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Sudden Increase and Regrowth of Fecal Coliforms and Escherichia Coli in Wastewater Biosolids After High Solids Centrifugation

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SUDDEN INCREASE AND REGROWTH OF FECAL COLIFORMS AND *ESCHERICHIA COLI* IN WASTEWATER BIOSOLIDS AFTER HIGH SOLIDS CENTRIFUGATION

by

Gordon G. Araujo Jr.

A Thesis

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Acronyms

AHL	acyl-homoserine lactone
AI	autoinducer
alum	aluminum sulfate
ATAD	aerobic thermophilic digestion
BFP	belt filter press
BLAST	Basic Local Alignment Search Tool
BP	Blue Plains
BU	Bucknell University
°C	degrees Celsius
C6-HSL	N-hexanoyl-L-Homoserine lactone
3-oxo-C6-HSL	3-oxo-N-hexanoyl-L-Homoserine lactone
C8-HSL	N-octanoyl-L-Homoserine lactone
3-oxo-C8-HSL	3-oxo-N-octanoyl-L-Homoserine lactone
CFR	Code of Federal Regulations
CST	capillary suction time
CTAB	hexadoecylmethylammonium bromide
DCWASA	District of Columbia Water and Sewer Authority
dsDNA	double-stranded DNA
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EPA	United States Environmental Protection Agency
FC	fecal coliforms
FedEx	FedEx Corporation
FISH	fluorescent *in-situ* hybridization
FR	Federal Registrar
HCl	hydrochloric acid
HSC	high solids centrifuge
HUS	hemolytic uremic syndrome
I.D.	internal diameter
JWPCP	Joint Water Pollution Control Plant

kHz	kilohertz
LACSD	Los Angeles County Sanitation District
LB	Luria-Bertani
LEE	locus of enterocyte effacement
Ler	LEE-encoded regulator
LSC	low solids centrifuge
LTB	Lauryl tryptose broth
MLSS	mixed liquor suspended solids
MPN	most probable number
MPN/g DS	most probable number per gram dry solid
MUG	4-methylumbelliferyl-β-D-glucuronide
NaCl	sodium chloride
NaOH	sodium hydroxide
NS	not studied
OPD	optimum polymer dose
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PSRP	Process to Significantly Reduce Pathogens
qPCR	quantitative real-time polymerase chain reaction
RAS	return activated sludge
RNA	ribonucleic acid
RNase	ribonuclease
ROX	reference dye
rpm	rotations per minute
rRNA	ribosomal RNA
qPCR	quantitative real-time polymerase chain reaction
SCM	standard culturing method
SDS	sodium dodecylsufate
SI	sudden increase
SM	Standard Method
sp.	species
SRT	solids retention time
TC	total coliforms
TPAD	temperature-phased anaerobic digester
UVB	ultraviolet-B

Virginia Tech	Virginia Polytechnic Institute and State University
VNC	viable non-culturable
WAS	waste activated sludge

Abstract

Treatment plants that operate either thermophilic or mesophilic anaerobic digesters with centrifugal dewatering processes have consistently observed densities of fecal coliform and *Escherichia coli*, both indicator bacteria, that decrease during digestion but then increase after dewatering and storage. The increases have been characterized as two separate phenomena to explain this observation: 1) “Sudden Increase,” or SI, which is defined as the increase that occurs immediately after dewatering and 2) “regrowth,” which is defined as an increase during storage of cake samples over a period of hours or days. The SI observation appears to be more prevalent with biosolids that are generated with thermophilic processes and dewatered by centrifugation. Both thermophilic and mesophilic digesters with centrifuge dewatering processes have observed the regrowth phenomena.

This research hypothesizes that the SI phenomenon is due to the presence of viable non-culturable (VNC) bacteria that are reactivated during dewatering. In other words, the bacteria were always present but were not enumerated by standard culturing methods (SCM). Analysis of the *E. coli* density in thermally treated solids by SCMs and quantitative real-time polymerase chain reaction (qPCR) indicated that *E. coli* densities are often underestimated by SCM. When analyzed with qPCR, the *E. coli* density after digestion can be 4-5 orders of magnitude greater than the non-detect levels identified by SCMs, which supports the non-culturable hypothesis.

The VNC state describes a condition where bacteria are alive but unable to sustain the metabolic process needed for cellular division. Supplements added to culturing media were investigated to determine if the resuscitation of VNC bacteria could be enhanced. The autoinducer molecules N-hexanoyl-L-Homoserine lactone (C6-HSL), 3-oxo-N-octanoyl-L-Homoserine lactone (3-oxo-C8-HSL), and norepinephrine were unable to induce the resuscitation of VNC *E. coli*.

Additional sampling was performed to determine if autoinducer molecules, peroxides, or other as of yet unknown inhibitory agents and toxins could be removed from biosolids during SCM. Culture media supplemented with the peroxide degrading compounds catalase, α-ketoglutaric acid, and sodium pyruvate was unable to resuscitate non-culturable *E. coli*. The additions of bentonite and exponential growth phase *E. coli* cell-free supernatant to culturing media were also unable to increase the culturability of *E. coli*. To remove inhibitory agents and toxins, a cell washing technique was employed prior to performing SCM; however, this cell washing technique may have increased cellular stresses that inhibited resuscitation since cell densities decreased.

A novel laboratory-scale dewatering process was also investigated to determine if the SI and regrowth phenomena observed in full-scale centrifugal dewatering could be mimicked in the laboratory using a lab shearing device. Fecal coliform and *E. coli* densities in laboratory prepared cake samples were observed to be an order of magnitude higher than full-scale dewatered cakes. Additionally, the laboratory-scale dewatering process was able to resuscitate fecal coliforms and *E. coli* in stored sludge such that the density increased by 4-5 orders of magnitude from non-detect values.

Lastly, the addition of aluminum sulfate during centrifuge dewatering at a full-scale utility produced an increased regrowth of fecal coliforms and *E. coli* that was sustained for 5 days.

Chapter 1: Introduction

1.1 Overview of a Wastewater Treatment Plant

Municipal wastewater treatment plants typically contain the same basic subunits. Figure 1–1 depicts a generalized process flow diagram for a typical municipal wastewater treatment plant. First, grit and sediment are removed during pre-treatment in a grit chamber which uses gravity to settle stones, sand, and silt. The influent then enters a primary sedimentation unit which removes a portion of the total suspended solids from the plant influent water. Secondary treatment consists of a biological treatment process which decreases the concentration of organic dissolved solids and organic suspended solids (Reynolds et al., 1996). In the biological or secondary treatment stage, microorganisms grow and multiply, which cause a buildup of biomass. The growth of microorganisms during biological treatment is mitigated by wasting a fraction of the activated sludge generated. The primary and secondary sludge is often then digested, which further reduces the amount of organic solids, prior to dewatering. The dewatering process is necessary to remove water so that the weight and volume of the biosolids are at a manageable value. Effluent and biosolids are required to meet regulatory requirements prior to discharge.

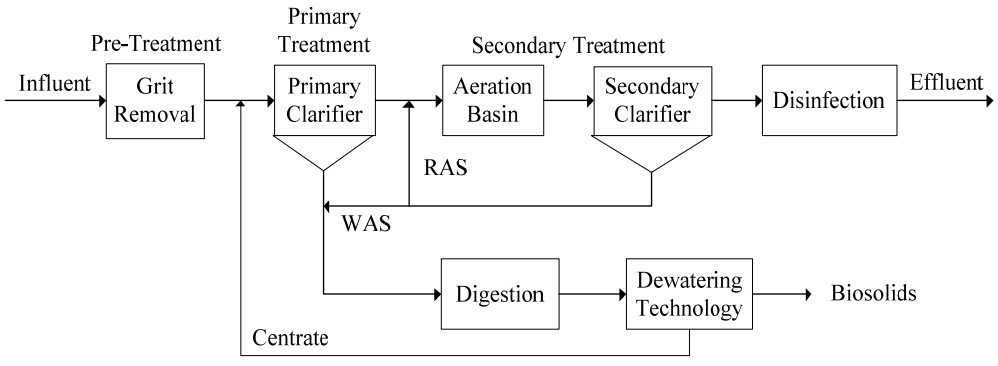

Figure 1–1: Schematic for Wastewater Treatment Plant

1.2 Overview of Sludge Treatment

The activated sludge, otherwise known as mixed liquor suspended solids, undergoes a sedimentation process whereby the solids concentrate and compact. The solids that leave from the bottom of the sedimentation tank (Figure 1–1) are termed waste activated sludge (WAS). A portion of the WAS is replenished into the aeration basin as the return activated sludge (RAS). The RAS is important since it maintains the microbial population within the biological treatment system.

Typically the WAS is combined with the primary clarifier solids and is digested either by anaerobic or aerobic digestion (Figure 1–1). The digestion process reduces the amount of degradable solids. Solids are made from three constituents: active biomass, inactive biomass, and fixed solids (Reynolds et al., 1996; Rittmann et al., 2001). To degrade the solids further, the digester is operated at relatively long solids retention times (SRT) of approximately 10-60 days. During this prolonged SRT, bacteria selectively compete for nutrients. As nutrients become scarce, bacteria either die, cannibalize one-another, or enter a suspended state. Through this process, the amount of organics are decreased leaving mostly fixed solids and residual organic biomass.

Typically, anaerobic digestion technologies are employed for solids reduction since anaerobic digestion does not require energy for aeration like aerobic digestion processes. Anaerobic digestion does have the drawback of requiring higher operating temperatures. An important family of anaerobic microbes in a wastewater treatment plant are the methanogens (Reynolds et al., 1996; Rittmann et al., 2001), a group of Archaea that are capable of producing methane. Since these microbes produce methane, many treatment plants burn the methane to produce the heat required to operate an anaerobic digester and some plants also use the methane to produce electricity.

After digestion, most utilities dewater the sludges to reduce hauling and disposal costs. The most common dewatering technologies are belt filter presses (BFPs) and centrifuges, low solids centrifugation (LSC) and high solids centrifugation (HSC). Dewatered sludge is called the cake while the liquid is termed centrate or filtrate. HSC typically achieves 22-30% solids while belt filter presses produce cakes with 12-20% solids (Reynolds et al., 1996).

1.3 Overview of Biosolids Regulations in the United States

The term biosolids can be somewhat misleading since some liquids, as well as the solids, are classified as biosolids (EPA, 1994). Biosolids are a federally regulated material that is produced when sewage sludge is treated. Biosolids are classified according to the United States Environmental Protection Agency (EPA) document titled "The Standards for the Use or Disposal of Sewage Sludge" (Title 40 of the Code of Federal Regulations [CFR], Part 503) as published in the Federal Register (58 FR 9248 to 9404) on February 19, 1993. The Part 503 rule, as this regulation is commonly known, describes biosolids as "…solid, semi-solid, or liquid residue generated during the treatment of domestic sewage in a treatment works" (EPA, 1994; EPA 1999). Therefore, sewage sludges that have been treated according to the methods outlined in the Part 503 rule are considered biosolids. This research will consider the Part 503 standards for digested sludge and cake from wastewater treatment plants.

The Part 503 rule applies to the uses for and disposal practices of biosolids. The subparts of the rule include the requirements for land application, surface disposal, pathogen and vector attraction requirements, and incineration. Because the biosolids discussed in this thesis only pertain to land applied and landfilled biosolids, this discussion will only detail the practices for the land application option for biosolids. Subpart D of the Part 503 rule defines criteria for pathogen and vector attraction requirements. Pathogens, such as certain bacteria, viruses, and parasites, can cause disease when introduced near a human population. Insects and rodents are some of

the vectors that transport and transfer pathogens to humans. The criteria listed in Subpart D aims to reduce the pathogenicity of biosolids and to minimize the attractiveness of vectors.

The pathogenicity of the biosolids are a concern to human health and the environment because of the potential diseases that can be contracted from exposure. In order to meet pathogen reduction requirements, Subpart D allows for the quantification of pathogens (*Salmonella* sp. bacteria, enteric viruses, and viable helminth ova) or pathogen indicators (fecal coliform and *Escherichia coli*).

1.3.1 EPA Class A and Class B Biosolids

Two biosolid classifications are commonly utilized as part of the Subpart D specifications: Class A and Class B. The Part 503 requirements for Class A and Class B biosolids are detailed in the following sections. Water and wastewater quality has been characterized though the detection of indicator bacteria. Commonly, the fecal coliform (FC) group of bacteria has served as the indicator bacteria for wastewater process as required by regulations in the United States of America (EPA, 1994). Although there are many definitions for the types of bacteria that are members of the total coliform (TC) group, the *Standard Methods for the Examination of Water and Wastewater* (Eaton, 1995) describe the coliform group as follows:

> *All facultative anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 hours at 35°C* (SM 9921A).

Included within the TC group are FC and *Escherichia coli*, typically members of the Enterobacteriaceae family of bacteria. Because bacteria within this family can be pathogenic and are used as indicators of fecal contamination, the specificity and accurate quantitation of these bacteria are needed.

1.3.2 EPA Class A Biosolids Requirements

The Class A biosolids classification pertains to treated materials that do not contain pathogens (*Salmonella* sp., enteric viruses, and viable helminth ova) at detectable levels (EPA, 1994). In order to meet the Class A biosolids classification, treatment plants need to meet the requirements specified in the Part 503 rule. Six alternatives exist for treating biosolids so they can be classified as Class A with respect to pathogens. For the purpose of this research, only Alternative 1 "Thermally Treated Biosolids" will be discussed.

In the early 1980s, Feachem et al. (1983) developed time and temperature plots for pathogen reduction including *Ascaris* ova, enteric viruses, *Vibrio cholarae*, and *Salmonella* sp. The plots, depicted in Figure 1–2, described how time and temperature related to inactivation of the selected pathogens. Feachem et al. (1983) used this data to delineate a "Zone of Safety," the requirements for digestion time and temperature that resulted in inactivation of pathogens.

However, as EPA was developing their Part 503 rules, the EPA determined that Feachem et al. (1983) did not provide enough detail when developing the time and temperature plots from the raw data (Willis et al., 2006). Thus, EPA created their own Time and Temperature Curves as depicted on Figure 1–2. The EPA Time and Temperature Curves were selected as the Part 503 rule requirement for Class A biosolids to be more conservative than Feachem et. al. (1983).

According to the Part 503 rules for Class A biosolids under Subpart D, four time-temperature regimes have been developed for different scenarios of treatment. For each of these regimes, time-temperature requirements are listed with time-temperature relationship equations. The four time-temperature regimes under Alternative 1 are listed in Table 1–1, and basically result in two curves, the regimes A-B-C curve and the regime D curve, as shown in Figure 1–2.

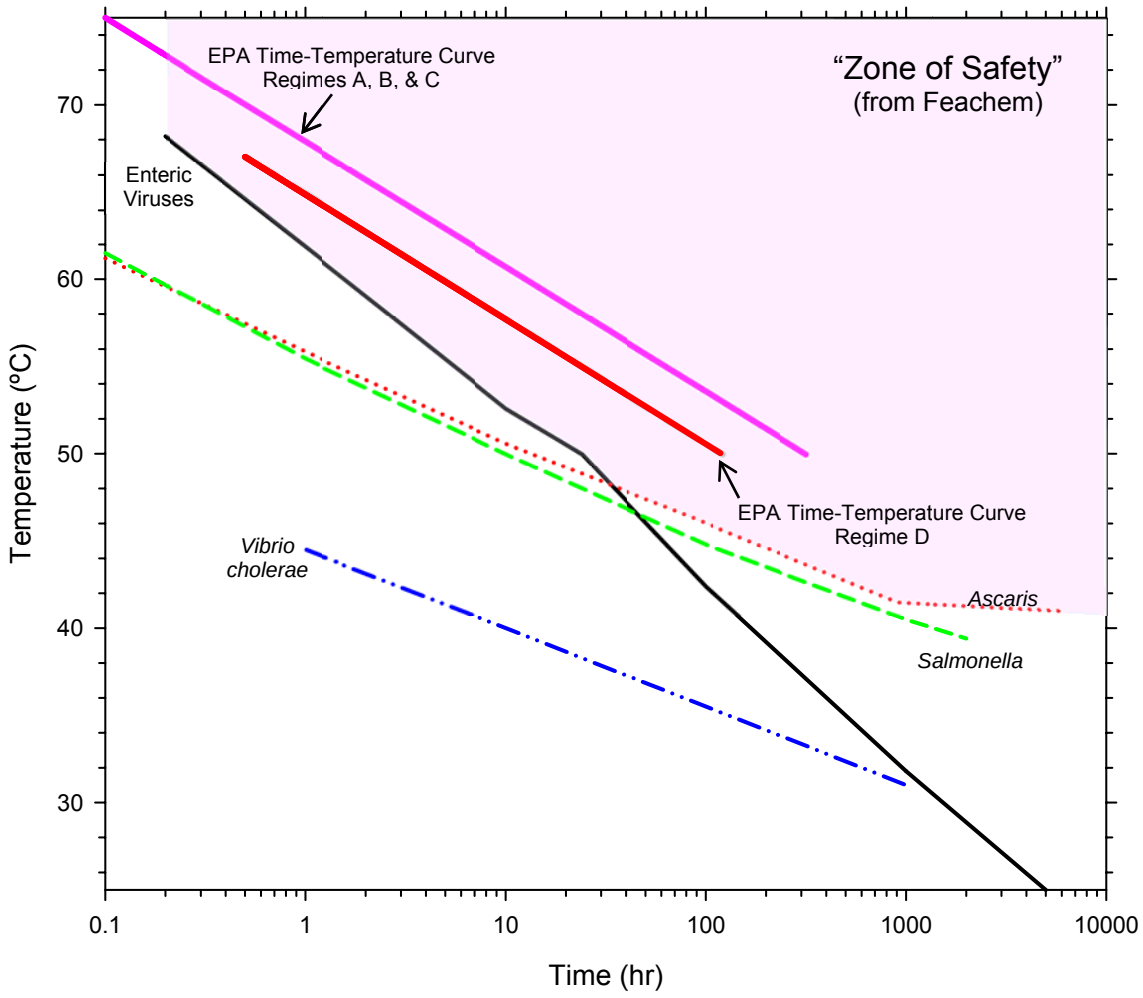

Figure 1–2: EPA Class A time and temperature curve and "Zone of Safety" adapted from Feachem et al. (1993)

Table 1–1: The four time-temperature regimes and equations for Class A pathogen reduction under Title 40 CFR Part 503, Subpart D, Alternative 1

Regime	Applies to:	Requirement	Time-Temperature Relationship*
A	Biosolids with 7% solids or greater (except those covered by Regime B)	Temperature of biosolids must be 50°C or higher for 20 minutes or longer	$D = \frac{131{,}700{,}000}{10^{0.14t}}$ (Equation 2 of Section 503.32)
B	Bioloids with 7% solids or greater in the form of small particles and heated by contact with either warmed gases or an immiscible liquid	Temperature of biosolids must be 50°C or higher for 15 seconds or longer	$D = \frac{131{,}700{,}000}{10^{0.14t}}$
C	Biosolids with less than 7% solids	Heated for at least 15 seconds but less than 30 minutes	$D = \frac{131{,}700{,}000}{10^{0.14t}}$
D	Biosolids with less than 7% solids	Temperature of sludge is 50°C or higher with at least 30 minutes or longer contact time	$D = \frac{50{,}070{,}000}{10^{0.14t}}$ (Equation 3 of Section 503.32)

* D = time in days; t = temperature in degrees Celsius. For example, if a plant was using Curve D and operated at 55°C, they would need to hold the solids for a minimum of 24 hours to meet Class A requirements.

Treatment plants that operate thermophilic digesters that follow the EPA Time and Temperature Curve requirements also need to meet bacterial requirements set by the Part 503 rule. A Class A biosolid must be treated with time and temperature requirements so that the density of the pathogen indicator FC is less than 1,000 most probable number (MPN) per gram dry solids (MPN/g DS) or the density of the pathogen *Salmonella* sp. is less than 3 MPN per 4 grams of total solids (dry-weight basis) (EPA, 1994; NRC, 2002). The objective of the law is that the biosolids are essentially pathogen free.

1.3.3 EPA Class B Biosolids Requirements

The Class B biosolids classification pertains to treated materials that contain pathogens (*Salmonella* sp. bacteria, enteric viruses, and viable helminth ova) at detectable levels that can be

managed with land application restrictions to prevent exposure to the biosolids after their use or disposal (EPA, 1994). Three alternatives exist in the Part 503 rule for meeting Class B pathogen requirements. Alternative 1 requires monitoring of indicator organisms such that the density of FC is less than 2 million (2×10^6) MPN/g DS, based on geometric mean of seven samples (EPA, 1994).

Alternatives 2 and 3 require that biosolids are treated in a Process to Significantly Reduce Pathogens (PSRP) or equivalent process. Under Appendix B of 40 CFR Part 503, PSRPs include aerobic digestion, air drying, anaerobic digestion, composting, and lime stabilization. Each PSRP has specific requirements for time and temperature. For example, the anaerobic digestion (studied in this research) PSRP requires that SRT shall be between 15 days at 35°C to 55°C (mesophilic) and 60 days at 20°C. When a PSRP is utilized, microbial monitoring for the regrowth of FC or *Salmonella* sp. bacteria are not required (EPA, 1994). Therefore, treatment plant operators are not required to monitor whether the density of FC is less than 2×10^6 MPN/g DS.

