observed before; in fact, low FC concentrations is a good indicator of the absence of *Salmonella* (G. Berg, 1978; Hood, Ness, & Blake, 1983). The decrease in numbers of *Salmonella* with lime was very similar to the decrease in fecal coliforms (average log reductions of 2.62 and 2.95, respectively). Overall, the behavior of *Salmonella* and fecal coliforms are similar each other and have similar sample variations across the sampling period.

3.2.3 Male-Specific Coliphages (MSCs)

3.2.3.1 Fate of Male-Specific Coliphages in Wastewater Effluents

The fate of Male-specific coliphages in an advanced wastewater treatment plant was investigated using a similar sampling schedule to the bacterial indicators above to ascertain the behavior of how viruses are transported through the wastewater treatment processes. In the liquid train portion of the study, the highest loads of MSCs occur in the influent, with an average daily load of 1.68×10^{17} (corresponding to 810 PFU/mL). In general, MSCs were not found after secondary treatment and no MSCs were found in any of the effluent samples tested. The detection limit for MSCs in liquids was 9.81×10^{12} which was calculated by multiplying the lowest PFU obtainable (1 PFU/500 ul) by the flow and recovery percentage.

Figure 3.8 Male-Specific Coliphage in the Liquids Compartment



The daily load of MSCs decreased throughout the liquid wastewater treatment train with all of the effluent samples below the detection limit. The average log reduction between the influent and effluent was at least 3.62 (calculated by substituting the effluent

load with the detection limit). Primary and secondary treatment both decreased MSC levels, with the greatest log decrease occurring in secondary treatment with an average decrease of 1.15 log units while the primary settling process removed 2.45 log units. The removal of MSCs in the primary treatment is primarily due to the sorption of MSCs onto solids and settling out. The reduction in secondary treatment is due to continued sorption onto flocs and the predation MSCs by ciliates and other microorganisms in the secondary reactors. A net log reduction of 0.83 to 3.28 of MSC in activated sludge was reported elsewhere (Hata, Kitajima, & Katayama, 2013) that is within our reported values of 1.15 log reduction in activated sludge.

Bacterial indicator concentrations were higher in concentration and survived further in the liquid treatment train than the viral indicator due to their higher initial concentrations. The reductions of bacterial and viral indicators were quite different, particularly in primary and secondary treatment. Both bacterial indicators decreased by less than half a log in the primary treatment and actually increased in numbers on some sample days, whereas virus indicators decreased by 2.45 log in the primary treatment. Although MSCs replicate by infecting coliform bacteria and therefore could potentially grow during primary treatment and in the primary solids, they are generally not believed to replicate under the observed environmental conditions. In the secondary treatment, bacterial indicators decreased by approximately two logs, whereas virus indicators decreased by 1.15 logs. This observation agrees with past research where significant reductions of viruses in activated sludge (0.49 – 3.39 log) were observed along with higher removals of *E. coli*, 4.74 logs (Zhang and Farahbakhsh 2007; Hata, Kitajima et al. 2013). Our observed results seem to show that viruses are inactivated more than bacteria

in the primary treatment where settling is the primary removal mechanism; versus secondary treatment where predation and bacterial growth shows a greater reduction in bacteria than in viruses. Temperature has been shown to influence virus inactivation, with temperatures greater than 20 oC correlating with decreased survival time (Yates, Gerba et al. 1985). Other factors such as solids content and microbial ecology can also play a role.

3.2.3.2 Fate of Male-Specific Coliphages in Wastewater Solids

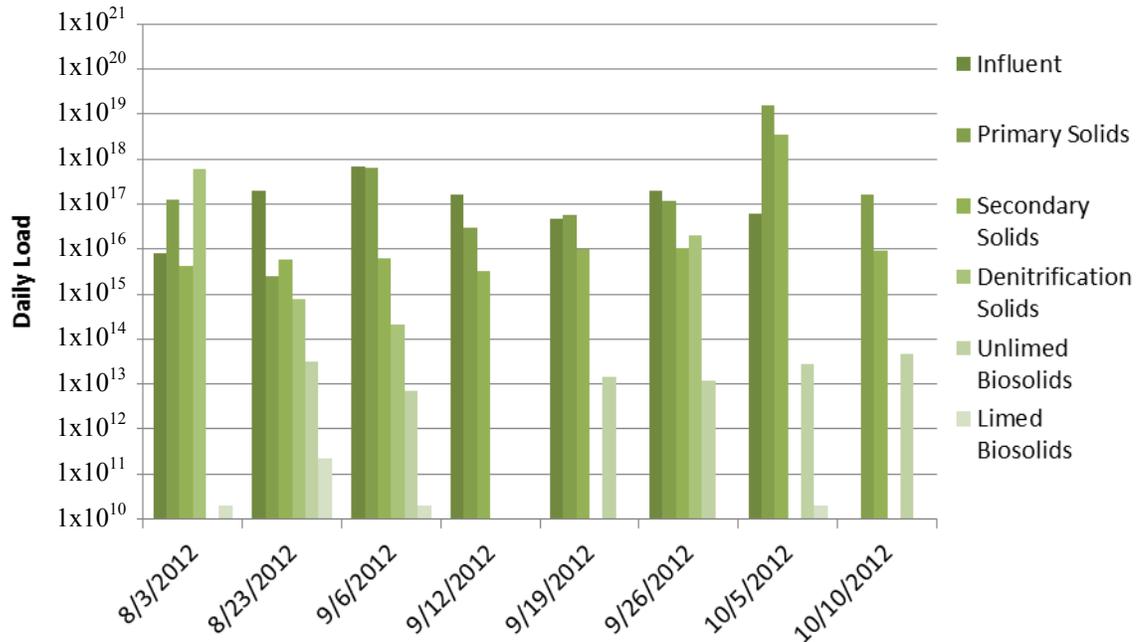
Figure 3.9 shows the daily load of MSCs in the solids component of the wastewater treatment process. The detection limit for the various solid types differ due to the varied solid contents of each stream (Table 3.1), the different recovery rates (Figure 3.1), and the different environmental conditions, such as the rich ecology of the activated sludge and the anoxic conditions in the nitrification/denitrification processes. The values were calculated by multiplying the measured concentration by the flow rate then divided by the recovery rate, if available for that date otherwise the average value was used (Appendix D). The detection limit for MSCs was calculated by multiplying the lowest PFU obtainable (1 PFU/500 ul) by the flow and average recovery percentage.

Table 3.1 Solids Concentration

	Influent	Primary MLSS	Primary Effluent	Secondary MLSS	Secondary Effluent	Tertiary MLSS	Tertiary Effluent
Concentration (mg/L)	338.27	42205	69.39	3474.69	8.34	1788.13	4.99

The loads of MSCs in the solids stream were generally higher than the liquids stream samples. The unlimed solids daily load shows a consistent presence of viruses with an average of 1.79×10^{13} virus particles per day. Although MSCs in limed biosolids were generally below the detection limit, there were occasional virus (n=3) measured in the limed biosolids (Figure 3.9).

