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PATHOGEN AND INDICATOR ORGANISM INACTIVATION AND QUANTIFICATION
FOR LOW-COST LOW-TECH BIOSOLIDS TREATMENT

By

Karina M. Eyre

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Environmental Engineering

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This thesis has been approved in partial fulfillment of the requirements for the Degree of
MASTER OF SCIENCE in Environmental Engineering.

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Preface

Chapter 4, Method Verification and Implementation for Coliphage Testing in Biosolids, is planned to be submitted for future publication. Researchers from the Metropolitan Wastewater Reclamation District of Greater Chicago will collaborate and provide supplemental data for this future publication, but have not directly contributed to any data presented in this thesis, with the exception of sharing the standard protocols for coliphages.

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Abstract

Biosolids are a by-product of municipal wastewater treatment which, when treated to reduce pathogens, can be land applied as a fertilizer. Class A biosolids must contain low pathogen levels and are unrestricted in their use. This thesis describes methods to identify pathogen and indicator organism (PIO) inactivation mechanisms and PIO inactivation rates for use in low-cost low-tech Class A processes. Procedures to control the levels of spiked bacteria and viruses, total solids, volatile fatty acids, pH, and temperature in biosolids are described for their use in laboratory experiments. Methods for coliphage in biosolids as a surrogate for total enteric viruses are also presented. The two-step enrichment method yields a most probable number estimate of coliphages and was found to have minimal interference from solids, limit background bacteria interference, and may be a useful process monitoring tool at wastewater treatment facilities.

1 Introduction

1.1 Project Background

1.1.1 What are Biosolids?

Domestic and industrial wastewaters are treated at municipal water resource recovery facilities (WRRFs) to protect human and environmental health. Typical treatment processes used for reducing the concentrations of organic matter, nutrients, total solids, and pathogens before discharge of the treated liquid effluent into the environment include: physical (e.g., settling), chemical (e.g., precipitation), and biological (e.g., activated sludge) processes. The solid residuals resulting from these treatment processes are treated, usually with a process such as mesophilic anaerobic digestion, to reduce pathogen levels, and stabilize the sludge by reducing the volatile solids content, thereby reducing their tendency to attract vectors (e.g., rats, flies, etc.). After they have been treated in a stabilizing process, they are termed biosolids [1]. Management and disposal of biosolids is a major cost and challenge for WRRFs, with roughly 6 million dry tons of biosolids annually produced in the U.S. alone. Importantly, biosolids are an organic and nutrient-rich material that has beneficial effects on soil properties and plant production [2]. Because of the high organic and nutrient content of biosolids, the United States Environmental Protection Agency (EPA) has promoted their beneficial reuse as a soil amendment. As a result, up to 60% of the biosolids produced in the United States are land-applied for a variety of beneficial uses, including application on farmland, mine reclamation sites, parks, personal garden use, and forested lands [3].

1.1.2 EPA Regulations for Biosolids

Land application of biosolids is regulated by the EPA via the Part 503 Rule. The Part 503 rule provides pollutant limits for the land application of biosolids, including pathogen and indicator organism (PIO) levels, which are the main focus of this work. The EPA classifies biosolids into two main categories based on the PIO levels: Class A and Class B. Class A biosolids are required to meet stricter standards than Class B for PIO content. Because the standards for Class B biosolids are more lenient with respect to the PIO levels, the land application of Class B biosolids is required to adhere to stringent guidelines. These

include restrictions on the consumption of food crops, restrictions for livestock grazing, and restrictions in public access to land where Class B biosolids have been applied.

As summarized in Table 1.1, the EPA has established three alternatives for treating biosolids so that they can be classified Class B. Alternative 1 is focused on a limit for fecal coliforms, whereas Alternative 2 and 3 require treatment by one of the Processes to Significantly Reduce Pathogens (PSRPs), or processes that have been proven to be equivalent to a PSRP, respectively.

Table 1.1 Alternatives provided in the Part 503 Rule for Class B Biosolids [4]

Alternative 1	Geometric mean of seven fecal coliform samples is less than 2,000,000 most probable numbers (MPN)/g total solids (TS)
Alternative 2	Treated with a process that is equivalent to process to significantly reduce pathogens (anaerobic digestion, aerobic digestion, air drying, composting, and lime stabilization)
Alternative 3	Process that is equivalent to process to significantly reduce pathogens

In comparison, there are six alternatives for producing Class A biosolids, which are required to demonstrate greater pathogen inactivation to ensure a safe end product. Alternative 1 is focused on four time-temperature regimes, whereas Alternative 2 requires that biosolids be treated in a high pH-high temperature process, and meet specific pH, temperature, and air-drying requirements. Alternative 3 covers treatment by other known processes that can reduce enteric viruses and viable helminth ova. Alternative 4 is focused on treatment in an unknown processes, where specific known mechanisms for pathogen inactivation are not present in a process, but the product can be tested for the adherence to Class A standards. Alternative 5 covers treatment of biosolids in one of the so-called Processes to Further Reduce Pathogens (PFRP), and Alternative 6 allows for treatment of biosolids in a process equivalent to a PFRP. The PFRPs that can be used to achieve Class A status are composting, heat drying, heat treatment, thermophilic anaerobic digestion, beta ray irradiation, gamma ray irradiation, or pasteurization [4].

Table 1.2 Alternatives provided in the Part 503 Rule for Class A Biosolids [4]

Alternative 1	Designated time and temperature requirements are met in a designed process
Alternative 2	The pH of the sewage sludge is raised above 12 and heated above 52 °C and remains there for 72 hours. After the treatment period, the sludge is then air dried to above 50%
Alternative 3	Enteric viruses and helminth ova are analyzed before and after treatment. The mechanism of pathogen destruction is determined and monitored during future operation of the process.
Alternative 4	Pathogen levels (enteric viruses and helminths) can be tested from an individual batch of biosolids, and if it meets the requirements can be distributed as Class A product.
Alternative 5	Biosolids are treated in a process to further reduce pathogens. (Composting, heat drying, heat treatment, thermophilic aerobic digestions, beta or gamma irradiation, and pasteurization)
Alternative 6	Biosolids are treated in a process that has been demonstrated as an equivalent to a process to further reduce pathogens.

Importantly, Alternative 6 for Class A allows for use of a treatment process equivalent to a PFRP, i.e., the process can consistently reduce pathogens to levels comparable to those achieved in the PFRPs. This allows for innovation in process development and for new technologies to be considered [4]. This is important because the alternatives for Class A biosolids production tend to be expensive, high-maintenance, and energy or chemical intensive processes. This poses a problem for the majority of publicly-owned WRRFs in the U.S., which serve small communities (defined as population of $\leq 10,000$ people and wastewater flow rates of $< 1 \times 10^6$ gal/day) [5]. These facilities typically have limited capital, operating, and personnel resources and, therefore, cannot implement conventional Class A technologies. They also frequently produce smaller quantities of biosolids, which makes processes intermittent and reduces the efficacy of solids treatment systems compared to facilities that can operate solids processing continuously. Nonetheless, these facilities still desire to produce Class A biosolids. Fortunately, low-cost, low-technology (LCLT) methods are available and being used successfully at WRRFs in the U.S. and elsewhere. A Water Environment Research Foundation (WERF) study evaluated four of these processes: long-term lagooning, air drying, combined lagooning/air drying, and cake

storage [6]. Unfortunately, there are insufficient data available to effectively implement and regulate these LCLT processes. More information is needed regarding the fate of PIOs in LCLT processes, the mechanisms that cause PIO inactivation, and process operations for LCLT processes to be successfully and broadly applied.

1.1.3 Regulated Pathogens and Indicator Organisms in Biosolids

1.1.3.1 PIO Levels for Class A and B Biosolids

Biosolids have the potential to contain a wide range of human PIOs, however, the Part 503 regulations are focused on the levels of bacteria, enteric viruses, protozoa, and helminths in Class A or Class B biosolids (Table 1.3). Specifically, the Class B standards are focused on fecal coliform numbers, whereas the Class A regulations require lower levels of fecal coliforms, as well as detection level quantities of enteric viruses and helminth ova.

Table 1.3 PIO standards for Class A and B biosolids

	Class A	Class B
Bacteria	1,000 MPN fecal coliforms/g TS Or 3 MPN <i>Salmonella</i> sp./4 g TS	2,000,000 MPN fecal coliforms/g TS
Enteric Virus	1 plaque forming unit (PFU)/4 g TS	NA
Helminth Ova	1 ova/4 g TS	NA

1.1.3.2 Bacteria

Individuals infected with pathogenic bacteria will shed those organisms in their feces, resulting in their presence in wastewater. However, wastewater also carries a number of harmless bacteria that colonize the intestinal tract of humans. Therefore, there are two different levels of acceptable bacterial levels for Class A biosolids. Fecal coliforms represent a mix of pathogenic (*Shigella*, *Salmonella*, enteropathogenic *E. coli*) and non-pathogenic bacteria (certain strains of *Bacteriodes*, *Enterococci* and *E. coli*), and because the test is non-specific to any particular species/strain, they can be present at relatively

high levels of 1000 MPN/g TS for Class A biosolids. There is also a standard for *Salmonella* sp., which are common in wastewater sources and must be below 3 MPN/g TS. *Salmonella* tests must meet this low level because the standard applies to a specific species. Enteric bacteria are also regulated in Class B biosolids, but only for fecal coliforms and at a much higher concentration. [1]

Fecal coliforms are typically enumerated using culture-based techniques, in which the organisms are either counted as colony-forming units (CFUs) on agar plates or filter disk EPA 1603 [7], or most probable number (MPN) by dilution of samples into tubes containing a nutrient solution [7, 8]. MPN methods that include a fermentation step, such as EPA methods 1680 and 1681, are typically used for biosolids because of their ability to handle solids and accommodate the high concentrations of fecal coliforms that can be present in biosolids samples. Filter methods cannot accommodate this and are, therefore, typically not used. While there is a trend in microbiology procedures towards molecular techniques for microbial detection, culture techniques remain favored by the EPA because they are established and easily implemented on a nationwide basis.

1.1.3.3 *Helminth Ova*

Helminths are parasitic worms, certain strains of which can infect humans. These include *Ascaris lumbricoides*, *Enterobius vermicularis*, and *Trichuris trichiura*. An infected individual will shed the ova of a parasitic worm, resulting in their presence in wastewater. The United States has had relatively low occurrence of helminth infections due to the advent of sanitation systems. Nonetheless, helminth ova can still be present within biosolids and are remarkably resistant to inactivation, withstanding processes such as mesophilic anaerobic digestion and chlorine disinfection. They can also persist in soils for many months and are generally considered as one of the PIOs that are most difficult to inactivate. [1]

Viable helminth ova are typically counted under a microscope [9]. Unfortunately, this culture-based microscopic counting technique is relatively challenging and time consuming, requiring almost a month of incubation time, and many hours of laboratory effort to interpret a single sample. Therefore, the goal of the individual work by another

student on the project, Tanner Keyzers, was to improve upon the current enumeration methods for quantifying helminth ova in biosolids.

1.1.3.4 Enteric Viruses

Enteric viruses are viruses that specifically infect the human gut. They are important to regulate because there are over 100 types of enteric viruses that may be shed by infected individuals and can, therefore, be present in wastewater. These can include coxsackie, adenovirus, norovirus, rotaviruses, and hepatitis A viruses. Specifically, enteric viruses are regulated in Class A biosolids because they can persist on crops for 15-60 days and in soil from 20-100 days during which they can remain infective, so their levels must be sufficiently low in Class A biosolids to merit their unrestricted use. [1]

Enteric viruses are quantified as plaque-forming units (PFU), where each PFU represents the location where a virus particle has infected a layer of animal cells. This process for quantifying enteric viruses is time intensive, with cell culture requiring 3 weeks to complete and 1.5-3 weeks to observe infection of cells. Viruses must also be removed from the sample via a beef extract elution prior to infecting the animal cells, a process which takes up to 8 hours per sample to complete [9]. A high level of skill is required for application of these steps. For example, for the pilot scale studies described below, only the elution of samples could be completed by personal at Michigan Technological University. Inoculation into animal cells was performed by a team at Michigan State University because of the challenges associated with the cell culture assays. Methods for improved virus testing are much needed and could improve safety by allowing more monitoring to occur and could also ease the burden of the testing needed for implementing new technologies.

1.2 Scope and Objectives of Study

The overall goal for the project, of which the research is apart, is to develop a rational and universal approach for the design of LCLT Class A biosolids treatment processes. As part of achieving the overall project goal, fundamental information on the impact of process parameters on the kinetics of inactivation of PIOs is being collected under a wide range of conditions in carefully controlled laboratory studies, and validated in pilot-scale studies

conducted at collaborating WRRFs that use different activated sludge configurations and produce Class B biosolids using anaerobic digestion. The author contributed significantly to the pilot-scale studies; however, because of the team nature of the pilot-scale effort, the work reported on in this thesis is focused on the laboratory-based studies led by the author.

During the field pilot-scale studies, key physical/chemical factors (e.g., temperature, total solids, ammonia, volatile acids) and PIO reduction were monitored to see if Class A treatment goals were met, even under the heterogeneous non-ideal conditions in the field. These pilot-scale studies are being supported by laboratory studies focused on improving our fundamental understanding of the physical, chemical, and biological stressors through which PIOs are inactivated during LCLT treatment of biosolids. Simultaneous examination of multiple stressors in biosolids creates several challenges. Therefore, the overall goal of this specific study, as part of the effort to evaluate the effect of long term biosolids storage on indicator organisms, was to perform preliminary experiments to develop the experimental design and procedures for the laboratory scale studies. Key procedures evaluated for the laboratory scale studies included methods for quantifying PIOs in biosolids, especially coliphage in biosolids. Specifically, the objectives of this research were as follows:

Objective #1: Preliminary Evaluation of Laboratory-Scale Study Design

The effect of the physical and chemical properties of the biosolids on PIO inactivation is believed to be significant, as are the synergistic effects of certain chemicals and physical parameters. In particular, based on the pilot scale studies, it was determined that temperature, pH, volatile fatty acids, and total solids were all important to PIO inactivation and merited further investigation. A factorial design was selected so that the statistical relationship of individual and dual factors could be evaluated; however, there was little precedent for how to perform the laboratory-scale experiments. Therefore, the first objective of this study was to perform a preliminary evaluation of the methods and procedures for the laboratory-scale experimental design.

Objective #2: Coliphage Method Development for Biosolids

Detection of PIOs is a critical procedure for the pilot- and laboratory-scale studies. Enumeration of enteric pathogens during the pilot-scale studies required use of an outside laboratory. As part of this work, coliphages, i.e., viruses that infect *Escherichia coli* and other coliforms, were examined as a potential PIO based on previous research [10-13], which suggests that coliphages could provide a useful tool for monitoring Class A processes as an indicator organism for enteric viruses. Coliphages are non-pathogenic to humans, are abundant in wastewater and biosolids, are typically more resistant to inactivation than enteric viruses, and can be enumerated more quickly and with less effort than required to quantify enteric viruses in biosolids samples. To date, there are two EPA approved methods for the analysis of coliphages in groundwater, and drafts of EPA methods for coliphages in wastewater and recreational water sources [14-17]. However, there are no approved methods for the monitoring of coliphage in biosolids. Therefore, the second objective of this study was to develop and verify coliphage testing in biosolids.

In the following chapter (Chapter 2), the background literature is reviewed in more detail for the LCLT technique of air drying, which was one of the techniques evaluated in the field pilot-scale studies. During air drying, the biosolids are dried through a combination of turning, internal heat production, solar radiation, and ambient temperatures. The subsequent chapters are focused on Objective 1, the preliminary evaluation of the experimental techniques to be used in the laboratory studies (Chapter 3), and Objective 2, the quantification of coliphage in biosolids (Chapter 4). Finally, the conclusions drawn from this research are presented in Chapter 5, along with recommendations for future work.

2 Air Drying Literature Review

2.1 Process Description

Air drying can be performed using either dewatered or liquid biosolids (Figure 2.1). Air drying of dewatered biosolids is initiated on impervious pads where biosolids are laid out until the total solids levels reach 30-35%. The biosolids are then formed into windrow piles approximately 2 feet tall that are periodically turned to facilitate drying and oxidation of biodegradable material. Biosolids slurries are similarly placed on pervious pads or a porous media bed in depths up to 1 foot and allowed to drain until windrows can be formed and turned. When air drying is applied as a stand-alone treatment process, treatment should continue until either sufficient desiccation has occurred or recommended time-temperature guidelines have been met. Farrell et al (2004) recommended that desiccation-based air-drying operations should maintain biosolids until the moisture content is $\leq 5\%$. These low moisture levels are needed to inactivate *Ascaris ova*, as discussed below. However, in practice achieving this level of drying may be challenging for most utilities for several reasons. In many regions of the U.S., precipitation and humidity will continuously reintroduce water to biosolids stored outdoors and increase their moisture content.

In regions where $\leq 5\%$ moisture can be attained, e.g., in the southwestern U.S., the desiccated biosolids are likely to cause operational problems due to dust formation. More commonly, air-drying systems are “time and temperature” based. General management guidelines suggest that in areas where summers are warm and winters are mild, air drying should be conducted for at least 250 days and should include the summer months, whereas 350 days of air drying is recommended for areas with warm summers and cold winters. To date, little information is available on the application of air drying in cold regions. In all cases, during the windrow stages, the biosolids should be turned at regular intervals to ensure that substrates are well-mixed and brought in contact with the microorganisms that degrade them. This is analogous to windrow composting in which the metabolic activity of the microorganisms generates heat, which contributes to pathogen inactivation as discussed below. Turning also introduces oxygen to facilitate biological processes and ensures uniform drying throughout the depth of the windrow [6].

Figure 2.1: The Air Drying Process

2.2 Mechanisms of Pathogen Destruction

Based on available literature, four potential mechanisms may contribute to pathogen reduction: drying, exposure to elevated temperatures, chemical inactivation, and competition for resources with non-pathogenic organisms.

2.2.1 Drying

Drying is thought to be one of the most effective mechanisms for reducing the concentration of pathogens during the air-drying. As explained by Farrell et al. (2004), when water evaporates from biosolids, dissolved salts are concentrated. This can be lethal to bacterial cells due to an osmotic effect and the low vapor pressure that draws water out of cells [6].