The assumption that the concentration of FC and other indicator bacteria do not increase during biosolids storage is a core assumption of the Part 503 rule. As demonstrated by a growing community of researchers, the density of indicator bacteria can increase after dewatering, and this has implications for meeting regulatory requirements (Iranpour et al., 2002; Qi et al., 2004; Monteleone et al., 2004; Estrada et al., 2004). Class A and Class B biosolids requirements were defined by the Part 503 rule to provide a reasonable confidence that the public would not encounter health consequences from the land application of biosolids.

1.4 Issue of Sudden Increase and Regrowth

“Sudden Increase (SI)” and regrowth are phenomena that have been observed in full-scale

wastewater treatment plants that operate anaerobic digesters with HSC dewatering technologies. This section discusses the current literature as it pertains to mesophilic and thermophilic digesters designed to achieve Class A or Class B biosolids requirements.

1.4.1 Impact of SI and Regrowth on Class A Biosolids

Iranpour et al. (2002) indicated that wastewater treatment plant operators that operate thermophilic digesters designed to meet Class A biosolids requirements have become aware that increases in FC densities are readily occurring in post-digestion biosolids. Higgins et al. (2006) described the increases as occurring in two distinct phases, "Sudden Increase" and "regrowth." This phenomenon is shown graphically as presented in Figure 1–3. The data is from a plant that utilized pre-pasteurization (66°C for 1 hour) to meet Class A time and temperature requirements followed by anaerobic digestion and centrifuge dewatering. The results show that after pre-pasteurization and digestion, the plant meets the Class A requirement of $<10^3$ FC/g DS, in fact, after digestion the FC density was below detection. However, after dewatering, the FC density increased to 10^5 FC/g DS, a 4-5 order of magnitude increase in less than 20 minutes. After storage, the FC density continued to increase to 10^7 FC/g DS.

The term SI refers to the sudden increase in indicator bacteria, mainly FC and/or *E. coli*, immediately after the dewatering process when compared to the enumerations prior to dewatering, a time frame of less than 20 minutes (Higgins et al., 2006). Regrowth refers to the growth of microbes during the storage of the dewatered biosolids, typically on the order of hours to days (Higgins et al., 2006).

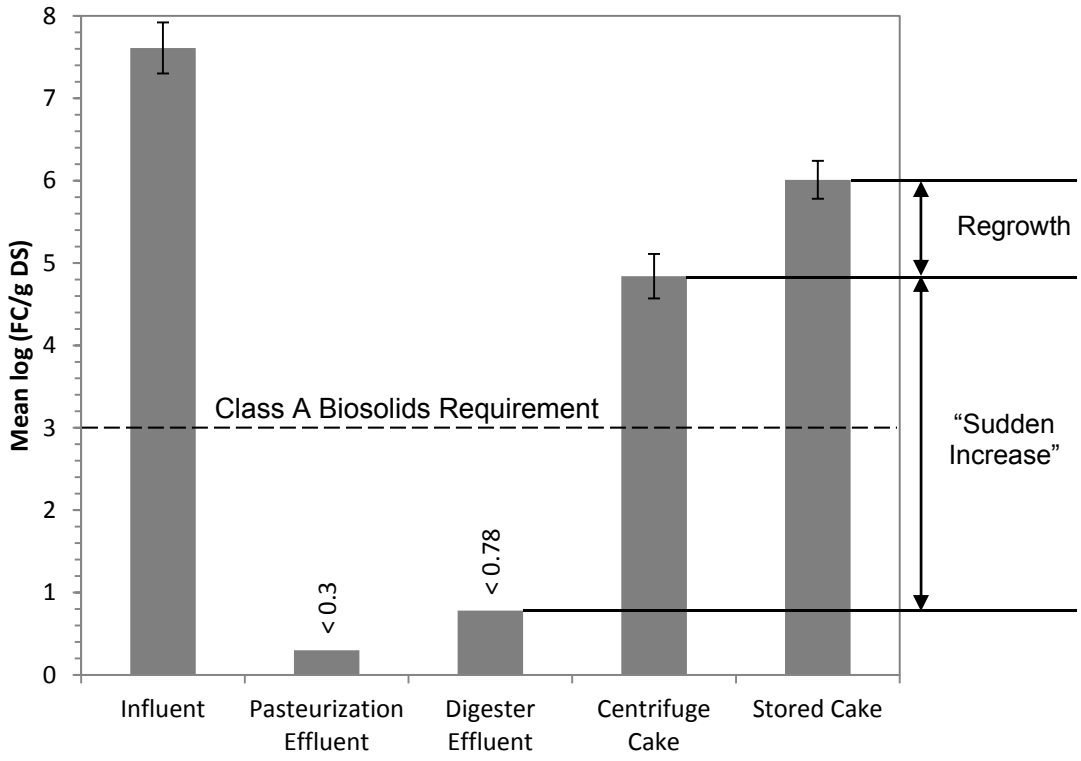

Figure 1–3: Fecal coliform counts from different locations before and after thermophilic digestion, after dewatering, and after on-site cake storage (Pre-Past-1) (Error bars represent one standard deviation)

1.4.2 Impact of SI and Regrowth on Class B Biosolids

At Class B mesophilic anaerobic wastewater treatment systems, SI has not been significantly observed; however, the regrowth phenomena has been observed (Erdal et al., 2003; Monteleone et al., 2004; Qi et al., 2004; Higgins et at., 2006). FC concentrations in mesophilic cake samples initially meet the 2 x 10^6 MPN/g DS bacteria requirements. However, after 24 hours of incubation at 20°C, FC concentrations increase by 2-3 orders of magnitude, making the cake samples no longer compliant with the Part 503 rule requirements for Class B biosolids classification.

1.4.3 Implications of SI and Regrowth

The SI and regrowth of bacteria are therefore a significant problem for wastewater treatment plants that produce biosolids for land application. Treatment plants typically meet the FC limits after digestion but no longer meet the requirements after dewatering. Because of the SI and regrowth phenomena, uncertainty exists about the true density of FC in biosolids being land applied.

1.5 Hypotheses for Sudden Increase

The term SI is defined as an increase in FC or *E. coli* density in cake solids collected immediately after a dewatering process compared to the feed to the dewatering process (Higgins et al., 2006). Typically, solids remains in a HSC for 1-3 minutes before exiting as dewatered cake and centrate. During this short period of time, the culturability of FC and *E. coli* has been observed to suddenly increase. These increases can be up to 4 or 5 orders of magnitude in this 1-3 minute time period (Iranpour et al., 2002; Monteleone et al., 2004; Qi et al., 2004; Higgins et at., 2006). Also, during cake storage, a one to two orders of magnitude increase is typically observed for FC and *E. coli* density within 48 hours as determined by standard culturing methods (SCMs).

The term “SI” has been utilized to describe the observation of sudden increase in microbial population by SCMs and not to describe a mechanism to the observed increase. Several theories have been proposed to explain the SI phenomena:

1. Floc breakup
2. Bacteria growth
3. Contamination

4. Resuscitation of viable non-culturable bacteria

These theories have been discussed in various literature sources, and a review of these theories is discussed in the following sections.

1.5.1 Floc Breakup

Many treatment plants rely on the flocculation process to thicken sludges that are wasted from secondary clarifiers. These floc particles are formed by the attractions between bacteria, inert and organic solids, and flocculants (typically polymers or chemical coagulants). The flocs that settle during sedimentation are further degraded during the digestion process. These digested flocs, which may have a different composition than when they entered the digester, are dewatered by various technologies, including HSC. As discussed previously, SI has been identified as a common occurrence with anaerobic, thermally treated sludges that are dewatered using HSC. The breakup of flocs during HSC may be one potential reason that SI occurs.

Qi et al. (2004) presumed that the intensive shearing forces associated with centrifuge dewatering may be a reason for the increased enumeration of FC. Qi et al. (2004) suggested that shearing forces were responsible for the breakup of flocs which would increase the surface area where bacteria could culture. Upon testing this hypothesis with a kitchen blender (maximum speed for 3 minutes at room temperature [25°C]), Qi et al. (2004) noted that the blended and unblended samples contained the same concentration of FC. Microscopy of the blended and unblended samples identified that the blending process produced smaller floc sizes. Nevertheless, the blending of digester feed sludge did not result in higher FC enumeration compared to unblended feed sludge (Qi et al., 2004).

Instead of producing shear forces using a kitchen blender, Monteleone et al. (2004) used a laboratory-scale centrifuge to determine if increasing centrifugal force resulted in higher

enumeration of FC. Unfortunately, the research did not indicate a simple relationship between centrifugal force and *E. coli* concentration. Low centrifugal force (below 500 x*g*) resulted in the highest concentration of *E. coli*. Increasing the centrifugal force beyond 500 x*g* resulted in diminished *E. coli* enumeration. When laboratory-scale data was compared to full-scale centrifugal dewatering, the immediate resuscitation of FC was not observed (Monteleone et al., 2004). Qi et al. (2004) and Monteleone et al. (2004) both concluded that floc breakup did not explain the SI of the FC and *E. coli* density.

1.5.2 Bacteria Growth

Qi et al. (2004) suggested bacterial growth, specifically regrowth, as an explanation for the increase in FC concentration after centrifugal dewatering. The MPN data collected in the Qi et al. (2004) experiments suggested that FC concentrations did not increase during centrifugation but rather after centrifugation. The regrowth after centrifugal dewatering was not able to be replicated using laboratory centrifugation techniques and the possibility that resuscitation occurred during centrifugation could not be dismissed (Qi et al., 2004). However, because the centrifugation process takes 1-3 minutes, which is much less than the doubling time for bacteria, the large increase in bacteria density immediately after centrifugation is not due to bacterial growth. Thus, the Qi et al. (2004) suggestion that regrowth explained the increase in FC concentration after centrifugal dewatering is likely not correct.

1.5.3 Contamination

Iranpour et al. (2002) suggested that the increase in FC density measured after a thermophilic digestion process and HSC may be a consequence of contamination from in-plant transfer (pipelines) and storage of biosolids. Specifically, they theorized that after digestion contamination and cooling of biosolids resulted in the increases of FC (Iranpour et al., 2002). It is hypothesized that contamination from process equipment, pipelines, and storage facilities may also provide a

bacteria source that increased bacteria densities. Contamination should be considered at plant, but researchers have eliminated this as a mechanism in several cases so other potential mechanisms should be explored.

1.5.4 Resuscitation of Viable Non-Culturable Bacteria

Another possible explanation for the SI phenomena is the presence of viable non-culturable (VNC) bacteria that are resuscitated and become culturable after centrifuge dewatering. Oliver (2005a) compiled literature referencing that bacteria, such as *E. coli*, are capable of entering the VNC state when subjected to environmental stresses (e.g., heat stress, nutrient starvation, white light, toxicity from inhibitory agents, etc.). Because the scientific community has yet to define which biochemical parameters are necessary for viable versus dead cells, the existence of the VNC state is controversial (Oliver, 2005a). Typically, however, a cell, which is metabolically active yet unable to experience sustained cellular division that produces a colony that is discernable using SCMs, is considered to be in the VNC state (Oliver, 1993). SCMs, which typically assay for enzymatic activity (e.g., β-galactosidase, β-glucuronidase, etc.) do not typically culture VNC bacteria since VNC bacteria do not have the same metabolic activity as a normal viable cell (Anglès d'Auriac, 2000). It is possible that after thermophilic treatment, a portion of the FCs enter the VNC state and are resuscitated after centrifuge dewatering.

As mentioned previously, many researchers have identified that the concentration of FC increases by 4-5 orders of magnitude after centrifugal dewatering of thermophilic sludges. Because the dewatering process only constitutes 1-3 minutes, which is too short a period of time for population doubling to occur, the resuscitation of VNC bacteria is one potential explanation for the observed SI phenomenon. Unfortunately, current SCMs have also been described as being incapable of correctly enumerating VNC bacteria (Oliver, 2005a). Thus, the true density of viable bacteria measured by SCMs is potentially biased low.

In the Part 503 rule, the application and use of biosolids are regulated in part based on the population of bacteria. If bacterial enumerations are biased-low, utilities may be underestimating viable organisms after thermophilic digestion. Oliver (2005a) has identified literature supporting the conclusion that the VNC bacteria retain their virulence.

This thesis proposes that resuscitated VNC bacteria are a potential cause for the SI of bacteria in thermophilic processes. As a result, the SCMs currently used to enumerate FC in biosolids underestimate the actual densities. If culturing methods can be identified to promote the resuscitation of bacteria, then more accurate enumeration of indicator bacteria can be achieved. It is not clear how the bacteria are resuscitated by centrifuges. In this research, the following two hypotheses for resuscitating VNC bacteria were investigated:

1. Removal of growth inhibitors produced during dewatering

2. Resuscitation of VNC bacteria by quorum sensing

These two hypotheses are discussed in further detail in the following sections.

1.5.4.1 Removal of Growth Inhibitors Produced during Dewatering

Mizunoe et al. (1999) reported that peroxides reduce the culturability of bacteria by promoting the VNC state. Peroxides are typically produced by bacteria during metabolic instabilities caused by heat stress, nutrient starvation, and other environmental changes. The same factors causing peroxide production are also theorized to promote the VNC state. Literature indicates that peroxide-degrading compounds such as catalase, sodium pyruvate, and α-ketoglutaric acid are capable of resuscitating bacteria in the VNC state (Mizunoe et al., 1999).

In experiments conducted by Mizunoe et al. (1999), *E. coli* O157:H- strain E32511/HSC was

subjected to low-temperature stress (4°C incubation for 21 days) to cause the VNC state. Luria-Bertani (LB) agar plates enhanced with enzymatic (i.e., catalase) and nonenzymatic (i.e., sodium pyruvate, α-ketoglutaric acid) peroxide degrading compounds were able to restore the culturability of stressed cells after incubation at 25°C for 48-hours. Culturability typically increased from non-detect to 10^4-10^5 colony forming units (CFU)/mL within 48-hours on LB agar plates supplemented with catalase, sodium pyruvate, or α-ketoglutaric acid.

This research proposes that peroxide-degrading compounds can be added to SCM to promote the resuscitation of non-culturable bacteria. Because peroxide inhibition should be neutralized by the addition of peroxide-degrading compounds, this research hypothesizes that improved enumeration of bacteria density will occur when culturing media is enhanced with peroxide-degrading compounds.

1.5.4.2 Resuscitation of VNC Bacteria by Quorum Sensing

Prokaryotic bacteria were once considered to live unicellularly, with only stimulation from environmental factors such as the presence of chemicals and physical changes (Reading et al., 2006). This simplistic view of prokaryotic life has been dismissed with the discovery of small "hormone-like" organic molecules called autoinducers, which allow bacteria to communicate with one another (Reading et al., 2006). These autoinducers provide a cell-to-cell signaling system that serves to regulate gene expression based on cell density. Because autoinducers signal for the expression of certain genes based on cell density, autoinducers are considered quorum sensing molecules (Reading et al., 2006).

Early research into quorum sensing molecules first occurred during a study into the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (Reading et al., 2006). Since then, many more quorum sensing signaling molecules and genes have been identified (Reading et al., 2006). These quorum sensing molecules are divided into three classes. In Gram-negative bacteria, the

LuxR/I-type system is utilized for quorum sensing using various acyl-homoserene lactone (AHL) molecules. A peptide signaling system (*lux*S/autoinducer-2 [AI-2]) is utilized by Gram-positive bacteria, and an interkingdom signaling system utilizing epinephrine/norepinephrine/ autoinducer-3 (AI-3) has also been identified for cross-species signaling (Reading et al., 2006).

Multiple studies have found that quorum sensing in *E. coli* is essential for regulating culturability based on both cell density and metabolic potential of the environment (Surette et al., 1998; Lyte et al., 1996; Reissbrodt et al., 2002; Valle et al., 2004). These studies reference both the AHL and norepinephrine quorum sensing pathways. For *E. coli*, which does not produce a known AHL, the ability to interpret signaling molecules from other bacteria provide *E. coli* with information about cell density and nutrient available in the environment. This may be important for pathogenic *E. coli* because dispersion is necessary for greatest pathogenicity. Signaling molecules produced within nutrient-rich environments could communicate to nearby cells that reproduction is possible.

This research theorizes that the presence of *E. coli* in a nutrient-rich environment could promote exponential growth of bacteria because of cell-to-cell signaling molecules. This research will evaluate the hypothesis that the addition of cell-to-cell signaling molecules to culturing media will promote the resuscitation of non-culturable bacteria.

1.6 Hypotheses for Regrowth

The term regrowth is defined as an additional increase of FC or *E. coli* density during storage of cake samples over a period of hours or days (Higgins et al., 2006). The regrowth of FC and *E. coli* is hypothesized to occur from the release of substrate (e.g., polysaccharides, proteins, etc.) during the shearing process of HSC (Higgins et al., 2006). This regrowth phenomena has been observed by Qi et al. (2004) and Monteleone et al. (2004) in full-scale wastewater treatment

plants with mesophilic digestion. However, Qi et al. (2004) and Monteleone et al. (2004) were unable to replicate full-scale observations using laboratory procedures to replicate centrifugation and shearing forces.

The research presented herein poses questions on how to mimic full-scale dewatering processes in the laboratory such that regrowth occurs. Qi et al. (2004) and Monteleone et al. (2004) proposed that shear and centrifugation independently promote the breakup of flocs. Qi et. al (2004) presumed that the intensive shearing forces associated with centrifugation may be a reason for the increased FC density.

Our theory is that the shearing forces imparted to the solids during HSC releases bioavailable materials that support microbial growth. Therefore, the dewatering process (including centrifugation, shear forces from screw conveyance, and pressure dewatering) contains features that, when combined, may result in the regrowth of FC and *E. coli.*

1.7 Research Needs

The current understanding of the SI and regrowth phenomena are not well understood based on the literature reviewed. Previous research has indicated that the concentration of FC and *E. coli* has been observed to decrease after digestion and to increase after the dewatering process. However, the mechanisms for these increases are not understood. Research is needed to determine a better method for enumerating FC and *E. coli* that are in a VNC state after thermophilic treatment. Additional research is required to determine if enhancements to SCM can be used to increase the culturability of FC and *E. coli* by laboratory culturing techniques. Alternatively, research needs to be performed to determine if novel laboratory methods can be employed to promote the culturability of FC and *E. coli*. In addition, approaches are needed to prevent or mitigate SI and regrowth such that utilities can meet regulatory requirements.

1.8 Research Objectives

This research was focused on evaluating the resuscitation and regrowth hypotheses and determining methods of enumerating FC and *E. coli* to more accurately estimate the density of VNC bacteria. The main objectives of the research are summarized as follows:

- Examine the impact of digestion and dewatering processes on the extent of the increases;
- Investigate the resuscitation hypothesis;
- Examine potential culturing media supplements to improve the enumeration of FC after thermophilic treatment;
- Investigate if quorum sensing molecules are capable of reversing the VNC state;
- Determine if inhibitor toxicity results in diminished culturability;
- Determine if substrate is required for SI and regrowth.
- Investigate methods for controlling SI and regrowth.

This research hypothesizes that shear during centrifuge dewatering promotes the release of polysaccharides and proteins which then become bioavailable to resuscitate bacteria. The release of substrates bound in flocs provides a nutrient source for stressed and non-culturable bacteria. Once non-culturable bacteria are exposed to bioavailable nutrients in biosolids samples, the bacteria resuscitate and regrowth occurs.

1.9 Report Organization

This thesis is organized into the following sections:

- Chapter 1 describes the background information pertaining to wastewater treatment, sludge treatment, and biosolids regulations in the United States. Additionally, Chapter 1 presents the issues of SI and regrowth and describes and the hypotheses for their causes.

- In Chapter 2, the SI and regrowth are examined for different digestion and dewatering processes that are presented for the wastewater treatment plants discussed in this thesis.

- The time and temperature effects on the destruction of pathogens in Class A biosolids are discussed in Chapter 3.

- An investigation into the effects of peroxide as an inhibitor to bacterial growth is presented in Chapter 4.

- In Chapter 5, the cell-to-cell signaling by quorum sensing molecules hypothesis is examined.

- Chapter 6 presents data examining whether inhibitory substances can be removed from wastewater samples prior to bacterial enumeration by SCM.

- The effect of shear during the dewatering of sludge samples is discussed in Chapter 7.

- In Chapter 8, a full-scale experiment was performed to determine if coagulant addition during centrifugal dewatering bound substrates thus reducing regrowth.

- Finally, the overall conclusions from this thesis research are presented in Chapter 9.

Chapter 2: Observation of Sudden Increase and Regrowth in Full-Scale Treatment Plants with Thermophilic and Mesophilic Anaerobic Digesters

2.1 Introduction

The SI phenomena observed after the centrifugal dewatering of thermally treated anaerobic sludge is an important concern for wastewater operators trying to meet Class A biosolids culturability requirements. As indicated in Section 1.4.1, the density of FC and *E. coli* has been found to increase by 4-5 orders of magnitude after centrifugal dewatering and cake storage. This rapid increase in FC and *E. coli* density typically causes biosolids to no longer meet Class A pathogen reduction requirements. One possible mechanism explaining the SI phenomenon is the resuscitation of VNC bacteria. As discussed in Section 1.5.4, VNC bacteria are metabolically inactive bacteria that do not culture by SCMs. The presence of VNC bacteria would provide a rationale for the underestimated population of viable bacteria when determined by SCMs.