Figure 3.9 Male-Specific Coliphages in the Solids Compartment



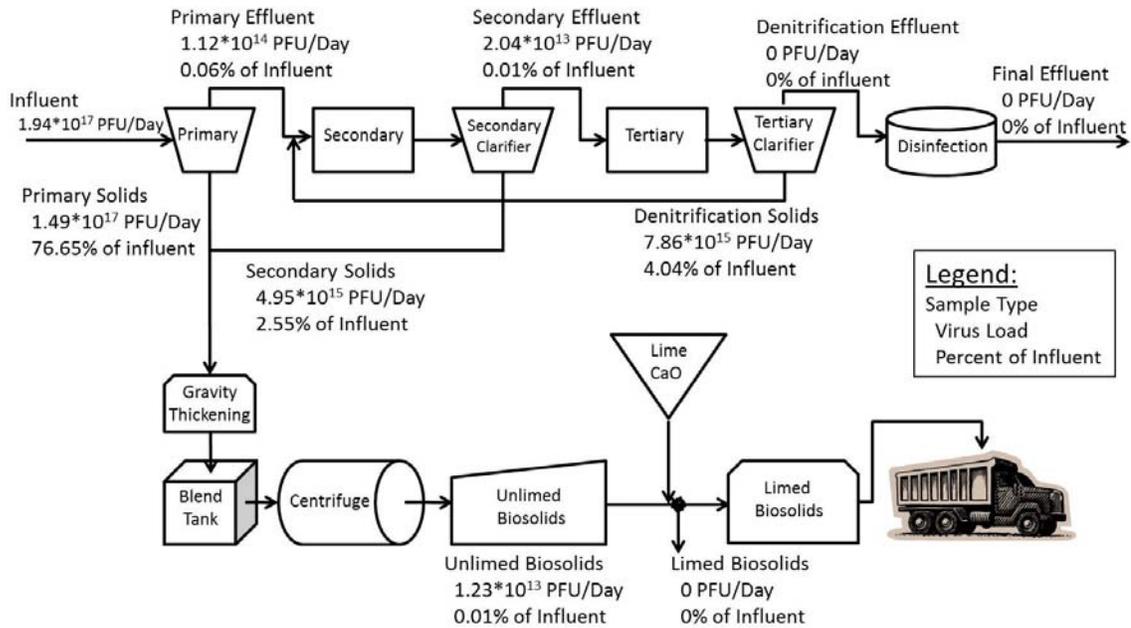
MSC loads in the solids generally decreased through the treatment plant (Figure 3.9). The average log reduction in MSCs from influent to limed biosolids was 6.19 (99.999%). MSCs were found in every one of the primary, secondary, and tertiary solids samples tested, but not in every biosolids sample tested. A large log reduction occurred when the solids from primary, secondary, and denitrification were combined to form unlimed biosolids (3.74 log reduction). This corresponded well with the reduction of fecal coliforms at this step (3.88 log). These reductions may be due to the dewatering processes involved in this step. Overall, the solids treatment process here appears to be effective in reducing the total amount of MSCs from the influent stream.

3.2.3.3 Plantwide Male-Specific Coliphage Concentrations in One Day

A holistic look at the MSC loads at a wastewater treatment plant provides insight into the fate of viruses through both the liquid and solid treatment trains simultaneously. The highest load on September 26 was found in the influent at 6.58×10^{16} CFU and no

viruses were detected in the final effluent and limed biosolids, 0 CFU. The load of MSCs in liquid reached the detection limit after the tertiary process; in the solids, MSCs were detected until the lime treatment.

Figure 3.10 Male-Specific Coliphage Snapshot: 9/26/12



In primary treatment, MSCs were found to be 3 logs greater in the solids than in the liquids. This demonstrates that MSCs partition into the solids that settle out in the primary treatment with those 3.24 logs removed from influent to primary effluent. In the secondary reactor, MSCs were found to be 1.92 logs greater in the solids than in the liquids with a removal of 0.74 logs from primary effluent to secondary effluent. There was a greater amount of MSCs detected in secondary solids than from the primary effluent; suggesting a possible growth of MSCs in the activated sludge which contains coliforms. A similar trend was seen in the denitrification processes with a greater load of MSC in the denitrification solids than in the secondary effluent; suggesting similar growth in the denitrification solids. The removal of MSCs in the liquids from secondary

to final effluent fell below the detection limit. On the solids, we see a decrease of MSCs through the dewatering processes as the solids are combined into unlimed processes; and after liming, the limed biosolids than fell below the detection limit. One previous study reported a higher concentration of coliphages in the return activated sludge that contains higher coliphage numbers than those present inside the secondary reactor (Tanji, et al., 2002). Others have also reported on the partitioning of MSCs into the solids (C. P. Gerba, Stagg, & Abadie, 1978; Malina, Ranganathan, Sagik, & Moore, 1975; Tanji, et al., 2002).

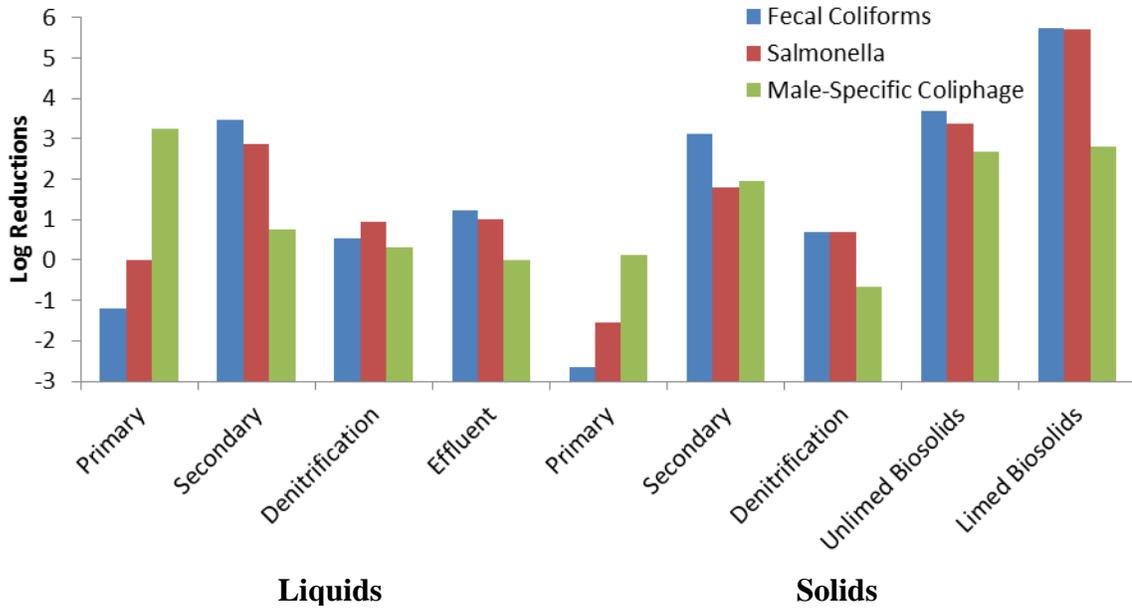
Overall, the daily viral load removal in liquids and solids were 3.83 logs and 6.52 logs, respectively. The log removal of solids may be greater due to the lower detection limit of the biosolids (2.0×10^{10}) versus that of the liquid (9.81×10^{12}).

3.2.4 Comparison Snapshot between Bacterial and Viral Indicators

Viral indicators were always detected at lower levels than the bacterial indicators. These higher bacterial indicator levels are in agreement with previous studies that compared *E. coli* to coliphages (John & Rose, 2005; Zhang & Farahbakhsh, 2007).

Figure 3.11 plots the log removal of each process in both liquids and solids. It should be noted that processes that exhibit negative log reduction values indicate microbial growth. In general, the log reductions are similar for the different indicators, with some noted difference between the MSCs and bacteria in primary treatment (more MSC removal) as well as less MSC removal during biosolids liming compared to bacterial removal.

Figure 3.11 Bacterial and Viral Indicator Log Reductions in Snapshot



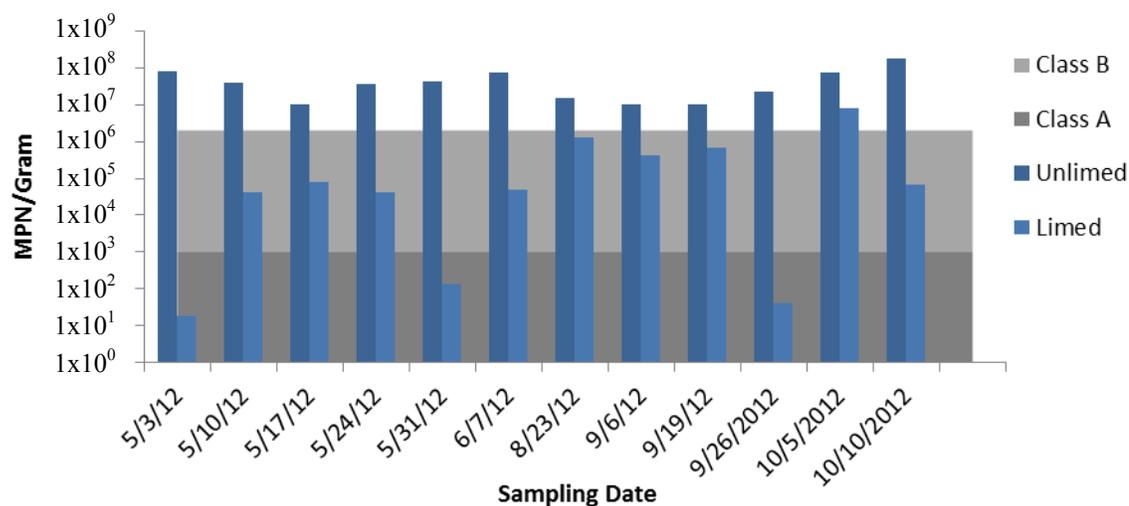
3.3 Liming

The limed biosolids that leave the plant are classified as Class B. Class B biosolids requires a fecal coliforms concentration below 2 million MPN/gram (light grey in Figure 3.12) while the stricter Class A requires fecal coliforms to be less than 1000 MPN/gram (dark grey in Figure 3.12). Samples of limed biosolids were measured to assess the extent to which current regulations are fulfilled.