Importantly, drying is one of the few mechanisms known to effectively inactivate *Ascaris* ova, which are particularly resistant to treatment. Reimers et al. (1986) quantified helminth ova densities at a number of full-scale facilities across the United States, and evaluated the relationship of the age of the sludge, type of digestion process used prior to air drying, and percent moisture to *Ascaris* viability. The only statistically significant parameter for inactivation, as determined through regression analysis, was the percent moisture of the drying bed [18]. Consistent with this analysis, Cram (1943) and Baxter et al. (1983) observed the viability of *Ascaris* ova to be greatly reduced at moisture contents <10%, with complete inactivation at moisture contents <5% [19], [20]. Interpreting pathogen reduction at very low moisture concentrations is complicated by the fact that this level of drying is usually accomplished, at least in part, through heating of the biosolids. This heating is caused largely by microbiological activity and makes it difficult to independently identify the effects of heat and drying on *Ascaris* ova inactivation. Studies that

independently examine the effects of drying and heat on *Ascaris* ova have apparently not been conducted.

The loss of moisture that occurs in air drying also inactivates other pathogens. Ward & Ashley (1977) performed a laboratory study in which they isolated the effect of drying on human poliovirus by keeping the temperature at 21 °C to eliminate temperature effects. In addition, the test was conducted in stabilized raw sludge to lower the concentration of ammonia and eliminate its virucidal effects. Poliovirus was inactivated as the moisture content dropped below 17% and the inactivation was determined irreversible because RNA molecules that were released during the drying process were excessively degraded [21].

Rapid drying appears to be less effective than slower drying. A full-scale study conducted by Wilson & Co. (1990) in Roswell, New Mexico analyzed 6-week drying time in low windrows that produced high bacterial counts even though the product was 90% solids. Nonetheless, long drying periods where the same solids percentage was reached slowly showed more favorable bacterial numbers, therefore suggesting the influence of other effects on bacterial viability. Time was shown to be one of the most influential parameters for bacterial inactivation [22].

2.2.2 Elevated Temperatures

Temperatures in biosolids undergoing air drying may become elevated relative to ambient temperature due to biological activity in the pile coupled with radiation effects. The amount of heating that occurs depends on how frequently the biosolids are turned and, thus, occurs to a greater extent in windrows compared to sand drying beds. In a windrow, frequent turning introduces oxygen to native bacteria, which in turn produce heat via their metabolic activity. Temperatures characteristic of composting can often be reached with adequate turning, sufficient pile depth, and favorable weather conditions.

Similarly, a study conducted by Song et al. (2014) in Arizona and Mexico showed that tilling of biosolids undergoing air-drying affected the temperature in the biosolids. Liquid biosolids were applied to sand drying beds in layers 20-30 cm thick and tilled using a hand tool three times a week. Tilled biosolids reached maximum temperatures of 60 °C whereas

un-tilled biosolids reached temperatures of approximately 50 °C during summer drying. Winter drying resulted in lower maximum temperatures for both tilled and un-tilled. The difference in maximum temperatures was also smaller, but the average temperature over the 50-day period remained higher when the biosolids were tilled (20 °C) compared to un-tilled (15 °C). As a result of the higher temperatures, the tilled beds also reached higher total solids contents and had faster drying rates [23].

Solar heat also helps with heating biosolids, but it is limited in its effect. If the biosolids are in a thin layer and exposed to a warm arid climate, solar heat contributes to more heat production through the pile. The study by Wilson and co-workers in Arizona experienced marked differences in the temperatures reached during the summer because the biosolids were only 20-30 cm thick, so the ambient temperatures of the environment had a greater impact on the bulk of the solids undergoing treatment. Tilling was concluded to have a larger effect on temperature in the winter when the air temperature was low. Solar drying can assist in windrows as well, but due to the size of the windrow necessary for increased biological temperatures the effects usually only impact the first few inches of the pile. However, frequent turning can help distribute this effect through the pile and result in drying [22].

2.2.3 Chemical Action

Chemical action could possibly aid in the inactivation of organisms of interest but it is likely not the single cause of inactivation in an air-drying operation.

2.2.4 Competition with Nonpathogenic Bacteria

Nonpathogenic bacteria are present in biosolids and compete with pathogens for the same nutrients. The effects of competition with nonpathogenic organisms on pathogen inactivation were evaluated directly by Mondal et. al., 2015 in samples of mesophilic anaerobically digested (MAD) biosolids and air dried biosolids using the indicator organism *Salmonella birkenhead*. The air-dried samples were sampled at beginning, mid and late points of the 420-day pan drying period. The sampled biosolids were divided into three categories: one sample was unaltered to act as a control, and the other two samples were either autoclaved or gamma irradiated to inactivate bacteria present in the sample.

All samples were inoculated with *Salmonella* bacteria after initial preparation. A nutrient broth was also introduced to an additional set of samples to investigate the possibility of nutrient limitation in the various samples. The samples were held at 37 °C for 24 hours and for 20 °C for 24 and 48 hours respectively. Bacterial growth of *Salmonella* in the magnitude of 2-3 log₁₀ was observed in the gamma irradiated and autoclaved biosolids but not in the control, which suggests that pathogen survival was negatively impacted by competing organisms. Air dried samples exhibited higher growth in sterile samples, but only in the mid and late air-dried materials. Early air-drying material exhibited a low growth rate, which the authors speculated was because of an unfavorable physiochemical environment. The addition of the nutrient broth caused the *Salmonella* population to increase in all samples. This suggests a nutrient limitation is present in the biosolids, which prevents bacterial growth. A nutrient broth inoculated with *Salmonella* was prepared as a standard in all of the experiments, and in all cases the nutrient broth exhibited higher growth. There was some discussion of the transformation of the nutrients in the sterilized biosolids which would make it more available to the *Salmonella* and contribute to the increased growth. However, the growth was substantial in the sterile biosolids compared to unsterile, which suggests that even if nutrient transformation contributed to higher growth, microbial competition remains an important consideration for pathogen inactivation [24]. A decrease in volatile solids is also often observed during air drying, which could indicate that nutrients are being consumed by competing organisms [25].

2.3 Treatment Considerations

2.3.1 Pretreatment

Pretreatment of biosolids via a digestion process appears to be critical for successful application of air drying. A study by Armin 1988 found that untreated sludge that was stored and air dried without aerobic or anaerobic digestion, contained high amounts of indicator bacteria as well the larva, eggs and hatched worms [26].

Both aerobic and anaerobic digestion prior to air drying appear to be effective pretreatment for air-drying. When anaerobically and aerobically digested biosolids were dried on sand beds by Song et al, 2014, similar levels of fecal coliform were detected initially. However,

Salmonella was practically undetectable in aerobically digested biosolids but was present in anaerobically treated biosolids. Air drying that underwent aerobic digestion as a pretreatment began below Class A limits ($>3\text{MPN}/4\text{ g dry biosolids}$) for *Salmonella* and remained there for the 30-55 days of air drying. Anaerobically pre-treated biosolids began air drying above Class A limits and were able to achieve Class A levels barring precipitation. When rewetting from rainfall occurred, anaerobically digested biosolids were able to recolonize and cross above the limit for Class A biosolids. Presumably, because the aerobically digested biosolids had low initial levels of microorganisms, regrowth was not as feasible [23].

2.3.2 Pathogen Regrowth

As noted above, pathogen regrowth can be a concern if bacteria are not completely inactivated during air drying and if conditions become favorable for growth, e.g., due to rewetting of biosolids from precipitation. In particular, bacterial regrowth is possible in air dried beds if the destruction of organic material during pretreatment is insufficient as concluded by Baxter et al, 1983 [20]. This is consistent with findings from Song et al, 2014 [23], which found that inadequate destruction in pretreatment caused regrowth. Aerobic digestion caused greater reduction of pathogens in the beginning of the air-drying phase and was more resilient to regrowth than anaerobic digestion. This was also due to higher total solids values for aerobic digestion, which resulted in less impact of rewetting from rain on the drying process in total solids values and bacterial regrowth.

Other recent studies indicate that with proper treatment, bacterial and viral levels will remain stable in air dried biosolids even when rewetted. A study in Melbourne, Australia conducted by Rouch et al. 2011, evaluated regrowth in biosolids that had been dried to 90-95% total solids for 13 or 25 months respectively. The biosolids samples were split and half were rewetted to 15-20% moisture content. 10^6 cfu/g of *Salmonella* and *E. Coli* were added to both wetted and dry samples and compared to un-inoculated wetted and dry samples. All samples were incubated for 4 weeks at $20\text{ }^{\circ}\text{C}$. Un-inoculated samples remained below detection limits for both *Salmonella* and *E. Coli* and inoculated samples did not experience growth during the 4-week period. Bacterial levels were constant in wetted samples whereas dry samples experienced anywhere from a 4-5 $\log_{10}$ reduction. The researchers speculated that no growth occurred because the well stabilized biosolids

lacked the un-stabilized organic matter necessary for regrowth of the indicator organisms. Their findings similarly indicate that dry biosolids do not provide a suitable environment for bacterial survival [27].

2.3.3 Climate

Not surprisingly, relatively rapid biosolids drying can be achieved when air drying is conducted in arid and semi-arid climates that enhance desiccation of the biosolids. In particular, three studies conducted in regions of the US that have an arid or semi-arid climate achieved solids contents ranging from 60-70% in as little as 15 to 20 weeks of air drying. However, it was often observed that the effective solids concentration did not indicate pathogen inactivation, and additional time was required to result in satisfactory pathogen reduction ([28]; [20]; [22]). The inland northwest has seen similarly favorable air drying results [29], with PFRP standards being met in one study as a results of the advantageous conditions [30]. In contrast, solids level >60% could not be achieved in Bahrain, despite ambient temperatures ranging from 57- 104 °F, because of high relative humidity of 83% and 67% in the winter and summer respectively. Air drying in this climate was variable in its results, obtaining anywhere from 20%-50% moisture, with one sample even drying for 9 months and only achieving 37% moisture. All of the samples analyzed were abundant in pathogenic microbes, so drastic process changes would be necessary for safe use as agricultural fertilizer [26]. However, an integrated experimental and model study evaluated the importance of several key parameters on the effectiveness of solar drying of biosolids, and found that air humidity (and biosolids mixing) did not have a clear impact on the evaporation rate. Instead the best predictors of evaporation rate were solar radiation, air temperature, and ventilation rate. Nevertheless, the researchers also speculated that their data set was not sufficient to observe the effect of air humidity on the evaporation rate and conclusively rule out its importance in biosolids drying [31]. These finding suggest that effective air drying may be achieved in more humid climates at least under certain conditions.

2.3.4 Mixing

In addition, an approach that has been used to accelerate the rate of which higher solids concentration are achieved in air drying is to combine air dried biosolids with freshly

dewatered biosolids mix and create a solids content of 30-40% [28]. This speeds the drying time of the freshly dewatered biosolids and can help create a more consistent product by combining different batches of biosolids.

Mixing of biosolids during air drying has the potential to enhance moisture evaporation by releasing water vapor and enhancing the availability of substrates to bacteria that generate heat as they metabolize the available nutrients. However, several studies highlight the need to carefully manage the timing of biosolids turning and its impact on the drying process. Frequent turning has the potential to reduce the radiant heat because of poor conductivity through the windrow. Mechanical turning is not necessarily more effective for air drying, as demonstrated by lower bacterial densities in sand drying beds in one study's findings [22]. Similar trends were observed in a study evaluating bio-drying, a process in which the heating and drying of biosolids by bacteria is enhanced by forced aeration. Mechanical mixing of the biosolids increased the rate of water evaporation and VSS degradation. However, mechanical mixing was the most effective when it was applied at four, rather than two-day, intervals. Presumably, the longer intervals in between mixing minimized the loss of heat needed to achieve drying and optimize bacterial destruction of VSS [32].

Frequent turning of biosolids in any operation will also increase the resilience of the operation to external elements such as rainfall. In an operation conducted by Song et al., 2014 where some biosolids were turned and other were not, solids concentration in all biosolids decreased as expected following rainfall events. However, solids levels in biosolids that were turned experienced a rebound quickly following rainfall and a relatively constant total solids level could be maintained. In contrast, a marked drop in total solids in the static biosolids occurring following rainfall and in some cases never recovered, dropping from nearly 100% total solids to below 50%. The tilled biosolids were able to return to nearly 100% solids within the same timeframe [23]. Another study noted that if the biosolids are sufficiently dried, rewetting due to rainfall is unlikely due to hydrophobicity the solids develop [30].

Reed beds can also be used as a porous bed for air drying. Reeds (or other wetland vegetation) are planted in layers of coarse sand and gravel then 3-4 inches of sludge is spread across with the reeds still protruding above the sludge layer. The movement of the

reeds in the wind facilitates mixing and allows water to seep down into the sand layers beneath where it can drain or nourish the reeds. The reeds facilitate passive composting as well as air drying by providing oxygen to the sludge via their root system [1]. The effectiveness of pathogen inactivation was investigated as compared to conventional sand beds and it was concluded that there was no significant difference in the effectiveness of either. Neither method was effective in reducing helminth ova densities [33].

Issues associated with odor are frequently encountered with air drying processes following turning, particularly if the facility is close to residences or businesses. However, odor complaints when biosolids were stockpiled for at least 6 months before turning were reduced. The nitrogen content of the biosolids during the storage period dropped from 4.5% to 0.8%. Presumably, the ammonia emissions and their associated odor decreased [28].

2.4 Conclusions

In summary, air-drying of biosolids has potential to effectively inactivate pathogens and can achieve high levels of total solids. While several parameters appear to impact the efficacy of these processes, areas with favorable climate, biosolids that have undergone pre-treatment, desiccation, and heat production all contribute significantly to process success.

3 Laboratory Scale Development

3.1 Introduction

To achieve the overall goal of developing a rational and universal approach for the design of LCLT Class A biosolids treatment processes, it is necessary to obtain fundamental information on the impact of key process parameters on the kinetics of PIO inactivation under a range of conditions in carefully controlled laboratory studies. The goal of this chapter is to present the rationale, methodology, and results of preliminary experiments that were performed in the process of developing the experimental approach for systematically evaluating the impact of key process variables on the inactivation of PIOs as a function of time. Therefore, the objectives of this chapter are to:

1. Briefly review the results of the pilot scale studies and how their results can inform the laboratory studies,
2. Outline the experimental approach used in the preliminary laboratory studies,
3. Present the methods used for the preliminary laboratory studies, and
4. Review the results of the preliminary lab studies and lessons learned.

3.2 Summary of Pilot Scale Results

The pilot scale studies were conducted at two small wastewater treatment facilities in the upper peninsula of Michigan: the Portage Lake Water and Sewer Authority (PLWSA) in Houghton, MI and the Gogebic-Iron Wastewater Authority (GIWA) in Ironwood, MI. Both plants are designed for wastewater flows of ~3 million gallons a day (mgd) and typically operate in the range of 1-3 mgd. Both facilities have solids processing trains that incorporate mesophilic anaerobic digestion (MAD) for vector attraction reduction. After MAD, the biosolids are dewatered via belt filter presses with polymer addition for a final solids level of 16-20%. The biosolids are stockpiled in uninsulated sheds during the winter months when land application is not possible. The biosolids are applied as a class B product when the biosolids thaw in the spring.

During the pilot scale studies at each WRRF, biosolids were subjected to the combined LCLT treatment of long-term storage followed by air drying. Specifically, the biosolids were

stored for 1 (PLWSA) or 2 (GIWA) years and in indoor or outdoor conditions and followed by air drying for 12 weeks. During each treatment phase, chemical and physical properties of the biosolids were monitored to observe their impact on PIO inactivation to determine the treatment mechanisms that could result in Class A biosolids.

During the study, several trends were observed. Total solids (TS) increased throughout storage due to freeze drying and desiccation. Solids levels started at 16% and approached 30-50% during storage, and 85% after air drying at GIWA. Ammonia was lost throughout storage due to nitrification, and measured pH levels indicate that ammonia would not be toxic (NH_3) form to microorganisms as the pH began at ~8 and approached 4.5 by the end of the study. However, volatile fatty acids (VFAs) were present throughout the study and would be present in their toxic protonated form at the observed pH levels. Finally, temperatures remained below 25 °C, and the biosolids were frozen for most of the winter. Nonetheless, despite the rather mild conditions, fecal coliforms reached Class A levels and experienced a 3-log reduction within one year of storage, human poliovirus achieved a 3-log reduction within 100 days of storage, and *Ascaris* ova saw a 2-log reduction, but only during air drying. Some regrowth of fecal coliforms was observed beyond the 1000 MPN/g TS standard during air drying however, and the final product unfortunately did not meet Class A levels at the end of treatment.

3.3 Factors of Importance

Based on the results of the pilot scale studies, and a review of the literature on PIO, the key factors expected to have the greatest effect on PIO inactivation during LCLT treatment of biosolids are expected to be: TS, temperature, VFAs, and pH. These factors are reviewed in detail in the following paragraphs.

3.3.1 Total Solids

In LCLT biosolids treatment studies, TS evidently plays an important role in the reduction of PIOs. High TS content in particular appears to increase pathogen inactivation [19, 21, 23]. Nonetheless, low solids treatment for an extended period of time may also yield favorable results [34]. TS increases may be facilitated by freeze thaw cycles [35], or

through air and solar drying [6, 29, 30] with evidence that turning increases drying through heat production and accelerated drying [23].

3.3.2 Temperature

Temperature is a well-established factor for inactivating pathogens, and is the driving stressor for inactivation for most approved Class A processes [9]. However, few studies have looked PIO inactivation under ambient temperatures. Warmer conditions appear more detrimental to PIOs than conditions near freezing [36]. However, freeze thaw cycles are thought to contribute to PIO reduction during long term storage [37].

3.3.3 Volatile Fatty Acids

VFAs are naturally present in biosolids and may play an important role in pathogen inactivation. In particular, VFAs appear to be significant in the inactivation of *Ascaris* ova [38-40] and enteric bacteria [41-43]. However, it is also well documented that bacteria that infects the human gut are relatively tolerant against high concentrations of VFAs [44-49]. However, many of these studies analyzed relatively short periods of exposure (less than a day) to these acids. Prolonged exposure to lower concentrations may be key to the inactivation efficacy in LCLT processes. In addition, the acids in the human gut are primarily in the range of 1-6 carbon chains [50]. Long chain fatty acids are thought to be more toxic to PIOs [41].