SCMs for detecting TC, FC, and *E. coli* include multiple tube fermentation and membrane filtration. These techniques allow for an aliquot of sample to be inoculated into standard media which promotes the growth of specific bacteria. Multiple tube fermentation and membrane filtration are commonly utilized because the equipment needed to perform the methods are inexpensive and because the methods themselves are not complicated and do not require highly skilled laboratory technicians. Nevertheless, there are problems associated with the traditional culturing techniques. Culturing techniques require the multiplication of bacterial cells so that quantification can be performed. Typically 1-2 days is required for TC sampling with confirmational sampling for FC and *E. coli* taking another 1-2 days. Additionally, antagonistic organisms interfere with the growth of target bacteria, thus diminishing the true number of target

bacteria (Rompré et al., 2002). Lastly, traditional culturing techniques do not have the specificity nor detection capabilities for VNC bacteria (Rompré et al., 2002).

The EPA requires the use of Method 1680 for enumeration of FC in bolsolids which is the same as Standard Method (SM) 9921. Bromocresol purple, a chromogenic pH indicator, changes color when the breakdown of lactose is catalyzed by β-D-galactosidase, which is an indication that TC are present (Rompré et al., 2002). In this breakdown, the pH of the culture media decreases as lactose is split into galactose and glucose. Similarly, the presence of *E. coli* is typically assessed through color change of a fluorogenic substance that is cleaved by β-D-glucuronidase (Rompré et al., 2002). SM 9921 utilizes the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), which fluoresces under long-wave UV light, to determine the presence of *E. coli*. Because both of these methods require enzymatically active bacteria, the culturing techniques utilized by SM 9921, and similar culturing methods can potentially underestimate the population of bacteria present because of VNC bacteria (Rompré et al., 2002). Therefore, traditional culturing techniques for the enumeration of coliforms and *E. coli* are potentially biased low because of the VNC population and problems associated with enumeration by traditional methods.

In addition to traditional culturing techniques to enumerate *E.coli*, direct enumeration can be performed using molecular-based techniques. Current molecular-based techniques include polymerase chain reaction (PCR) and fluorescent in-situ hybridization (FISH). As our understanding of DNA and RNA increase, the specificity, time and cost for analytical methods has decreased. Likewise, molecular-based techniques have become more mainstream within laboratories for microorganism enumeration. Nevertheless, because of the cost associated with the requisite equipment and the technical knowhow and precision required to complete these techniques, molecular-based methods are not likely to become standardized methods for use in non-laboratory environments (Rompré et al., 2002).

The quantification of TC by quantitative real-time polymerase chain reaction (qPCR) has been possible because the known DNA sequence for the *lac*Z gene which encodes β-galactosidase (Rompré et al., 2002). *E. coli* density has been quantified by qPCR using the *uid*A gene which encodes β-glucuronidase and the homologous gene pair *gad*A and *gad*B which encode glutamate decarboxylase (Rompré et al., 2002; McDaniels et al., 1996). Both *uid*A and *gad*A/B have been identified to be specific to *E. coli* (McDaniels et al., 1996). However, differences in the expression of the *uid*A and *gad*A/B genes have been identified for various *E. coli* strains (Rompré et al., 2002; McDaniels et al., 1996; Venkateswaran et al., 1996). Venkateswaran et al. (1996) indicates that pathogenic serogroups of *E. coli* (i.e., Enterohaemorrhagic *Escherichia coli* [EHEC] serogroup O157:H7) typically do not produce β-glucuronidase even though they possess the *uid*A gene. The absence of β-glucuronidase is the result of catabolic repression by lactose (Venkateswaran et al., 1999), which results in VNC EHEC bacteria when cultured on MUG enhanced media while qPCR still detects EHEC bacteria because the *uid*A gene is present. For phenotypic analysis using culturing techniques, detection of glutamate decarboxylase activity was more specific for *E. coli* than β-glucuronidase. Additionally, culturing methods that test for glutamate decarboxylase activity did not yield detections for false positive bacteria (i.e., *Shigella* spp. and *Citrobacter freundi*) like culturing methods that test for β-glucuronidase activity (McDaniels et al., 1996). Genotypic analysis for *E. coli* was equivalent when PCR primers were designed to amplify sequences of either the *uid*A or *gad*A/B genes (McDaniels et al., 1996).

As discussed with traditional culturing techniques, the *lac*Z, *uid*A, and *gad*A/B genes encode enzymes (i.e., β-galactosidase, β-glucuronidase and glutamate decarboxylase, respectively) that are specific to quantify TC and *E. coli*. Nonetheless, qPCR techniques still lack the specificity required to determine if DNA present in environmental samples is attributable to live or dead bacterial cells since bulk DNA is collected from the sample prior to analysis (Panutdaporn et al., 2006). Consequently, qPCR techniques can result in an *E. coli* quantification that is biased high.

Although more complex and less common among molecular-based techniques, FISH has been used to overcome the problem associated with live/dead specificity in qPCR. The FISH technique utilizes an oligonucleotide probe to detect complementary sequences of 16S rRNA molecules within phylogenetically identified micro-organisms (e.g., Enterobacteriaceae, *E. coli,* etc.). FISH cannot, however, be used to detect the coliforms as a group since the oligonucleotide probe is highly specific and will not detect mismatches from phylogenetically diverse microorganisms (Rompré et al., 2002). Additionally, because of their low metabolic activity, VNC bacteria typically have a low ribosome content, which results in a low quantity of 16S rRNA targets for hybridization and induces a weak fluorescent hybridization signal (Rompré et al., 2002). Another problem caused by VNC bacteria and dead cells is residual rRNA molecules that exist after the cell is no longer physiologically intact (Rompré et al., 2002). Thus, the quantification of bacteria using FISH is also problematic and typically yields data that is biased low.

2.2 Research Needs

Molecular techniques are currently able to more accurately determine the order of magnitude concentration for *E. coli*. However, the *E. coli* density determined by molecular techniques potentially result in an overestimation because of non-specificity for live and dead cells. SCM potentially result in an under-estimation of FC and *E. coli* populations because of diminished culturability caused by VNC bacteria or other mechanism yet to be understood. Research needs to be conducted to evaluate if molecular techniques are capable of estimating the SI and regrowth phenomena in Class A and Class B biosolids.

2.3 Research Objectives

The SCM available are not capable of adequately quantifying potentially VNC FC and *E. coli*

when compared to molecular techniques. Molecular techniques, however, might overestimate the number of cells because genetic material remains regardless of whether the cell is live or dead. This difference between enumerating FC and *E. coli* by molecular techniques and SCM creates a situation where potentially neither method accurately enumerates the quantity of FC and *E. coli* present.

This research will evaluate the density of FC and *E. coli* using both SCM and molecular techniques from various configurations of full-scale wastewater treatment plants utilizing HSC. The data from SCM and molecular techniques will be compared to determine if the SI and regrowth phenomena can be predicted.

2.4 Methods and Procedure

To support the research contained in this thesis, six wastewater treatment plants were studied because of the SI and regrowth phenomena observed after dewatering by HSC. As summarized in Table 2–1, two wastewater treatment plants were designed to meet EPA Class A biosolids requirements while four plants were designed to meet Class B biosolids requirements. Various digester configurations and time-temperature regimes were studied as they pertain to the SI and regrowth phenomena. This treatment plant data is presented to demonstrate the commonality of SI and regrowth in different configurations of wastewater treatment plants. Samples were collected after various process elements for evaluation by SCM and molecular based techniques as described in Sections 2.4.1 and 2.4.3.

Table 2–1: Summary of plant processes and operating conditions

Plant	Digestion				Dewatering
	Series/Parallel	SRT	Temperature	EPA Class A or B	
TPAD-1	Thermophilic Mesophilic (in Series)	15 days 20 days	58 °C 36 °C	A	HSC
Pre-past-1	Pre-Pasteruization Mesophilic (in Series)	1 hour 19 days	66 °C 35 °C	A	HSC
Thermo-1	Continuous Stir Tank Reactors (in Parallel)	15-20 days	55 °C	B	HSC
Meso 1	Mesophilic (in Parallel)	32 days	38 °C	B	HSC
Meso-2	Mesophilic (in Parallel)	22 days	36 °C	B	HSC
Meso-3	Mesophilic (in Parallel)	22 days	36 °C	B	HSC

2.4.1 Multiple Tube Fermentation Culturing Technique

Samples were shipped overnight on ice to preserve bacterial activity in accordance with SM 9060B (Eaton, 1995).

2.4.1.1 Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B. Cake samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile phosphate-buffered saline (PBS) dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). For liquid samples, 300 mL of sludge were homogenized in a sterile blender for 2 minutes (EPA Method 1680). Serial dilutions (1:10) were prepared for each sample. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media. Five replicate tubes were inoculated for each dilution. Presumptive cultures were

incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive Lauryl tryptose broth (LTB) media, the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under ultraviolet-B (UVB) lighting.

2.4.1.2 Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2006). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

2.4.1.3 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

2.4.2 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the

MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

2.4.3 Enumeration of *E. coli* using Molecular Methods

Samples were collected in triplicate, and bacteria were quantified by molecular methods using the following methodology:

1. Collection, preparation, and storage of biosolid samples in triplicate
2. Extraction and purification of DNA from triplicate samples
3. Quantification of total DNA extracted
4. Enumeration of *E. coli* using qPCR
5. Comparison of DNA results to traditional culturing methods

Because this methodology uses DNA to directly quantify *E. coli*, the culturability of the target bacteria does not affect quantification.

2.4.3.1 Biomass Sampling for Molecular Analysis

Samples were shipped overnight on ice to preserve bacterial activity in accordance with SM 9060B (Eaton, 1995). The total solids of the biomass was determined according to SM 2540D (Eaton, 1995). Liquid sludge samples were centrifuged at 14,000 *xg* for 5 minutes in an attempt to remove suspended naked DNA. Approximately 200 mg of homogenized, centrifuged pellet or 100 mg of homogenized, dewatered cake were prepared in triplicate for DNA extraction. This mass of biosolids was weighed into a Lysing Matrix E tube (QBIOgene, Carlsbad, CA) and stored at -80ºC until DNA extraction was performed.

2.4.3.2 DNA Extraction from Biosolids

This DNA extraction protocol was developed from Garbor et al. (2003) with some modifications as

discussed in detail herein. Biosolids were weighed directly into a Lysing Matrix E tube (QBIOgene, Carlsbad, CA) as described in Section 3.4.5.1. Biomass was homogenized with 750 µL sterile lysis buffer (100 mM Tris-HCl, 100 mM Sodium EDTA, 1.5 M NaCl, 1% hexadecylmethylammonium bromide (CTAB), pH 8) in a FastPrep® Instrument (QBIOgene, Carlsbad, CA) for 30 seconds at a speed of 5.5. A 5 µL aliquot of 20 mg/mL protease K was added to the Matrix E tube and incubated at 55°C for 30 minutes. A 200 µL aliquot of sterile 20% sodium dodecylsulfate (SDS) was added to the Matrix E tube and incubated at 65°C for 2 hours. The tube was shaken by hand every 30 min. The Matrix E tube was centrifuged at 14,000 *xg* for 10 minutes, and the supernatant was removed for further extraction. This extraction method was repeated two more times by adding 500 µL of lysis buffer to the pellet and homogenizing in the FastPrep® Instrument for 30 seconds at a speed of 5.5 as indicated previously. The homogenized tube was incubated at 65°C for 10 minutes during each re-extraction. The suspension was centrifuged at 14,000 *xg* for 10 minutes to pelletize the solids. The supernatant was removed and combined with the previous extracts.

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant. The tube was mixed by inversion and incubated for 10 minutes at room temperature. After incubation, the tube was centrifuge at 10,000 *xg* for 10 minutes at 4°C. The organic phase was removed into a new tube. The phenol/chloroform/isoamyl alcohol addition, mixing, incubation, centrifugation, and removal were repeated once and combined with the first organic phase extraction. An equal volume of chloroform was added to the combined organic phase extract. The tube was mixed by inversion and incubated for 10 minutes at room temperature. After incubation, the tube was centrifuged at 10,000 *xg* for 10 minutes at 4°C. The organic phase was removed and wasted. Next, 0.6 volumes (compared to the volume of organic phase wasted) of 2-propanol (-20°C) was added to the pellet and incubated overnight at 4°C. After incubation, the tube was centrifuged at 16,000 xg for 10 minutes at 4°C. Supernatant was decanted and the pellet was washed with 500 µL of 70% ethanol. Next, the tube was centrifuged at 10,000 *xg*; and

the supernatant was removed. The pellet was air dried for 15 minutes and resuspended with 200 µL of TE buffer (10 mM Tris-Cl, 1 mM Sodium EDTA, pH 7.5). Humic substances were removed with a Promega Wizard® Genomic DNA Purification Kit (Madison, WI). Extracted DNA was stored at -80°C until DNA quantification and PCR could be performed.

2.4.3.3 Total DNA Quantification

Total genomic DNA was quantified using a fluorescence-based method that combines PicoGreen® dsDNA Quantitation Reagent (Molecular Probe, Eugene, OR) in equal proportion with the sample DNA. A Turner TBS-380 Mini-Fluorometer (Turner BioSystems Inc., Sunnyvale, CA) was used to measure fluorescence response. Known concentrations of Calf Thymus DNA (Sigma Aldrich Co., St. Louis, MO) were prepared with PicoGreen® to determine the concentration of genomic DNA.

2.4.3.4 E. coli Quantification using Real-Time PCR

E. coli was quantified using the qPCR technique cited by Chen et. al. (2006). In brief, since the *gad*A/B gene, which encodes glutamate decarboxylase, has been shown to have sensitivity and specificity to *E. coli* (Gabor et al., 2003; McDaniels et al., 1996; Smith et al., 1992), qPCR primers were constructed to amplify the *gad*A/B sequence. The PrimerQuest^{SM} software provided by Integrated DNA Technologies (Coralville, IA) was used to design the forward primer (5'-GCG TTG CGT AAA TAT GGT TGC CGA-3') and reverse primer (5'-CGT CAC AGG CTT CAA TCA TGC GTT-3') sequences (Chen et al., 2006), which yield a 305 bp PCR product. These primers were analyzed against BLAST to confirm their specificity to *E. coli*. Triplicate PCR reactions with 12.5 µL of Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA), 0.5 µM of each primer, 30 nM of reference dye (ROX), and 10 µL of 10 ng sample DNA, all diluted to a final volume of 25 µL with DNase/RNase free water, were quantified with the Stratagene MX3005P qPCR system (La Jolla, CA) (Chen et al., 2006). The PCR program contained a 10-minute initial

denaturation at 95ºC, followed by 40 cycles each of denaturation at 95ºC for 30s, annealing at 59ºC for 1min, and extension at 72ºC for 30s (Chen et al., 2006). The program then conducted a final dissociation curve analysis of the PCR products. Serially diluted *E. coli* DNA ranging from 2 to 7,620 copies was used as an external DNA standard for each qPCR analysis (Chen et al., 2006). *Pseudomonas putida* DNA (10 ng) was included in each standard as background DNA (Chen et al., 2006).

2.5 Results and Discussion

The culturing and molecular data collected from six full-scale wastewater treatment plants is discussed in the following sections.

2.5.1 Temperature-Phased Anaerobic Digester Plant 1

This temperature-phased anaerobic digester (TPAD-1) utilized a 58°C thermophilic reactor with a 15 day SRT followed in series by a 36°C mesophilic reactor with a 20 day SRT to achieve the EPA Time and Temperature requirements for Class A biosolids. After digestion, solids are dewatered by HSC to produce cake with 22-23% solids. Digester effluent contained 3-4% solids.

The culturing data presented in Figure 2–1 indicated that the SI phenomena occurs after HSC since the concentration of FC and *E. coli* increased immediately after the dewatering process by HSC. Digester effluent contained FC and *E. coli* densities that were non-detect. After the dewatering process, FC and *E. coli* enumerations increased to 5 x 10^3 MPN/g DS. Additionally, culturing data from the TPAD-1 operator indicated that regrowth occurs after cake storage (data not presented). The FC concentrations identified in TPAD-1 cake samples were present at concentrations above the Class A biosolids limit.

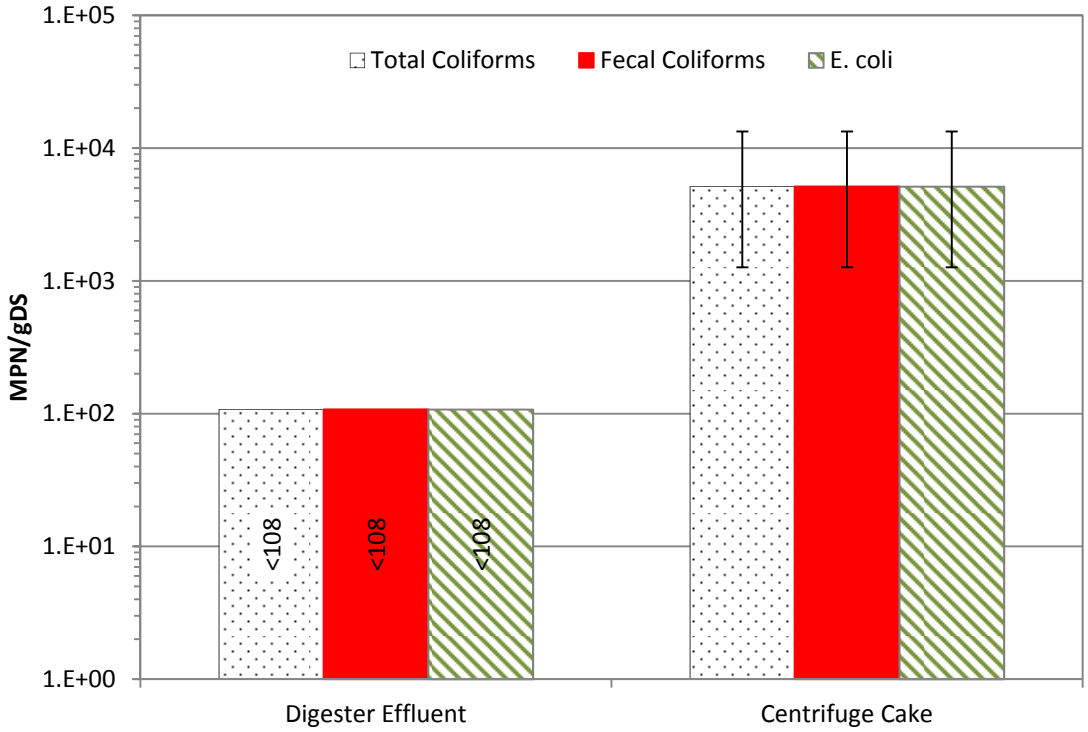

Figure 2–1: Total coliform, fecal coliform, and *E. coli* densities measured after the TPAD-1 digestion and dewatering process (Error bars represent 95% confidence interval)

2.5.2 Pre-Pasteurization Plant 1

This pre-pasteurization/mesophilic plant (Pre-past-1) utilized a 66°C thermophilic reactor with a 1 hour SRT followed by a 35°C mesophilic reactor with a 19 day SRT to achieve the EPA Time and Temperature requirements for Class A biosolids. Digester effluent sludge contains 1-2% solids. After digestion, sludge is dewatered by HSC to produce cake with 27-29% solids. A scroll conveyor transports dewatered cake to storage hoppers.

Digester influent FC and *E. coli* density were observed to be approximately 10^7 MPN/g DS by both SCM and qPCR methods as presented in Figure 2–2. Culturing and qPCR data depicted in Figure 2–2 was collected in cooperation with Dr. Yen-Chih Chen of Bucknell University. After

pre-pasteurization, FC and *E. coli* enumerations decreased to non-detect by SCM but remained at 10^7 MPN/g DS by qPCR (*E. coli* density only). After mesophilic digestion, FC and *E. coli* concentrations remained non-detect by SCM. *E. coli* enumerations by qPCR decreased from 10^7 to 10^5 MPN/g DS after mesophilic digestion.