3.3.1 Fecal Coliform Concentration in Biosolids

Figure 3.12 shows the concentration of fecal coliforms in unlined and limed biosolids on different sampling dates in relation with the biosolids classification outlined in Sludge Rule 503. All the sampling events showed a reduction of fecal coliforms with the addition of lime.

Figure 3.12 Fecal Coliforms Concentrations in Biosolids



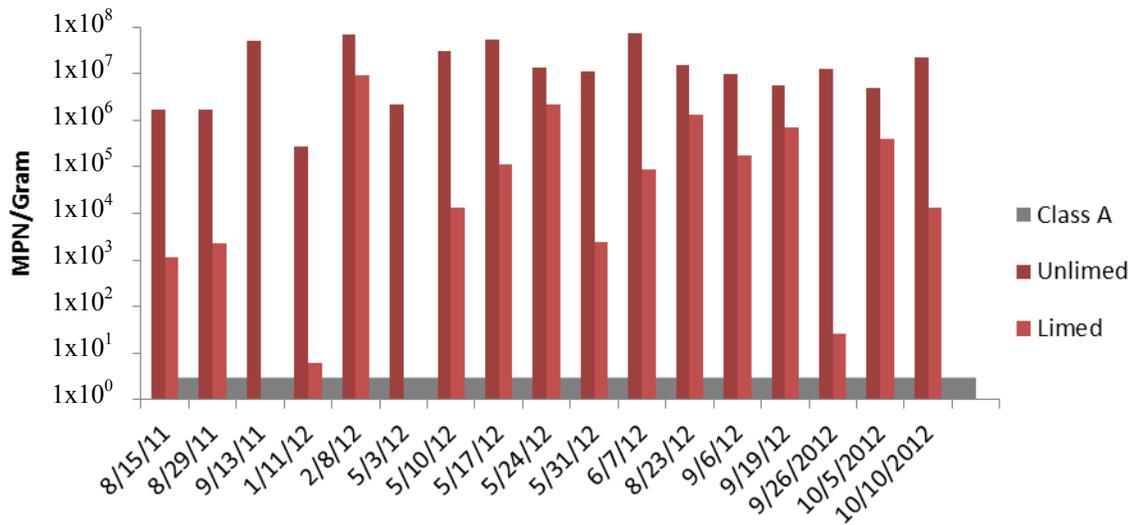
The concentrations of fecal coliforms before the introduction of lime exceed Class B classification limits by at least 10×, with an average concentration of 4.92×10^7 MPN/g. The average limed biosolid concentration was 8.74×10^5 . All of the limed biosolids met the Class B standard of 2 million MPN/gram except for one sampling event that took place on 10/5/12. It should be noted that the limed samples were taken right after the introduction of the lime with minimal mixing and reaction time (a worst case scenario). In fact, further reaction time typically takes place at the site as the samples travel from the liming station to bunkers awaiting final disposal. Some sampling events (5/3/12, 5/31/12, and 9/26/12) resulted in levels of fecal coliforms in the limed biosolids that actually meeting the Sludge Rule 503 Class A standard of 1000 MPN/gram.

3.3.2 *Salmonella* Concentrations in Biosolids

Salmonella has a stricter rule under Sludge Rule 503 than Fecal Coliforms (FC) and is only outlined for Class A biosolids which require *Salmonella* to be at less than 3 MPN/Gram to meet Class A standards. Figure 3.13 shows the concentration of

Salmonella in limed and unlimed biosolids in comparison with the Class A classification standard of Sludge Rule 503 (3 MPN/gram) at various sampling events (n=17). On two dates (9/13/11 and 5/3/12), the biosolids falls under Class A classification. Overall, alkaline treatment averages a log reduction of 2.87. If the plant wishes to achieve Class A certification additional treatment will be required.

Figure 3.13 *Salmonella* Concentrations in Biosolids



Comparing Figures 3.13 and 3.14 indicates that *Salmonella* has similar susceptibility to alkaline treatment as fecal coliforms. FC levels are higher in the unlimed biosolids, but only by 0.38 logs. Liming reduced both bacterial indicators to the same concentration levels.

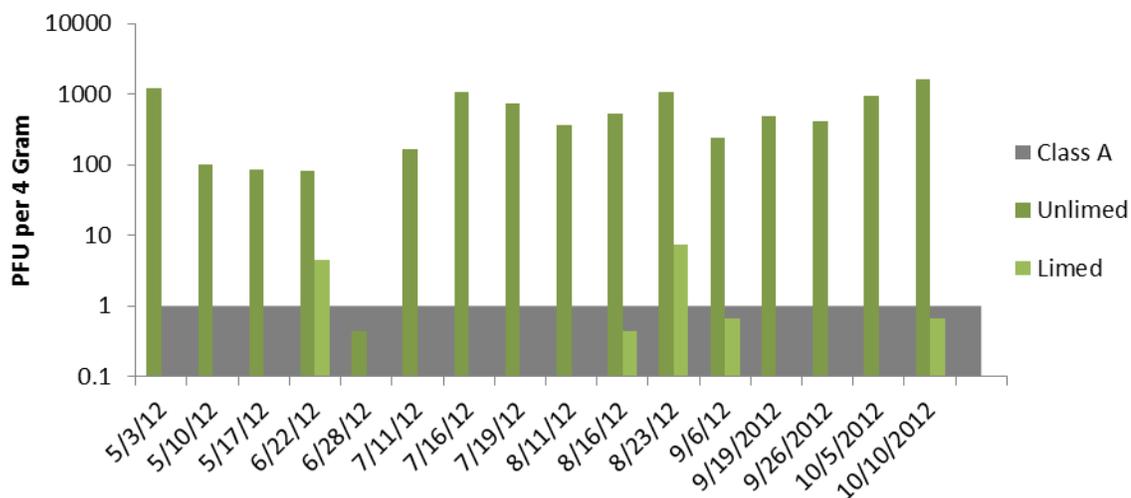
In terms of variability, the unlimed biosolids data varied within the first few days of sampling (5/3-5/17, n=3); the remaining data (5/24-10/10, n=9) exhibit similar trends with a rise in both bacterial indicator concentrations (5/24-6/7) followed by a slight decrease (6/7-9/19) and then finally a small rise (9/19-10/10). For the limed biosolids data, we see a much closer relationship between the FC and *Salmonella*. This provides

further evidence that FC and *Salmonella* behave similarly when exposed to lime. Looking carefully at the reduction data between both bacterial species (Figure 3.15), we can see that the log reductions between them are not consistent. The reason for the inconsistency is likely due to the sampling point and the liming process. The liming process at this particular plant utilizes ground up flecks of quicklime (CaO) stored in silos that are then mixed into the biosolids. The differences in log reduction within the sampling events may be partly explained by the fact that various sizes of quicklime are introduced into the biosolid giving it a heterogeneous distribution of lime. These variances in lime mean that the percent lime may vary drastically especially as the sampling size decreases. As the sampling size gets smaller, the proportion of lime in the biosolid fluctuates (i.e., the possibility that a sample only contains lime increases).