3.3.4 pH

pH is the master chemical variable, dictating the toxicity of VFAs and ammonia to PIOs. In particular, VFA effectiveness against microbes changes with pH because the speciation of the acid changes to its protonated form at pH levels below 4.5 and allows for transfer across cellular membranes [39, 41]. pH is also significant for ammonia speciation, as ammonia is only toxic to microbes above pH when it is predominately in the NH_3 form [43, 51, 52]. However, because stored biosolids lose ammonia [25, 29] and tend to acidify during storage, ammonia is not likely a major source of chemical inactivation. Therefore, the relationship between pH and VFAs is believed to be more significant during long term storage of biosolids.

3.4 Selected Parameters

The ultimate goal of the laboratory study is to develop a multivariate model that predicts PIO inaction as a function of the four factors selected for analysis: pH, temperature, TS, and VFAs. A factorial design was selected to analyze the effects of the controlled parameters because of the ability to identify PIO inactivation rates, isolate interactions between key factors, and conduct experimental tests in a streamlined fashion [53]. These factors and their factor amounts were selected at the conclusion of pilot scale studies that took place at PLWSA and GIWA. In the first rounds of experiments, the goal is to use the factorial design to identify the most important factors affecting PIO inactivation. In these experiments PIO inactivation was monitored at different combinations of the key factors of higher and low values (Table 3.1).

Table 3.1: Summary of Selected Experimental Parameters and Values

	Low	High	Tolerance
pH	4.5	8	+/- 1
Temperature (°C)	10	25	+/- 1
TS (%)	4	20	+/- 2%
VFA (mg/kg TS)	---	+20000	+/- 500

3.5 Experimental Methodology

Many of the methods used in the following sections are official EPA methods. For details, method number used for each test and well as simplified procedures used in daily laboratory use, see Appendix A.

3.5.1 Microbial Testing

3.5.1.1 *Fecal Coliforms*

For all tests described below, native fecal coliforms present in the biosolids after anaerobic digestion were measured as indicator organisms using EPA method 1681 [54]. The levels

of fecal coliforms were fairly constant for all biosolids used for testing (10^5 - 10^6 MPN/g TS), and it was not necessary to spike organisms.

3.5.1.2 *Escherichia coli* NRRL B-59838 (Green Fluorescent Protein (GFP))

In addition to fecal coliforms, *Escherichia coli* are another important indicator of fecal contamination [55]. In particular, one of the major waterborne pathogenic bacteria of concern is *E. coli* O157:H57, which is an enterohemorrhagic *E. coli*. Therefore, a derivative of the *E. coli* O157:H57 strain, NRRL B-59838, was obtained from the Agricultural Research Service Culture Collection. This organism is serotype O157:H57, but lacks Shiga toxins and therefore is non-pathogenic. The strain is genetically modified to contain a green fluorescent protein, so it can be used as a traceable bacterial indicator. It will fluoresce underneath short wave UV light, which allows colonies to be easily identified [56]. It will be referred to hereon as GFP.

GFP cannot be released to the environment because it has been genetically engineered. It is therefore treated as a BSL2 organism even though it does not pose a substantial infectious risk. Accordingly, all materials used in this study that come in contact with the culture and any remaining culture were destroyed by autoclaving, 10% bleach, or 70% ethanol. See standard operating procedures in Appendix B for details.

After it was acquired, GFP was maintained for laboratory use via streaking on Luria-Bertani (LB) (BD 244610) 1.5% agar plates containing 100 ug/L ampicillin (Sigma A9518). The plates were incubated at 37 °C and then stored at 4 °C until needed. The culture was transferred to fresh plates approximately once a month. For longer storage, GFP was grown in LB (Miller) (BD 244610) broth overnight then transferred to fresh broth for 3 hours to enter exponential phase. After exponential phase was reached, the culture was centrifuged at 2000 x g for 10 minutes, broth was decanted, and the pellet was re-suspended in buffer water (BW) (Hach 2143166). The centrifuging, decanting and re-suspension was repeated twice more before mixing 1:1 with sterile 50% glycerol/water mix and freezing at -80 °C for long term storage [57].

To quantify GFP cell concentration, the spread plate technique was used. Samples were first serially diluted in BW and spread on LB (BD 244610) plates containing 1.5% agar and

100 µg/L of ampicillin (Sigma A9518). The plates were inverted and incubated at 37 °C overnight before quantifying the colony forming units (CFU). A UV light wavelength 365 nm was used to check fluorescence of the colonies, particularly for samples with biosolids present. Any colonies that did not fluoresce were not counted because they were considered to be antibiotic resistant background bacteria, not GFP. [57]

3.5.1.2.1 Growth Curve

To establish a growth curve, 0.5 mL overnight GFP was transferred to 50 mL of LB (BD 244610) broth + 100 ug/L ampicillin (Sigma A9518) in a 250 mL side arm nephelo flask and incubated in a shaker water bath at 37 °C and 100 rpm. Every 30 minutes, a sample was removed from the flask, diluted in sterile BW, and analyzed using the spread plate technique. The optical density (OD) was simultaneously recorded using the arm flask in a spectrophotometer (Thermo Fisher Scientific Model # 4001/4) at 600 nm. A typical growth curve is pictured in Figure 3.1. The relative relationship between cell numbers and OD₆₀₀ is shown in Figure 3.2. The growth curves are similar to those produced by other work with the organism [58] and provided a useful check prior to inoculation into biosolids.

Figure 3.1 Optical Density of GFP vs Time

Figure 3.2 Colony Forming Units per mL vs Optical Density 600

3.5.1.2.2 Fecal Coliform Test Interference

To determine if the addition of GFP would interfere in the quantification of fecal coliforms, GFP cells washed in BW were added to A1 medium (BD 218231) at dilutions of 10^{-1} through 10^{-8} and enumerated using EPA 1681. No positive tests were found. This is likely due to the fact that *E. coli* O157:H57 is a poor fecal coliform, because it does not grow well at 45 °C [11, 59]. This suggests an advantage for using GFP as an indicator organism for biosolids because it is an easily identified, is non-pathogenic, represents a pathogen of concern accurately and is independent of fecal coliform numbers.

3.5.1.3 Coliphage

3.5.1.3.1 Test Spike Propagation

Coliphages derived from biosolids were obtained for testing and spiking using a procedure described in Chapter 4.

Male specific and somatic coliphages were analyzed in accordance to a version of EPA 1601 modified for biosolids using an MPN approach. This modification of Method 1601 is detailed in Chapter 4.

3.5.1.4 Microbial Additions

In experiments requiring spiking with GFP and male specific and somatic coliphages, the organisms were propagated as described above. Then, to inoculate reactors, the organisms were diluted to the desired level in sterile buffered BW and added to the reactors. For liquid reactors, diluted microbes were directly added to individual reactors. For reactors with solids contents above 20%, diluted microbes were added to the biosolids mixture and mixed on low speed for 1 minute using a Kitchen Aid mixer.

3.5.2 Total Solids Adjustment

Drying was selected as the mechanism for raising the TS and allow for aqueous reagents to be added. This approach was selected over other dewatering mechanisms, such as centrifuging and pressing, because it prevented regrowth of fecal coliforms, and allowed higher solids concentrations to be achieved. Drying is a reproducible process, and the desired TS can be achieved relatively quickly.

3.5.2.1 Drying Rates

Biosolids were dried in sterile 9"x13" glass baking pans in a 37 °C oven. The solids were turned daily to release moisture and homogenize the solids. During method development, TS were monitored daily to determine drying rates (Figure 3.3). It was observed that pressing the biosolids into the pan resulted in an exponential increase in TS and resulted in more reproducible drying than when batches were not pressed into the pan.

Figure 3.3 Biosolids Drying Rates when held at 37 °C for 140 hours

3.5.2.2 Fecal Coliform Survival

To confirm that fecal coliforms were not inactivated during the drying process, fecal coliforms were sampled daily over 140 hours of drying at 37 °C. Over the course of the test, fecal coliform levels remained stable at levels of 10^4 to 10^5 MPN/g TS as illustrated in Figure 3.4. The survival of the fecal coliforms under the drying conditions is presumably due to the fact that fecal coliforms are heat tolerant up to 45 °C.

Figure 3.4 Fecal Coliform Survival in Biosolids dried at 37 °C

3.5.2.3 Dry-Adjust-Rehydrate (DAR) Procedure

For most of the laboratory scale treatments described below, a process referred to as the dry-adjust-rehydrate (DAR) procedure was used. To dry the biosolids and achieve the desired TS level the biosolids were dried at 37 °C to a TS level beyond the desired end level. Then, any chemical additions were made (e.g. hydrochloric acid or sodium hydroxide to adjust pH) to adjust the conditions in the biosolids. Then, distilled water was then added as needed to bring the biosolids to the desired TS level. The prepared biosolids were placed in sealed reactors to maintain their solids levels. All adjustments were performed on a mass basis.

3.5.2.4 50% TS Testing

Because of the high TS levels achieved at GIWA, the high factor amount for TS (Table 3.1) was originally set to 50%. To achieve 50% TS using the DAR procedure required drying biosolids to 80-85% TS to allow for aqueous reagent additions. At these high TS levels, the biosolids became hydrophobic and would not rehydrate. This was remedied by breaking the biosolids chunks apart in a blender and sieving through a #16 sieve, because smaller particles have superior moisture absorption. The biosolids were first blended for one minute and sieved. Then, any material retained on the sieve was returned to the

blender to repeat the process. After 3 successive blending cycles, 70% of the original material passed through the sieve. The retained and sieved materials can be seen in the photograph shown in Figure 3.5.

Figure 3.5 Sieved biosolids (left) vs retained (right)

Unfortunately, this method resulted in a great deal of dust formation and the blender operation created elevated noise levels. Hoods that could accommodate the dust were investigated, but no suitable ones were located. A micro-deval device that is typically used for aggregate crushing was also investigated, but it was determined that it would be too difficult to disinfect after use and it was not effective at crushing the biosolids pieces.

In addition, challenges were experienced with the sieved 85% biosolids when rehydrated with reagents during the DAR procedure. Specifically, the biosolids with a TS set point of 50% rehydrated well, but those rehydrated to 25% turned into a slurry. This observation is presumably due to physical changes that the biosolids underwent when dried to high levels. Because the biosolids could not be uniformly adjusted between solids levels, and the process to adjust the biosolids to a point they could be usable at 50% was arduous, the 50% adjustment level was abandoned. Accordingly, biosolids adjustments were limited to 30% TS.

3.5.3 VFA Adjustment

VFA adjustments (table 3.2) were made adding a composite VFA solution which was prepared so that multiple acids could be added in a single step. The VFAs selected and their proportions were sourced from Rojas-Oropeza et al, who looked at the impact of mixtures of VFAs found in anaerobic digestion of *Ascaris ova*. They found one solution saw a greater reduction in *Ascaris ova* was found, which was used in this study [42]. The solution was concentrated to limit the amount of reagent added and minimize changes in TS.

Table 3.2 VFA Solution Preparation

Acid Type	Concentration (mg/L)	Concentration (M)	Amount Concentrate for 1 L
Acetic	42000	0.7	40
Propionic	22000	0.3	22
Valeric	12000	0.12	13
Total	76000	1.11	75

3.5.4 Temperature Adjustment

Temperature adjustments were made by using incubators. A cold temperature incubator (Fisher Model 307) was used for 10 °C tests and a heating incubator (VWR Scientific Model 1535) was used for 25 °C tests. The temperatures were periodically spot checked to ensure temperature was maintained.

3.5.5 pH Adjustment

pH was adjusted by adding 1 M sodium hydroxide or 1 M hydrochloric acid as appropriate to the biosolids during the DAR procedure. However, there were significant difficulties with maintaining pH at the level the biosolids were adjusted to. For example, the biosolids typically started in the pH 7-8 range and subsequently trended towards pH 7 throughout tests. Methods to control this were therefore explored.

3.5.5.1 Buffering

Because pH 8 was selected as the high pH value and most incubated biosolids remained in the pH 7-8 range throughout testing, it was determined that no buffer would be used for the biosolids. Instead, the natural buffer capacity of the biosolids was used for these scenarios.

Potassium hydrogen phthalate (KHP) was selected as the buffer for the low pH value of 4.5. Of the buffers in the pH 4-5 range, KHP is one of the few that is not a volatile acid and thus would have the least interference with biological processes and set parameters. KHP buffer for pH 4.5 was prepared according to Robinson and Stokes [60].

To test the buffer efficiency of KHP in biosolids, biosolids were prepared using the DAR procedure to 30%, the pH was adjusted to 4.5, and finally the TS adjusted to 4% or 20% TS. During the solids adjustment process, 10 mL (high buffer) or 1 mL (low buffer) was added for every 150 g of wet weight solids. The biosolids were distributed into individual reactors and incubated at 10 or 25 °C for one week, after which the pH values were checked again. See table 3.3 below for pH values after one week.

Table 3.3 pH after 1 week at High buffer (HB) vs low buffer (LB) low solids (LS) or high solids (HS), and high temp (HT) or low temp (LT)

	HB-HS- HT	LB-HS- HT	HB-LS- HT	LB-LS- HT	HB-LS- LT	LB-LS- LT	HB-HS- LT	LB-HS- LT
A	6.25	6.12	4.19	6.72	4	6.57	6.09	6.23
B	6.09	6.13	4.73	6.67	2.43	6.44	6.15	5.95

Although some buffering occurred, most of the reactors were already above pH 6 after just 7 days. One set of reactors, the high buffer, low solids and high temperature condition stayed within range, but it was suspected that the pH was possibly over adjusted to a pH of 2. This is supported by one of the reactors for the high buffer, low solids, low temperature condition remaining at that level.

Based on further calculations, it was concluded that the amount of buffer needed to control a pH change to ≥ 1 pH unit would require the biosolids be re-hydrated completely with a 1

M solution. This would alter the ionic strength of the solutions, which would change the impact of chemicals added to the reactors. Because the biosolids already have an ionic strength, altering the ionic strength from their natural state would likely confound the results and be difficult to account for.

It was decided that buffering the biosolids was an impractical approach for buffer biosolids. Therefore, other approaches were investigated for pH that are described in the following section.

3.5.6 Individual Reactor Designs

In the first attempt to simultaneously adjust multiple factors, a series of batch reactors (8 oz. Boston Round bottles) were set up with 150 g of wet weight solids per bottle for each combination of conditions. Subsequently, replicate reactors were sacrificed at each sampling point and analyzed for the constituents of interest.

3.5.6.1 50% TS with pH adjustment

50% TS reactors were prepared according the DAR procedure and the pH was adjusted to give an initial pH of ~8 or 4.5, as discussed above. In all cases, pH changes were observed in the batch reactors (Table 3.4). The pH changes in duplicate reactors were very reproducible over the 40-day period, and in reactors initially adjusted to pH 8, the pH remained very close to the initial adjusted value. However, the reactors with biosolids adjusted to an initial pH of 4.5 were observed to undergo significant, albeit similar increases in pH. No TS data was taken because of the accuracy of the DAR procedure.

Interestingly, mold growth was observed only in reactors with an initial pH of 4.5 and iron was observed to leach from the biosolids. A similar phenomenon was observed during the pilot scale studies at GIWA during long term storage when the pH and TS were at similar levels to those in the batch reactors. The iron is presumably from ferric chloride added to the treatment process at GIWA and PLWSA for chemical phosphorus removal.

Table 3.4 pH changes in 50% TS reactors over 40 days held at 25 °C

	Initial pH	Final pH	pH change
A1	7.8	8.08	0.28
A2	7.8	8.12	0.32
B1	4.41	6.49	2.08
B2	4.41	6.44	2.03
C1	4.7	7.06	2.36
C2	4.7	7.07	2.37
G1	8.41	8.37	0.04
G2	8.41	8.04	0.37

3.5.6.2 4% TS with pH and Temperature Adjustments

To simulate lagoon storage at low TS values 4% TS trial reactors were prepared [34]. The solids levels at PLWSA in the digesters were between 1-2%, which was determined to be too low to adjust up to 4%. Therefore, dewatered cake (~16% TS) was rehydrated using the DAR procedure, with either DI water or filtrate collected off the belt filter presses as the aqueous phase used for rehydration. Duplicate batch reactors were prepared with biosolids diluted using each solution. At each sampling event, duplicate reactors were sacrificed. For 6 sampling points, 2 samples from each temperature incubation (10 °C and 25 °C) were sacrificed, therefore a total of 48 reactors were prepared for each rehydration medium (DI water or filtrate).

The filtrate, which was stored at 4 °C between collection and use, was tested as dilution mechanism because it would be representative of the liquid phase of 4% TS biosolids placed in lagoon storage that have not undergone a dewatering step. However, DI water provides a more reproducible rehydrating fluid because filtrate collected at different times could contain variable amounts of ammonia and VFAs.

In these experiments the solids were adjusted on a mass basis with no chemical alterations. In between filling each reactor with 150 g wet weight biosolids, the Kitchen Aid

mixer was used to consistently mix the slurry. Subsequently, the TS results were very consistent, remaining at the adjusted TS level $\pm 0.5\%$ throughout the test as shown in Figure 3.6.

Figure 3.6 TS measurements for 4% trial reactors

Points represent the average of single measurements from four reactors. Reactors diluted with distilled water vs filtrate were not separated

Incubation at the two tested temperatures affected the native fecal coliform levels (Figure 3.7). The levels in the 10 °C reactors stayed fairly constant and high, whereas in reactors at 25 °C the fecal coliforms declined over time. There was some variability between fecal coliform levels in each reactor, but they generally were within the same order of magnitude and small compared to total changes. Spiking the reactors with known amounts of bacteria may provide a way to minimize the reactor-to-reactor variability.

Interestingly, when comparing the fecal coliforms between biosolids rehydrated with filtrate or distilled water, there was very little difference between the two (Figure 3.7). There may be a slight impact of the filtrate at the higher temperature, as the fecal coliform levels decreased more quickly in the reactors diluted with filtrate. However, with additional incubation time, the results obtained using the different rehydrating solutions were comparable to each other. There was no difference observed in fecal coliform levels with the two rehydrating solutions in the low temperature reactors.

Figure 3.7 Fecal Coliform Levels in 4% trial reactors using native fecal coliforms

Points represent the average of single measurements from duplicate reactors. Error bars represent the standard deviation. F or DI refers to filtrate or distilled water as a dilution medium respectively and 10 or 25 refers to the incubated temperature in degrees Celsius.