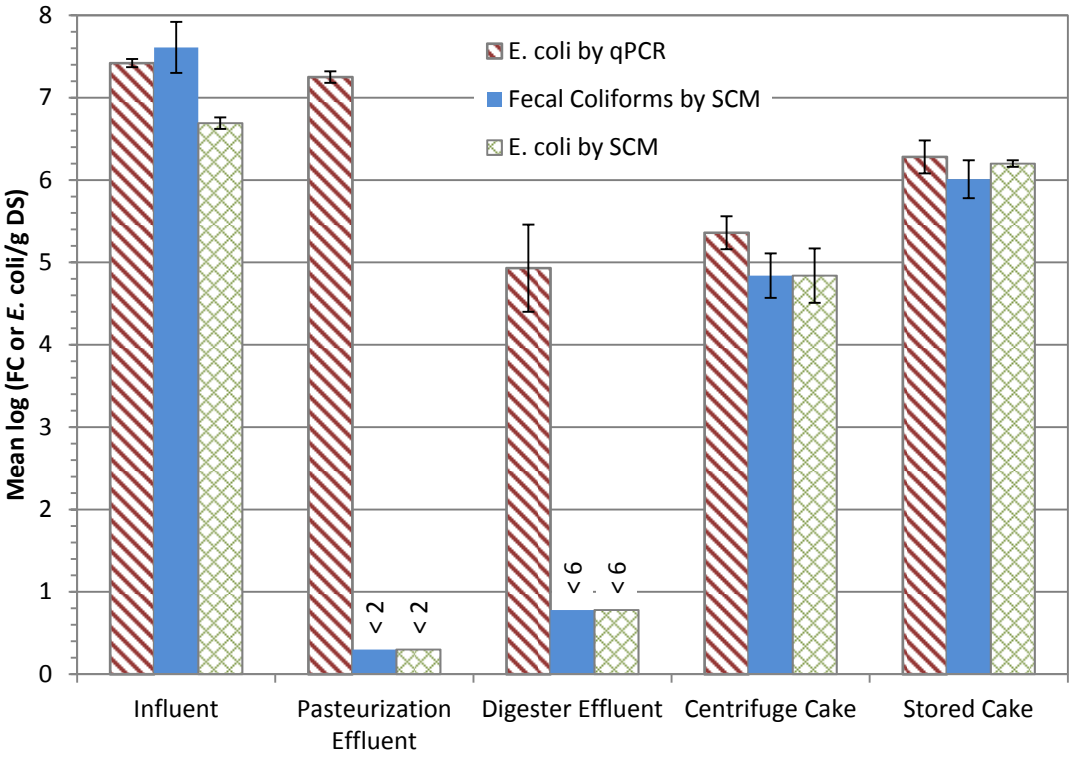

Figure 2–2: Fecal coliform and E. coli density measured using qPCR and standard culturing methods in pre-pasteurization (Pre-past-1) followed by mesophilic digestion process with high solids centrifugation dewatering (Error bars represent one standard deviation)

The culturing data presented in Figure 2–2 indicated that the SI phenomenon occurs after HSC since the concentration of FC and *E. coli* increased immediately after the dewatering process by HSC. Digester effluent contained FC and *E. coli* densities that were non-detect by SCM. However, *E. coli* quantification by qPCR indicated densities 4 orders of magnitude higher than SCM observations. After the dewatering process, FC and *E. coli* enumerations increased to 10^5

MPN/g DS, which was comparable to qPCR enumerations for *E. coli*. The FC concentrations identified in Pre-past-1 cake samples were present at concentrations above the Class A biosolids limit. After cake storage, FC and *E. coli* regrowth was observed by both SCM and qPCR (*E. coli* quantification only). The regrowth phenomenon was observed to increase FC and *E. coli* densities by 1-2 orders of magnitude to 10^6 MPN/g DS.

2.5.3 Thermophilic Plant 1

The anaerobic, thermophilic digester at Thermophilic Plant 1 (Thermo-1) utilized two 55°C thermophilic continuous stir tank reactors (CSTR) in parallel with a 15-20 day SRT to achieve the requirements for Class B biosolids. Digester effluents are stored prior to dewatering. The dewatering process utilizes HSC to produce mean cake solids of 34.5%.

Digester influent FC and *E. coli* density were observed to be approximately 5 x 10^7 MPN/g DS by both SCM and qPCR methods as presented in Figure 2–3. Culturing and qPCR data depicted in Figure 2–3 was collected in cooperation with Dr. Yen-Chih Chen of Bucknell University. Digested sludge was then stored in a tank. FC and *E. coli* densities were 5 x 10^2 MPN/g DS by SCM. *E. coli* quantification by qPCR indicated that DNA copies were about 10^5 copies/g DS. The SI phenomena occurred after HSC since the concentration of FC and *E. coli* increased immediately after the dewatering process by HSC. After the dewatering process, FC and *E. coli* densities increased to 5 x 10^4 MPN/g DS, which was comparable to qPCR enumerations for *E. coli*. After cake storage, FC regrowth was observed by SCM and *E. coli* regrowth was observed by qPCR. The *E. coli* density by SCM was unknown (>464 MPN/g DS). Unfortunately, not enough dilutions were performed to determine the actual density. The FC concentrations identified in Thermo-1 cake samples were present at concentrations below the Class B biosolids limit.

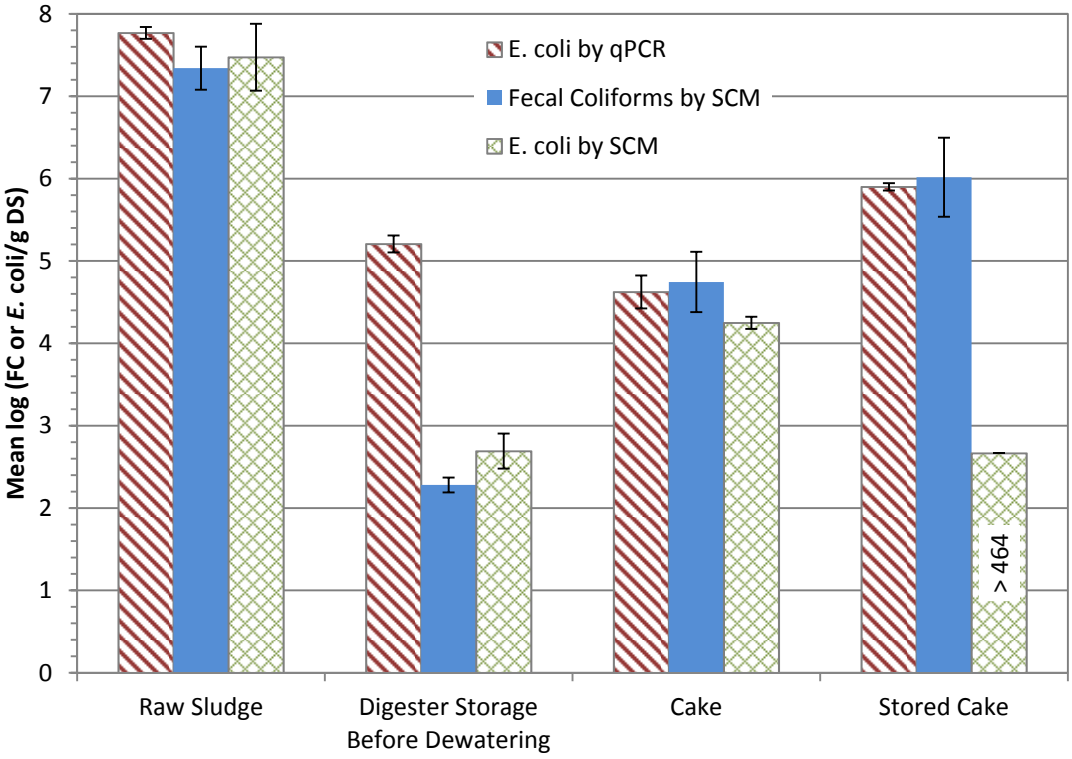

Figure 2–3: Fecal coliform and E. coli density measured using qPCR and standard culturing methods in a single stage thermophilic digestion (Thermo-1) process with high solids centrifugation dewatering (Error bars represent one standard deviation)

2.5.4 Mesophilic Plant 1

Two, single stage mesophilic anaerobic digesters operate at 38°C in parallel with a 32 day SRT to achieve the requirements for Class B biosolids. Digester effluents are stored prior to dewatering. The dewatering process utilizes a HSC to produce mean cake solids of 24.3%.

Digester influent FC and *E. coli* density were observed to be approximately 10^8 MPN/g DS by both SCM and qPCR methods as presented in Figure 2–4. Culturing and qPCR data depicted in Figure 2–4 was collected in cooperation with Dr. Yen-Chih Chen of Bucknell University. After the first mesophilic digester, FC and *E. coli* densities decreased to 10^6 MPN/g DS by SCM and qPCR (*E. coli* density only). After the second mesophilic digester, FC and *E. coli* densities remained

consistent with those after the first digester. Digested sludge was then stored in a tank and FC and *E. coli* densities remained consistent with digester effluent concentrations.

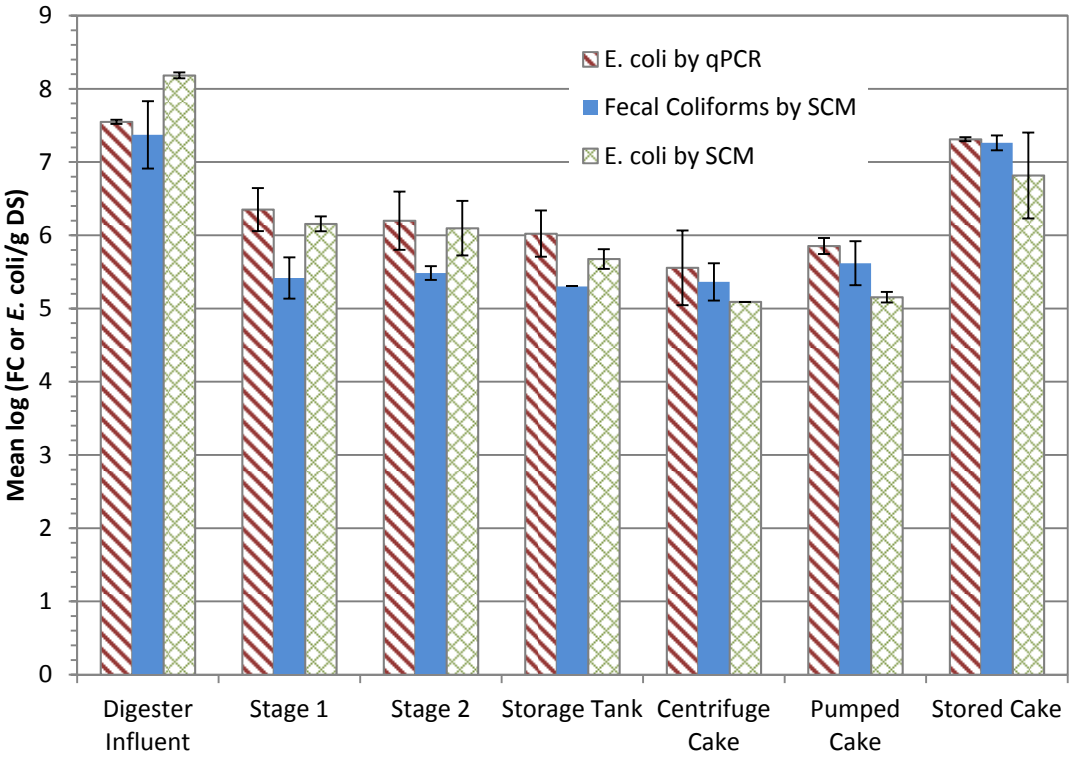

Figure 2–4: Fecal coliform and E. coli density measured using qPCR and standard culturing methods in mesophilic (Meso-1) digestion process with high solids centrifugation dewatering (Error bars represent one standard deviation) Data provided by Higgins et al. (2006).

After the dewatering process by HSC and pumping, FC and *E. coli* densities decreased slightly when compared to digester effluent and storage tank densities. After cake storage, FC and *E. coli* regrowth was observed by SCM and *E. coli* regrowth was observed by qPCR as shown by increased densities from 10^5 to 10^7 MPN/g DS. The FC density after HSC was identified to be at concentrations below the Class B biosolids limit. However, once these dewatered cakes were stored, the FC concentrations at Meso-1 were greater than the Class B biosolids limit due to regrowth.

2.5.5 Mesophilic Plant 2

Two, single stage mesophilic anaerobic digesters operate at 36°C in parallel with a 22 day SRT to achieve the requirements for Class B biosolids. The dewatering process utilizes a Humboldt HSC. The HSC typically produces cakes in the range of 30-33% solids. Culturing and qPCR results from Meso-2 are presented as Figure 2–5.

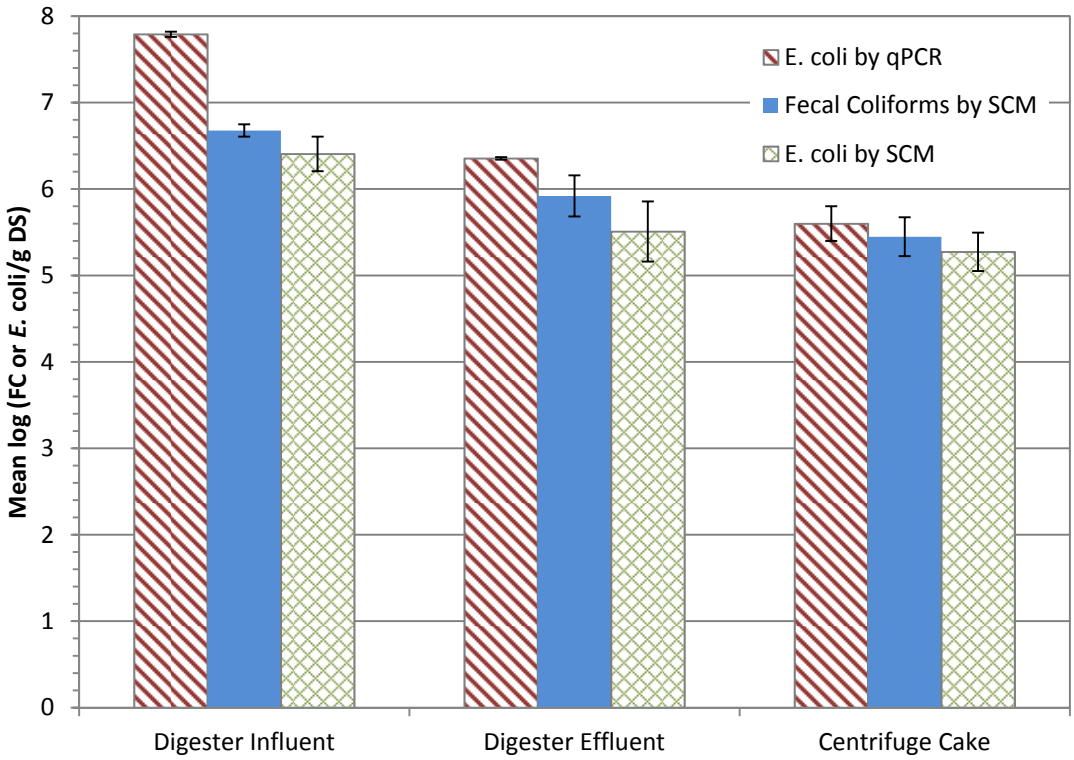

Figure 2–5: Fecal coliform and E. coli density measured using qPCR and standard culturing methods in mesophilic (Meso-2) digestion process with high solids centrifugation dewatering (Error bars represent one standard deviation)

Digester influent FC and *E. coli* density were observed to be approximately 5 x 10^6 MPN/g DS by SCM, and *E. coli* concentration was nearly 10^8 MPN according to qPCR methods as presented in Figure 2–5. The qPCR data depicted in Figure 2–5 was collected by Dr. Yen-Chih Chen of Bucknell University. After the mesophilic digester, FC and *E. coli* densities decreased below the

Class B biosolids limit when quantified by SCM and qPCR (*E. coli* only). After the dewatering process by HSC, FC and *E. coli* densities again decreased to a value of 5 x 10^5 MPN/g DS as determined by SCM and qPCR. Stored cake samples were not obtained from Meso-2 during this experiment; however, data from previous experiments by Dr. Yen-Chih Chen and Dr. Matthew Higgins indicate that the FC and *E. coli* densities increase in stored cake samples due to regrowth.

2.6 Research Significance

The consequences of SI and regrowth were investigated at multiple full-scale thermophilic and mesophilic digesters that utilize HSC dewatering. The following sections discuss the significance of the SI and regrowth phenomena.

2.6.1 Discussion of Thermophilic Treatment and the SI and Regrowth Phenomena

Three important observations were consistent in thermally treated sludge dewatered using HSC. These three observations, related to the SI and regrowth phenomena and to the use of qPCR, are discussed below.

- A sudden increase in FC and *E. coli* density was observed immediately after centrifugal dewatering of thermally treated sludge. The SI observation was determined by assessing the culturability of FC and *E. coli* in samples collected after thermal treatment and centrifugal dewatering. These samples represented a 3-4 minute period of process time whereby the density of FC and *E. coli* increased 2-3 orders of magnitude.

- After storage of cake solids, the FC and *E. coli* density continued to increase within 24 hours by 1-2 orders of magnitude. This increase in population, occurring after cake storage has been identified as the regrowth phenomenon. Regrowth was demonstrated

to occur consistently with stored cake samples that were generated from thermally treated sludges dewatered by HSC.

- SCM and qPCR yielded different values for *E. coli* density after thermal treatment and before dewatering. After thermal treatment, SCM determined that the *E. coli* was below detection while qPCR results indicated that the *E. coli* density was equivalent to the *E. coli* density in dewatered cake samples. After centrifugal dewatering, the *E. coli* density was equivalent when determined by SCM and qPCR. Therefore, qPCR may be a reasonable predictor for the SI phenomenon since SCMs appear to underestimate the *E. coli* population after thermophilic treatment.

- The qPCR results support the resuscitation of VNC bacteria hypothesis developed to explain the SI phenomena. The results for *E. coli* density determined by molecular techniques predicted the magnitude of SI that occurs after centrifuge dewatering.

2.6.2 Discussion of Mesophilic Treatment and the SI and Regrowth Phenomena

Sludge treated by mesophilic, anaerobic digesters and dewatered by HSC yielded different observations pertaining to FC and *E. coli* densities throughout the solids treatment process when compared to thermally treated sludge. After mesophilic digestion, FC and *E. coli* density in Meso-1 and Meso-2 did not decrease to non-detect values when determined by SCMs. Additionally, SCM and qPCR results were equivalent for *E. coli* density after digestion and centrifugal dewatering. The FC and *E. coli* density was observed to only increase after storage of dewatered cake. The *E. coli* density, determined by both SCM and qPCR, increased by 1-2 orders of magnitude after cake storage for 24 hours.

Based on the mesophilic, anaerobic treatment data, the following observations can be made in reference to the SI and regrowth phenomena.

- After centrifugal dewatering, the FC and *E. coli* densities did not experience SI when compared to densities from digester effluent samples. SCM and qPCR both produced comparable results for *E. coli* density. Because an immediate increase in FC and *E. coli* population was not observed after dewatering, the SI phenomenon does not appear to occur in mesophilic treated sludges.

- An increase in FC and *E. coli* density was observed after dewatered cake was stored for 24 hours. *E. coli* density was equivalent when determined by SCM and qPCR. This increase in FC and *E. coli* population of only 1-2 orders of magnitude is characteristic of the regrowth phenomenon.

- The qPCR results also suggested that SI was not likely to occur since there was little difference between qPCR and SCM results for *E. coli* density after mesophilic digestion.

- The results from the testing suggest qPCR is a reliable method for predicting SI.

2.6.3 Frequency of SI and Regrowth Phenomena

The SI and regrowth phenomena have been observed at wastewater treatment facilities operating both thermophilic and mesophilic anaerobic digesters and using both centrifuge and belt filter press dewatering technologies. To further understand the processes associated with SI and regrowth, the SI and regrowth observations from the utilities studied in Chapter 2 and the literature reviewed in Chapter 1 have been summarized as Table 2–2.