3.3.3 Male-Specific Coliphage Concentrations in Biosolids

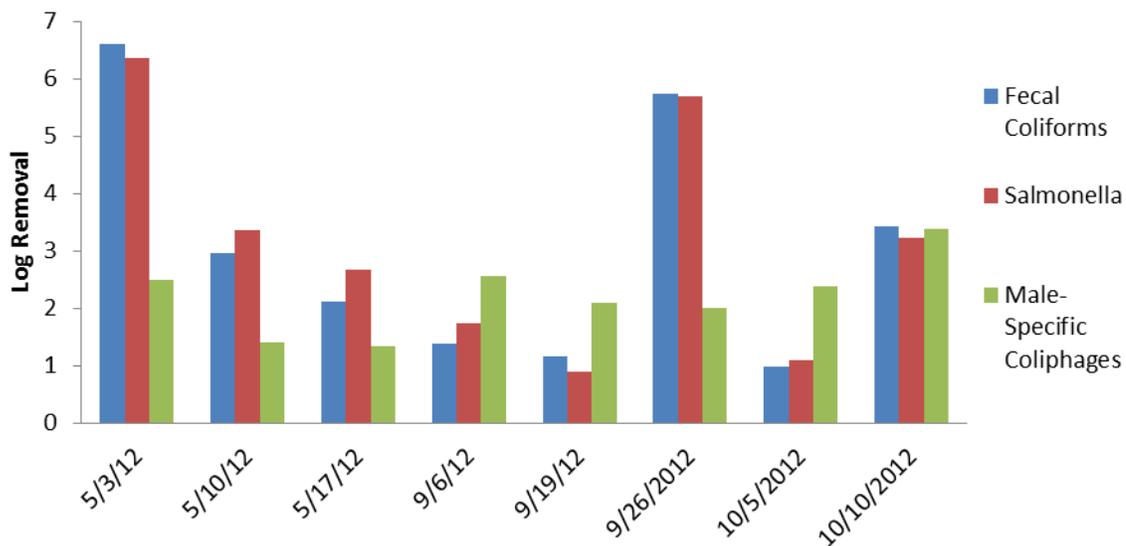
The previous experiments looked at how regulations and bacterial indicators survive the liming processes, but did not take into account the possible pathogenicity in biosolids due to viruses. In this experiment we measured the concentration of MSCs in both unlimed and limed biosolids. Figure 3.14 shows the concentration of MSCs at various sampling events (n=16) in comparison to the Class A biosolids classification outlined in Sludge Rule 503. It is important to note that these values are raw data and do not take into account the recovery percentages (Figure 3.1). The average concentration of MSCs in unlimed biosolids was 573 PFU/ 4 grams while the average of the limed biosolids was 0.84 PFU/4 grams. 88% (14 of 16) of the limed biosolids samples met the Class A biosolids classification of 1 PFU/4 grams.

Figure 3.14 Male-Specific Coliphage Concentrations in Biosolids



Although the MSCs were detected in unlimed solids at all sampling dates, in limed biosolids only five dates showed positive presence of MSCs. This suggests that MSCs are very susceptible to alkaline treatment. The concentrations of MSCs did not follow the trends seen in the bacterial indicators. The reason for this may be attributed to the inherent different characteristics between viruses and bacteria.

Figure 3.15 Comparative Indicator Log Reductions in Biosolids



In order to determine if liming has the same effect on bacteria as on viruses, the log removal values for all three indicator species were compared. By measuring the change in log concentration of the unlimed and limed samples, we can ascertain a few key points: 1) the log reductions between Fecal Coliforms (FC) and *Salmonella* are closely related, 2) Male-Specific Coliphage (MSC) log reductions do not follow those of the bacterial indicators, and 3) the log removals are not consistent over the sampling period.

As mentioned above, the log reduction trends are different between the bacterial indicators and viral indicators. This was expected due to the different characteristics between the microorganisms. For instance, the concentrations of bacteria in the unlimed biosolids were multiple logs higher than the concentration of viruses for an equivalent volume. Therefore the observed reduction in viruses may be greater but limited by the amount of viruses present and the detection limit for viruses that were reached in the denitrification processes in the effluent and the liming process in the solids. The smaller size of the viruses may contribute to the lower reduction rates seen in the viruses.

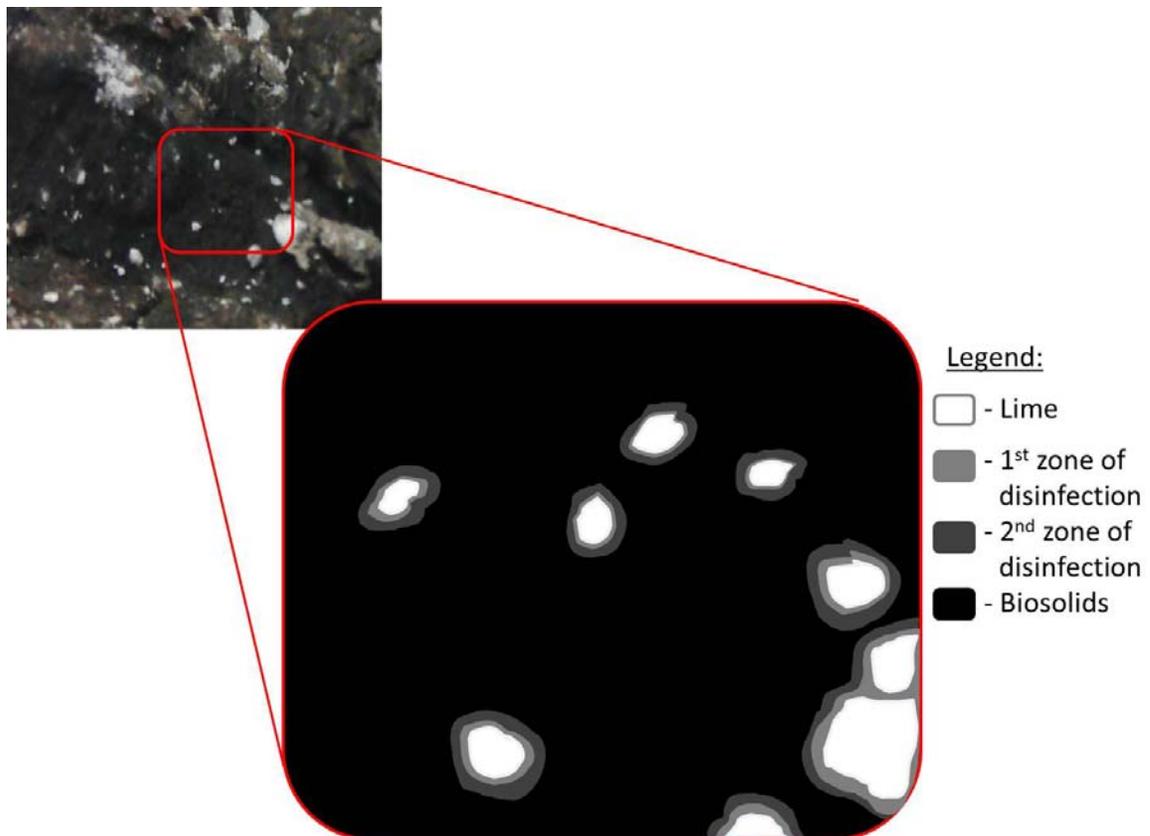
3.4 Heterogenicity of Biosolids

While investigating the concentrations of Male-specific coliphages (MSCs) in the biosolids, the possibility of obtaining a non-representative sample became evident while looking at the distribution of lime within the biosolids. The impact of liming heterogeneity on virus concentrations was therefore explored.

3.4.1 Proposing Zones of Disinfection in Limed Biosolids

Figure 3.16 shows a cross section of limed biosolids and the flecks of lime present in the biosolid matrix. In this study we propose that there are areas around the lime in which the pH is dramatically increased creating what we termed “zones of disinfection”. However, due to the high solid content of the biosolid itself, the lime may not be able to fully diffuse through the biosolid. This diffusion is dependent upon the surface area of the lime exposed to the biosolid, the mixing that takes place during the liming process, and the water content of the biosolid. Different zones of diffusion occur around the biosolid with the high pH occurring on or very near the pieces of lime. This zone effect could potentially create places within the biosolid where a pH of 12 is not met, and thus microorganisms may survive.