The pH changed very little during incubation, presumably because it was not adjusted from the natural pH. Reactors incubated at the higher 25 °C exhibited a greater decrease in pH than 10 °C, likely because of increased biological activity.

Figure 3.8 pH in trial 4% reactors

Points represent the average of single measurements from duplicate reactors. Error bars represent the standard deviation. F or DI refers to filtrate or distilled water as a dilution medium respectively and 10 or 25 refers to the incubated temperature in degrees Celsius.

Ammonia concentrations were checked at 24 days for 4 of reactors (Table 3.5). The duplicate reactors incubated at 25 °C were within 10% of each other. One key difference was that the samples hydrated with filtrate had ammonia levels approximately 1500-2000 mg/kg TS higher than those diluted with distilled water. This result is not surprising, and highlights one of the reasons why filtrate was considered for use as the rehydrating solution. However, one concern is that the concentrations of constituents, like ammonia, in the filtrate may vary depending on when the sample was collected.

Table 3.5 Ammonia levels after in 4% trial reactors 30 days.

Individual measurements of duplicate reactors are reported.

	Ammonia (mg NH ₃ -N/kg TS)
Filtrate 1	6387.5
Filtrate 2	6183.33
DI 1	4841.66
DI 2	4404.16

Another concern with using filtrate as the rehydrating solutions is that the filtrate could also be high in VFAs, and the ratio of VFAs to ammonia could vary as well. If the ratio of VFAs to ammonia was variable, one factor would have to be prioritized over the other when adjusting the filtrate to always be consistent. To gain some insight into this, VFAs were tested after 30 days. VFAs were relatively similar between DI and filtrate reactors, but more pronounced differences were observed between the 25 and 10 °C reactors (Table 3.6). The relatively similar VFA values for the four reactors may be a result of a balance in the production and consumption of VFAs that could have occurred because the reactors were left for 30 days before testing. It should also be noted that all VFA levels had substantially increased from initial levels, which are typically around ~4000 mg Ac/kg TS. This increase is likely a result of fermentation that occurred in the anaerobic environment.

Table 3.6 VFA levels in 4% trial reactors after 30 days.

Reactors held at different temperatures and different hydration solutions were tested after 30 days

	VFA (mg Ac/kg TS)
DI 25	19725.18
F 25	17896.72
DI 10	20667.11
F 10	21498.23

3.5.6.3 4% TS with pH, temperature, and VFA adjustment and PIO spiking

In the next round of tests with the batch reactors, the spiking of the biosolids with known levels of PIOs was investigated. To create individual spiked reactors, batches of biosolids were first adjusted to the desired conditions using the DAR method. Then, 8 oz Boston round bottles were then filled with 150 g wet weight of the adjusted biosolids. The bottles were filled in triplicate and each time a reactor was sampled, the entire bottle was sacrificed. Native fecal coliforms were used, while GFP and (MS and somatic) coliphages were spiked in at 10^6 and 10^5 organisms/g TS respectively. Two sets of conditions were tested: (1) high temperature, low solids, high pH, and low VFAs; (2) low temperature, low solids, low pH, and low VFAs. These are in accordance to the factorial matrix (Table 3.6), which was revised based on experiments described previously.

Table 3.7 Factorial Design Matrix

Minus signs represent low values and plus signs represents high values

Count	Temperature	VFA	pH	Total Solids
1	-	-	-	-
2	+	-	-	+
3	-	+	-	+
4	+	+	-	-
5	-	-	+	+
6	+	-	+	-
7	-	+	+	-
8	+	+	+	+

3.5.6.4 High Temp, Low Solids, High pH, Low VFA

The high temp, low solids, low pH, and low VFA scenario was conducted according to the factorial matrix (Table 3.7). The biosolids were adjusted using the DAR procedure, with DI water as the rehydrating solution to adjust the TS to 4%. Sodium hydroxide was used to

adjust the pH to 8 and no buffer was added. The VFAs were left at the background levels, and the reactors were incubated at 25 °C.

The results obtained from microbial testing under the given conditions are presented in Figure 3.9. Fecal coliforms and coliphages remained stable throughout the test period. GFP was initially present at 10^5 CFU/g TS, it was non-detect after 9 days. The loss of GFP is presumably because of the poor survival of GFP at that temperature [61]. Future experiments increased the density of the spike to 10^6 CFU/g TS and a sampling point at 3 days was added to capture any initial reductions.

Figure 3.9 Measured organisms per g TS over time for HT, LS, HP, LV

Values are log adjusted and the points represent the average of single measurements from triplicate reactors, error bars represent the standard deviation. Fecal coliforms and coliphages are verified in a most probable number format, GFP was directly evaluated in colony forming units.

The pH did not remain as stable as hoped (Figure 3.10), which likely was a result of the lack of a buffer addition and limited capacity of natural buffer in the biosolids. The pH likely trended downward as a result of acid production, which naturally occurs under anaerobic conditions.

Figure 3.10 pH measurements over time for HT, LS, HP, LV

Points represent the average of single measurements from triplicate reactors, error bars represent the standard deviation.

3.5.6.5 Low Temp, Low Solids, Low pH, Low VFA (LT, LS, LP, LV)

A low temp, low solids, low pH, and low VFA scenario was conducted according to the factorial matrix (Table 3.7). The biosolids were adjusted using the DAR procedure, with DI water as the rehydrating solution to adjust the TS to 4%. Hydrochloric acid was used to adjust the pH to 4.5, and KHP buffer [60] was added at a level of 10 mL per reactor in an attempt to control pH. No extra VFAs were added, and the reactors were incubated at 10 °C.

The effect of the conditions on the sampled microbes is presented in figure 3.11. Overall, little change was observed in the PIOs. This observation is not particularly surprising as the selected conditions were relatively mild. GFP was stable with only a small decrease observed (~1 log). This is consistent with previous observations for GFP inactivation as a function of temperature [61] and verified the decision to increase the spike and sample more frequently and earlier to capture inactivation trends.

Figure 3.11 Measured organisms per g TS over time for LT, LS, LP, LV

Values are log adjusted and the points represent the average of single measurements from triplicate reactors, error bars represent the standard deviation. Fecal coliforms and coliphages are verified in a most probable number format, GFP was directly evaluated in colony forming units.

One of the most concerning aspects of the results for this scenario was that pH did not remain stable, even with the addition of buffer (Figure 3.12). By the first day, the pH level had already increased from 4.5 to above 5 and was approaching 6 by the end of 12 days. This is a problem for the experimental design concerning because pH affects all chemical processes and with such changes observed, the results would not accurately reflect the desired effect of pH on the system.

Figure 3.12 pH measurements over time for LT, LS, LP, LV

Points represent the average of single measurements from triplicate reactors, error bars represent the standard deviation

Ultimately, while other factors could be controlled, the fact that pH could not be controlled in the low and high temperature scenarios led to the individual reactor approach to be abandoned. It should be noted that although many studies that investigate pH effects have been performed, generally experiments have been conducted in buffered water, or only the initial pH was reported, with no continuous monitoring over the study [38, 39, 51].

3.5.7 Large-Scale Batch Reactors

3.5.7.1 Method

Because of the difficulties encountered with controlling the pH in the small batch reactors described above, a larger scale batch reactor approach was adopted that permitted the periodic adjustment of the pH in the biosolids slurry. This approach would not be possible with a high solids matrix. Therefore, the TS was fixed at 5% for all runs to simulate a lagoon environment and TS was eliminated as a factor in the study.

The larger batch reactors were created using 2 L wide mouth solution bottles (Fisherbrand #FB8002000). The caps of the bottles were modified by drilling a hole so that an ISFET

pH probe (Hach PHW77-SS) could be inserted to the reactor and a sampling port could be accessed via a 25 mL serological pipette. See Figure 3.13 for a photo of the setup. The reactor was designed to be sealed at all times, except when pH was adjusted or samples were retrieved. The goal of this approach was to simulate a semi-anaerobic environment that biosolids experience in a lagoon storage environment. Accordingly, the reactor contents were left unmixed except when the pH was adjusted or the reactors sampled. Mixing was achieved via a 3-inch magnetic stir bar and stir plate to fully suspend and mix the reactor contents. This was done once a day.

Figure 3.13 Reactor Set up for Large Scale reactors

To initiate these experiments, the bottles were filled with 2 L of biosolids that had been adjusted 5% TS on a mass basis. VFAs were added at a level of 20,000 mg/kg TS, and GFP and male specific and somatic coliphages were added as described above. For each sampling event, 50 mL were pulled from each reactor using a 25 mL sterile pipette. Two samples were collected each time a reactor was sampled.

To evaluate the new experimental approach, a test run was performed using two of the large scale reactors. For this test run, a high pH, high VFA, and high temperature scenario was selected. This scenario was selected because fewer pH adjustments would be needed and the behavior of VFAs could be observed. The latter was important since previous investigations had not monitored VFA behavior over time. 1 M Sodium hydroxide was added daily to maintain a pH of 8. For temperature control, the reactors were incubated at 25 °C.

3.5.7.2 Results

The pH data for the trial run is summarized in Figure 3.14. The starting pH for the reactors prior to any pH adjustment was approximately 7.4. Subsequently, an adjustment period was observed for approximately 5 days as the pH approached the target pH of 8, after which point the pH changes became smaller and much more consistent. This suggests that the natural buffer capacity was consumed and a stable system was achieved.

Figure 3.14 Starting pH when adjusted daily

Points represent the average of two reactors, error bars represent the standard deviation

As the pH stabilized, the volume of sodium hydroxide added decreased to about 2 mL per day (Figure 3.15). This is a relatively small amount considering the reactor size of 2 L, but

over time the addition of the solution impacted the TS levels, which saw a gradual decline (Figure 3.16).

Figure 3.15 Amount of sodium hydroxide needed daily to adjust pH to 8

Points represent the average of two reactors, error bars represent the standard deviation.

Figure 3.16 TS values over time in batch reactors

Points represent the average of duplicate measurements from duplicate reactors, error bars represent the standard deviation

GFP, male specific and somatic coliphages and fecal coliforms saw declines throughout the experiment (Figure 3.17). Overall, fairly consistent inactivation trends were observed, suggesting that spiked large scale batch reactors allows for a better determination of the PIO inactivation constants than the small scale reactors. Both GFP and fecal coliforms were more susceptible than the coliphages, suggesting that coliphage are a relatively conservative indicator.

Figure 3.17 Measured organisms per g TS over time for batch reactors

Values are log adjusted and the points represent the average of duplicate measurements from duplicate reactors, error bars represent the standard deviation. Fecal coliforms and coliphages are verified in a most probable number format, GFP was directly evaluated in colony forming units.

VFA concentration in the biosolids slurry was also monitored (Figure 2.18). This was important because there was concern as to whether this approach would maintain consistent levels of VFAs in the reactors. VFAs decreased steadily with time, likely because of background biological activity.

Figure 3.18 VFA values over time in batch reactors

Points represent the average of duplicate measurements from duplicate reactors, error bars represent the standard deviation

To investigate the cause of VFA losses, follow up experiments were performed. In the first experiment, the consumption of VFAs via methanogens naturally present in the biosolids was investigated. The second experiment investigated if the VFA loss was physical from mixing the reactors daily.

3.5.7.3 VFA Loss via Methanogen Consumption

To perform the investigation of VFA loss due to consumption by methanogens, freshly pressed biosolids were collected from the Portage Lake Water and Sewer authority using a sterile container and then stored at 4 °C until use. The moisture content was evaluated using EPA Method 1684 and then the TS level was adjusted to 5% TS by diluting with deionized water and mixing until smooth using a Kitchen aid mixer. Subsequently, 100 mL aliquots of 5% sludge was dispensed into 160 mL serum bottles. Then, three different treatments were applied, each in triplicate: a) no VFA supplement b) VFA supplement c) VFA supplement added every few days using a needle and syringe. In the reactors receiving VFA supplements, the previously described VFA solution was added to result in the same concentration used in the large scale batch reactors. Once the adjustments were complete, the bottles were sealed with a rubber stopper and clamp and were not purged

prior to sealing. The goal was to create a semi anaerobic environment. The bottles were incubated at either 25 or 10 °C without shaking for 16 days.

Prior to sampling, the bottles were removed from their incubation and placed on a shaker table at 120 rpm for 2 minutes before using a gas tight syringe to collect a 0.5 mL head space sample for methane analysis. The sample was analyzed on a gas chromatogram for methane only using a PCE program [62] that had been calibrated for methane analysis. Henry's Law was used to convert the measured methane concentration into methane produced per bottle. Bottles were vented when the pressure reached 30 psi for safety reasons. The methane per bottle was measured prior to venting as well as after venting to quantify methane losses.

The results for methane production at 25 °C for the three treatments are summarized in Figure 3.19. The treatment with no VFA supplement served as the baseline level for comparing the effects of adding VFAs. These un-supplemented reactors yielded a steady production of methane, whereas supplemented reactors produced higher methane yields.

Figure 3.19 Methane Production per Bottle over time at 25°C

Points represent the average of single measurements from triplicate reactors. Error Bars represent the standard deviation and are sometimes covered by the data. No VFA refers to bottles with no added VFAs, VFA refers to a one time supplement and VFA+ refers to bottles that were replenished with VFAs on days 0, 5 and 12.

Some insight into the effect of VFA spikes can be gained by comparing methane production in the reactors with serial supplements of VFAs to methane production in the reactors with a single initial spike of VFAs. The methane production rates for the supplemented reactors with VFAs added on days 0, 5 and 12 are shown in Figure 3.20.

Figure 3.20 Methane production rates of bottles supplemented with VFAs

Points represent the average of single measurements from triplicate reactors. Error Bars represent the standard deviation and are sometimes covered by the data.

The rates of methane production were determined using linear regression with data that represented the average of three replicates. The rates are very similar following each VFA supplement. This suggests that the microbial community responded consistently and significantly (nearly 3.5 times the baseline production) to each VFA supplement. In contrast, a different pattern of methane production was observed for the one-time supplement (Figure 3.21). This treatment had a similar initial methane production rate to the constantly supplemented rate. However, after 4 days, the methane production rate dropped to one comparable to the observed rate in the un-supplemented treatments. This suggests that the VFAs aid in methane production but are quickly depleted. Final measurement of the VFAs was not conducted to confirm this as it was assumed that the only source of methane production would be due to VFA consumption.

Figure 3.21 Methane production rates of bottles supplemented once with VFAs

Points represent the average of single measurements from triplicate reactors. Error Bars represent the standard deviation and are sometimes covered by the data.

Furthermore, the amount of methane produced in the VFA supplemented reactors was proportional to the amount of VFAs that were added to each reactor. Methanogenesis will only convert acetate into methane, and the solution added to the reactors was a combination of propionate, acetate and valerate. It was presumed during computations that all propionate and valerate would be broken down into acetate prior to transformation into methane. It was determined that all of the VFAs added were converted into methane for the reactors incubated at 25 °C.

The methane production rates for the 10 °C scenarios are pictured in Figure 3.22. Methane production was much lower because methanogenesis was inhibited at the low temperature. However, after 2 weeks, methane production increased dramatically suggesting that VFA consumption could still be an issue even at unfavorable low temperatures. Interestingly, the reactor with 3 sequential VFA additions had a lower methane production than the reactors with a single initial VFA addition. This suggests the repetitive VFA supplements may have an inhibitory effect, such as occurs with soured digesters, because the methanogens could not accommodate the supplemented acids.

Figure 3.22 Methane Production per Bottle over time at 10°C

Points represent the average of single measurements from triplicate reactors. Error Bars represent the standard deviation and are sometimes covered by the data. No VFA refers to bottles with no added VFAs, VFA refers to a one-time supplement and VFA+ refers to bottles that were replenished with VFAs every few days.

3.5.7.4 Physical VFA Losses

To verify that physical losses (e.g. via stripping) were not contributing to the observed VFA losses, the physical effects were isolated in another experiment. This investigation was prepared using freshly pressed biosolids collected from the Portage Lake Water and Sewer authority using a sterile container, and stored at 4 °C until use. The initial moisture content was evaluated using EPA Method 1684 prior to adjusting the biosolids to 5% TS by diluting with deionized water and mixing until smooth with a Kitchen aid mixer. The two 2 L wide mouth solution bottles were filled with 2000 g wet weight of the 5% solids mixture and autoclaved at 121 °C for 60 minutes on 3 consecutive days to reduce biological activity. After the third autoclaving cycle, the reactors were brought to room temperature and 15 mL of the VFA mixture was added to give an initial concentration of 20,000 mg/kg TS. TS were then checked again using EPA Method 1684. The bottles were incubated at 25 °C and mixed gently once a day using a magnetic stir plate and large stir bar to homogenize the mixture. Samples for VFA analysis were collected every 7 days for 3 weeks.

Figure 3.23 Volatile Acids over time for Volatile Acid Stripping Test

Points represent the average of single measurements from duplicate reactors. Error Bars represent the standard deviation

Based on the VFA data shown in Figure 3.23, any losses of VFAs due to stripping were masked by the production of VFAs which resulted in an increase in VFA concentration in the reactors. This observation is likely a result of incomplete inactivation of acidogenic microbes coupled with the natural decay of organic matter in the reactors. VFAs did decrease slightly between 7 and 14 days, possibly as a result of regrowth of methanogens and consumption of the abundant acids.

3.6 Conclusions

Several key insights can be drawn from the preliminary laboratory-scale studies that can be used in the further development of the experimental methodology. One, it was found that GFP could be used as an indicator organisms in biosolids that is representative of a pathogen of concern yet nonpathogenic and is easily measured independent of fecal coliforms. Two, methods were developed for adjusting the TS, pH, and VFA levels in biosolids. However, application of the factorial experimental design requires not only that the key factors be adjusted, but that they hold their desired level for the duration of the experiment. While temperature and TS could easily be maintained, maintaining pH and

VFAs at the desired values proved problematic using the selected experimental system. In particular the low pH condition was difficult to maintain, even with the addition of buffer. While physical losses of VFAs were not observed, the consumption of VFAs resulted in too many complications for application of the factorial design. Based on these findings combined with frequent mixing issues as a result of biosolids viscosity it is recommended the biosolids slurry approach explained in these experiments be abandoned in on-going laboratory investigations.