Table 2–2: Summary of SI and regrowth incidence at various wastewater utilities

Plant	Digestion	EPA Class A or B	Dewatering Technology	Sudden Increase	Regrowth
TPAD-1	Thermophilic Mesophilic (in Series)	A	HSC	NS	Yes
Pre-past-1	Pre-Pasteruization Mesophilic (in Series)	A	HSC	Yes	Yes
Iranpour et al. (2002)	Thermophilic	A	HSC	Yes	Yes
Higgins et al. (2007) Thermophilic Plant 1	Thermophilic (in parallel)	A	HSC	Yes	Yes
Higgins et al. (2007) Thermophilic Plant 2	Thermophilic (in series)	A	HSC	No	No
Higgins et al. (2007) ATAD	Thermophilic aerobic (in series)	A	HSC BFP	Yes No	Yes NS
Cheung et al. (2003)	Mesophilic	B	HSC	Yes	NS
Thermo-1	Continuous Stir Tank Reactors (in Parallel)	B	HSC	Yes	Yes
Meso 1	Mesophilic (in Parallel)	B	HSC	No	Yes
Meso-2	Mesophilic (in Parallel)	B	HSC	No	Yes
Meso-3	Mesophilic (in Parallel)	B	HSC	No	Yes
Erdal et al. (2003)	Mesophilic	B	HSC	Yes	Yes
Erdal et al. (2003)	Mesophilic	B	BFP	No	Yes
Qi et al. (2004) Plant 1	Mesophilic	B	BFP LSC HSC	No Yes Yes	No Yes Yes
Qi et al. (2004) Plant 2	Mesophilic	B	HSC	Yes	Yes
Qi et al. (2004) Plant 3	Mesophilic	B	HSC	No	Yes

BFP = belt filter press, HSC = high solids centrifugation, LSC = low solids centrifugation, HSC = high solids centrifugation, NS = not studied

Table 2–2 (Continued): Summary of SI and regrowth incidence at various wastewater utilities

Plant	Digestion	EPA Class A or B	Dewatering Technology	Sudden Increase	Regrowth
Qi et al. (2004) Plant 4	Mesophilic	B	LSC HSC	Yes Yes	Yes Yes
Monteleone et al. (2004) Site 1	Mesophilic (in Series)	B	HSC	No	NS
Monteleone et al. (2004) Site 2	Pasteurization and Mesophilic (in Series)	A	HSC	Yes	NS
Monteleone et al. (2004) Site 3	Pasteurization and Mesophilic (in Series)	A	BFP	No	NS
Monteleone et al. (2004) Site 4	Mesophilic (in Series)	A	HSC	Yes	NS
Monteleone et al. (2004) Site 5	Mesophilic (in Series)	B	HSC	No	NS
Higgins et al. (2007) Meso-2	Mesophilic	B	BFP	No	No
Higgins et al. (2007) Meso-4	Mesophilic	B	BFP	No	No
Higgins et al. (2007) Meso-5	Mesophilic	B	BFP	No	No
Flemming et al. (2009) WWTP Code #1	Mesophilic	B	HSC	No	No
Flemming et al. (2009) WWTP Code #2	Mesophilic	B	BFP/LSC	No	Yes
Flemming et al. (2009) WWTP Code #3	Mesophilic	B	HSC	Yes	No
Flemming et al. (2009) WWTP Code #4	Mesophilic	B	BFP	No	No
Flemming et al. (2009) WWTP Code #5	Mesophilic	B	HSC	No	Yes
Flemming et al. (2009) WWTP Code #6	Mesophilic	B	HSC	Yes	Yes

BFP = belt filter press, HSC = high solids centrifugation, LSC = low solids centrifugation, HSC = high solids centrifugation, NS = not studied

In order to understand the frequency of SI and regrowth, the observations presented in Table 2–2 were then separated into four categories: thermophilic anaerobic digestion, mesophilic anaerobic digestion, centrifuge dewatering, and belt filter press dewatering. The frequencies of these SI and regrowth occurrences are tabulated as Table 2–3.

Table 2–3: Frequency of SI and regrowth occurrence at utilities utilizing anaerobic digestion and dewatering technologies

Digestion Type	Dewatering Technology	Sudden Increase	Regrowth
Thermophilic (EPA Class A)	Centrifugation	6 /7	5 / 6
	Belt Filter Press	0 / 2	Not Studied
Mesophilic (EPA Class B)	Centrifugation	10 / 19	14 / 16
	Belt Filter Press	0 / 6	1 / 6

The data presented in Table 1–1 identifies the reactor and dewatering conditions where the SI and regrowth phenomena are observed. At the utilities that operated thermophilic digesters, SI and regrowth were observed at nearly all of the utilities where centrifuge dewatering was performed. Belt filter press dewatering of sludges produced by an aerobic thermophilic digestion (ATAD) process and by a pasteurization and mesophilic digestion process did not appear to promote the SI phenomena. The regrowth phenomena was not studied for this utility treatment configuration.

At centrifuge dewatered mesophilic sludge utilities, the regrowth phenomena was observed in nearly all of the utilities studied. The SI phenomena, was only observed at half of the utilities studied, which operated mesophilic digesters and centrifuge dewatering processes. The SI and

regrowth phenomena do not appear to be prevalent at utilities operating mesophilic digesters with belt filter press dewatering technologies.

This data indicates that the SI phenomenon is most frequently observed at utilities operating thermophilic digesters and centrifuge dewatering technologies. The regrowth phenomenon is typically observed after centrifuge dewatering technologies with both thermophilic and mesophilic digested sludges.

2.7 Conclusions

Based on the observation of FC and *E. coli* density after process elements in full-scale anaerobic wastewater treatment plants utilizing HSC, the following conclusions were identified:

- SI was observed mainly in full-scale treatment plants utilizing thermal treatment and centrifuge dewatering.

- The regrowth phenomena was observed in full-scale treatment plants designed to meet both Class A or Class B biosolids requirements and it is associated mainly with centrifuge dewatering.

- The use of qPCR was effective in predicting the *E. coli* density after the SI phenomena when compared to SCM enumerations.

- The accuracy of the *E. coli* density, as determined by qPCR, is unknown since qPCR is non-specific for live and dead cells, but the results suggest it may be a reliable method for estimating densities of *E. coli* in sludge. In fact, the pattern of densities measures by SCM and qPCR is the expected outcome if the non-culturable/reactivation hypothesis was correct.

- The resuscitation of non-culturable bacteria may be a possible explanation for the SI phenomena observed with biosolids collected from full-scale treatment plants.

Chapter 3: Evaluating the Validity of the Time-Temperature Curves for Class A Biosolids Treatment: A Discussion on the Presence of VNC Bacteria and the Resuscitation and Regrowth of Indicator Bacteria

3.1 Introduction

A number of wastewater treatment plants are upgrading to Class A biosolids and are using thermal treatment to achieve Class A requirements. For this case, wastewater treatment systems are designed so that sludge is processed for a specific detention time and at an appropriate reactor temperature as defined by the EPA time and temperature curve (Section 1.3.2). This correlation between time-temperature has received much attention lately because of the SI and regrowth phenomena which results in increased FC and *E. coli* densities greater than the Class A requirement after dewatering by HSC.

In the early 1980s, Feachem et al. (1983) developed time and temperature plots for pathogen reduction including *Ascaris* ova, *Enteric* viruses, *Vibrio cholarae*, and *Salmonella* sp. The plots, depicted in Figure 3–1, described how time and temperature related to the destruction of the selected pathogens. Feachem et al. (1983) used this data to delineate a "Zone of Safety," the time-temperature combination that would result in the destruction of pathogens. However, as EPA was developing the Part 503 rules, the EPA determined that Feachem et al. (1993) did not provide enough detail when developing the time-temperature plots from the raw data (Willis et al., 2006). Additionally, the U.S. Food and Drug Administration established pasteurization requirements for eggnog which provided time and temperature requirements for a slurry (Willis et al., 2006). With the information from Feachem et al. (1993) and the U.S. Food and Drug Administration, EPA created their own Time and Temperature Curve as depicted on Figure 3–1.

The EPA Time and Temperature Curve was selected as the Part 503 rule requirement for Class A biosolids to be more conservative than Feachem et al. (1983).

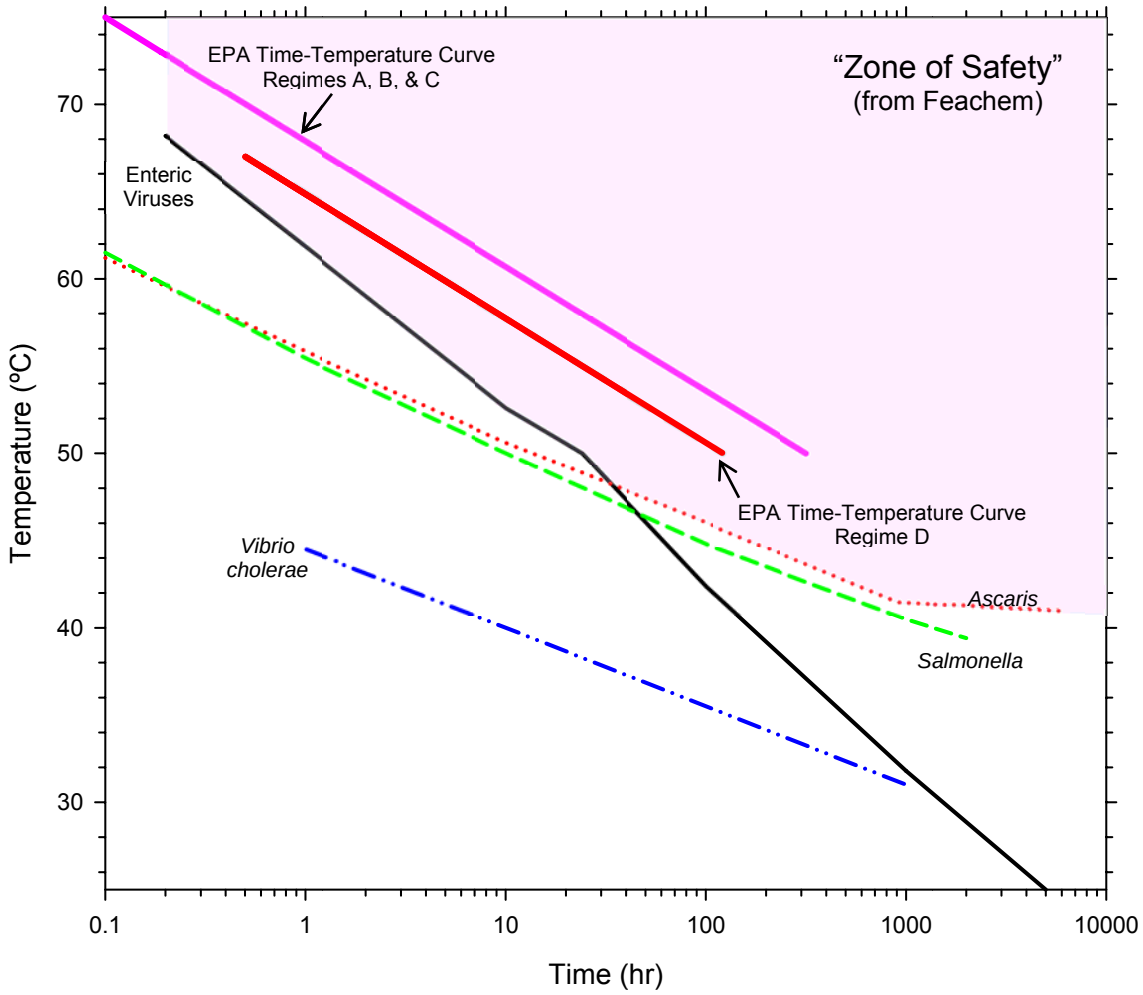

Figure 3–1: EPA Class A time and temperature curve and "Zone of Safety" adapted from Feachem et al. (1993)

The conclusions presented in Chapter 2 described that indicator bacteria destruction may not be complete even when solids treatment processes are designed to meet EPA Time and Temperature requirements. At full-scale treatment plants designed to meet Class A biosolids

requirements, FC and *E. coli* densities were observed to suddenly increase after centrifugal dewatering to levels in excess of the Class A biosolids requirement. This resuscitation of VNC bacteria hypothesis was further substantiated by qPCR data that predicted the quantity of VNC bacteria.

3.2 Research Needs

When the EPA Time and Temperature Curve was created, the idea of a non-culturable bacteria was not included. It was assumed that if a sample had non-detectable densities of FC or *E.coli*, no viable organisms were present. Because SCMs have been observed to undercount FC and *E. coli*, alternate techniques need to be investigated to determine if the EPA Time and Temperature Curve is sufficient for pathogen and indicator destruction. Molecular based methods have been demonstrated in Chapter 2 as a good predictor of potentially viable bacteria that do not culture by SCMs. Additional research is needed to determine if the EPA Time and Temperature Curve represents a sufficient amount of thermal treatment to destroy the bacteria and not just put some in a VNC state.

3.3 Research Objectives

A laboratory scale experiment was performed to examine the impact of time and temperature on FC and *E. coli* destruction. This experiment was necessary to understand the impacts of VNC bacteria on the EPA Time and Temperature Curve requirements for Class A biosolids. To determine the impacts of VNC bacteria on time and temperature requirements, three thermostatically controlled bench-top digesters were operated by Chris Wilson and John Novak at Virginia Tech. These reactors were first inoculated and fed weekly with District of Columbia Water and Sewer Authority (DCWASA) Blue Plains (BP) Advanced Wastewater Treatment Facility sludge over the course of 17 weeks prior to this experiment to establish a stable bacterial

consortium. *E. coli* were enumerated at prescribed times by both SCM and qPCR to determine a time and temperature relationship that evaluated the destruction of VNC bacteria. Data was collected from these reactors over time for evaluation by SCM and qPCR. *E. coli* data was compared to Class A biosolids requirements to determine if the EPA Time and Temperature Curve adequately accounts for VNC bacteria as determined by qPCR.

3.4 Methods and Procedure

The following methods and procedures were utilized in this experiment.

3.4.1 Batch Reactor Design

Three high-density polyethylene batch fermentation reactors supplied by Hobby Beverage Equipment Company (Temecula, California) were utilized for this study. The nominal volume of each vessel was 6.5 gallons (25 liters). Each reactor operated with an active volume of 22.5 liters. A threaded stainless steel thermometer, supplied by Hobby Beverage Equipment Company, was installed into each reactor. Temperature control was achieved by circulating heated water through an external jacket (0.5-inch I.D. vinyl tubing wrap). Reactors were operated at 49°C, 53°C, and 57.5°C (± 0.2) throughout this study.

At startup, 17 weeks prior to this experiment, the three digesters were seeded with approximately 15 liters of mesophilic anaerobic digested sludge from Pepper's Ferry Regional Wastewater Treatment Facility (Radford, Virginia). After approximately 15 days of acclimation to the desired digestion temperature, daily feeding in the absence of wastage was used to bring the digester contents up to its final operating volume of 22.5 liters. Each reactor was fed with a 1:1 ratio of primary and secondary solids from BP sludge, measured on a mass basis, and diluted to approximately 3% solids. The reactor was fed 1.5 liters of blended sludge using a draw-fill procedure to achieve the desired SRT. After 15-weeks of inoculations with BP sludge, the three

reactors were starved for 2 weeks to reduce the FC density to non-detect levels. The reactors were then inoculated with sludge provided by Pepper's Ferry Regional Wastewater Treatment Facility (Radford, Virginia). No further feeding or wasting was performed during this experiment.

3.4.2 Reactor Sampling

Samples of reactor contents were collected at 0, 1, 3, 6, 12, 24, and 48 hours, and 7, 14, 21, and 28 days. No additional inoculums were added to the reactors during this experiment. Samples were processed for MPN analysis using Method 1680 (Section 3.4.3) and DNA analysis (Section 3.4.5). Culturing activities for the 0, 1, 3, 6, 12, 24, and 48 hour samples were performed at Virginia Tech by Bucknell University. Culture samples collected on days 7, 14, 21, and 28 were processed at Bucknell University. DNA samples were collected on days 14, 19, 28, and 34 and analyzed at Bucknell University.

3.4.3 Multiple Tube Fermentation Culturing Technique

Samples were shipped overnight on ice to preserve bacterial activity in accordance with SM 9060B (Eaton, 1995).

3.4.3.1 Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B. Cake samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). For liquid samples, 300 mL of sludge were homogenized in a sterile blender for 2 minutes (EPA Method 1680). Serial dilutions (1:10) were prepared for each sample. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media. Five replicate tubes were inoculated for each dilution. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath.

At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive Lauryl tryptose broth (LTB) media, the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under ultraviolet-B (UVB) lighting.

3.4.3.2 Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2006). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

3.4.3.3 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

3.4.4 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

3.4.5 Enumeration of *E. coli* using Molecular Methods

Samples were collected in triplicate, and bacteria were quantified by molecular methods using the following methodology:

1. Collection, preparation, and storage of biosolid samples in triplicate
2. Extraction and purification of DNA from triplicate samples
3. Quantification of total DNA extracted
4. Enumeration of *E. coli* using qPCR
5. Comparison of DNA results to traditional culturing methods

Because this methodology uses DNA to directly quantify *E. coli*, the culturability of the target bacteria does not affect quantification.

3.4.5.1 Biomass Sampling for Molecular Analysis

Samples were shipped overnight on ice to preserve bacterial activity in accordance with SM 9060B (Eaton, 1995). The total solids of the biomass was determined according to SM 2540D (Eaton, 1995). Liquid sludge samples were centrifuged at 14,000 *xg* for 5 minutes to remove suspended naked DNA. Approximately 200 mg of homogenized, centrifuged pellet or 100 mg of homogenized, dewatered cake were prepared in triplicate for DNA extraction. This mass of biosolids was weighed into a Lysing Matrix E tube (QBIOgene, Carlsbad, CA) and stored at -80ºC until DNA extraction was performed.

3.4.5.2 DNA Extraction from Biosolids

This DNA extraction protocol was developed from Garbor et al. (2003) with some modifications as described in detail herein. Biosolids were weighed directly into a Lysing Matrix E tube (QBIOgene, Carlsbad, CA) as described in Section 3.4.5.1. Biomass was homogenized with 750

µL sterile lysis buffer (100 mM Tris-HCl, 100 mM Sodium EDTA, 1.5 M NaCl, 1% CTAB, pH 8) in a FastPrep® Instrument (QBIOgene, Carlsbad, CA) for 30 seconds at a speed of 5.5. A 5 µL aliquot of 20 mg/mL protease K was added to the Matrix E tube and incubated at 55°C for 30 minutes. A 200 µL aliquot of sterile 20% sodium dodecylsulfate (SDS) was added to the Matrix E tube and incubated at 65°C for 2 hours. The tube was hand-shook every 30 min. The Matrix E tube was centrifuged at 14,000 *xg* for 10 minutes, and the supernatant was removed for further extraction. This extraction method was repeated two more times by adding 500 µL of lysis buffer to the pellet and homogenizing in the FastPrep® Instrument for 30 seconds at a speed of 5.5 as indicated previously. The homogenized tube was incubated at 65°C for 10 minutes during each re-extraction. The suspension was centrifuged at 14,000 *xg* for 10 minutes to pelletize the solids. The supernatant was removed and combined with the previous extracts.

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatant. The tube was mixed by inversion and incubated for 10 minutes at room temperature. After incubation, the tube was centrifuge at 10,000 *xg* for 10 minutes at 4°C. The organic phase was removed into a new tube. The phenol/chloroform/isoamyl alcohol addition, mixing, incubation, centrifugation, and removal were repeated once and combined with the first organic phase extraction. An equal volume of chloroform was added to the combined organic phase extract. The tube was mixed by inversion and incubated for 10 minutes at room temperature. After incubation, the tube was centrifuged at 10,000 *xg* for 10 minutes at 4°C. The organic phase was removed and wasted. Next, 0.6 volumes (compared to the volume of organic phase wasted) of 2-propanol (-20°C) was added to the pellet and incubated overnight at 4°C. After incubation, the tube was centrifuged at 16,000 xg for 10 minutes at 4°C. Supernatant was decanted and the pellet was washed with 500 µL of 70% ethanol. Next, the tube was centrifuged at 10,000 *xg*; and the supernatant was removed. The pellet was air dried for 15 minutes and resuspended with 200 µL of TE buffer (10 mM Tris-Cl, 1 mM Sodium EDTA, pH 7.5). Humic substances were removed

with a Promega Wizard® Genomic DNA Purification Kit (Madison, WI). Extracted DNA was stored at -80°C until DNA quantification and PCR could be performed.

3.4.5.3 Total DNA Quantification

Total genomic DNA was quantified using a fluorescence-based method that combines PicoGreen® dsDNA Quantitation Reagent (Molecular Probe, Eugene, OR) in equal proportion with the sample DNA. A Turner TBS-380 Mini-Fluorometer (Turner BioSystems Inc., Sunnyvale, CA) was used to measure fluorescence response. Known concentrations of Calf Thymus DNA (Sigma Aldrich Co., St. Louis, MO) were prepared with PicoGreen® to determine the concentration of genomic DNA.

3.4.5.4 E. coli Quantification using Real-Time PCR

E. coli was quantified using the qPCR technique cited by Chen et al. (2006). In brief, since the *gad*AB gene, which encodes glutamate decarboxylase, has been shown to have sensitivity and specificity to *E. coli* (Gabor et al., 2003; McDaniels et al.,1996; Smith et al., 1992), qqPCR primers were constructed to amplify the *gad*AB sequence. The PrimerQuest^{SM} software provided by Integrated DNA Technologies (Coralville, IA) was used to design the forward primer (5'-GCG TTG CGT AAA TAT GGT TGC CGA-3') and reverse primer (5'-CGT CAC AGG CTT CAA TCA TGC GTT-3') sequences (Chen et al., 2006), which yield a 305 bp PCR product. These primers were analyzed against BLAST to confirm their specificity to *E. coli*. Triplicate PCR reactions with 12.5 µL of Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA), 0.5 µM of each primer, 30 nM of reference dye (ROX), and 10 µL of 10 ng sample DNA, all diluted to a final volume of 25 µL with DNase/RNase free water, were quantified with the Stratagene MX3005P qqPCR system (La Jolla, CA) (Chen et al., 2006). The PCR program contained a 10-minute initial denaturation at 95ºC, followed by 40 cycles each of denaturation at 95ºC for 30s, annealing at 59ºC for 1min, and extension at 72ºC for 30s (Chen et al., 2006). The program then conducted

a final dissociation curve analysis of the PCR products. Serially diluted *E. coli* DNA ranging from 2 to 7,620 copies was used as an external DNA standard for each qPCR analysis (Chen et al., 2006). *Pseudomonas putida* DNA (10 ng) was included in each standard as background DNA (Chen et al., 2006).

3.5 Results and Discussion

Virginia Tech operated a set of three thermostat thermophilic reactors with a 15 day SRT. The three reactors were operated at 49ºC, 53ºC, and 57.5ºC independently. At 1, 3, 6, 12, 24, and 24 hours and 7, 14, 21, and 28 days, sludge samples were collected for SCM. DNA analysis was performed on samples collected on days 0, 14, 19, 28, and 34.