Figure 3.16 Zones of Disinfection within the Biosolid Matrix

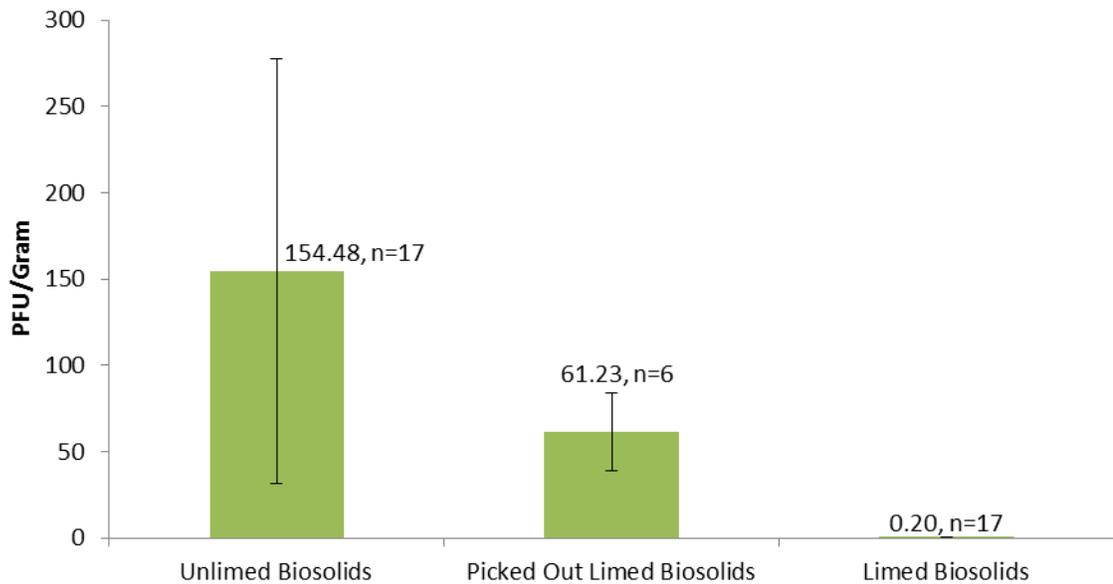


Here, the impact of sample size on the detected MSC levels was assessed; to do this limed biosolid samples were taken and separated into two groups for analysis. The first group functioned as the control, where the extraction and concentration of viruses were performed following the standard procedure. In the second group, the limed biosolids were taken apart and lime was removed—these samples were called the Picked Out Limed Biosolids.

3.4.2 Determining Virus Survivability in Limed Biosolids

High levels of MSCs were detected in the unlimed biosolids, while a reduced number was found after picking out the lime from the limed biosolids, and very few MSCs were found in limed biosolids (Figure 3.17). The biosolids were measured and normalized to comparable infectious viral particles per gram.

Figure 3.17 Male-Specific Coliphage Concentration in Various Biosolids



The unlimed biosolid sample measurements were taken immediately prior to lime introduction, the “picked out limed biosolids” were limed biosolids that had lime hand removed, and the limed biosolids were taken from the same sampling port as the picked out limed biosolids. Samples were transported to the environmental lab at the University of Maryland, allowing time for the biosolids pH to increase, but assayed within two hours after collection. A significant reduction in MSCs was observed when the lime was not picked out from the sample. The reduction from unlimed biosolids to picked out limed biosolids was at 0.37 logs, whereas the difference in reduction from picked out limed biosolids to limed biosolids was 2.49 logs, and the reduction directly from unlimed biosolids to limed biosolids was 2.86 logs.

These results indicate that the mixing and dissolution of the lime during sampling most likely contributes to the overall inactivation values observed. In other words, the limed biosolids likely have more infective viruses than are determined through standard virus samples methods. This result has important implication for biosolid regulations, which are based on methods that underestimate infective virus levels after liming.

3.5 Sampling Variation in Biosolids

After determining the concentration of bacterial indicators in the unlimed biosolid samples, a key question to consider was whether the biosolid matrix was heterogeneous or homogeneous in terms of bacterial indicator distribution.

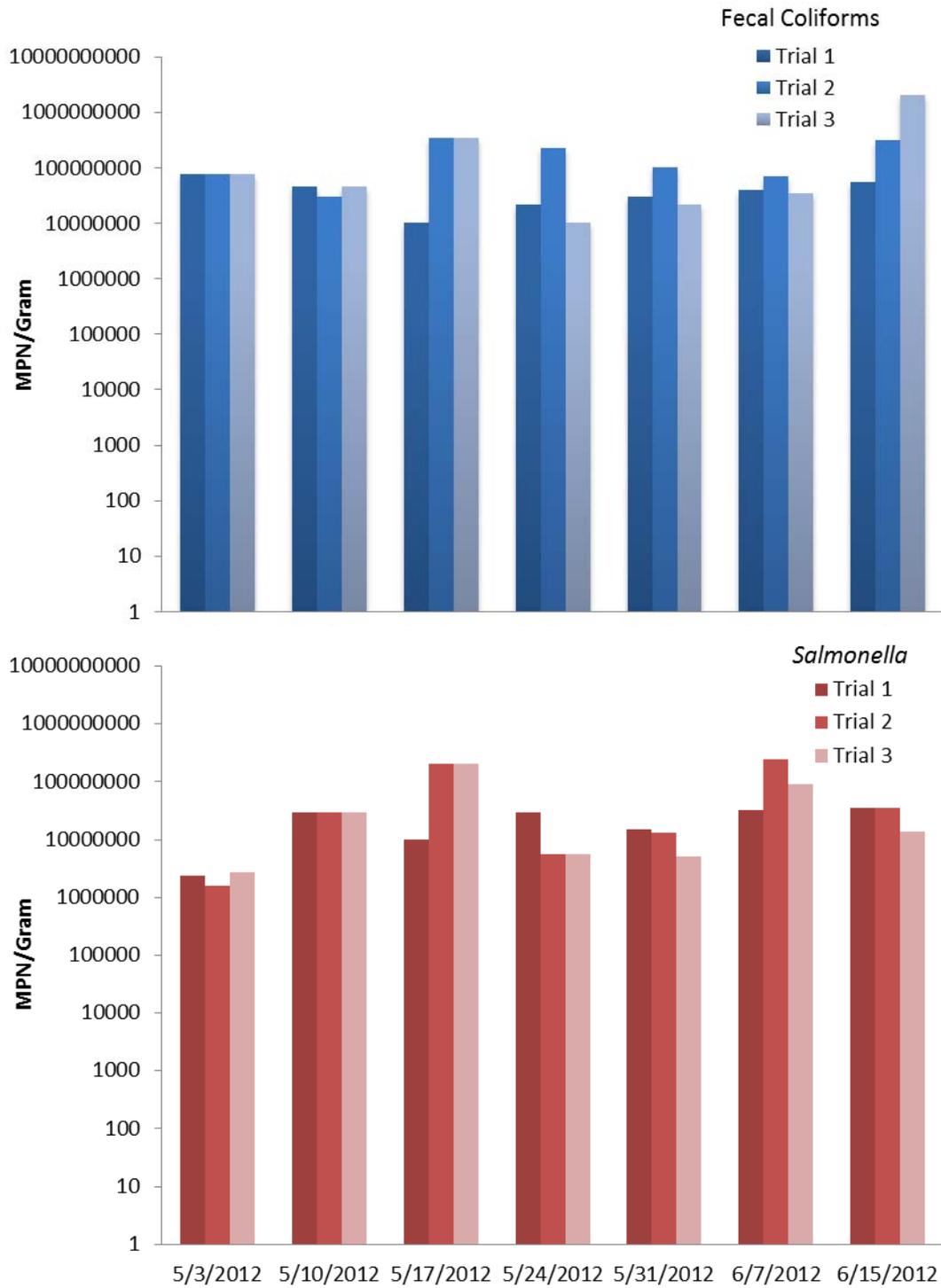
3.5.1 Bacterial Homogeneity Unlimed Biosolids

If the biosolid matrix has a heterogeneous distribution of bacterial indicators, replicate samples would be variable within each of the sampling dates, while a

homogenous distribution would have consistent triplicate sample within each of the sample dates. Figure 3.18 shows the triplicate values of fecal coliforms (blue) and *Salmonella* (red) from the sampling events.

Triplicate values in each sampling date look relatively close to each other. All values were between 6 and 8 log units in both FC and *Salmonella* samples, with higher values occurring more frequency in the fecal coliforms data. This data suggests that there is homogeneity in the samples both across the biosolids themselves and across the sampling date for the bacterial indicators. These triplicates support the sampling method carried out in this study and the sampling sized used for bacterial indicators.