4 Method Verification and Implementation for Coliphage Testing in Biosolids

4.1 Introduction

During treatment of municipal wastewater at water resource recovery facilities (WRRFs), solid residuals are produced. The solids contain organic matter and nutrients, which can be sustainably reused via land application (e.g., agricultural land, mine reclamation sites, etc.). However, these solid residuals also contain enteric human pathogens. In the United States (US), Part 503 of Title 40 of the Code of Federal Regulations requires that the solids be treated to reduce pathogen and indicator organism (PIOs) levels prior to land application. Treated solids that achieve the required PIO reductions are termed biosolids. Biosolids with non-detectable PIO levels are designated as Class A biosolids, and have few restrictions on their use. One of the categories of PIOs included in the Part 503 regulations are human enteric viruses. Methods for detection and quantification of enteric viruses in biosolids require three key steps: sample collection, elution/concentration, and assay/identification. The assays used include the plaque and quantal methods [4]. Unfortunately, there are several challenges associated with the application of these methods. For example, the current method can yield non-detect concentrations in wastewater and sludge samples [63, 64], and because viral infections typically occur in humans at certain times of the year, there may be seasonal variations in enteric virus numbers [65]. In addition, biosolids samples can be toxic to the cellular host used for the virus assay [66], and recoveries during the elution/concentration step can be variable, with actual virus levels up to 10,000 times greater than that measured [67] and typical recoveries of 18-25% [68]. Finally, the assay methods can be expensive to conduct and require a high level of expertise to perform, which is typically beyond the capabilities of most WRRF laboratories [69].

Alternatively, bacteriophages, i.e., viruses that infect bacteria, have a morphology that is similar to human viruses and have been suggested as a potential indicator for enteric viruses. They are found in all environments where bacteria are present, including the human microbiome and, therefore, wastewater [11]. Accordingly, bacteriophage have

been suggested as tools for process monitoring of wastewater systems [70, 71]. In particular, there has been a focus on use of coliphages, which are a subset of bacteriophages that infect coliform bacteria such including *Escherichia coli*, as fecal indicator viruses. Coliphages are divided into two major functional types: somatic coliphages, which infect the bacterial host cell via direct attachment to the cell wall; and male-specific (also known as F+) coliphages, which infect their bacterial host cell by attachment to the cell's F-pilus and, therefore, only infect hosts that contain the F+ plasmid and produce F-pili [11]. Somatic coliphages are typically more abundant than male specific coliphages in wastewater treatment [65, 72, 73], and in some studies somatic coliphages have been detected in all raw wastewater samples [63].

Coliphages have been demonstrated to correlate well with the presence of other fecal indicator organisms, particularly in ambient water [10, 72]. In wastewater systems they typically have stronger correlations to other measured viruses than bacterial indicators [63, 71], although this has not always been found to be the case [74]. There have been a few studies that investigated coliphage levels in biosolids, and promising correlations between coliphage and enteric viruses have typically been observed in anaerobic digestion and biosolids [12, 65, 67, 73, 75]. As a result, Western Australia has adapted standards for coliphages in their highest quality biosolids product [13].

Unfortunately, there is no standard unified method for the quantification of coliphage in biosolids. The U.S. Environmental Protection Agency (EPA) has developed two methods for coliphage quantification in groundwater: the two-step enrichment EPA method 1601 (ENR), and the single agar layer EPA method 1602 (SAL). The ENR method is only certified as a presence/absence test by the EPA, whereas the SAL method quantifies coliphages in plaque forming units (PFU) [14, 15]. The ENR method can be quantified as a most probable number (MPN) test through quantification of multiple sample volumes, (ENR/MPN) and while not EPA certified, this modification has been used by others to make the method quantitative [76]. In addition, draft methods have been published for coliphage detection in recreational waters by the ultrafiltration and single agar layer EPA method 1642 [16], and in secondary wastewater by the single agar layer EPA method 1643 [17]. However, EPA methods 1601 and 1602 specifically state that they are not intended for use in biosolids samples, and provide no guidelines for the adaptation to

biosolids, whereas EPA methods 1642 and 1643 make no mention of biosolids samples. This has led to a variety of methods being applied in studies of coliphage in biosolids, which may lead to disparate results. Modified versions of EPA method 1602 appear to have been successfully implemented, but only for low solids digester samples [67]. Double agar layer (DAL) methods have also been used [77], but often require a solids extraction step for their efficacy [64, 66, 71, 78]. Beef extract elutions are commonly used because of their current application in enteric virus testing. However, because coliphages have a strong affinity for particles [79], these methods can provide variable results. Several studies have attempted to optimize these protocols [65, 66, 80], but the studies vary greatly in their final recommendations and report recoveries of spiked coliphages of only 16% [81, 82].

Biosolids present several challenges as a sample medium for coliphage analysis using direct plate count methods like SAL and DAL. For example, the presence of the solid particles can cause visual interference when looking for plaques via direct observation because solid particles can look similar to plaques and can mask small plaques due to agar discoloration. Elution procedures can be used to reduce this interference by facilitating the removal of coliphages from the solids matrix, but these techniques are time consuming and have drawbacks that were discussed previously. Detection of low concentrations of coliphage is also difficult with biosolids samples because the solid medium cannot be directly tested and must be diluted for direct analysis. Because of these challenges associated with using the SAL and DAL methods for coliphage analysis in biosolids, the overall goal of this study was to evaluate use of the ENR/MPN method for the detection of male specific and somatic coliphages in biosolids. This two-step enrichment procedure is capable of quantifying a wide range of values, has a lower detection limit than the SAL and DAL direct plate counts, and is less subject to interference from the presence of solids. Specific objectives of the study were as follows: (1) further develop the modified ENR/MPN method for application to biosolids and perform quality control tests on the modified method; (2) compare the SAL, DAL, and modified ENR/MPN methods for the detection of male specific and somatic coliphages in biosolids; and (3) demonstrate application of the modified ENR/MPN method at one small and one large water resource recovery facilities in the United States—the Portage Lake Water and Sewer Authority (PLWSA), Houghton, MI, and the Stickney Water Reclamation Plant

(SWRP), operated by the Metropolitan Water Reclamation District of Greater Chicago, IL (data to be added prior to publication).

4.2 Methods

4.2.1 Test Materials Preparation

4.2.1.1 Media Preparation

The nalidixic acid (NA) antibiotic solution, Ampicillin+ Streptomycin (AS) antibiotic solution, Tryptic Soy Broth (TSB), double agar plates, were prepared according to EPA method 1601 [14]. The TSB+antibiotic solution was prepared by adding 1 mL of AS or NA antibiotic for every 100 mL of prepared TSB. AS antibiotic was used for male specific testing, and NA antibiotic was used for somatic coliphage testing.

4.2.1.2 Host Bacteria Stock Cultures

Host bacterial stock cultures *E. coli* CN-13 (Cat. No. ATCC#700609) for somatic testing and *E. coli* F_{amp} (Cat. No. ATCC#700891) for male specific testing were obtained from the ATCC. Frozen, overnight, and log-phase host bacteria stock cultures were prepared in accordance to EPA method 1601 [14].

4.2.1.3 Coliphage Stock Propagation Method

Male-specific and somatic coliphage stocks for use as spiking suspensions during method testing and for QC samples were developed following an approach adapted from the EPA method 1601 instructions for sewage filtrate [14]. First, 2.5 mL of biosolids diluted in TSB at a 10⁻¹ ratio (g wet weight/mL), 1.25 mL of appropriate log-phase host bacteria stock culture, and 25 mL of TSB+antibiotic were incubated overnight. After incubation, the mixture was vortex mixed, and then centrifuged at 2,000 x g for 10 minutes. The supernatant was filtered through a 0.22 µm syringe filter (MilliporeSigma Millex SLGS033SB) into cryogenic vials (Thermo-Fisher 5000-0020) and stored at -80 °C for up to one year or at -20 °C for up to one month.

4.2.2 SAL and DAL Methods

For SAL analysis, 10 mL of 1.5% TSA+antibiotic with 0.5 mL of log phase host bacteria culture, and 1 mL of diluted sample were combined in a 15 mL round bottom culture tube. The contents were mixed and poured into a 100 mm petri dish [77]. For DAL analysis, 3 mL of 0.7% TSA with 0.1 mL of log phase host bacteria culture, and 1 mL of diluted sample were combined in a 15 mL round bottom culture tube. The contents were mixed and poured into a 100 mm petri dish with 10 mL of pre-solidified 1.5% TSA+antibiotic [15]. After the agar solidified, the plates were sealed with parafilm, inverted and incubated overnight at 37 °C. After incubation, plaques were quantified as visible spots that formed in the bacterial lawn.

4.2.2.1 Biosolids in SAL and DAL Methods

To confirm the visual interference in the SAL and DAL methods, biosolids samples with approximately 16% TS were tested using the procedure described above. The results were compared to positive controls to determine if plaque visualization could be realized.

4.2.3 Development of the ENR/MPN method for Biosolids Samples

The ENR/MPN method as modified for biosolids samples was performed per EPA method 1601, with modifications as described below. In addition, in the process of modifying the method, comparisons were made with some of the modifications, and quality control (QC) checks were made.

4.2.3.1 Two-Step Enrichment Method, ENR/MPN- Michigan Tech Team

Collected biosolids samples were first diluted in sterile buffered water (BW) (Hach 2143166) at a ratio of 10 g wet weight solids to 90 mL BW. The sample was then blended for 1 minute in a sterile blender (Oster) and serially diluted in BW. A minimum of three dilutions were tested. Round bottom screw top culture tubes (10-15 mL) were filled with 5 mL TSB+antibiotic, 0.25 mL log-phase bacteria, and 0.5 mL of diluted sample. Five tubes were prepared per dilution, for both male specific and somatic coliphage types. The incubation tube caps were loosened and the tubes incubated for 16-24 hours at 37 °C.

Following overnight incubation, the tubes were vortex mixed for 2-3 seconds to homogenize the mixture before spotting the suspension onto prepared agar plates, with one spot per enrichment tube, five spots per plate, and 10 μ L of suspension per spot. The plates were inverted and incubated overnight at 37 °C. After 16-24 hours, the spots were inspected for infection. Any form of coliphage infection was recorded as a positive result. The number of positive results was input into a Thomas Formula solver by iteration workbook in Excel [83] to provide an MPN of coliphages present. This value was divided by the total solids value to quantify the amount of coliphages per gram total solids. Total solids were quantified via EPA 1684 [84]. A complete list of the modifications made to EPA method 1601 is provided in Appendix C, along with rationale for the modifications.

4.2.3.2 Two-Step Enrichment Method, ENR/MPN- Stickney Team

The Stickney team followed the same two-step enrichment method as the Michigan Tech team, with the following exceptions: (1) samples were diluted in TSB instead of BW; (2) enrichment tubes contained double the volume, but the same proportions; and (3) the tubes were incubated in 50 mL centrifuge tubes and centrifuged at 1000 x g for 10 minutes before spotting. To ensure that the differences in the application of the modified ENR/MPN method at the two facilities did not significantly impact the results, the effect of using BW instead of TSB for sample dilution, and the impact of centrifuging of the sample were evaluated.

4.2.3.3 BW vs TSB

To evaluate the impact of the dilution step solution, biosolids samples were diluted in TSB and BW, each in triplicate. Then the two-step enrichment method was used to quantify the coliphage concentration in each solution, and the results obtained using the modified ENR/MPN method were compared for the two dilution methods.

4.2.3.4 Enrichment Growth Curve

To examine the impact of different coliphage levels on the results of the modified ENR/MPN method, verify that low detections are possible, observe the time required for infection to host, and determine the concentrations of coliphage achieved under the

modified enrichment conditions, a series of enrichment growth curves were performed. Sterile 100 mL bottles were filled with 50 mL of TSB+antibiotic, 2.5 mL of log phase bacteria, and 5 mL of biosolids sample diluted in BW, with dilutions ranging from 10^{-1} through 10^{-4} (g wet weight biosolids/mL dilution solution). The bottles were subsequently incubated at 37 °C and sampled every hour for the first 6 hours of incubation then again at 24 hours. At each sampling point, 1 mL was removed from each incubation bottle and serially diluted in BW. Then, 10 µL of each dilution was spotted 3 times on a prepared double agar plate before incubating overnight at 37 °C. The dilution that provided visualization of individual plaques numbering from 5 to 35 was used to quantify the number of plaque forming units (PFU) per enrichment tube.

4.2.3.5 Methods for Removing Interfering Bacteria

Sometimes background bacteria or host bacteria in the enrichment culture material will grow over the circular spot area, making it difficult to determine if lysis of the host bacterial culture has occurred [14]. When this problem occurs, one solution is to remove the interfering bacteria from the enrichment culture by filtration or centrifugation. The Stickney modified ENR/MPN procedure included a centrifugation step, but Michigan Tech's procedure did not. To examine the effect of centrifugation, a comparison of the two techniques was made. In addition, an alternative approach of diluting the sample to reduce the numbers of interfering bacteria was also evaluated.

4.2.3.5.1 Low Speed Centrifuge

Samples that were not centrifuged were incubated in glass round bottom culture tubes whereas centrifuged samples were incubated in 50 mL centrifuge tubes. After overnight incubation of the enrichment culture, the centrifuged samples were centrifuged for 5 minutes at 2000 x g prior to spotting on double agar plates. Both treatments were performed in triplicate.

4.2.3.5.2 High Speed Centrifuge

The same procedure for low speed centrifuge was followed, with the following changes: enrichment tubes were changed from 50 mL to 15 mL centrifuge tubes and the centrifuge speed was increased to 4000 x g for 10 minutes.

4.2.3.5.3 Dilution

Biosolids were diluted from 10^{-1} through 10^{-3} and enriched overnight, following the enrichment procedure described above. After overnight incubation, each enriched tube was serially diluted in BW at dilutions ranging from 10^{-1} through 10^{-3} . 10 μ L of each dilution was spotted onto prepared plates. The position of the spot on the agar plate for each dilution of each enrichment culture was maintained to enable visual observation of the effect of dilution on the lysis zone.

4.2.3.6 *Quality Assurance/Quality Control*

Method blanks and positive controls were run with each coliphage test as part of the laboratory assurance program. Additionally, the following techniques were tested for additional QA/QC monitoring.

4.2.3.6.1 Plaque Confirmation

Although not required by EPA method 1601, some laboratories follow a spot plate coliphage confirmation procedure to confirm lysis zones if there are one more questionable zones on a spot plate [14]. To apply the plaque confirmation procedure to the modified ENR/MPN method, the method was run in triplicate with PLWSA biosolids. After spotting the plates, the enrichment tubes were stored at 4 °C. When the spotted plates were examined, 10 spots from male specific plates and 10 spots from somatic plates were selected for confirmation, with 5 negative enrichments and 5 positive enrichments chosen in each case. These enrichments then underwent the following confirmation procedures:

- a) A flamed loop was used to gently scrape a plaque from the selected lysis zone. This sample was then diluted in a tube containing 5 mL of TSB with no

antibiotics, and allowed to stand for 5 minutes. The tube was then vortex mixed and 10 µL was spotted on a prepared double agar plate.

- b) The enrichment tube for the selected spot was vortex mixed, then a flamed loop was used to collect a small sample, which was in turn used to inoculate a tube containing 5 mL of TSB with no antibiotics that was allowed stand for 5 minutes. The tube was then vortex mixed and 10 µL was spotted on a prepared double agar plate.
- c) The enrichment tube for the selected spot was vortex mixed, and then a 1 mL sample was collected and dispensed into a 1.5 mL sterile centrifuge tube. The aliquot was centrifuged for 10 minutes at 5,000 x g. Subsequently, 10 µL of the supernatant was spotted on a prepared double agar plate.

All checked spots were inverted and incubated at 37 °C overnight.

4.2.3.6.2 Matrix Spike and Matrix Spike Duplicate

Consistent with the QC requirements for EPA method 1601, several QC samples were analyzed, including a matrix spike and matrix spike duplicate. To spike the biosolids, the coliphage stocks derived from biosolids were diluted in BW before spotting 100 µL onto biosolids. Un-spiked biosolids, duplicate spiked biosolids and the diluted coliphage spike were all quantified using the enrichment method to compute a percent recovery of the spiked biosolids. Percent recovery and relative percent difference between samples were computed in accordance to EPA 1602 [15].

4.2.3.6.3 Milorganite Spiked Samples

Parallel to QC requirements for the fecal coliform test [54], Milorganite was examined as a possible generic matrix for QC requirements. To perform the evaluation, coliphage stock derived from biosolids were diluted in BW before spotting 100 µL onto Milorganite. Coliphage levels in the un-spiked Milorganite, duplicate spiked Milorganite and the diluted coliphage spike were all quantified using the modified ENR/MPN enrichment method to compute a percent recovery of the spiked biosolids.

4.2.3.6.4 SAL, DAL and MPN/ENR Accuracy Comparison

The coliphage levels enumerated in spiked aqueous samples using the modified ENR/MPN method were compared to the results obtained using the SAL and DAL methods. The diluted sample used for the comparison was prepared by diluting coliphage stocks in sterile BW. Each method was run in triplicate. The coliphage concentration in each sample was determined using the approach outlined for each specified method.

4.2.4 Application of the ENR/MPN Method at PLWSA

Samples of primary sludge, return activated sludge (RAS), primary digester contents, and biosolids cake were collected at PLWSA in sterile quart sized mason jars. All samples were collected on the same day within 20 minutes of each other. Biosolids cake at PLWSA is produced intermittently (every 1-2 weeks) via a belt filter press. This schedule is a result of the low volume of sludge produced at the facility, and their use of a secondary digester to hold digested sludge until the presses can be run. Accordingly, samples were only collected on days the presses were run. After collection, the samples were transported on ice to the laboratory at Michigan Tech where they were held at 4 °C until analysis. Samples were processed within 24 hours of collection.

4.3 Results and Discussion

4.3.1 Biosolids in SAL and DAL Methods

It was determined that solids interference was too great to accurately observe plaques in methods which directly incorporate the biosolids sample into the agar layer. This was concluded from comparison of the types and sizes of plaques that formed on positive controls, which if present on the plates containing biosolids would have been indistinguishable from the solids. It should also be noted that 16% is a fairly low level of solids for some biosolids treatment methods, so the issue would be exacerbated with increased solids levels. The ENR/MPN method was therefore determined superior because of the ability to handle the solids without the necessity of an extraction step.