The TC results, after inoculation with Pepper's Ferry Regional Wastewater Treatment Facility sludge, indicated a rapid decline in concentration at the three thermophilic reactors (Figure 3–2). Within three hours, the TC concentration in the 57.5°C reactor decreased below 1,000 MPN/g DS. Although the Class A biosolids requirements do not regulate TC, the 1,000 MPN/g DS provides a point of reference for discussion. The TC concentration in the 53°C reactor decreased below 1,000 MPN/g DS after 6 hours. The 49°C reactor contained TC at levels above 1,000 MPN/g DS until 24 hours after inoculation. The time and temperature for TC indicates that longer time requirements are needed for reactors operating at lower temperatures which is consistent with the results from Feachem et al. (1983).

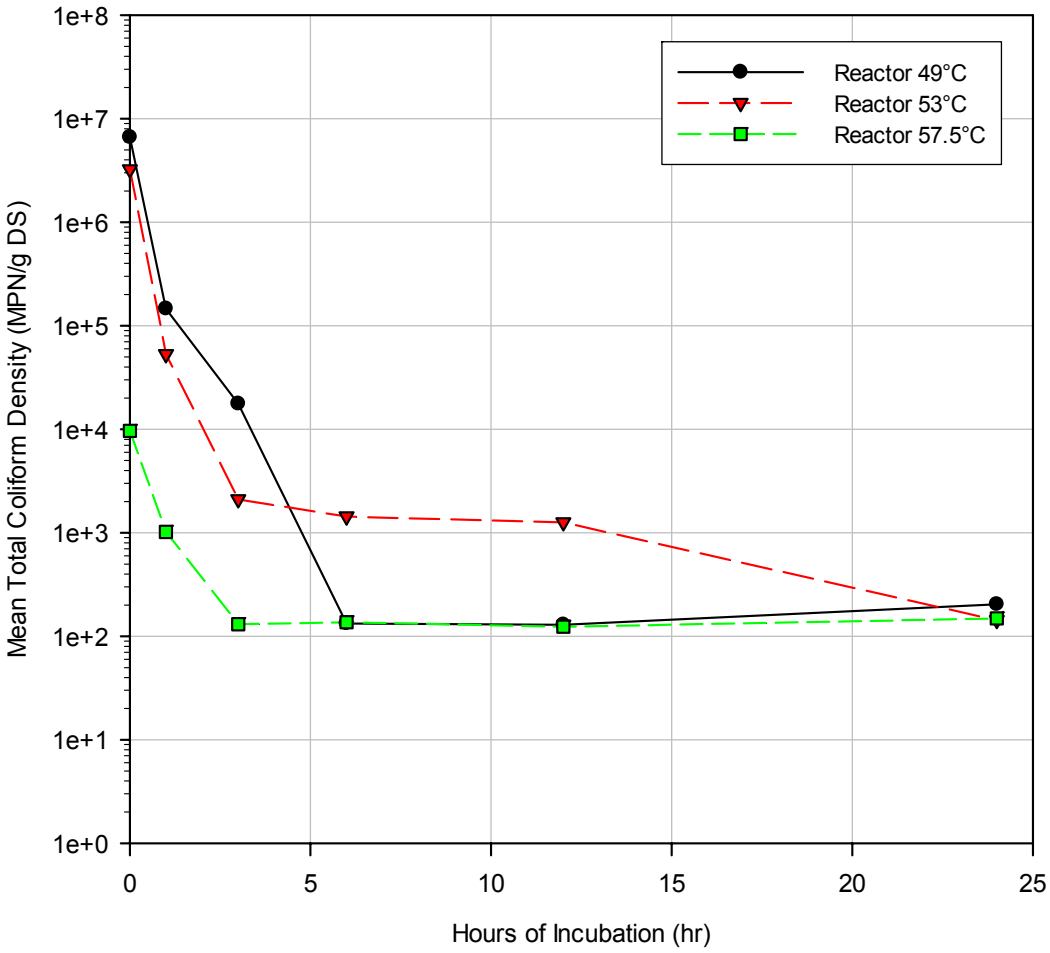

Figure 3–2: Total coliforms densities measured by SCM during batch digestion at different temperatures

The FC results, presented as Figure 3–3, indicated a similar decreasing trend discussed for the TC results. However, the FC values after 6 hours of incubation for all reactor temperatures are an order of magnitude lower than the TC values. Since the Part 503 rule for Class A biosolids applies to FC, screening of the FC data against the 1,000 MPN/g DS requirement is meaningful. Figure 3–3 presents the FC data in reference to the Class A biosolids requirement. FC levels below 1,000 MPN/g DS were observed after 0.5 hours for the 57.5°C reactor, 1 hour for the 53°C reactor, and 4 hours for the 49°C reactors.

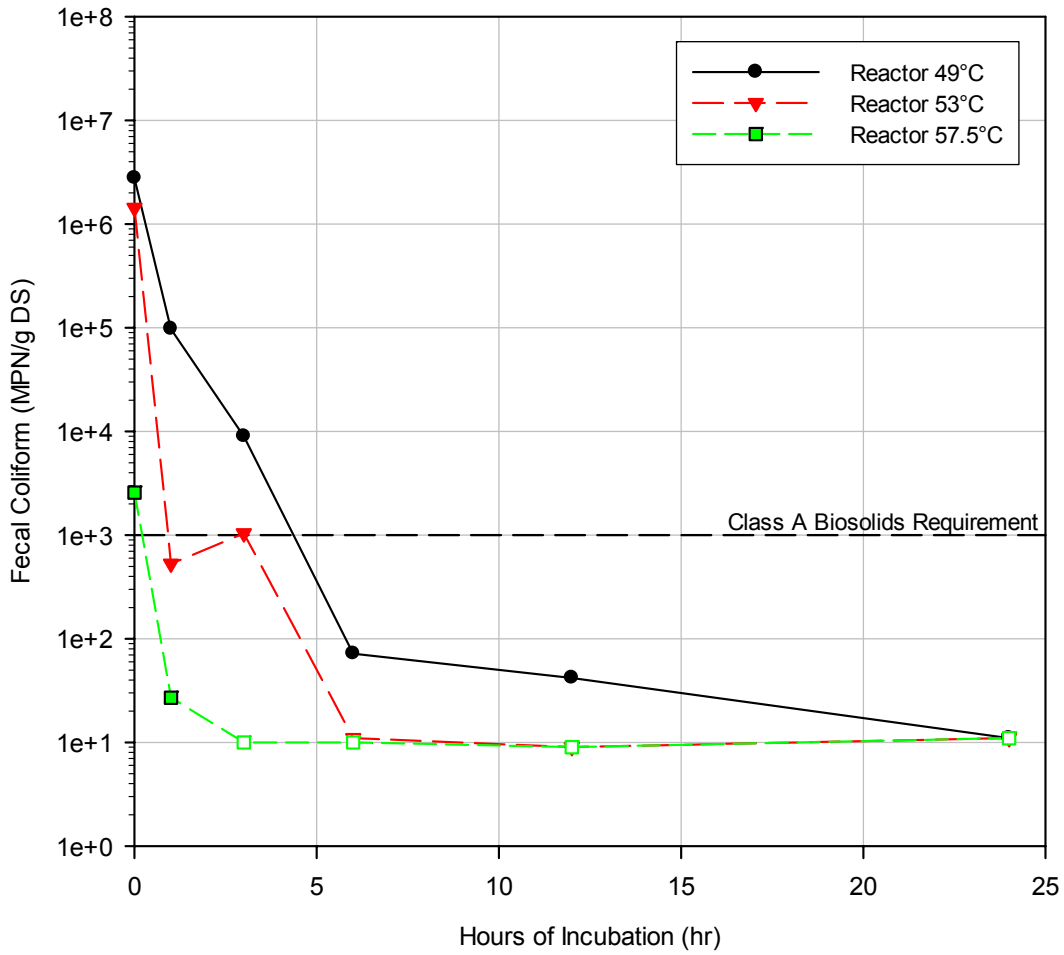

Figure 3–3: Fecal coliforms densities measured by SCM during batch digestion at different temperatures (open symbols indicate non-detect value)

E. coli data collected for the thermally treated sludge is presented in Figure 3–4. For the three reactor temperatures, the *E. coli* inactivation trends were observed to be similar to the FC trends. *E. coli* levels below 1,000 MPN/g DS were observed after 0.5 hours for the 57.5°C reactor, 1 hour for the 53°C reactor, and 4 hours for the 49°C reactors.

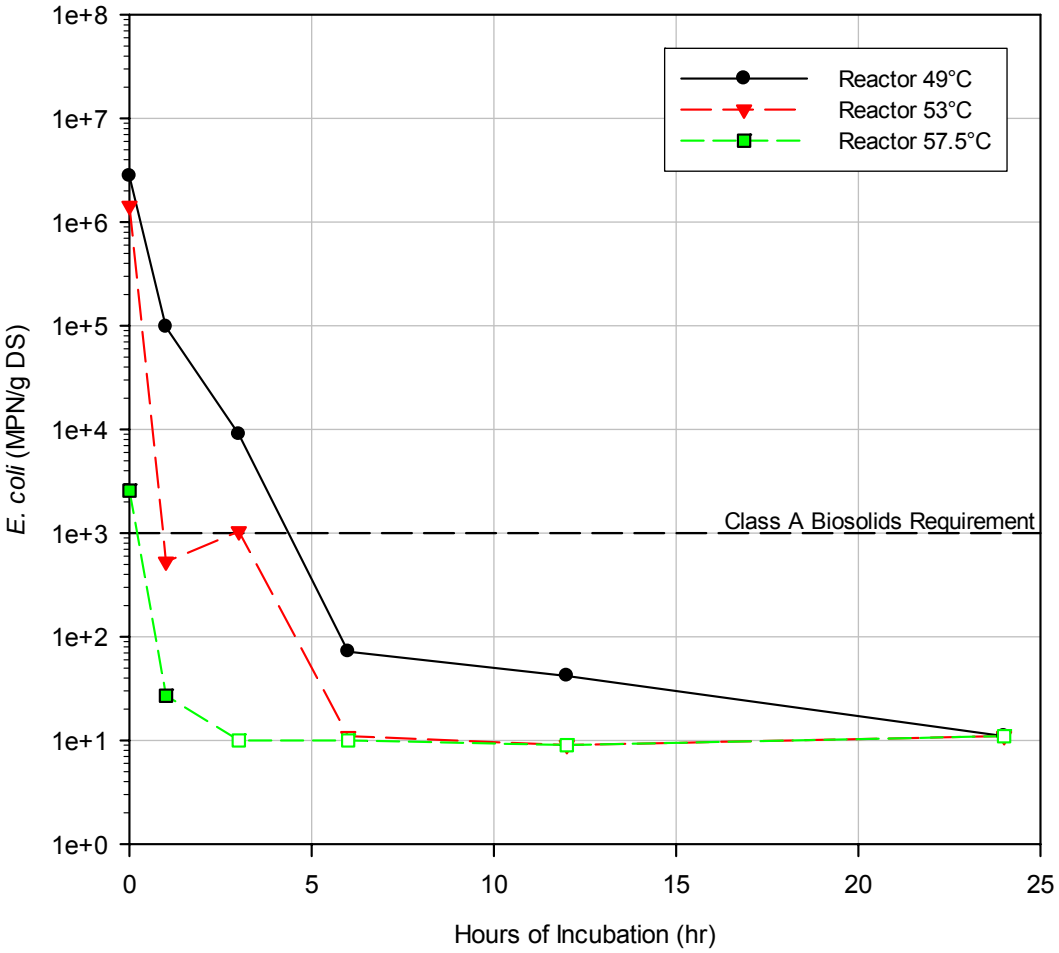

Figure 3–4: *E. coli* densities measured by SCM during batch digestion at different temperatures (open symbols indicate non-detect value)

E. coli were also quantified using qPCR on days 0, 14, 19, 28, and 35 (Figure 3–5). DNA analysis of *E. coli* indicated a different observation compared to the SCM data presented in Figure 3–4. *E. coli* concentrations decreased below Class A biosolids requirements (10^3 MPN/g DS) by SCM within 6 hours at all reactor temperatures. The qPCR data indicated that the *E. coli* density only decreased from 10^8 to 10^6 MPN/g DS after 14 days for all temperatures, and remained near that level for the next 20 days.

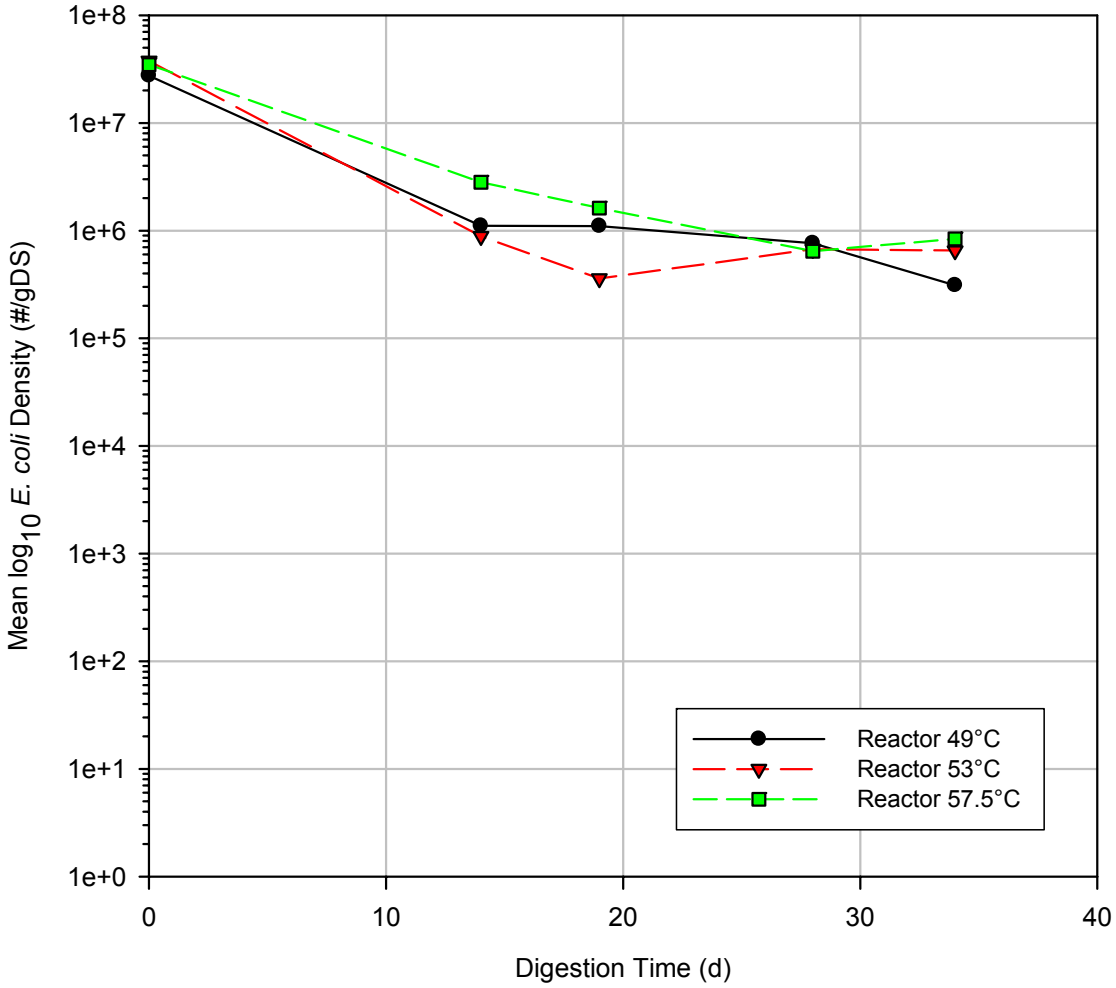

Figure 3–5: *E. coli* densities measured by qPCR during batch digestion at different temperatures.

The higher *E. coli* density determined by qPCR indicates that SCM potentially underestimate the density of viable *E. coli*. The DNA numbers need to be qualified however, since qPCR quantifies both live and dead cells; it is not clear how many dead cells are being enumerated by qPCR. However, the previous results in Chapter 2 indicated that qPCR was a good predictor for bacteria population SI and regrowth. Therefore, even though an overestimation may occur because of dead cells, qPCR is effective in predicting the appropriate order of magnitude for *E. coli* density.

In order to understand the implications of the SCM and qPCR data collected from the thermally controlled laboratory reactors, the data was incorporated into the context of the EPA Time and Temperature requirements. Figure 3–6 presents the time and temperature relationship at which non-detect results for *E. coli* density were observed. This figure also presents the qPCR predicted *E. coli* density for comparison with SCM results.

The solid green circles identified on Figure 3–6 depict the SCM results for each reactor temperature in relation to the EPA Time and Temperature Curve. Each solid green circle references the time at which the Class A biosolids limit was achieved for the given reactor temperature. The reactors time-temperature relationship based on culturing method indicates that the Zone of Safety observed by Feachem et al. (1983) is a conservative limit for wastewater treatment plant designers. Likewise, the EPA Time and Temperature Curve is more conservative than the results from this experiment.

However, this is not true when culture independent results are used. The *E. coli* population, when analyzed by qPCR, is plotted on Figure 3–6 as solid red squares. The solid red squares represent the *E. coli* detections because the *E. coli* density never decreased below 1,000 MPN/g DS, the Class A biosolids requirement. The difference between SCM and molecular based quantification of *E. coli* results in uncertainty as to whether digested sludge has achieved Class A biosolids classification.

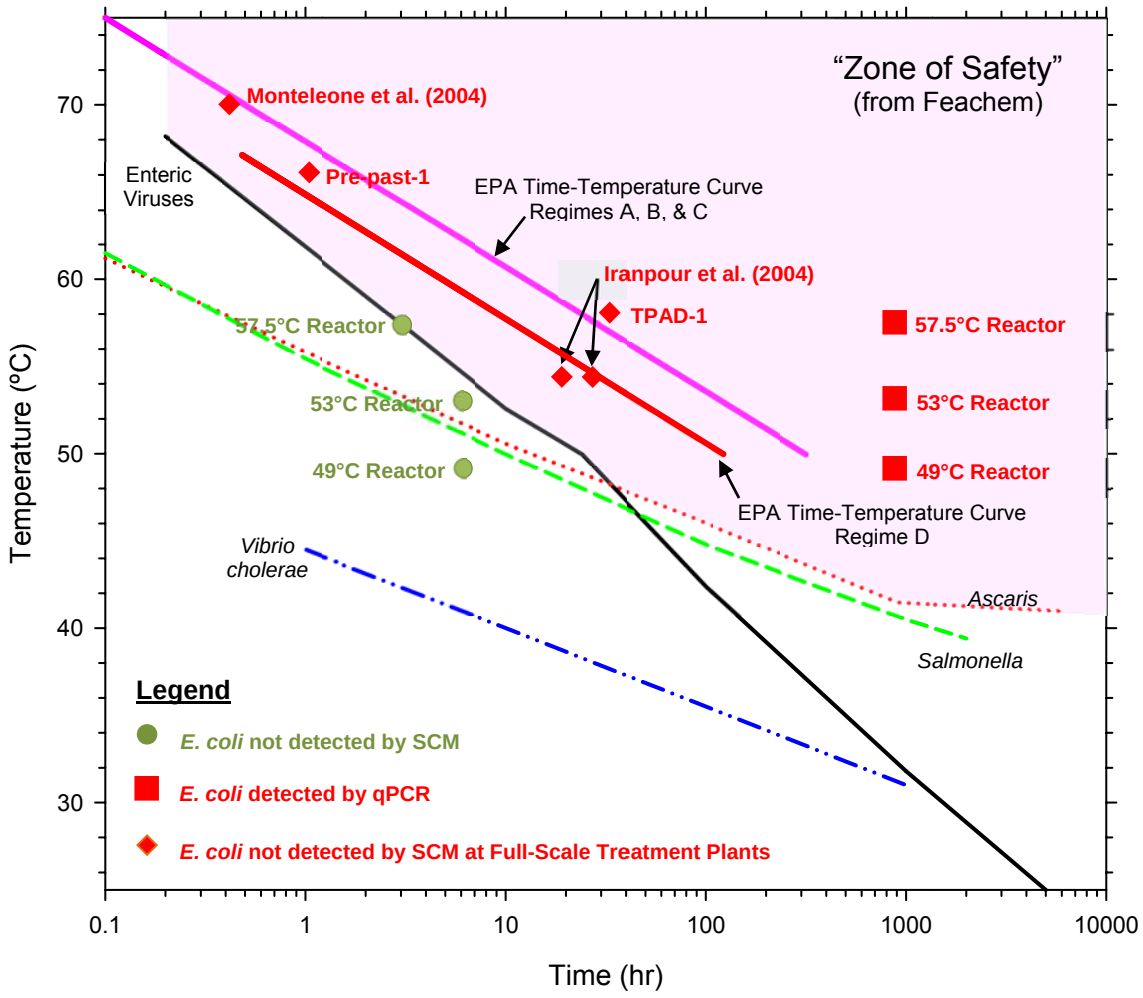

Figure 3–6: Results for *E. coli* density as determined by SCM and qPCR and compared to the EPA Class A time-temperature curve (Solid green circles symbolize batch digestion results at each reactor temperature by SCM; Solid red squares symbolize batch digestion results at each reactor temperature by qPCR at Day 34; Solid red diamonds symbolize full-scale anaerobic digestion treatment plant configurations designed to achieve Class A biosolids requirements)

In addition to the laboratory time-temperature results, the full-scale results discussed in Chapter 2 are also included in Figure 3–6. The solid red diamonds on Figure 3–6 provide time and temperate relationship used at the Pre-past-1 and TPAD-1 full-scale treatment plants discussed in Sections 2.5.1 and 2.5.2. The operating time and temperature are more conservative than the

EPA Time and Temperature Curve D, which is the curve that applies in this case. Nevertheless, the SI and regrowth phenomena were identified at both Pre-past-1 and TPAD-1. At TPAD-1, the SI and regrowth phenomena did cause *E. coli* density to increase past 1,000 MPN/g DS by SCM. Enumeration by qPCR was not performed on TPAD-1 biosolids. However, at Pre-past-1, the SI and regrowth phenomena did cause the *E. coli* density to increase beyond the Class A biosolids requirements after HSC and storage. As determined by SCM, non-detect results were observed after pasteurization and mesophilic digestion but not after centrifugation and storage. The SI and regrowth phenomena caused the *E. coli* density to increase to 10^6 MPN/g DS after centrifugation and storage. *E. coli* density as determined by qPCR adequately predicted the density of *E. coli* after SI. Therefore, the *E. coli* density, when determined by SCM, does not accurately enumerate non-culturable *E. coli*, whereas qPCR effectively predicts the density of *E. coli* after SI and regrowth.