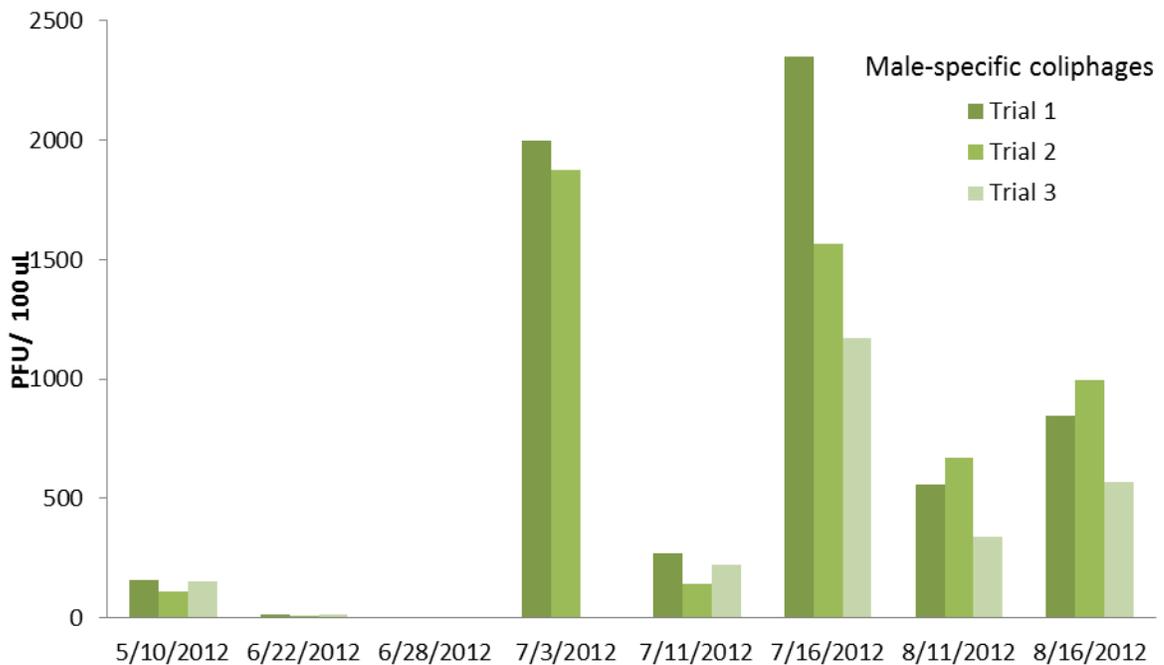
Figure 3.18 Bacterial Variance of Unlined Biosolid Samples



3.5.2 Viral Homogeneity in Unlined Biosolids

After determining that the biosolids matrix was homogeneous for bacterial indicators, a similar study was conducted for the MSCs. Similarly to bacterial indicators, if the biosolid matrix has a heterogeneous distribution of MSCs the triplicate samples would be variable and a homogenous distribution would be shown by consistent replicate samples. Figure 3.19 shows the triplicate values of MSCs at the various sampling dates.

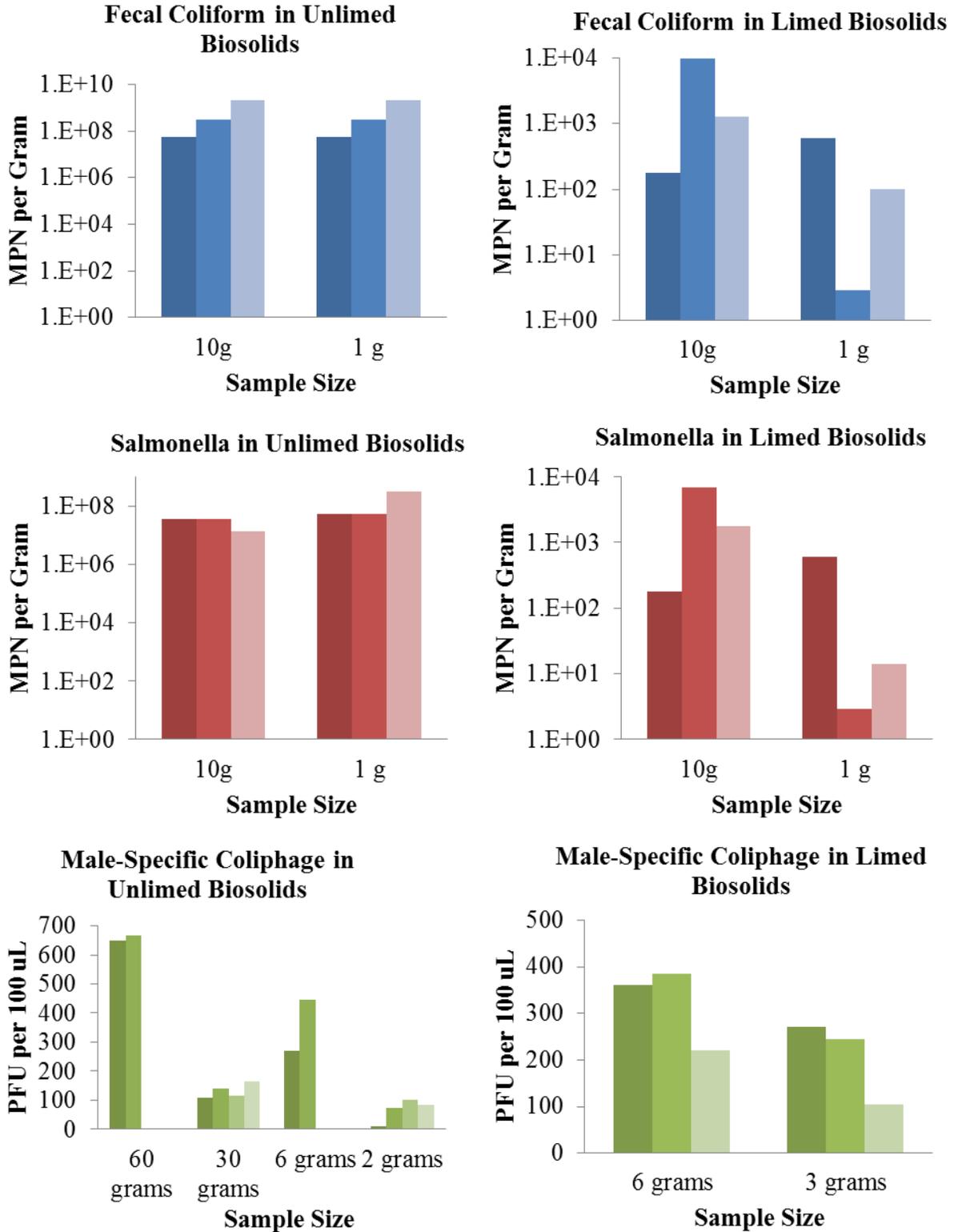
Figure 3.19 Viral Variance in Unlined Biosolids



From the data shown (Figure 3.19), we can see that there is some variance amongst the replicates. The variations between the highest value and the lowest value differ by approximately 3.5 logs (6/28 and 7/3). This variance is much greater than any of the values seen in the bacterial indicators. This suggests that the behavior of MSCs is drastically different in both inactivation and distribution within the biosolid matrix compared to bacterial indicators.

3.5.3 Homogeneity of Bacteria and Viruses in Limed Biosolids

Figure 3.20 Sampling Size Variation for Indicators in Unlimed and Limed Biosolids



The EPA 1680 method for assaying bacterial indicators calls for liquefying the biosolids through liquefying 30 grams of biosolid in 270 mL of water to get an equivalent of 0.3 grams per mL. For the MSC assay, the 6 g of biosolids were measured and liquefied with 10% beef extract solution. Using these sample sizes as a guide, the bacterial indicators were tested for heterogeneity at smaller sample sizes (10 grams and 1 gram); MSCs were also tested at both a range of sample sizes (60 grams, 30 grams, and 3 grams) as shown in Figure 3.20. The bacterial indicators in unlimed biosolids showed homogeneity regardless of sample size. The MSC concentrations, on the other hand, varied with sample size. In the limed biosolids, we hypothesized that due to the large fluctuations in lime particle size; samples would be more variable than unlimed biosolids due to the differences in concentration of lime in each sample of limed biosolids taken. This hypothesis could be tested by taking a smaller sample and determining the contained bacteria and viruses. If the hypothesis holds true, the small samples would have high inter-variability which would decrease as the sample size gets bigger due to the effect of regression to the mean. From Figure 3.20 we can see that, this effect is happening not only for MSCs but for bacterial indicators as well. The results demonstrate that pockets of bacteria or viruses may be present in the limed biosolid matrix.