4.3.2 Development of the ENR/MPN Method for Biosolids Samples

4.3.2.1 *BW vs TSB*

The ENR/MPN method as originally modified by the Stickney team for biosolids samples used TSB as their dilution medium for testing. However, the Michigan Tech team switched the dilution medium to BW so that the same sample dilutions could be used for testing fecal coliforms [54] and coliphages using the ENR/MPN method. In addition, the switch to BW also minimized foaming that occurred during blending, which was a problem encountered when using TSB. When tested side by side, the choice of the dilution medium did not have a statistically significant effect ($\alpha > 0.05$) on the results when the samples were randomized (Figure 4.1).

There was concern that the use of BW as the dilution medium would dilute the TSB in the enrichment tubes and impact host bacterial growth. However, blank tubes to which only BW was added in place of sample showed no impairment of bacterial growth. Furthermore, only 1 mL of sample is added for every 10 mL of TSB nutrient broth, and an equivalent amount is added to every tube, so any dilution effects would be equally realized across all enrichments. Additionally, the fact that similar results were achieved with the two dilution media indicates that there was likely no impairment of the coliphage infecting the bacteria. Either dilution medium was therefore determined to provide equivalent coliphage results, allowing data obtained with either method to be directly compared.

Figure 4.1 Effect of Dilution Medium on Method Results

Measurements were conducted in triplicate and the values presented represent the average.

Error bars represent the standard deviations

4.3.2.2 Enrichment Growth Curve

The results for the somatic and male specific coliphage growth curves are shown in Figures 4.2 and 4.3. The estimated number of coliphages per enrichment is reported in Table 4.1 and is based off of a previous measurement for the biosolids used for this experiment.

Figure 4.2 Somatic Overnight Enrichment Growth Curve

D1, D2, D3, and D4 represent the dilutions of biosolids 10^{-1} through 10^{-4} respectively.

Measurements were conducted in triplicate and the error bars represent the standard deviation.

Some symbols exceed the magnitude of the presented error bars

Figure 4.3 Male Specific Overnight Enrichment Growth Curve

D1, D2, D3, and D4 represent the dilutions of biosolids 10^{-1} through 10^{-4} respectively.

Measurements were conducted in triplicate and the error bars represent the standard deviation.

Some symbols exceed the magnitude of the presented error bars.

Table 4.1 Estimated Number of Coliphages Present in each Enrichment Bottle

	Coliphage Concentration per Enrichment Bottle (MPN/bottle)	
Dilution	Male specific	Somatic
10^{-1}	133.58	114.06
10^{-2}	13.36	11.41
10^{-3}	1.34	1.14
10^{-4}	0.13	0.11

For both male specific and somatic enrichments, enrichments that contained initial concentrations of 10-100 coliphages quickly displayed coliphage enrichment. Within 2-3 hours, the enrichment had reached levels of 10^8 to 10^{10} pfu/mL, where they remained for the remainder of the enrichment. In comparison, enrichments with lower initial levels took longer to exhibit high coliphage levels. In particular, the enrichments containing the 10^{-3} and 10^{-4} dilutions for male specific coliphage exhibited no response during the first 6 hours of incubation but displayed positive responses when tested at 24 hours. This suggests that low levels of coliphage may take longer to exhibit positive responses, and a 24-hour enrichment time should be used to ensure that all coliphages are accurately captured.

Another observation within the lower dilutions is a lower total concentration of coliphages in the final enrichment. This is likely a result of a longer lag time for coliphage infection than in the more concentrated enrichments. The coliphage concentrations in all enrichments appear limited by the concentration of bacteria present in the enrichment. Therefore, when the enrichment takes longer to reach highly concentrated levels, the bacteria present in the enrichment will have likely passed through their log phase of growth and began entering their stationary or death phase. Therefore, there are fewer healthy bacteria for the coliphage present to infect than in enrichments where the coliphage concentrations increase more rapidly. Coliphages will only infect actively growing bacteria [85], so a bacterial population with a portion in stationary or death phase would be expected to produce fewer coliphages.

Nonetheless, the amount of coliphage present in these lower incubations are sufficiently concentrated to produce a positive result. Furthermore, modified enrichment achieves

concentrations 2 orders of magnitude greater compared to water enrichments [86]. This suggests that purification methods that reduce the coliphage concentration (i.e. dilution) will still exhibit robust positive results and that the incubation time prior to final plate observation may be able to be reduced.

4.3.2.3 Methods for Removing Interfering Bacteria

4.3.2.3.1 Low Speed Centrifuge

When centrifuged at 2000 x g in 50 mL centrifuge tubes, the results displayed a statistically significant result (p value=0.069) for somatic coliphages (Figure 4.4). This was likely a result of somatic coliphages attaching to their bacterial host when centrifuged, which is more likely for somatic coliphages because they infect via the cell wall. Centrifuging of high concentrations of somatic coliphages in the 100-2000 x g range is often accompanied by an addition of Freon to combat this issue [87, 88].

It was also observed that even though the bacterial cells and biosolids were removed via the process, the observed spots did not show much difference from those that were not centrifuged. Confluent bacterial lawns were still frequently present, which negated the purpose of centrifuging. Higher speeds would likely be required to effectively remove the bacteria from these, which was investigated next.

Figure 4.4 Low Speed (2000x g) Centrifuge Comparison

Measurements were conducted in triplicate and the values presented represent the average.

Error bars represent the standard deviation

4.3.2.3.2 High Speed Centrifuge

When enrichments were centrifuged at 4000 x g in 15 mL centrifuge tubes, there was no observed difference in the final computed concentration of the two treatments (Figure 4.5). A small amount of bacterial growth was observed in negative spots, but high speed centrifugation was much more effective at purifying the enrichments than the lower centrifuge speed. No adherence of the coliphages was observed to particles as evidenced by the lack of a statistically significant difference of the observed concentrations for the two treatments, which may be another advantage to centrifuging at higher speeds. However, centrifuging is time intensive, requires specialized equipment, and forces disposable centrifuge tubes to be used. Therefore, sample dilution was explored as an alternative approach for removing bacterial interference.

Figure 4.5 High Speed (4000x g) Centrifuge Comparison

Measurements were conducted in triplicate and the values presented represent the average.

Error bars represent the standard deviations

4.3.2.3.3 Dilution

Dilution was found to be remarkably effective for removing interfering bacteria from enrichments. As illustrated in Figures 4.6 and 4.7, all enrichments that were diluted exhibited the same positive or negative results across all dilutions and the results became clear between the 10^{-2} and 10^{-3} dilutions. Both positive and negative spots had virtually all host and background bacteria removed and their respective results were much clearer. Compared to centrifugation, with dilution the entire sample is preserved and no manipulation occurs that could potentially alter the positive/negative result, which could be an advantage given issues with coliphages adhering to particles and their hosts. Additionally, because such high concentrations of coliphage are achieved during enrichment compared to water enrichments, dilution also has an advantage of reducing the concentration of coliphage spotted, which could reduce the risk of contaminating potentially negative spots on the same plate.

Figure 4.6 Dilution as a purification technique male specific coliphage

Each enriched biosolids dilution is pictured in rows, the columns represent the dilution of each enrichment from 10^{-1} through 10^{-3} in buffered saline

Figure 4.7 Dilution as a purification technique somatic coliphage

Each enriched biosolids dilution is pictured in rows, the columns represent the dilution of each enrichment from 10^{-1} through 10^{-3} in buffered saline

4.3.2.3.4 6 hour and 24 hour check

Another effective technique for reducing interfering bacteria is to check the plates after 6 hours and at 24 hours of incubation. Based on the results from the growth curve analysis, high concentrations of coliphage between 10^8 to 10^{10} coliphages/mL are present in positive enrichments. These high concentrations, combined with the observation of infections within 3 hours for initial concentrations of 100 coliphages per enrichment, suggest positive responses could be observed within 6 hours. The host bacteria on the plate have also been observed to form a lawn within the 6 hour incubation. In fact, a similar recommendation is outlined in EPA method 1601, but as an initial check, not a final determination of infection [14].

In this study, all plates that were checked at 6 and 24 hours exhibited identical positive and negative responses (Figure 4.8). The positive and negative results were also clearer at 6 hours than at 24 hours, presumably because background bacteria not adequately controlled by antibiotics during enrichment has the potential to grow during the 24 hour incubation. This is particularly true for male specific enrichments, which typically experience more background contamination and have a higher potential for less clear results. This suggests the AS antibiotic may be less effective than NA antibiotic for controlling the microflora found in biosolids.

Figure 4.8 Biosolids enrichment spots after for male specific biosolids 10^{-1} concentration at 6 hours (left) and the same plate after 24 hours (right)

One documented disadvantage of EPA method 1601 is that the procedure takes an additional day for analysis than its counterpart EPA method 1602 [11]. However, checking the plates at 6 hours allows the complete procedure to be conducted in a little over 24 hours, therefore, making the analysis time equivalent to EPA method 1602. Others have investigated methods to shorten the two-step enrichment procedure for other sample media, they have focused on accelerating the first enrichment, not the second incubation [89, 90].

4.3.2.4 Quality Assurance/ Quality Control

4.3.2.4.1 Plaque Confirmation

Plaque confirmation is an important QC check for questionable plaques. Of the 20 spots confirmed during this study, 4 had inverse results (Figures 4.9 and 4.10, Table 4.2). This is likely higher than would typically be experienced in any given test run because questionable plaques were specifically chosen.

Of the three methods for confirming plaques, those that confirmed from the enrichment tube were more accurate than those that used the plate to confirm. The confirmations sourced from the plate confirmed the result from the plate, but for 3 positive samples and one negative sample, both confirmations sourced from the tube yielded the opposite result from the plate confirmation. The centrifuge and loop confirmations always agreed with one another as testing from the tube eliminates the possibility that the spot has been contaminated on the plate and is, therefore, recommended. This contamination is likely a result of the high concentration of coliphages in the enrichment tubes as well as the possibility of background bacteria from the sample contaminating other spots on the plate. It should be noted that although confirmations often did clarify the coliphage infections, these techniques did not necessarily remove all background and host bacteria.

The male specific plates had more false positives. This is likely a result from the overgrowth of background bacteria that more frequently occurs with male specific plates. The biosolids used to test the confirmation techniques seemingly had more background contamination than usually experienced, which may have exacerbated the issue. Some of the issues with overgrowth can also be mitigated via the techniques outlined previously.

Figure 4.9 Confirmation Techniques for Somatic Plates

Centrifuge (a,c), loop from enrichment (b,e) and picking plaque from plate(c,f). Positive confirmations pictured above (a,b,c) and negative confirmation (d,e,f) below. Circles represent enrichments where different techniques provided disparate results.

Figure 4.10 Confirmation Techniques for Male Specific Plates

Centrifuge (a,c), loop from enrichment (b,e) and picking plaque from plate(c,f). Positive confirmations pictured above (a,b,c) and negative confirmation (d,e,f) below. Circles represent enrichments where different techniques provided disparate results.

Table 4.2 Results for confirmation techniques for selected plaques

	Male specific				Somatic			
	Sample	Centrifuge	Loop	Pick Plaques	Sample	Centrifuge	Loop	Pick Plaques
Positive	STAND	+	+	+	STAND	+	+	+
	C1A	+	+	+	B1D	+	+	+
	A2E	-	-	+	C2B	-	-	+
	B2C	+	+	+	C2C	+	+	+
	B2D	-	-	+	C3A	+	+	+
Negative	BLANK	-	-	-	BLANK	-	-	-
	B2B	-	-	-	B2E	-	-	-
	A3E	-	-	-	C2D	-	-	-
	B3E	-	-	-	A3C	-	-	-
	C3B	-	-	-	B3D	-	-	-

4.3.2.4.2 Matrix Spike, Matrix Spike Duplicate and Milorganite Spikes

The matrix spike results (un-spiked sample, spike, and spiked sample) are summarized in Table 4.3 for male specific and somatic coliphages. Because EPA method 1601 is not verified for an MPN format, only presence absence, there are no reported percent recoveries available for comparison. Therefore, preliminary comparisons were made to EPA method 1602 and EPA method 1643, because they are coliphage techniques. Comparisons to EPA method 1681 were also evaluated because it utilizes an MPN format and biosolids as a sample. The standards for Class A levels for fecal coliforms were used for this because they are a similar concentration to coliphage concentrations found in biosolids [54] (Table 4.4). The percent recoveries for male specific coliphage in the current study exceeded the standards in EPA method 1602 and 1643, although were within the range reported for the method 1681 MPN format. Somatic coliphages met EPA method 1602 and 1643 standards.

Table 4.3 Matrix Spike for Somatic and Male specific, all concentrations are in MPN/g TS

	Un-spiked	Spike Concentration	Spiked Biosolids	% Recovery
Male specific	9.34E+02	1.53E+04	2.72E+04	172%
Somatic	5.38E+02	3.03E+04	2.49E+04	80%

Table 4.4 Matrix Spike Recovery Standards for EPA Methods 1602, 1643 and 1681

	Male Specific		Somatic		Fecal Coliforms (Class A)	
EPA Method	% Recovery	Relative % Difference	% Recovery	Relative % Difference	% Recovery	Relative % Difference
1602	0-120%	57%	48-291%	28%	---	---
1643	8-54%	35%	45-368%	30%		---
1681	---	---	---	---	2-541%	182%

When tested in duplicate, the relative percent difference results similarly agreed with recovery levels reported in EPA methods 1602 and 1643 (Table 4.5). Larger percent differences were realized with the MPN format than were reported with direct observation. However, the MPN format typically reports lower precision between duplicate samples (Table 4.4) [54], so the relatively large percent differences may not be of concern. Further testing with multiple labs would be required to set standards for the tolerances of this method.

Table 4.5 Matrix Spike Duplicate Results, all concentrations are in MPN/g TS

	Un-spiked	Spike Concentration	Spiked Biosolids	% Recovery	Mean % Recovery	Relative % Difference
MS1	2.03E+03	2.38E+04	1.53E+04	56%	66.5%	32%
MS2	2.03E+03	2.38E+04	2.03E+04	77%		
S1	5.38E+02	1.26E+04	4.10E+04	320%	238%	69%
S2	5.38E+02	1.26E+04	2.03E+04	156%		

Milorganite appears to be an effective standard test matrix for coliphages (Tables 4.6 and 4.7). Percent recoveries were within expected ranges based off of reported recoveries in EPA methods 1602 and 1681. However, because Milorganite is an extremely dry product, there were issues with breaking the solids up effectively using the blender alone. To mitigate this, the original dilution of Milorganite was soaked for 3 hours prior to blending and the spikes re-tested. This resulted in a consistent emulsion with increased percent recoveries, and is recommended for extremely dry biosolids samples. When soaked prior to testing, not only were the percent recoveries improved, but also the relative percent differences were smaller. In addition, both male specific and somatic coliphages were detected in un-spiked Milorganite when the samples were soaked, but were not detected when no soaking step was involved. Test blanks were satisfactory during the test that un-spiked Milorganite contained coliphage. However, these levels were very low. Further testing of soaked and not soaked Milorganite yielded negative results, as did further test blanks. It is not entirely conclusive whether or not Milorganite contains coliphages, but if it does, the concentration is low and would likely not interfere with spiking procedures.

Table 4.6 Matrix Spike Duplicate Milorganite, not soaked. Concentrations are in MPN/g TS

	Un-spiked	Spike Concentration	Spiked Biosolids	% Recovery	Mean % Recovery	% Difference
MS1	0	2.38E+04	1.53E+04	166%	166%	166%
MS2	0	2.38E+04	2.03E+04	166%		
S1	0	4.20E+05	4.84E+04	53%	74%	58%
S2	0	4.20E+05	4.84E+04	96%		

Table 4.7 Matrix Spike Duplicate Milorganite, soaked for 2 hours before testing.
Concentrations are in MPN/g TS

	Un-spiked	Spike Concentration	Spiked Biosolids	% Recovery	Mean % Recovery	% Difference
MS1	25.51	4.20E+05	3.45E+03	83%	94%	25%
MS2	25.51	4.20E+05	4.42E+03	106%		
S1	18.62	1.58E+05	3.45E+03	216%	176%	46%
S2	18.62	1.58E+05	2.17E+03	136%		

4.3.3 Comparison of the SAL, DAL and ENR/MPN Methods for Aqueous Samples

Because biosolids impair the visualization of plaques in direct count assays, only aqueous coliphage samples were used to evaluate the comparison between direct count and ENR/MPN quantifications. While the SAL approach is effective for water applications, the DAL is likely the best representation of the actual concentration of coliphages. Based on the results summarized in Figure 4.11, and using the DAL as the point of comparison, the ENR/MPN approach likely resulted in an overestimation of coliphage concentration whereas the SAL likely underestimated the coliphage concentration. Others have reported slight overestimations for coliphage quantification via the MPN approach in water [76, 89]. Therefore, because the ENR/MPN validation likely overestimates the amount of coliphage present by 10-15%, the standards for their acceptable levels in biosolids would need to be appropriately adjusted to reflect this.

Figure 4.11 Double agar layer (DAL) Single Agar Layer (SAL) and MPN/ENR Comparison for Aqueous Samples

Measured values represent the average and error bars represent the standard deviation

4.3.4 Application of ENR/MPN Method at PLWSA

The coliphage results obtained at different points in the solids process train at PLWSA are summarized in Figure 4.12. Coliphages were relatively consistent throughout the nine month study period, despite substantial changes in flow and seasonal variations in operations. Little inactivation of coliphage was seen through the treatment process until after anaerobic digestion, which resulted in an approximately 1 log reduction for male specific and somatic coliphages. Total log reductions through treatment are reported in Table 4.8, with total average log reductions of less than 2.0. This relatively low level of reduction may be explained by the fact that coliphages appear to be predominately removed from wastewater through their adhesion to particles and, therefore, thus persist in solids treatment [79]. Nonetheless, mesophilic anaerobic digestion appears to mildly inactivate coliphages which is congruent with published bench scale tests [67].

In addition to looking at the average results, the temporal trends were also examined. Comparing the results in Figures 4.13 and 4.14, the numbers of somatic coliphage were more consistent over time than male specific coliphage. It also appears that coliphage

levels may tend to be higher during the summer months. This observation may in part be due to the increase in flow in the fall, when the student population returns to the collection area. Finally, it is interesting to note that the majority of the variability in the coliphage data is at the influent end of the solids process train (e.g., the primary sludge), whereas the coliphage levels in biosolids were relatively consistent throughout the year.