3.6 Research Significance

The results from this work suggest that the EPA Time and Temperature Curve is not adequate for complete inactivation of the indicator bacteria. Instead, some of the thermally treated bacteria are entering a VNC state and are therefore not being enumerated by the SCMs, giving a false indication that no viable indicators are present. As a result, utilities are not meeting Class A FC requirements even though thermophilic digestion meets the time and temperature requirements.

The underestimation of *E. coli* density by SCM and the possible overestimation of *E. coli* density by qPCR yield a significant problem for treatment plant designers and operators attempting to achieve Class A biosolids requirements. SCM do not adequately quantify bacteria present prior to SI and regrowth as determined by qPCR. Therefore, further research needs to be performed to determine if SCM can be enhanced to quantify non-culturable *E. coli* during anaerobic thermophilic digestion.

Higgins et al. (2006) proposed three possibilities for the difference between SCM and qPCR quantification of *E. coli*:

1. A portion of the *E. coli* DNA measured by qPCR stems from undegraded, free DNA that remains in solution after cell lysis.

2. A fraction of the *E. coil* DNA quantified by qPCR is due to dead or nonviable cells that have not yet lysed.

3. Part of the *E. coli* DNA enumerated by qPCR is due to VNC *E. coli* that are still viable.

Although Higgins et al. (2006) does not resolve which phenomena is occurring, the discussion of the literature supports their three possibilities; and the results presented in Chapter 2 and Chapter 3 support the third possibility. In this thesis, the third possibility will be examined to determine if non-culturable bacteria can be resuscitated through enhancements to SCMs. The following report sections will investigate various methods to enhance SCMs such that *E. coli* density will be enumerated at a magnitude similar to *E. coli* density determined by qPCR.

3.7 Conclusions

The FC and *E. coli* results presented for the time-temperature experiment yielded the following observations:

- The EPA Time and Temperature Curve is a conservative design standard that results in enumerations of FC and *E. coli* below the Class A requirements, when determined by SCM.

- Molecular techniques do not support the conclusion that *E. coli* are destroyed when time-temperature design standards are achieved.

- The “Zone of Safety,” described by Feachem et al. (1983), do not adequately account for non-culturable bacteria that may still be viable.

- Full-scale anaerobic thermophilic wastewater treatment plants have the potential to produce biosolids that do not meet EPA Class A biosolids requirements when *E. coli* density is determined by qPCR instead of SCM even though they meet the EPA Time and Temperature requirement.

Culturing data and DNA data collected for *E. coli* density do not provide consistent results during anaerobic thermophilic digestion as hypothesized. The non-detect *E. coli* results, as determined by SCM and observed during anaerobic thermophilic digestion, do not account for the non-culturable *E. coli* density characterized by qPCR data. Research needs to be performed to determine if viable non-culturable *E. coli* are responsible for the discrepancy SCM and qPCR *E. coli* densities.

Chapter 4: Investigation of Peroxide Scavengers to Enhance the Recovery of Thermally Treated Indicator Bacteria by Standard Culturing Methods

4.1 Introduction

Bacteria, such as *E.* coli, are capable of entering a reversible, VNC state when subjected to environmental stresses such as heat stress or nutrient starvation. Because the scientific community has yet to define which biochemical parameters are necessary for viable versus dead cells, defining the VNC state is controversial. Typically, however, a cell, which is metabolically active yet unable to experience sustained cellular division that produces a colony that is discernable using standard culturing methods, is considered to be in the VNC state (Oliver, 1993).

VNC bacteria are one of the hypotheses explaining the SI of FC and *E. coli* as discussed in Chapter 2 and Chapter 3. As discussed in Chapter 3, heat stress is one possible cause for the VNC state. Because thermal treatment of sludge causes heat stress on bacteria, the VNC state is a plausible cause for non-culturable bacteria. Fundamentally, if the VNC hypothesis is correct, the reason VNC bacteria are non-culturable with SCMs is that culturing methods are providing false negative results.

Unfortunately, heat stress alone does not describe the mechanistic change required to influence culturability by SCMs indicative of the VNC state. One possible explanation for the VNC state has been discussed by Bloomfield et al. (1998) who suggests that the generation of H_2O_2 compounds is a result of a metabolic imbalance caused by a change in environment. The transfer of thermally treated biosolids from an anaerobic environment to standard culturing media results in significant redox, temperature, and nutrient changes which may promote the generation

of H_2O_2 compounds. These peroxides are produced when stressed VNC cells are transferred into nutrient-rich LB plates at optimal growth and enzymatic activity temperatures. Cellular metabolism causes the rapid production of superoxide and free radicals. Because the cell is not phenotypically adapted to the new environment, detoxification of the superoxides and other free radicals cannot keep up with cellular growth and cell death typically occurs. VNC cells are not capable of degrading peroxides because of their diminished metabolic state. The metabolic pathways that typically exist in the cell are not functioning because of the stressed state. Normal, viable cells would not have difficulty detoxifying peroxides in their environment.

Research indicates that peroxide-degrading compounds such as catalase, sodium pyruvate, and α-ketoglutaric acid are capable of resuscitating bacteria in the stressed state or preventing their death when placed in rich media (Mizunoe et al., 1999). In one experiment, *E. coli* O157:H- strain E32511/HSC was subjected to low-temperature stress (4°C incubation for 21 days) to cause cells to enter the VNC state. LB agar plates enhanced with enzymatic (i.e., catalase) and nonenzymatic (i.e., sodium pyruvate, α-ketoglutaric acid) peroxide degrading compounds were able to restore the culturability of stressed cells after incubation at 25°C for 48-hours. Plates without peroxide-degrading compounds were not able to resuscitate VNC bacteria. Culturability typically increased from non-detect to 10^4-10^5 CFU/mL within 48-hours on LB agar plates supplemented with catalase, sodium pyruvate, or α-ketoglutaric acid.

Similar research performed by Czechowicz et al. (1996) found that sodium pyruvate was able to resuscitate *E.* coli O157:H7 strain 933 that was subjected to heat stress (57°C for up to 60 minutes). Czechowicz et al. (1996) identified that plate count agar supplemented with 1% sodium pyruvate was able to increase the resuscitation of heat-stressed *E. coli* by up to 10^3 CFU/mL when compared to the tryptic soy agar control media with heat-stressed *E. coli* inoculation. Plate count agar contained glucose and yeast extract which were not present in the tryptic soy agar. The presence of glucose and yeast extract in the plate count agar in addition to supplementation

with 1% sodium pyruvate may have had a synergistic effect on the resuscitation of heat-stressed *E. coli* cells.

Literature related to the effects of catalase supplementation on the enumeration of *Staphylococcus aureus* and *E. coli* were also reviewed in addition to Mizunoe et al. (1999). In research presented by Flowers et al. (1977), *S. aureus* cells were heat stressed for varying periods of time at 52°C and enumerated on various selective media. Media supplemented with 780 units/plate of bovine catalase were able to resuscitate stressed *S. aureus* by up to 1,100-fold. Collaborative research by Martin et al. (1976) investigated the resuscitation effects of catalase with *E. coli*. Instead of using heat-stressed *E. coli* cells, Martin et al. (1976) studied the effects of catalase on the enumeration of acid injured *E. coli* cells (cells were incubated for 1 hour at 32°C in 300 mM sodium acetate buffer, pH 4.2). The acid injured *E. coli* cells incubated on violet red bile agar supplemented with 0.1 mL of a 0.02% catalase solution produced a 10^2 CFU/mL increase compared to the control vile red bile agar plates.

Based on the research of Mizunoe et al. (1999), it was identified that 0.1% sodium pyruvate and 1000 units/plate of catalase were optimal for *E. coli* resuscitation. Mizunoe et al. (1999) found that the 0.1% concentration of sodium pyruvate was capable of a greater resuscitation rate when compared to the 1% sodium pyruvate concentration suggested by Czechowicz et al. (1996). The effects of the α-ketoglutaric acid supplement on *E. coli* resuscitation were not as pronounced; however, the 0.1% α-ketoglutaric acid supplemented medium appeared to have the highest plate counts after resuscitation (Mizunoe et al., 1999).

Mizunoe et al. (1999) performed additional experimentation to determine if the supplements were merely serving as a nutrient source or if their H_2O_2 degrading properties were utilized during cellular resuscitation. The resuscitation of *E. coli* on 0.1% sodium pyruvate supplemented media was compared to 0.1% acetic acid supplemented media; acetate is a closely related metabolite

for sodium pyruvate (Mizunoe et al., 1999). Additionally, plates with catalase supplemented LB agar were compared to plates with heat-denatured catalase supplement. In both instances, the metabolizable substrate did not enhance the culturability of *E. coli*. Therefore, the resuscitation of cells was attributable to the destruction of H_2O_2 compounds.

Because of the peroxide-degrading properties of sodium pyruvate and catalase, the supplemented culture media in the Mizunoe et al. (1999) experiments were able to resuscitate stressed VNC cells. The peroxide-degrading compounds were fundamental in protecting stressed cells against oxidative stresses caused by resuscitation in nutrient-rich media at optimal growth temperatures.

4.2 Research Needs

The VNC state is a plausible explanation for diminished culturability after thermal treatment. As observed previously, qPCR data suggests that the density of viable *E. coli* is significantly higher than the concentration determined by SCM. Research needs to be performed to determine if enhancements to SCMs can be performed to resuscitate VNC bacteria. Specifically, peroxide degrading compounds have been identified to resuscitate VNC bacteria in pure cultures. Research needs to be performed to determine if peroxide degrading compounds can resuscitate FC and *E. coli* in wastewater biosolids.

4.3 Research Objective

The objectives of this experiment were to determine if improvements to SCMs will result in improved resuscitation of VNC indicator bacteria in thermally treated biosolids. In this study, the effect of adding peroxide-degrading compounds to the standard culturing media to enhance recovery of *E. coli* in thermally treated sludges was examined. Catalase, sodium pyruvate, and

α-ketoglutaric acid were supplemented to traditional culturing methods for wastewater sludge and cake samples.

Cake and sludge samples were received from the TPAD-1 wastewater facility, which follows the EPA Time and Temperature Curve to achieve Class A biosolids. Sampling of this plant has shown that it experiences SI and regrowth in that the *E. coli* density is below detection after digestion, but after dewatering cake densities are 10^4-10^5/g DS. Cake and sludge samples were cultured using both traditional and enhanced presumptive media during the multiple tube fermentation culturing technique. The enhanced presumptive media investigated whether sodium pyruvate, α-ketoglutaric, or catalase addition to traditional presumptive media yielded higher enumerations of FC and *E. coli*.

The generation and presence of peroxides in wastewater samples is hypothesized to prevent the enumeration of VNC FC and *E. coli* from thermally treated sludges. This research will determine if the addition of peroxide-degrading compounds can effectively resuscitate VNC bacteria such that enhanced SCM adequately account for the SI and regrowth phenomena identified in Class A biosolids.

4.4 Methods and Procedure

Class A sludge and cake were provided by the TPAD-1 wastewater facility. This facility utilizes the EPA time-temperature to achieve Class A biosolids. Samples were shipped on ice by overnight FedEx shipment and were stored at 4°C prior to the start of the experiment which commenced on same day as sample receipt. MPN analysis was performed on the sludge and cake using both traditional and enhanced presumptive media as described herein. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B.

4.4.1 Multiple Tube Fermentation Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Cake samples (30.0 ± 0.1 g) and sludge samples (30.0 ± 0.1 mL) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). Serial dilutions (1:10) were prepared for liquid and solid samples. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media (traditional and enhanced). Five replicate tubes were inoculated for each dilution. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive LTB media (traditional and enhanced), the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under UVB lighting.

4.4.1.1 Traditional Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2006). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

4.4.1.2 Enhanced Presumptive Media

The traditional presumptive media, as described in Section 4.4.1.1, was augmented with either

sodium pyruvate, α-ketoglutaric acid, or catalase for use as the enhanced presumptive media. LTB media augmented with either 0.1% sodium pyruvate [Sigma Aldrich] or 0.1% α-ketoglutaric acid [Sigma Aldrich]) was sterilized in an autoclave at 121°C, 15 psi for 15 minutes. Before sterilization, the pH was adjusted with 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide (NaOH) until a pH of 6.8 was obtained. The volume of acid or base used to adjust the pH of the media was less than 10% of the total volume of solution. Because of the enzymatic quality of catalase, 1000 units/tube catalase, which was filter sterilized using a 0.22 μm sterile filter, was aseptically transferred into sterile traditional presumptive media.

4.4.1.3 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

4.4.2 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

4.5 Results and Discussion

Sludge and cake samples were collected from TPAD-1 and cultured using standard LTB media and LTB media supplemented with either catalase, sodium pyruvate, or α-ketoglutaric acid. The media supplements were added because they have been shown to degrade peroxide compounds (Bloomfield et al., 1998; Mizunoe et al., 1999). Based on the literature from Bloomfield et al.

(1998), it is proposed that peroxide and other oxidative compounds are released when cells are stressed. The following discussion evaluates whether the addition of peroxide scavengers to standard culturing media improves the culturability of TC, FC, and *E. coli*.

Coliform and *E. coli* densities for the sludge samples collected at TPAD-1 were cultured on standard and enhanced LTB media and the culturing data are presented as Figure 4–1. Sludge samples cultured with traditional media (control) were observed to contain non-detect values for TC, FC, and *E. coli*. Likewise, neither catalase or α-ketoglutaric acid amended LTB media were able to resuscitate target bacteria; the densities for TC, FC, and *E. coli* were non-detect. Only the sludge sample cultured on sodium pyruvate amended LTB media was observed to have TC and FC densities greater than the quantification limit of 10^2 MPN/g DS. The density of TC increased to greater than 10^4 MPN/g DS while the FC density increased to 10^3 MPN/g DS. Confirmation sampling using EC-MUG media did not detect *E. coli* above the quantification limit of 10^2 MPN/g DS.

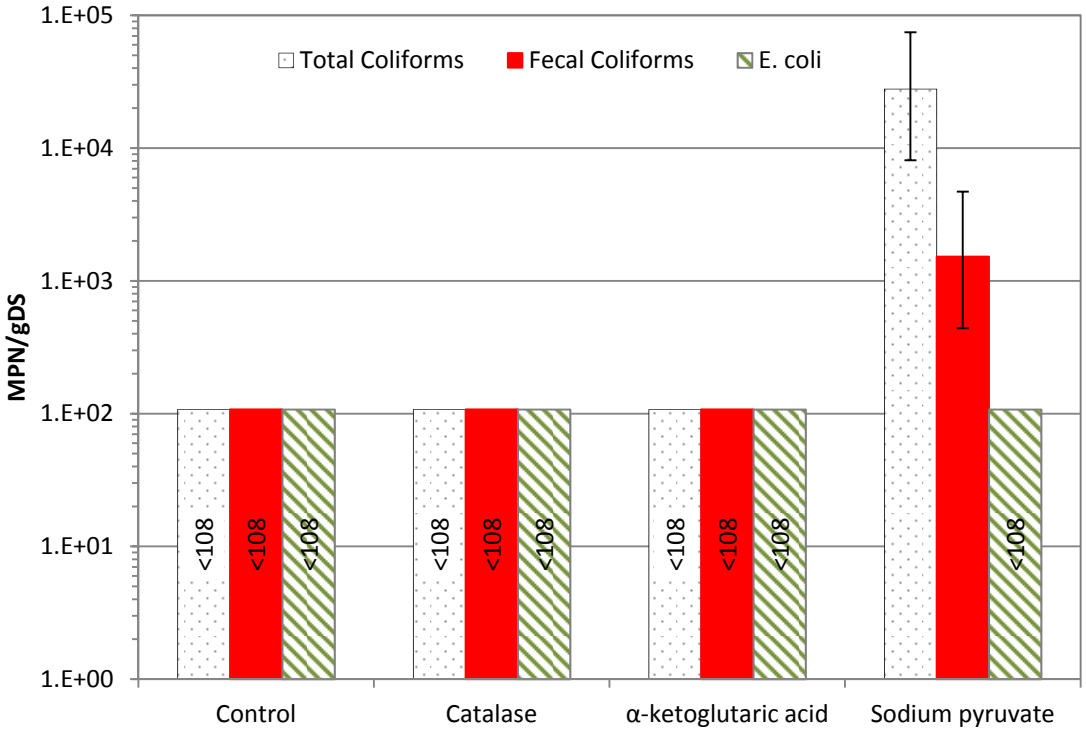

Figure 4–1: Culture results for thermophilic treated sludge from TPAD-1 and cultured on traditional and enhanced media (Error bars represent upper and lower 95% confidence intervals)

Coliform and *E. coli* densities for the cake samples collected at TPAD-1 were cultured on standard and enhanced LTB media and the culturing data are presented as Figure 4–2. Cake samples cultured with traditional media (control) were observed to contain TC, FC, and *E. coli* densities of nearly 5 x 10^3 MPN/g DS. The addition of catalase to the LTB media yielded densities for TC, FC, and *E. coli* that were comparable to the control cake density of 5 x 10^3 MPN/g DS. LTB media amended with α-ketoglutaric acid yielded the lowest TC, FC, and *E. coli* densities (3 x 10^3 MPN/g DS). Only the cake sample cultured on sodium pyruvate amended LTB media resulted in an increase in TC, FC, and *E. coli* densities greater than the control cake. The TC density was 3 x 10^4 MPN/g DS while the FC and *E. coli* densities were 10^4 MPN/g DS.

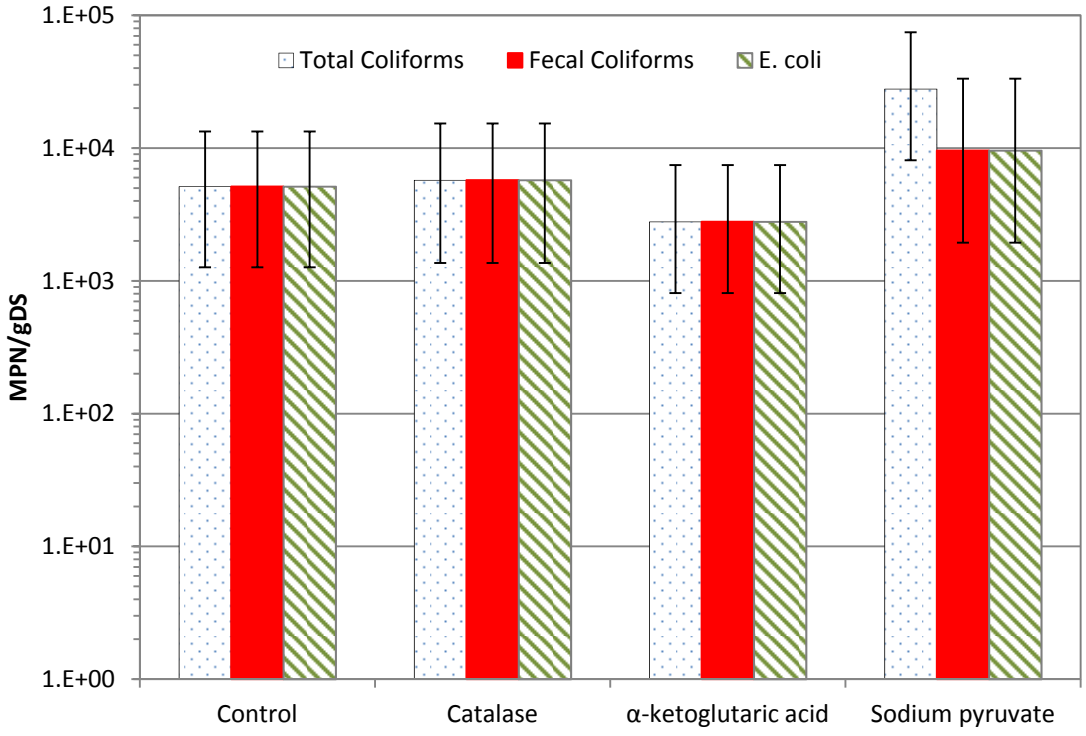

Figure 4–2: Culturing results for thermophilic treated cake from TPAD-1 dewatered by HSC and cultured on traditional and enhanced media (error bars represent upper and lower 95% confidence intervals)

As depicted in Figure 4–1 and Figure 4–2, the resuscitation of bacteria using enhanced presumptive media with catalase and α-ketoglutaric acid had a minimal effect on the culturability of *E. coli* in TPAD-1 wastewater samples. Sludge samples cultured in sodium pyruvate enhanced LTB media yielded the highest densities of bacteria; however, only TC and FC experienced regrowth, not *E. coli*. The TC density from the sodium pyruvate supplemented LTB media was comparable for both cake and sludge samples. Unlike the sludge samples, cake samples yielded quantifiable densities of *E. coli*. Marginal difference in TC, FC, and *E. coli* density were observed between the control cake and cake cultured in supplemented LTB media. Additionally, no TC, FC, or *E. coli* were cultured from the sludge using the EPA Method 1680 without any

enhancements (control sample). The control cake sample had TC, FC, and *E. coli* densities of 5 x 10^3 MPN/g DS.