Chapter 4: Conclusions

In this study, we attempted to answer certain gaps in our knowledge regarding microorganisms and the wastewater treatment process. First, we analyzed the partitioning and removals of bacteria and viruses at the wastewater treatment processes. Both bacteria and viruses preferentially partitioned into the solids in each of the primary, secondary, and tertiary treatment processes. From the snapshot surveys conducted at the plant, bacteria indicators grew in the primary reactors and then partitioned into the solids and were removed through the treatment processes. The total log removals of fecal coliforms, *Salmonella*, and Male-specific coliphages were 4.51, 5.17, and 6.19, respectively for the solids. The total log removal of fecal coliforms, *Salmonella*, and Male-specific coliphages in liquids was 4.47, 5.16, and 3.62, respectively. In the liquids, the indicator removal may be higher than reported due to detection limit.

The effectiveness of the liming processes was affirmed for both bacteria and viruses. The high alkalinity of the solids reduced the concentrations of both bacterial indicators and viral indicators. The log reduction of the biosolids due to liming of fecal coliforms, *Salmonella*, and Male-specific coliphages were 1.79, 1.72, and 2.12, respectively. Only one of the limed biosolids sampled had indicator levels above Class B limits set under Sludge Rule 503.

Lastly, the heterogeneity of bacterial and viral distribution was determined with various solid sample sizes and types. In unlimed biosolids, both bacterial indicators were homogeneously distributed; MSCs were heterogeneously distributed. In limed biosolids, all indicators were heterogeneously distributed and their levels dependent upon the percent of lime present in the subsample taken.

Appendix A Unlimed and Limed Biosolids Data

Unlimed Biosolids			Limed Biosolids			
Date	Type	MPN/gram	Date	Type	MPN/gram	
5/3/2012	Fecal Coliforms	78000000	5/3/2012	Fecal Coliforms	13	
		78000000			26	
		78000000			18	
	Salmonella	2400000		5/3/2012	Salmonella	0
		1600000				0
		2700000				0
	MSC	4840		5/3/2012	MSC	Failed
		1713.33				Failed
		3183.33				Failed
	5/10/12	Fecal Coliforms		45000000	5/10/12	Fecal Coliforms
30000000			69			
45000000			130000			
Salmonella		30000000	5/10/12	Salmonella		260
		30000000				26
		30000000				40000
MSC		157.6666667	5/10/12	MSC		0
		108.6666667				0
		155.3333333				0
5/17/2012		Fecal Coliforms	10000000	5/17/2012		Fecal Coliforms
	35000000		4000			
	35000000		100000			
	Salmonella	10000000	5/17/2012		Salmonella	260000
		20000000				6900
		20000000				69000
5/24/12	Fecal Coliforms	22000000	5/24/12	Fecal Coliforms	700000	
		23000000			1300000	
		10000000			7800000	
	Salmonella	30000000		5/24/12	Salmonella	410000
		5500000				1800000
		5500000				4300000
5/31/2012	Fecal Coliforms	30000000	5/31/2012	Fecal Coliforms	3	
		100000000			400	
		22000000			3	
	Salmonella	15000000		5/31/2012	Salmonella	6.7
		13000000				240
		5100000				6900

		39000000			4000
	Fecal Coliforms	71000000		Fecal Coliforms	130000
6/7/12		35000000			13000
		32000000			2600
	Salmonella	240000000		Salmonella	260000
		90000000			4000
		55000000			180
	Fecal Coliforms	320000000		Fecal Coliforms	10000
6/15/2012		2000000000			1300
		35000000			180
	Salmonella	35000000		Salmonella	6900
		14000000			1800
		15			0
	MSC	7		MSC	6
6/22/12		13			0
	Salmonella	10000000		Salmonella	260
		0			0
6/28/12	MSC	1		MSC	0
		1			0
		2000			3450
7/3/12	MSC	1875		MSC	530
		270			0
7/11/2012	MSC	140		MSC	0
		220			0
		0			0
7/13/12	MSC	0		MSC	0
		0			0
		2350			0
7/16/2012	MSC	1566.666667		MSC	0
		1170			0
		0			0
7/18/12	MSC	0		MSC	0
		0			0
		600			346
	MSC	2720		MSC	485
7/19/2012		976.6666667			149
	Fecal Coliforms	200000000		Fecal Coliforms	24
	Salmonella	10000000		Salmonella	260

		560
8/11/12	MSC	670
		340
		845
8/16/2012	MSC	995
		570
	Fecal Coliforms	15000000
8/23/2012	Salmonella	15000000
	MSC	1635
	Fecal Coliforms	10000000
9/6/12	Salmonella	10000000
	MSC	355
	Fecal Coliforms	10000000
9/19/2012	Salmonella	5500000
	MSC	745
	Fecal Coliforms	22000000
9/26/2012	Salmonella	13000000
	MSC	620
	Fecal Coliforms	74000000
10/5/2012	Salmonella	5100000
	MSC	1430
	Fecal Coliforms	180000000
10/10/2012	Salmonella	22000000
	MSC	2435

		0
8/11/12	MSC	0
		0
		0
8/16/2012	MSC	0
		1
	Fecal Coliforms	1300000
8/23/2012	Salmonella	1300000
	MSC	11
	Fecal Coliforms	410000
9/6/12	Salmonella	180000
	MSC	1
	Fecal Coliforms	700000
9/19/2012	Salmonella	700000
	MSC	0
	Fecal Coliforms	40
9/26/2012	Salmonella	26
	MSC	0
	Fecal Coliforms	7800000
10/5/2012	Salmonella	410000
	MSC	1
	Fecal Coliforms	69000
10/10/2012	Salmonella	13000
	MSC	0

Appendix B Holistic Population Balance Data

Daily Load of Fecal Coliforms

Date (2012)	Influent per Day	Primary Effluent per Day	Primary Solids per Day	Secondary Effluent per Day	Secondary Solids per Day	Tertiary Effluent per Day	Tertiary Solids per Day	Effluent per Day	Unlimed Biosolids per Day	Limed Biosolids per Day
8/3	1.40E+18	4.46E+17	2.31E+21	6.21E+15	1.30E+19	4.27E+14	1.81E+16	3.69E+13	2.18E+17	2.61E+10
8/23	9.05E+17	3.58E+17	1.18E+21	0.00E+00	2.70E+18	6.03E+16	1.48E+15	6.03E+13	1.63E+16	1.42E+15
9/6	1.24E+18	4.59E+17	4.53E+19	3.19E+15	8.70E+18	7.19E+14	4.36E+19	4.59E+13	1.09E+16	4.46E+14
9/12	7.18E+15	1.10E+18	1.08E+21	7.18E+15	5.95E+18	4.59E+16	6.49E+15			
9/19	7.71E+18	2.35E+17	1.41E+19	1.33E+17	2.46E+17	3.43E+16	4.17E+16	4.50E+13	1.09E+16	7.62E+14
9/26	7.74E+17	1.26E+19	6.73E+21	1.24E+16	7.91E+17	1.24E+15	5.44E+16	7.33E+13	2.39E+16	4.35E+10
10/5	7.12E+17	6.75E+16	4.96E+19	1.14E+16	7.79E+17	3.93E+15	4.44E+16	4.31E+13	8.06E+16	8.49E+15
10/10		2.92E+17	3.17E+21	6.56E+15	1.92E+17	6.56E+14	1.82E+14	4.19E+13	1.96E+17	7.51E+13