Figure 4.12 Coliphage Levels throughout Treatment at PLWSA.

Measurements were taken from July 2018 through March 2019. Single samples were taken each time, error bars represent the standard deviation.

Table 4.8 Total Log Reductions of male specific and Somatic Coliphages through Solids Process Train at PLWSA

	Total Average Log Reduction	Maximum Log Reduction	Minimum Log Reduction
Male specific	1.80	2.53	1.08
Somatic	1.72	2.04	0.85

Figure 4.13 Somatic Coliphages at Different Treatment Points over time

Figure 4.14 Male Specific Coliphages at Different Treatment Points over time

Coliphage data compiled from similar studies are summarized in Tables 4.9 and 4.10, along with the results from the current study. This compilation suggests that variations of 1-2 log may be observed through the treatment processes, suggesting that while coliphages will be abundant in wastewater treatment, their actual level may fluctuate widely. In addition, these results indicate that the concentrations of coliphages present in wastewater treatment facilities are typically high enough that the ENR/MPN method will

provide consistent results. This simplifies analysis and allows for process monitoring of multiple process steps to be directly compared to one another.

When compared to existing literature, the results obtained with the modified ENR/MPN enrichment method are consistent with previously documented coliphage levels. This suggests that the other methods that have been developed for coliphage enumeration may correlate reasonably well with one another. Nonetheless, certain aspects of the method described in this paper, (i.e. consistency, minimization of sample manipulation, ability to test multiple mediums, comparable time for analysis) suggest that it merits use. In particular, a distinct advantage of the modified ENR/MPN enrichment method presented in this paper is that it can be conducted without modification across multiple sample media, including wastewater samples and biosolids samples

Table 4.9 Somatic Coliphage Log₁₀ Values from Literature

	Raw Wastewater ^Ψ			Raw Sludge			Primary Sludge			Activated Sludge		
	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max
Sidhu et al 2017 [74]	5.33		5.54									
Rose et al 2004 [71]	3.00		6.00									
Mandilara et al 2006* [78]		5.50			5.30							
Lasobras et al 1999* [66]					4.85			5.04			3.20	
Gutzman et al 2007 [‡] [75]				7.45	7.59	7.54						
Pillai et al 2011 [‡] [65]				3.10	5.23	7.84						
This Study [◊]							3.92	4.61	5.22			
	RAS			Anaerobic Digestion (no dewater)			Dewatered Sludge			Digested Dewatered		
	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max
Mandilara et al 2006* [78]					4.40							
Lasobras et al 1999* [66]											3.91	
Gutzman et al 2007 [‡] [75]							6.23	6.52	6.90	4.41	4.86	5.52
Pillai et al 2011 [‡] [65]												
Rhodes et al 2015 [‡] [73]					4.25						4.00	
Carroll 2011 [‡] [91]				4.94		6.53						
This Study [◊]	3.92	4.59	6.98	3.37	4.12	6.12				2.62	2.89	3.46

Ψ Denotes that the concentration is in PFU/100 mL, * denotes PFU/g solids, ‡ denotes PFU/g TS and ◊ denotes MPN/g TS

Table 4.10 Male Specific Coliphage Log₁₀ Values from Literature

	Raw Wastewater ^Ψ			Raw Sludge			Primary Sludge			Activated Sludge		
	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max
Rose et al 2004 [71]	2.00		8.00									
Mandilara et al 2006* [78]		4.00			4.50							
Lasobras et al 1999* [66]					4.46			4.64			3.20	
Gutzman et al 2007 [‡] [75]				6.40	7.08	7.40						
Pillai et al 2011 [‡] [65]				2.02	4.74	6.88						
This Study [◇]							3.86	4.92	6.05			
	RAS			Anaerobic Digestion (no dewater)			Dewatered Sludge (no digestion)			Digested Dewatered		
	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max	Min	Avg.	Max
Mandilara et al 2006* [78]					2.50							
Lasobras et al 1999* [66]											2.53	
Gutzman et al 2007 [‡] [75]							4.66	5.23	5.66	6.54	6.64	6.54
Pillai et al 2011 [‡] [65]												
Rhodes et al 2015 [‡] [73]					3.00						2.00	
Carroll 2011 [‡] [91]				3.07		6.12						
This Study [◇]	3.64	4.67	6.91	3.45	4.20	5.45				1.79	3.12	4.04

Ψ Denotes that the concentration is in PFU/100 mL, * denotes PFU/g solids, ‡ denotes PFU/g TS and ◇ denotes MPN/g TS

4.4 Conclusions

The developed coliphage method provides many advantages. High concentrations of coliphages were realized during overnight enrichment. The MPN approach may slightly overestimate the coliphages present, but provided relatively similar values to reported coliphage concentrations in similar mediums using different methods. Techniques to reduce interferences due to background and host bacteria were useful for ensuring unambiguous results were interpreted. Of the techniques to reduce interference used, dilution when necessary and checking the plates at 6 hours are recommended. These techniques provided the same quality as other purification results, but did so in a time effective manner, and provided minimal manipulation to the sample or enrichment. Biosolids matrix samples spiked with coliphages exhibited percent recoveries of 80-320% for somatic and 56-172% for male specific, which aligns with currently outlined standards for the same organisms. Milorganite was found to be a suitable standard test medium with 53%-216% percent recovery for somatic and 83-166% percent recovery for male specific. Process monitoring at PLWSA revealed that there was a slight seasonal variation in the influent primary solids and RAS. A 1-2 log inactivation was experienced throughout solids treatment, with male specific and somatic coliphage relatively similar abundance, and consistent with other studies were found. Solids levels in the different tested media varied from 0.2-21% yet provided consistent and comparable results, demonstrating the versatility of the procedure.

5 Summary and Conclusions

Key findings and conclusions from this research are summarized in this chapter, along with recommendations for future work.

5.1 Preliminary Evaluation of Lab Scale Study Design

Several key observations were made during the preliminary laboratory testing that should inform the next round of testing.

5.1.1 Microbial Inoculations

- GFP is an effective trackable bacterial indicator in biosolids, because it does not interfere with fecal coliform testing and is easily identified under short wave UV light.
- Native fecal coliforms are abundant for use as an indicator when the biosolids are not overly manipulated to set the test parameters.
- Coliphages will be fairly resistant throughout treatments, but wild type inoculations will exhibit some reductions. Individual strains may provide more consistent inactivation results, but because test methods cannot distinguish exact strains from those naturally present in the biosolids, the wild type strains may provide more realistic results.

5.1.2 Total Solids Adjustment

- Drying is effective for achieving high total solids levels but the dried biosolids can only be effectively re-hydrated to low TS within certain limits.
- Drying will not cause fecal coliforms to reduce or increase if dried at temperatures less than 37 °C

5.1.3 pH adjustment

- Sodium hydroxide and hydrochloric acid were effective at initially adjusting biosolids pH.

- Biosolids appear to have a natural buffering capacity that prevents adjustment below 4.5 and subsequently results in pH trends back towards 6-7 within a few days.
- No buffers were effective at resisting the pH changes experienced.
- Daily adjustment to liquid reactors was the most effective and most consistent means for maintaining consistent pH, but resulted in issues with maintaining VFA levels.

5.1.4 VFA Additions

- Under low solids conditions (TS~4-5%), methanogens present in the biosolids consumed all VFAs added. This rendered the effects of VFAs on inactivation un-measurable due to the losses.
- No VFA volatilization was experienced due to mixing in liquid reactors.

5.1.5 Temperature

- Based on the preliminary results, temperature seemed to have a notable effect on the survival of indicator organisms, even at a relatively moderate level of 25 °C.
- Temperature had a notable effect on VFA consumption, likely because of near optimal conditions for methanogenesis.

5.2 Modified ENR/MPN Coliphage Method

The modified ENR/MPN method that was developed and tested in the research shows potential as a technique for coliphage in biosolids.

5.2.1 Detection

- Enrichment is an effective method for use with biosolids because it limits the solids interference, results in high concentrations of coliphages during enrichment, and resulted in consistent detection.
- Blending samples is essential for detection.

- The MPN approach may overestimate the number of coliphages present by 10-15% as compared to the DAL method.
- Both TSB and BW provide comparable results when used for dilution of sample. BW does have an advantage by allowing several other tests to be run from the same sample.

5.2.2 Reductions in interferences

- Centrifuging must be conducted at high speeds to effectively reduce interferences in enrichments, however, it is also supply and time intensive
- Dilution of enrichments after incubation in BW and checking the plates at 6 and 24 hours after spotting were the most effective methods for reducing interferences.

5.2.3 Quality control

- Matrix spike and matrix spike duplicates ranged from 56-172% recovery for male specific and 80-320% for somatic coliphage. Relative percent differences for matrix spike duplicates were 69-32% different for male specific and somatic coliphage respectively.
- Milorganite was an effective standard test medium with percent recoveries of 53-166%. Soaking is recommended for better results and provided mean percent recoveries between 94-176% with between 25-46% relative percent differences for duplicates.
- Some coliphages were detected in pure Milorganite samples, but this was likely due to contamination. Subsequent testing revealed Milorganite to be coliphage free.

5.2.4 PLWSA Results

- 1-2 log reduction in coliphage was experienced throughout treatment, mostly in between anaerobic digestion and biosolids.
- Relatively similar values were observed in the current study for coliphage levels compared to what others have reported in similar studies for Class B biosolids.

- A slight seasonal variation was observed in coliphage levels with higher values for primary solids and RAS in summer than in winter.
- Biosolids coliphage levels were the most consistent, suggesting that the digester performed consistently when it came to coliphage inactivation.

5.3 Recommendations for Future Work

5.3.1 Lab Scale

- Better pH control and adjustment are required for application of the factorial analysis.
- An increase in total solids may limit VFA consumption by methanogens.
- An increase test run time from 3 weeks to 2 months should be made to better capture inactivation of PIOs.

5.3.2 Coliphage

- The sampling of coliphages in biosolids samples should be expanded to more treatment plants.
- A survey of coliphage levels in different types of Class A treatments could be useful for establishing coliphage survival rates on a full scale and describing correlations to enteric viruses.
- The simultaneous detection of male specific and somatic coliphage via a singular host should be investigated as a means of simplifying testing and potential future standards. [76].

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A Methods

A.1 Fecal Coliform (EPA 1681)

1. Aseptically fill 100 mL dilution bottles with 90 mL of dilution buffer water using the 100 mL graduated cylinder
2. Measure out 10 grams of biosolids onto sterile metal plate using a sterile spoon.
3. Mix 10 grams of biosolids with 90 mL of buffer water in blender until uniformly mixed. This is the 10^{-1} dilution.
4. Using a sterile pipette, prepare serial dilutions by transferring 10 mL of previous sample to 90 mL of buffer water in the next dilution bottle.
5. Prepare a minimum of 4 dilutions. Use previous data to select dilutions.
6. Dispense 10 mL of each dilution into tubes containing 2-3 mL of A1 media. Prepare 5 tubes per dilution.
  - a. **Blank:** Fill a tube with 10 mL of sterile buffer water
  - b. **Standard:** Fill a tube with 10 mL of buffer water. Flame a loop and allow to cool. Scrape standard *E. coli* culture off of the plate and inoculate tube.
7. After filling tubes, invert to mix the sample and media. Make sure no bubbles are present in the tubes.
8. Place the tubes in the incubator at 35 °C for 3 hours.
9. Remove the tubes and invert to release any bubble that have formed in the inner vials.
10. Place tubes in a 44.5 °C incubator for 21 +/- 2 hours. Keep tubes upright for the remainder of the test.
11. After the second incubation, remove the tubes and record positives as the presence or absence of a bubble in the inner vial.
12. Select significant dilutions according to EPA 168.1

13. Use the table to determine MPN index for each sample. Convert to MPN/g TS by dividing by the percent total solids (as expressed as a decimal).

A.2 Spread Plate for GFP Analysis (Standard methods)

1. Dry plates in 37°C incubator overnight so that approx. 1 g of moisture is lost. Do not invert
2. Dilute biosolids in sterile dilution buffer water.
3. Flame hockey stick by dipping in ethanol and running through the flame.
4. Pipette 0.1 mL or 1 mL of sample onto plate. Spread with sterile hockey stick.
5. Sterilize hockey stick after spreading.
6. Let plates dry before sealing with parafilm and incubating inverted at 37 °C overnight.
7. Count colonies after 16 hours. Viable counts are between 30 and 300 colonies.

A.3 Coliphage by Two Step Enrichment (Adapted from EPA 1601)

Antibiotic Preparation

1. For nalidixic acid (NA), add 1 g of nalidixic acid sodium salt to 100 mL DI water. For ampicillin-streptomycin (AS), add 0.15 g of ampicillin and 0.15 g of streptomycin to 100 mL of DI water.
2. Filter with 0.22 µm filter and syringe into sterile container. (Cryovials to freeze or 15 mL centrifuge tubes, leave room for expansion) Perform in the BSC or change filter each time new solution is drawn into syringe if working on the lab benchtop.
3. Freeze at -20 °C for long term storage or at 4 °C for up to 3 weeks.

Do not freeze/thaw a single vial more than 3 times.

Bacteria Host Preparation

1. Aseptically add 50 mL of TSB to 2 sterile Erlenmeyer Flasks
2. Aseptically add 0.5 mL of appropriate antibiotic (MS=AS, S=NA)
3. Aseptically add 0.5 mL of appropriate stock culture (MS or S)
4. Cover flasks with parafilm and place on shaker table for 16-18 hours at 37 °C at 100 rpm
5. After incubation, repeat steps 1 and 2 with the remaining flasks
6. Add 0.5 mL of appropriate overnight culture
7. Cover flasks with parafilm and incubate on a shaker table for 3 hours at 37 °C at 100 rpm
8. Check the optical density using the spectrophotometer in 850 to ensure the bacteria are in log-phase growth.
9. Store cultures immediately on ice or at 4 °C. Can be stored at 4°C for up to one week.

Double Agar Plates

1. Fill water bath with DI H₂O and turn on. Place a thermometer in bath and ensure bath is at correct temperature (50°C 122°F)
2. Grab screw top bottle with caps. The bottle should be double the volume of the agar you are making.
3. **Bottom Plates:** For each 100 mL of agar needed, add 3 g of Tryptic Soy broth and 1.5 g of agar to each bottle. Dilute with correct DI water volume.
4. **Top Plates:** For each 100 mL of agar needed, add 3 g of tryptic soy broth and 0.7 g of agar to each bottle. Dilute with correct DI water volume.
5. Add a stir bar and place on magnetic stir plate on high heat. Loosen the cap on the screw top.

6. Heat until all powder has dissolved and the liquid turns clear. Take caution to not let the agar boil over.
7. Adjust pH to 7.2-7.4 using 1 M NaOH or HCl
8. Autoclave for 15 minutes at 121°C on a liquid cycle. DO NOT let the agar sit in the autoclave any longer than it takes to finish the cycle.
9. Place in water bath and allow temperature to stabilize at ~50°C.
10. Bleach and Ethanol countertops
11. Once temperature has stabilized, add 1 mL of appropriate antibiotic for every 100 mL agar
(NA for somatic, AS for F+)
12. To the **TOP AGAR ONLY** add 10 mL of appropriate log phase bacteria
13. Using a plastic pipette, pour the agar onto a labeled plate and swirl to ensure full coverage on bottom of plate.
  - a. Pour 10 mL of bottom agar and let solidify.
  - b. Pour 5 mL of top agar and let solidify
14. Let plates cool with lids slightly off to prevent condensation build up
15. Store plates inverted at 4°C for up to a week.
16. Empty water bath and leave the lid off until next use. Pour extra agar in a tip box or used container. Do not let it harden in the bottle or put it down the drain.

Biosolids Assay

1. Take 10g (or 10mL for liquid) Biosolids and add it to 90 mL of buffer water (BW) in a blender
  - a. Blend until uniformly mixed, this makes the 10^{-1} dilution
2. Prepare 18 mL dilution bottles of sterile BW
3. Prepare 3-5 dilutions of each sample using serial dilutions.

4. In sterile culture tubes, add 5 mL of TSB+ Antibiotic, along with 0.25 mL of log phase bacteria. Prepare 5 per dilution.
5. Add 0.5 mL of each diluted sample to a labeled culture tube and invert to mix.
  - a. **Blank:** Add 0.5 mL of TSB with no sample.
  - b. **Standard:** Add 10 uL of coliphage stock to incubation tubes.
6. Place culture tubes in 37°C incubator overnight, making sure the caps are loose.

Plate Spotting

1. After incubation, remove tubes. Vortex for 1-2 seconds to mix.
2. Spot 10 µL from each tube onto a double agar plate. One plate is used per dilution per sample per coliphage type. Spot 5 times on the plate.
  - a. Create a blank and control plate for each coliphage type using the prepared blank and standard tubes.
3. Let drops air dry, uncovered.
4. Once dry, wrap plates in parafilm and incubate inverted at 37°C overnight (16-18 hours)
5. Next day, take plates from incubator and record if the spot is a positive or negative clearing zone for each dilution. Record the amount of positives and negative.
6. Use MPN program to determine MPN per sample

A.4 Double Agar Pour Plate (Adapted from EPA 1602)

1. Prepare 1.5% tryptic soy agar plates with 1 mL antibiotic stock per 100 mL. Pour 10 mL agar per plate.
2. Prepare 0.7% tryptic soy agar. Add 1 mL of antibiotic stock per 100 mL.
3. Dispense 3 mL into sterile test tubes and hold them in a 45 °C water bath to prevent from solidifying.
4. To each tube, add 0.1 mL of host log phase culture.