Daily Load of Salmonella

Date (2012)	Influent per Day	Primary Effluent per Day	Primary Solids per Day	Secondary Effluent per Day	Secondary Solids per Day	Tertiary Effluent per Day	Tertiary Solids per Day	Effluent per Day	Unlimed Biosolids per Day	Limed Biosolids per Day
8/3	6.21E+17	4.27E+16	2.31E+21	6.21E+15	5.00E+18	1.40E+14	3.02E+16	1.05E+13	1.81E+15	4.72E+10
8/23	1.36E+18	7.92E+16	3.71E+20	0.00E+00	1.01E+18	4.15E+14	1.48E+16	6.03E+12	2.72E+15	2.36E+14
9/6	1.86E+17	1.22E+17	1.17E+19	3.19E+15	8.70E+18	2.20E+14	3.69E+15	4.99E+11	1.81E+15	3.27E+13
9/12	2.19E+15	1.10E+18	2.77E+19	4.59E+15	5.95E+18	7.18E+15	1.02E+15			
9/19	2.35E+18	2.35E+17	5.45E+18	1.31E+16	2.46E+17	1.31E+15	4.17E+16	4.92E+12	9.98E+14	1.27E+14
9/26	4.89E+17	4.89E+17	3.27E+20	1.89E+15	7.91E+17	7.33E+13	5.44E+16	7.33E+12	2.36E+15	4.72E+09
10/5	4.50E+17	4.31E+16	7.80E+18	6.75E+15	3.02E+17	1.14E+15	4.44E+17	6.75E+12	9.25E+14	7.44E+13
10/10		2.00E+17	1.34E+21	2.92E+15	3.84E+16	4.19E+13	5.39E+15	6.56E+12	3.99E+15	2.36E+12

Daily Load of Male-Specific Coliphages

Date (2012)	Influent per Day	Primary Effluent per Day	Primary Solids per Day	Secondary Effluent per Day	Secondary Solids per Day	Tertiary Effluent per Day	Tertiary Solids per Day	Effluent per Day	Unlimed Biosolids per Day	Limed Biosolids per Day
8/3	8.23E+15	0	1.27E+17	0	4.30E+15	0	6.14E+17	0	0	1.99E+10
8/23	1.96E+17	8.72E+14	2.59E+15	3.25E+14	5.75E+15	0	7.88E+14	0	3.25E+13	2.18E+11
9/6	6.77E+17	1.61E+15	1.63E+17	9.98E+12	1.08E+16	0	8.19E+14	0	7.05E+12	1.99E+10
9/12	1.65E+17	7.68E+14	1.42E+16	2.99E+13	2.56E+15	0	0	0		
9/19	4.77E+16	1.18E+14	5.66E+16	0	1.88E+15	0	0	0	1.48E+13	0
9/26	1.94E+17	1.12E+14	1.49E+17	2.04E+13	4.95E+15	0	7.86E+15	0	1.23E+13	0
10/5	5.96E+16	5.34E+14	1.19E+16	9.37E+12	2.07E+15	1.87E+13	0	0	2.84E+13	1.99E+10
10/10		2.31E+15	8.96E+16	2.83E+14	6.31E+15	0	0	0	4.84E+13	0

Appendix C Snapshot Indicator Data

Bacterial and Viral Indicator Daily Load Values on 9/26/12						
	Influent	Primary Effluent	Secondary Effluent	Denitrification Effluent	Effluent	
Fecal Coliforms	7.74E+17	1.26E+19	4.21E+15	1.24E+15	7.33E+13	
<i>Salmonella</i>	4.89E+17	4.89E+17	6.41E+14	7.33E+13	7.33E+12	
Male-Specific Coliphage	1.94E+17	1.12E+14	2.04E+13	0	0	
		Primary Solids	Secondary Solids	Denitrification Solids	Unlimed Biosolids	Limed Biosolids
Fecal Coliforms		6.73E+21	2.68E+17	5.44E+16	2.39E+16	4.35E+10
<i>Salmonella</i>		3.27E+20	2.68E+17	5.44E+16	2.36E+15	4.72E+09
Male-Specific Coliphage		1.49E+17	1.68E+15	7.86E+15	1.23E+13	0

Appendix D Raw Recovery Data

Sampling Date		Log Dilutions				Calculated Concentration	Recovery Calculated/Spike
Spike Concentration							
8/31/2012							
Spike		N0	N1	N2	N3		
100	Primary Solid	67	8			73.5	73.50%
	Secondary Solids	63	14	0	0	101.5	101.50%
	Denitrification Solids	1	0	0	0	1	1.00%
9/7/2012							
Spike		N0	N1	N2	N3		
100	Primary Solid	8	0			4	4.00%
	Secondary Solids	59	3	0	0	44.5	44.50%
	Denitrification Solids	6	1	0	0	8	8.00%
9/12/2012							
Spike		N0	N1	N2	N3		
90	Primary Solid	14	0	-	0	7	7.78%
	Secondary Solids	25	1	0	0	17.5	19.44%
	Denitrification Solids	1	0	0	0	1	1.11%
9/19/2012							
Spike		N0	N1	N2	N3		
320.67	Secondary Solids	20	1	0		15	4.68%
	Denitrification Solids	6	1	0	0	8	2.49%
9/27/2012							
Spike		N0	N1	N2	N3		
320.67	Primary Solid	52	4	1	0	64	19.96%
	Secondary Solids	56	2	0	0	38	11.85%
	Denitrification Solids	5	0	0	0	2.5	0.78%
10/3/2012							
Spike		N0	N1	N2			
6910000	Primary Solid	300+	64	11		870	0.01%
	Secondary Solids	300+	109	10		1045	0.02%
	Denitrification Solids	300+	261	24		2505	0.04%
10/10/2012							
Spike		N0	N1	N2	N3		
830	Primary Solid	72	5	1	0	74	8.92%
	Secondary Solids	126	21	1	0	145.33	17.51%
	Denitrification Solids	5	1	0	0	7.5	0.90%

10/24/2012									
Spike		N0	N1	N2	N3	N4			
	Unlimed Recovery 1	300+	300+	38	4	2	9266.67		0.37%
2533333	Unlimed Recovery 2	300+	300+	62	4	1	6733.33		0.27%
	Unlimed Recovery 3	300+	300+	45	4	0	4250		0.17%
10/31/2012									
Spike		N0	N1	N2	N3	N4			
	Secondary Solids Recovery 1		21	3	0	0	255		2.17%
11730	Secondary Solids Recovery 2		42	2	1	0	540		4.60%
	Secondary Solids Recovery 3		22	2	0	0	210		1.79%
11/2/2012									
Spike		N0	N1	N2	N3	N4			
	Unlimed Recovery 1		187	15	3	0	2123.33		0.38%
552333	Unlimed Recovery 2		165	15	2	0	1716.67		0.31%
	Unlimed Recovery 3		300+	23	1	0	1650		0.30%
11/7/2012									
Spike		N0	N1	N2	N3	N4			
	Unlimed Recovery 1	300+	300+	121	12	3	18033.33		3.28%
550000	Unlimed Recovery 2	300+	300+	69	6	1	7633.33		1.39%
	Unlimed Recovery 3	300+	300+	94	10	1	9700		1.76%
	Influent Recovery 1	300+	42	6	0	0	510		0.93%
55000	Influent Recovery 2	300+	55	0	0	0	550		1.00%
	Influent Recovery 3	300+	30	3	0	0	300		0.55%
11/16/2012									
Spike		N0	N1	N2	N3	N4	N5		
	Primary Solid Recovery 1	300+	300+	300+	210	66	2	356666.67	0.01%
675000000	Primary Solid Recovery 2	300+	300+	300+	104	8	0	92000	0.00%
	Primary Solid Recovery 3	300+	300+	300+	37	2	1	52333.33	0.00%
	Influent Recovery 1	300+	300+	300+	300+	78		780000	0.12%
675000000	Influent Recovery 2	300+	300+	300+	300+	89		890000	0.13%
	Influent Recovery 3	300+	300+	300+	300+	74		740000	0.11%

Average Recoveries					
Date	Influent	Primary Solids	Secondary Solids	Denitrification Solids	Unlimed Biosolids
8/31/2012		73.50%	101.50%	1.00%	
9/7/2012		4.00%	44.50%	8.00%	
9/12/2012		7.78%	19.44%	1.11%	
9/19/2012			4.68%	2.49%	
9/27/2012		19.96%	11.85%	0.78%	
10/3/2012		0.01%	0.02%	0.04%	
10/10/2012		8.92%	17.51%	0.90%	
10/24/2012					0.27%
10/31/2012			2.86%		
11/2/2012					0.33%
11/7/2012	0.82%				2.14%
11/16/2012	0.12%	0.00%			
Average	0.47%	16.31%	25.29%	2.05%	0.91%

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