5. Prepare biosolids dilutions by adding 10 g of biosolids to 90 mL of sterile dilution water. Blend for 30-60 seconds. Serially dilute in dilution buffer water so that 30-80 PFU/mL are present.
6. Add 1 mL of diluted sample to each tube.
7. Immediately mix the contents of the tube and pour onto the bottom agar plate.
8. Let solidify.
9. Seal with parafilm, invert and incubate at 36 °C overnight before observing plaques

A.5 Single Agar Pour Plate (Adapted from Standard Methods)

1. Prepare 1.5% tryptic soy agar plates with 1 mL antibiotic stock per 100 mL
2. Dispense 10 mL of agar into sterile test tubes and hold them in a 45 °C water bath to prevent from solidifying.
3. To each tube, add 0.5 mL of host log phase culture.
4. Prepare biosolids dilutions by adding 10 g of biosolids to 90 mL of sterile dilution water. Blend for 30-60 seconds. Serially dilute in dilution water so that 30-80 PFU/mL are present.
5. Add 1 mL of diluted sample to each tube.
6. Let the mixture stand for 3 minutes before pouring into 100 mm plate.
7. Let solidify.
8. Seal with parafilm, invert and incubate at 36 °C overnight before observing plaques

A.6 Volatile Fatty Acids by Hach TNT

1. Set the right side of the DRB200 to VFA 1 (130 °C for 2 hours). Allow at least 10 minutes to warm up.

2. Pipet **1 mL of DI water** into M portion of user filled collector tubes and cover with the loose membrane, sealing with the collector tube cap.
3. Weigh **3 g** or pipette **3 mL of the biosolids** sample into a sample tube
  - a. For higher solids samples, 0.6 g of biosolids can be added and 6 mL 1.02 M phosphoric
4. Prepare a standard by adding **3 mL of 3000 mg/L** standard solution.
5. Add **3.4 mL of 1.74 M phosphoric acid** to the sampling tube.
6. Immediately push the D end of the collector tube over the open end of the sampling tube to start the seal. Use the press to seal the distillation column.
7. Place the sample tube and D end of each tube into the preheated block. Distill the sample for **2 hours**.
8. After **2 hours**, put on heat resistant gloves and remove the tube from the block. Immediately pull the sample tube off using a gentle side to side motion. Dispose of the sample tube in the trash.
9. Invert each collector tube and allow to cool for at least 10 minutes.
10. For each collector tube, hold the tube horizontally and rinse its walls with the distillate in order to homogenize it. Roll the distillate around the tube to gather all droplets clinging to the tube into the bulk of the distillate the return the tube to an upright position so the D end is still up. If not all of the distillate can be collected, discard the tube and retest sample.
11. When ready to analyze the samples, break the tube in half. Discard the D end.
12. Dilute the sample in the M end of the tube to the 6.0 ml mark with DI water. Use the pipette to mix the sample.
13. Analyze sample according to Hach Volatile Acids method, or prepare samples in volatile organic analysis for GC analysis.
14. Use the standard prepared during distillation to determine the percent recovery of the distillation.

A.7 Ammonia (Equivalent to EPA 1690)

1. Acidify the biosolids sample with 0.2 M H_2SO_4 . Use 35 mL of acid to 6 grams of biosolids. Record the weight of acidified biosolids. Ensure the pH is less than 2 using a pH strip.
  - a. This solution can be held for up to 3 weeks until analysis can be performed.
2. Set the right side of the DRB200 to AMM1 (120 °C for 30 minutes). Allow at least 10 minutes to warm up.
3. Add a stir bar to the acidified sample. Mix on a stir plate COVERED at 350 rpm for 5 minutes.
4. Pipet **1 mL of 0.016 H_2SO_4** into M portion of user filled collector tubes and cover with the loose membrane, sealing with the collector tube cap.
5. Measure out **5 mL of mixed acidified sample** with glass pipette and dilute to the line of a 100 ml volumetric flask with DI water.
  - a. To limit volatilization of ammonia, keep the sample solutions capped as much as possible.
6. Invert diluted solution 10 times.
7. Pipet **6 mL of the diluted biosolids** sample into a sampling flask. Ensure the solution is well mixed and taken from the bottom of the flask.
  - a. **High Range Standard:** Pipette 6 mL of 10 mg/L standard solution into user fill tube and proceed.
  - b. **Low Range Standard:** Pipette 6 mL of standard solution diluted to 2 mg/L (10 mL of 10mg/L solution into 50 mL volumetric flask)
  - c. **Blank:** Add 6 mL of DI water.
  - d. **Fortified Matrix:** To a biosolids sample diluted to the 100 ml mark, add 1 mL of 1000 mg/L standard solution. Add 6 mL of this sample.
8. Add **1.0 mL of 0.025 M borate buffer** to the sampling tube.

9. Immediately push the D end of the collector tube over the open end of the sampling tube to start the seal. Use the assembly in press and seal the distillation column.
10. Place the sample tube and D end of each tube into the preheated block. Distill the sample for 30 minutes.
11. After 30 minutes, put on heat resistant gloves and remove the tube from the block. Immediately pull the sample tube off using a gentle side to side motion. Dispose of the sample tube in the trash.
12. Invert each collector tube and allow to cool for at least 10 minutes.
13. For each collector tube, hold the tube horizontally and rinse its walls with the distillate in order to homogenize it. Roll the distillate around the tube to gather all droplets clinging to the tube into the bulk of the distillate then return the tube to an upright position so the D end is still up. If not all of the distillate can be collected, discard the tube and retest sample.
14. Prepare TNT832 vials for analysis. (or TNT831 vials for low range)
15. When ready to analyze the samples, break the tube in half. Discard the D end.
16. Dilute the sample in the M end of the tube to the 6.0 ml mark with DI water. Use the pipette to mix the sample.
17. Pipette appropriate sample amount of sample into TNT vial. Flip over TNT cap and screw it onto the vial.
18. Shake until the white powder denigrates.
19. Let the reaction set for 15 minutes. Read in DR 6000 after cleaning with a kimwipe. Record the results.

A.8   pH (EPA Method 9045D)

1. Calibrate the pH probe with a 3 point calibration.
2. Weigh 20 g of biosolids into a 100 mL beaker using a sterile spatula.
3. Dilute with 20 mL of water.

4. Let the solution sit for 15 minutes.
5. Insert pH probe into the slurry. Gently stir and record the pH when the meter stabilizes.

A.9 Total Solids (EPA 1684)

1. Fire crucibles in the muffle furnace for 1 hour. Move to desiccator and let cool completely.
  - a. Note: If only performing total solids (no volatile solids) 1 hour in the oven is sufficient for preparation.
2. Place cooled crucible on scale and record weight.
3. Fill with at least 25 grams of biosolids and record weight.
4. Fill a standard and blank with 40 mL of DI water or standard solution respectively.
5. Place in oven for at least 12 hours at 103-105 °C.
6. After 12 hours, remove crucibles and immediately transfer to a desiccator using tongs.
7. Let cool in desiccator at least 30 minutes before weighing.
8. Return to oven for an additional hour.
9. Repeat step 7.
10. If the samples have not changed by more than ± 0.04 grams, record a final weight. If not, return to the oven for another hour until the change is lower than 0.04 grams.

A.10 Volatile Solids (EPA 1684)

4. After the solids have been analyzed for total solids, place in a 550 °C muffle furnace for 1 hour.

5. Remove biosolids from furnace and transfer to desiccator using crucible tongs.
6. Let the biosolids cool in the desiccator for 30 minutes.
7. Record the weight of the ignited biosolids.
8. Return to furnace for another 30 minutes.
9. Repeat steps 2 through 4.

If the sample has not changed ± 0.04 g record weight. If the change is greater than that, return and repeat steps 6-7 until the weight changes less than ± 0.04 g.

B Standard Operating Procedure for GFP E. Coli

Title: Quantification of *Escherichia coli* serotype O157:H7 added to wastewater treatment solids

Code: Becker Research Group SOP 5.00

Main hazards: Release of green fluorescent protein gene into the environment

Objective: Quantification of *Escherichia coli* serotype O157:H7 added to wastewater treatment biosolids.

Scope: This SOP will be conducted by properly trained occupants of the Becker Research Group.

Abbreviations:

ARS: Agricultural Research Service

ATCC: American Type Culture Collection

CDC: Center for Disease Control

CFU: Colony-forming unit

NRRL: Northern Regional Research Laboratory

PBS: Phosphate-buffered saline

PPE: Personal protective equipment

Appropriate disinfectant: 10% bleach or 70% ethanol

Genetically modified organism: *Escherichia coli* serotype O157:H7

Culture collection numbers: ARS NRRL (B-59838). ATCC (43888), CDC (B6914-MS1)

Source: ARS NRRL

<https://nrnl.ncaur.usda.gov/cgi-bin/usda/prokaryote/report.html?nrnlcodes=B%2d59838>)

General description: This SOP details the procedures that will be used to culture *Escherichia coli* serotype O157:H7 (NRRL B-59838), add it to wastewater treatment solids (biosolids) in laboratory experiments, and quantify it in the biosolids during storage under different conditions. The strain will be obtained from the ARS NRRL. It has been engineered to contain a green fluorescent protein gene (Fratamico et al., 1997), which will facilitate visualization of its growth and inactivation without interference from background coliforms in the biosolids. Although it is of the O157:H7 serotype, it lacks the shiga toxins. Therefore, the unmodified strain is classified as BSL-1.

Location: Experiments involving *Escherichia coli* serotype O157:H7 (NRRL B-59838) will primarily be conducted on the bench top in Dow 839.

Propagation:

1. Inoculate stock culture into Luria-Bertani broth containing 100 µg ampicillin/mL
2. Incubate in shaker water bath at 100 rpm and 37 °C overnight.
3. Transfer overnight culture to fresh broth and incubate under same conditions until exponential phase reached.
4. Centrifuge cells at 2000 x g for 10 minutes.
5. Decant broth into waste container to be autoclaved.
6. Suspend cells in phosphate buffered saline
7. Repeat centrifuging, decanting, and suspension.

Addition of *Escherichia coli* serotype O157:H7 (NRRL B-59838) to biosolids:

E. coli serotype O157:H7 (NRRL B-59838) will be added to biosolids at a targeted concentration of 10⁶ CFU/g total solids. Experiments will be conducted using two different solids concentrations, 4% and 20% on a weight basis. Biosolids containing 4% solids behave as a thin slurry and are easily mixed by inversion. Biosolids containing 20% solids are cakelike. The following procedures will be followed when adding the NRRL B-59838 strain to the biosolids.

1. Dry un-amended biosolids at 40 °C until appropriate solids concentration (30 to 35% TS) is reached.
2. Measure physical and chemical biosolids properties
3. Add appropriate wet weight of biosolids to Kitchen Aid mixing bowl.
4. Add volatile fatty acid solution to biosolids.
5. Add NaOH or HCl to adjust pH
6. Add buffer solution to fix pH
7. Add solution containing wild-type coliphage suspended in buffer
8. Add solution containing *E. coli* (NRRL B-59838) in buffer
9. Mix using paddle attachment on low speed with silicone splash guard in place to minimize splashes.
10. For 20% solids samples: add deionized water to achieve necessary total solids and mix again at slow speed. Aliquot 150 g of wet biosolids to each Boston round bottle and seal.
11. For 4% solids: Aliquot 37.5 g of wet biosolids to each Boston round bottle. Add 112.5 g of deionized water to each bottle; seal; and invert to mix.
12. Incubate bottles at either 25 °C or 10 °C.

Enumeration:

1. Dilute biosolids sample in PBS. For samples spiked with $\sim 10^6$ CFU/g TS, the lowest dilution will likely be 10^{-8} to 10^{-11} .
2. Spread dilution on LB agar plates containing 100 µg ampicillin/mL
3. Seal plates with parafilm, invert, and incubate at 37 °C overnight
4. After incubation, count the colonies
5. To distinguish NRRL B-59838 colonies, illuminate the plates with a handheld long-wave UV lamp (365 nm) held approximately 4 to 6 inches directly over an opened plate.

Storage:

BSL-2 agents will be stored in sealed containers in the -80°C freezer in Dow 411.

Surface decontamination:

All surfaces will be wiped down with either 10% bleach or 70% ethanol after use of the NRRL B-59838 strain (*E. coli* O157:H7) and at the end of the day when work with this strain has been performed.

Waste disposal: All spread plates and solid waste exposed to the NRRL B-59838 strain will be autoclaved. All liquid waste will be treated with bleach to a concentration of 10% and allowed to sit for 20 minutes before disposing down the drain. The only exception is wastes generated during chemical analyses that expose the biosolids to physical or chemical conditions that will inactivate any NRRL B-59838 cells in the biosolids. These include: solids analyses in which biosolids are heated to 105 °C for 12 hours; volatile fatty acid analyses in which the biosolids are acidified and digested at 130 °C for 2 hours; and ammonia analyses in which the biosolids are acidified to pH < 2.

Equipment decontamination: All glassware and equipment that comes into contact with the NRRL B-59838 strain will either be autoclaved, treated with 10% bleach for at least 20 minutes, or treated with 70% ethanol.

Spills/splashes: Spills/splashes involving NRRL B-59838 strain will be decontaminated with a 10% bleach solution for 20 minutes. The 10% bleach solution and a biological spill kit will be available in the laboratory at all times.

For full clean-up response, see the Emergency Incident and Response Plan in the Biosafety Manual.

Personal Protective Equipment:

All lab occupants will wear long pants, close toed-shoes, and safety glasses in the lab. Other PPE requirements for working with BSL-2 agents will include:

- Lab coat
- Nitrile gloves

Appropriate PPE must be worn by all individuals in the room while the handheld UV unit is operating. The PPE must protect the eyes and skin from exposure to the UV light. Appropriate PPE to be worn when the handheld UV unit is in use includes:

- Nitrile gloves
- Lab coat with no gap between the cuff and the glove
- UV resistant face shield

Additional Special Handling Procedures: For full clean-up response, see the Emergency Incident and Response Plan in the Biosafety Manual.

Training and Qualifications: Only lab personnel who have completed BSL-1 and BSL-2 biosafety training may perform these procedures.

Documentation: All documentation relating to this procedure will be stored in a Google Drive folder entitled "Environmental Biotechnology Biosafety" and will be shared with all workers in the Becker Research Group.

References and documents:

Centers for Disease Control and Prevention. Emerging Infectious Diseases, 14 (1), Jan 2008. www.cdc.gov/eid

Fratamico, P. M.; M. Y. Deng; T. P. Strobaugh; and S. A. Palumbo. 1997. Construction and characterization of *Escherichia coli* O157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. J. Food Production. 60(10):1167-1173.

C Modifications for EPA 1601 for Use in Biosolids

Overnight Incubation/Tube Preparation

	Current	Biosolids Use	Rationale
Sample size	100 mL or 1 L samples of groundwater	0.5 mL of diluted biosolids sample	Biosolids are a more concentrated sample than water samples, so smaller volumes are ideal for analysis
TSB Use	10X TSB added to diluted sample	1 X TSB+antibiotic, 5 mL per tube	Smaller biosolids volume analyzed, the TSB is only used for the host bacteria support. Less concentrated because it is not being diluted in a large volume of water
Buffer Addition	Magnesium chloride stock added to water sample	No buffer added	If TSB is used, then buffering is not needed to support the bacteria during enrichment because there is not a large volume of un-buffered water.
Dilution Media	None	Dilution buffer water/PBS	Does not encounter the issues with foaming that TSB does and can be used in tandem with other microbial sampling (i.e. fecal coliforms)

Overnight Incubation/Tube Preparation (continued)

Bacterial propagation	16-18 hours overnight at 100 rpm and 36 °C transferred to fresh media to reach log phase in 3-4 hours	No change	Bacterial cultures are independent from the form of sample
Bacterial addition to overnight incubation	0.5 mL per 100 mL	0.25 ml per 5 mL TSB	Maintains the same ratio of TSB to bacteria for overnight enrichment
Antibiotic addition to overnight incubation	1 mL per 100 mL of sample	1 mL per 100 mL of TSB	Same total concentration of antibiotic in overnight enrichment
Mixing prior to incubation	Invert 5 times to mix	No mixing prior to incubation	Smaller volume of total liquid allows sample and bacteria easier contact. Vortexing each tube could be done, but does not appear to be required
Overnight Incubation	16-24 hours at 36 °C Caps loosened	No change	

Double Agar Plates

	Current	Biosolids Use	Rationale
Preparation of Double Agar Plates	10 mL 1.5% bottom agar 5 mL 0.7% top agar with 10 mL per 100 mL of log phase bacteria	No Change	Plates perform the same function in both methods and so change is needed.

Spotting/Second Incubation

	Current	Biosolids Use	Rationale
Sample mixing after	25 or more inversions	Vortex for 1-2 seconds	Vortexing can dislodge coliphages from their host and with smaller volumes is more practical and faster than inverting
Spotting volume	10 μ L	No change	Optimal volume for absorbing onto plate
Number of spots per plate	Up to 15	5	Used in an MPN format, allows for one plate to be used per dilution. Also allows enough space between spots (sometimes coliphage infections can clear the bacterial lawn around the spot area)

Spotting/Second Incubation (continue)

Second Incubation Time	16-24 hours	Check at 6 hours and at 24 hours	Excessive growth can occur overnight, and coliphage infections are usually evident at 6 hours and can be recorded as positives at that time

Recommendations for Highly Contaminated samples

	Current	Biosolids Use	Rationale
Filtration	Filter a small amount of 0.45 μm pore size filter	Optional but not recommended	Would be difficult with most biosolids samples due to solids presence and high amounts of bacteria
Centrifuging	0.5-1 mL of enrichments in microcentrifuge tube for 5000 - 10000 g for 10 minutes. Spot supernatant	Optional	Smaller volume could make more practical. Stickney centrifuges larger volumes, but this takes quite a bit of time given how many replicates there are. There is risk that coliphages may adhere to particles during centrifugation

Recommendations for Highly Contaminated samples (continued)

Picking plaques	Pick plaque with sterile pipette and then transfer to 0.5 mL TSB and let stand for 5 minutes before spotting again	Optional/recommended	Simplest and requires the least expensive equipment. An additional day may be required. Picking from the plate may result in contamination from other spots if not conducted carefully.
Dilution	Dilute 10 μL of each enrichment into 10 mL of sterile PBS	Recommended	Preserves entire sample, reduces coliphage concentration and reduces the presence of background bacteria
Note: Enriched samples may be saved for up to 48 hours at 4 °C. Therefore, if a sample is questionable after inspecting the spot one of the above methods can be performed and the plate re-spotted			

Data Interpretation

	Current	Biosolids Use	Rationale
Evaluation	Presence/absence	MPN	There will be by default high amounts of coliphage in biosolids, so quantitate evaluation is the most useful and can reliably detect coliphages in a solids matrix.

D   Copyright documentation

No material with a copyright was used in the preparation of this thesis