Based on these results, sodium pyruvate supplemented LTB media was the only enhanced media to produce increases in target bacteria culturability. This observation that sodium pyruvate supplemented media resuscitated target bacteria may indicate that peroxide concentrations in solution inhibited bacterial growth as hypothesized by Mizunoe et al. (1999). Nevertheless, only TC and FC, not *E. coli*, was shown to resuscitate with sodium pyruvate enhanced presumptive media. This research did not identify a reason to explain why *E. coli* densities were not comparable to FC densities.

4.6 Research Significance

The application of the media enhancements suggested in Mizunoe et al. (1999) on wastewater culturing techniques did not have a comparable effect to the laboratory, pure culture experimentation performed by Mizunoe et al. (1999). Several differences in the execution of this experimental method and the Mizunoe et al. (1999) experiments may have caused different outcomes. Because wastewater samples constitute a large consortium of bacteria, the mixed bacterial community may have growth requirements different than the *E. coli* O157 pure culture utilized by Mizunoe et al. (1999). Additionally, Mizunoe et al. (1999) utilized agar plate to culture bacteria, the EPA Method 1680 and SM 9221 methods for wastewater samples utilize liquid media for the multiple tube fermentation technique. The change in culture matrix may have influenced the results. Consequently, the resuscitation of *E. coli* utilizing enhanced media, as described by this experiment and Mizunoe et al. (1999), should be investigated further to determine if the culturing matrix influences resuscitation. Direct plating of wastewater samples on enhanced nutrient agar plates should be considered in future research efforts.

4.7 Conclusions

Data collected during this experiment indicated the following observations:

- SI was observed in cake samples after centrifuge dewatering that were greater than the Class A requirements for FC density.

- The sludge sample cultured in sodium pyruvate supplemented media yielded a TC density greater than that of the control cake sample.

- The density of FC from the sludge sample cultured in sodium pyruvate supplemented media was comparable to FC densities identified in the control cake samples.

- *E. coli* concentrations were not increased by the three media supplements for both cake and sludge samples.

The resuscitation effects promoted by the detoxification of peroxide compounds, as presented by Mizunoe et al. (1999), only appeared to be effective in causing the SI of indicator bacteria in sludge samples cultured with sodium pyruvate addition. This experiment identified that TC and FC density increased by 1-2 orders of magnitude from non-detect to 10^4 MPN/g DS and 10^3 MPN/g DS, respectively. Elevated densities of TC, FC, and *E. coli* in cake samples were also observed in sodium pyruvate supplemented media compared to the control cake. The addition of catalase and α-ketoglutaric acid to presumptive media does not appear to encourage the resuscitation if VBC bacteria as described by Mizunoe et al. (1999).

Chapter 5: Effects of Autoinducer and *E. coli* Cell-Free Supernatant Addition to Standard Culturing Media and of Cell Washing Technique to Determine whether Quorum Sensing Molecules Promote the Resuscitation of VNC Bacteria

5.1 Introduction

If the non-culturable hypothesis is correct, several possible reasons could explain why bacteria transition from a non-culturable to a culturable state after centrifuge dewatering such as:

- Removal of inhibiting substances;
- Release of autoinducers or quorum sensing molecules;
- Release of other substances that promote growth;
- Changes in environmental conditions.

This experiment will expressly study two possible mechanisms for SI:

1. The release of autoinducers.
2. The removal of inhibitory substances.

Although the current understanding of autoinducers is not complete, research has identified several different methods bacteria communicate within species and across species (Reading et al., 2006). Additionally, several bacteria have been shown to respond to mammalian chemical

signaling molecules (Reading et al., 2006). Many of these signaling molecules have been identified to promote or reduce bacterial growth. The growth effects caused by signaling molecules may allow researchers to control the growth of bacteria in laboratory methods using these signaling molecules. In one regard, the culturability of bacteria is important to correctly classify biosolids. From a culturability standpoint, determining a microbiologic technique which causes resuscitation of bacteria will provide accurate quantification of pathogenic indicator bacteria, *E. coli*. On the other hand, because *E. coli* can be pathogenic, the resuscitation of specifically Enterohemorrhagic *E. coli* (EHEC) could require changes to regulations for the use of biosolids as described by the Part 503 rule. Because current microbiology techniques lack the ability to resuscitate VNC bacteria effectively, the true quantification of indicator bacteria remains biased low, if the non-culturable hypothesis is correct. Low detection capabilities for *E. coli* and other indicator bacteria could potentially pose a direct human health risk for workers handling and utilizing biosolids and for residents of nearby land application.

5.1.1 Cell-to-Cell Signaling Molecules

Prokaryotic bacteria were once considered to live unicellularly, with only stimulation from environmental factors such as the presence of chemicals and physical changes (Reading et al., 2006). This simplistic view of prokaryotic life has been dismissed with the discovery of small “hormone-like” organic molecules called autoinducers, which allow bacteria to communicate with one another (Reading et al., 2006). These autoinducers provide a cell-to-cell signaling system that serves to regulate gene expression based on cell density. Because autoinducers signal for the expression of certain genes based on cell density, autoinducers are considered quorum sensing molecules (Reading et al., 2006).

Early research into quorum sensing molecules first occurred during a study into the regulation of bioluminescence in *Vibrio fischeri* and *Vibrio harveyi* (Reading et al., 2006). Since then, many

more quorum sensing signaling molecules and genes have been identified (Reading et al., 2006). These quorum sensing molecules are divided into three classes. In Gram-negative bacteria, the LuxR/I-type system is utilized for quorum sensing using various AHL molecules. A peptide signaling system (*lux*S/autoinducer-2 [AI-2]) is utilized by Gram-positive bacteria, and an interkingdom signaling system utilizing epinephrine/norepinephrine/ autoinducer-3 (AI-3) has also been identified for cross-species signaling (Reading et al., 2006). The following sections discuss the LuxR/I-type signaling system and the epinephrine/norepinephrine/AI-3 signaling system since there are applicable for Gram-negative *E. coli*.

5.1.1.1 The LuxR/I-type Signaling System

In Gram-negative bacteria, the LuxI-type proteins produce AHL autoinducers which in turn activate LuxR to increase gene expression. *Escherichia coli*, a Gram-negative bacteria, contains a LuxR homolog, SdiA (Wang, 1991), but does not contain a *lux*I gene that is capable of synthesizing AHLs (Swift, 1999; Michael, 2001). The SdiA receptor, according to Houdt et al. (2006), is capable of responding to synthetic AHL molecules. Specifically, Houdt et al. (2006) investigated the use of synthetic N-hexanoyl-L-Homoserine lactone (C6-HSL) and 3-oxo-N-hexanoyl-L-Homoserine lactone (3-oxo-C6-HSL), the most widespread AHLs, in effecting promoter expression in *E. coli* MG1655. In addition to the C6-HSLs, with or without an oxo group in the 3-position, Yao et al. (2006) found that SdiA also recognizes and folds when N-octanoyl-L-Homoserine lactone (C8-HSL) and 3-oxo-N-octanoyl-L-Homoserine lactone (3-oxo-C8-HSL) are present. The folding process is necessary for the *Sdi*A receptor to recognize quorum sensing molecules.

Because SdiA is capable of detecting various AHLs and utilizing these signaling molecules as switches for its folding, the *E. coli* SdiA protein is a LuxR-type quorum sensing molecule that is used to detect the presence of other bacterial species (Yao et al., 2006). When the *sdi*A gene

was encoded on a plasmid and overexpressed in *E. coli*, the *fts*QAZ gene for cell division was activated and further improved when AHL was added (Houdt et al., 2006). This implies that the addition of AHLs may promote the cell division required for regrowth.

5.1.1.2 The Epinephrine/Norepinephrine/AI-3 System and EHEC

In 1982, following an outbreak of an Enterohemorrhagic *E. coli* (EHEC) strain in the United States, the public health community recognized that EHEC was a significant problem within the food industry (WHO, 2005). Unlike most *E. coli*, a bacterium commonly found in the gut of humans and warm-blooded animals, strains of EHEC can cause severe foodborne illnesses (WHO, 2005). EHEC serotype O157:H7 has been identified as one of the most important serotypes with regard to public health concerns (WHO, 2005). In humans, EHEC O157:H7 infection typically results in abdominal cramps and diarrhea but can quickly progress to bloody diarrhea and the life-threatening hemolytic uremic syndrome (HUS) (WHO, 2005). The typical incubation period for EHEC infection is 3-4 days with recovery taking 10 days typically. Of patients with EHEC infection, an estimated 10% will develop HUS and 3-5% will die.

Physiologically, EHEC causes the formation of lesions on intestinal epithelial cells in a process termed attaching and effacing (AE), which results from the destruction of the intestinal microvilli and the production of Shiga toxins (Sperandio et al., 2003). Genes associated with the AE lesion are encoded by a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (Sperandio et al., 2003; Walters et al., 2006). The 41 genes associated with the LEE are organized into 5 operons and have been demonstrated to be responsive to quorum sensing signaling (Sperandio et al., 2003; Reading et al., 2006; Walters et al., 2006).

The AI-3 quorum sensing signal has been shown to activate transcription of the LEE type III secretion system, which is responsible for extensive cytoskeletal rearrangements resulting in AE lesions (Walters et al., 2006). AI-3 is able to activate the LEE-encoded regulator (Ler), which is

the first gene encoded on *LEE*1, and causes the activation of the LEE genes (Walters et al., 2006; Reading et al., 2006). Cross talk between AI-3 and the mammalian hormones epinephrine and norepinephrine have been observed to regulate *LEE*1 (Sperandio et al., 2003). Consequently, the AI-3 quorum sensing signals can be substituted by epinephrine and norepinephrine and still activate the Ler in EHEC.

5.1.2 Resuscitation of VNC Bacteria by Autoinducer Addition

The majority of research into the effects of autoinducer addition have been performed with pure cultures under laboratory control conditions. Valle et al. (2004), however, published a study whereby the bacteria population of an industrial wastewater facility was well characterized through molecular and microbiological techniques and AHLs were added to fresh sludge samples from the aerobic continuous flow reactors. The wastewater facility, designed to degrade phenolic cocktails generated by steel coking ovens, sporadically loses biological activity thus preventing the degradation of phenolic compounds. Although the industrial wastewater facility studied does not possess high concentrations of *E. coli*, the application of AHLs in mixed environmental cultures is important to this research. According to Valle et al. (2004), AHL-mediated gene expression was found to return non-phenolic culturing conditions to normal. Specifically, the addition of C6-HSL and 3-oxo-C6-HSL, both AHLs, were found to shift the predominance from non-phenol assimilating bacteria (i.e., *Thiobacillus* and *Acidobacteria*) to phenol degrading bacteria (i.e., *Thauera*, *Acidovorax*, and *Rhodopseudomonas* genera). The influence of AHL regulated gene expressions appears to be the most likely hypothesis to support the shift in bacterial population (Valle et al., 2004). Because AHLs were capable of initiating a regulatory pathway that promoted one phenotype over another, AHL-mediated gene expression likely initiated cell-to-cell signaling which changed the composition and function of the mixed bacteria culture.

Multiple studies have found that quorum sensing in *E. coli* is essential for regulating culturability based on both cell density and metabolic potential of the environment (Lyte et al., 1996; Surette et al., 1998; Reissbrodt et al., 2002; Valle et al., 2004). These studies reference both the AHL and norepinephrine quorum sensing pathways. For *E. coli*, which does not produce a known AHL, the ability to interpret signaling molecules from other bacteria provide *E. coli* with information about cell density and nutrients available in the environment. Surette et al. (1998) identified that possible quorum sensing signals exist from *E. coli* which are degraded before stationary phase and indicate that low a cell density bacterial population is beneficial for *E. coli* growth. This may be important for pathogenic *E. coli* because dispersion is necessary for greatest pathogenicity; therefore, stationary phase growth will not be beneficial to pathogenic *E. coli* attempting to infect as much area as possible. Likewise, signaling molecules produced within nutrient-rich environments could communicate to nearby cells that a favorable environment exists and that reproduction is possible. The presence of pathogenic *E. coli* and a nutrient-rich environment could therefore promote exponential growth of bacteria because of cell-to-cell signaling molecules.

Because the presence of the AHLs and autoinducers may provide a switch for the resuscitation of VNC bacteria, it is possible that the shear forces associated with HSC could release these compounds which result in the SI of FC and *E. coli*. As discussed by Surette et al. (1998), quorum sensing signals may increase the growth of *E. coli* when high nutrient levels and low cell densities are present which are theorized to be possible after HSC dewatering. As discussed in Chapter 7, the shear forces produced during HSC dewatering break up floc particles thus increasing the surface area for growth and possibly increasing the cell density, as discussed by Qi et al. (2004), and releasing nutrients that promote growth as presented by Higgins et al. (2006). Therefore, the shearing process may result in the release of AHLs and autoinducers from bacterial cells signaling for increased growth. The release of these signaling molecules may explain the SI phenomena.

5.1.3 Resuscitation of VNC Bacteria by Cell Washing Detoxification Technique

The SI phenomenon describes the increase in FC and *E. coli* density that occur immediately after centrifuge dewatering. One possible explanation for the depressed FC and *E. coli* density in sludge samples immediately before centrifuge dewatering is the presence of cellular toxins in solution. Research into copper toxicity was described by Grey et al. (2001) who identified that cellular toxicity to copper induced the VNC state in *E. coli*. In their experiment, Grey et al. (2001) amended LB plates with varying concentrations of copper sulfate (0 to 25 mM) and incubated mid- to late-exponential-stage *E. coli* strains ED8739 and ES80 on the plates for 2 days at 37°C. Using live/dead analysis, Grey et al. (2001) identified that even though no colonies were detected on the LB plates amended with 6 and 25 mM copper sulfate plates, significant concentrations of viable *E. coli* cells persisted, which is an indication for the presence of VNC cells. However, as discussed in Section 2.1, live/dead analysis is not a conclusive test when VNC bacteria may be present since VNC bacteria have low concentrations of rRNA which presents biased low results using the FISH methodology. To determine if the cells had entered the VNC state or died from the toxic levels of copper, Grey et al. (2001) investigated whether a cell washing technique could be used to detoxify the cellular environment and resuscitate VNC cells. To perform the cell washing technique, Grey et al. (2001) membrane filtered suspended *E. coli* and washed the cells with 3 to 5 volumes of 500 µM EDTA before resuspending the cells in the original volume of 0.9% NaCl. The cell washing technique described by Grey et al. (2001) successfully resuscitated *E. coli* that were subjected to copper toxicity. Culturability was able to be restored within two weeks of loss of culturability using the cell washing technique (Grey et al., 2001).

Similar studies have been performed using cell washing techniques to detoxify cellular environments which were exposed to saline stress, chlorination, heat stress, and oxidative stress (Ohtomo et al., 2001; Oliver et al., 2005b; Dukan et al., 1997). Ohtomo et al. (2001) harvested saline stressed *E. coli* cells by membrane filtration and washed with two volumes of PBS (which

contained 0.9% NaCl). Chlorine stressed *E.* coli cells were centrifuged (14,000 *xg* for 5 minutes) into a pellet before being resuspended in PBS (Oliver et al., 2001). Research by Dukan et al. (1997) on the resuscitation of chlorine stressed *E. coli* in PBS indicated that significant populations of VNC cells could be resuscitated after 1 day of incubation in PBS. The chlorine stressed cells were pelleted by centrifugation (3,000 *xg* for 10 minutes at 4°C) before being washed twice with 50 mM phosphate buffer (pH 7.1) and resuspended in the original volume of phosphate buffer (Dukan et al., 1997). In each case, the researchers observed a resuscitation of VNC *E. coli* cells when washed and resuspended in a PBS.

5.2 Research Need

Research needs to be performed to evaluate the effect of AHLs and norepinephrine addition on the resuscitation of FC and *E. coli* in wastewater samples. Since *E. coli* does not produce an AHL of its own, AHL molecules which are known to bind to the *E. coli* protein receptor (SdiA) should be studied. The norepinephrine pathway should be studied in a similar fashion to the AHL pathway.

Additionally, inhibitory effects from mixed quorum sensing molecules or other inhibitory compounds like copper may further promote the VNC state. Research should be performed to determine if quorum sensing molecules can be diluted from wastewater samples, thus removing potential inhibitory effects resulting in resuscitation.

5.3 Research Objective

Quorum sensing molecules are one possible mechanism promoting the VNC state. In this experiment, known autoinducer molecules were added to presumptive media to enhance SCM. The resuscitation effects on VNC bacteria present in Class A digested sludge and dewatered

cake samples were determined. It is hypothesized that the addition of autoinducer molecules to culture media will promote resuscitation in FC and *E. coli* populations. This experiment aims to determine if quorum sensing molecules are effective in reversing the non-culturability of VNC bacteria.

Alternatively, this experiment investigates whether quorum sensing molecules or other inhibitory substances may play an inhibitory role on cellular growth. A cell washing technique was employed to determine if inhibitory quorum sensing molecules hinder the resuscitation of VNC bacteria.

5.4 Methods and Procedures

Class A sludge and cake were provided by the TPAD-1 wastewater facility. Samples were shipped on ice by overnight FedEx shipment and were stored at 4°C prior to the start of the experiment which commenced on same day as sample receipt. Multiple tube fermentation culturing techniques were performed on the sludge and cake using both traditional and enhanced presumptive media as described herein. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B.

5.4.1 Multiple Tube Fermentation Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Sludge samples (30.0 ± 0.1 mL) and biosolid pellet (cake) samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). As described in Section 5.4.1.1, the biosolids pellet was generated by cell washing to remove inhibitory substances. Serial dilutions (1:10) were prepared for liquid and solid samples. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media (traditional and enhanced). Five replicate tubes were

inoculated for each dilution. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive LTB media (traditional and enhanced), the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under UVB lighting.

5.4.1.1 Detoxification of Inhibitory Agents (Cell Washing)

Liquid sludge samples (30.0 ± 0.1 mL) were centrifuged at 14,000 *xg* for 5 minutes using a tabletop mini-centrifuge. The supernatant was removed, and the biosolids pellet was re-suspended to a total volume of 30.0 ± 0.1 mL with sterile PBS dilution water (1:10 dilution) using a tabletop vortex mixer. The re-suspended pellet was then re-centrifuged at 14,000 *xg* for 5 minutes using a tabletop mini-centrifuge. The supernatant was removed, and the biosolids pellet was again re-suspended suspended to a total volume of 30.0 ± 0.1 mL with sterile PBS dilution water using a tabletop vortex mixer.

5.4.1.2 Traditional Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2005a). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

5.4.1.3 Enhanced Presumptive Media

The traditional presumptive media, as described in Section 5.4.1.2, was augmented with C6-HSL, 3-oxo-C8-HSL, or norepinephrine for use as the enhanced presumptive media. Because of the enzymatic qualities of C6-HSL, 3-oxo-C8-HSL, and norepinephrine, these supplements were dissolved into a stock solution (2.5 mM) prior to filter sterilized using a 0.22 µm sterile filter and aseptic transfer (200 µL) into sterile traditional presumptive media. A final concentration for each supplement in LTB media was 50 µM.

LTB presumptive media augmented with 5 g / 300 mL bentonite was sterilized in an autoclave at 121°C, 15 psi for 15 minutes. The LTB-bentonite slurry was mixed prior to autoclaving and transferred into each tube. The LTB-bentonite slurry was mixed during the transfer process to minimize variations in slurry density.

E. coli supernatant additive was prepared by inoculating LTB media with *E. coli* (ATCC# 25922) and culturing at 35°C ± 0.5°C. *E. coli* growth phase was monitored by optical density measurements at 600 nm every 30 minutes until exponential growth phase was encountered. *E. coli* supernatant was generated from *E. coli* cultures prior to lag phase. *E. coli* supernatant was generated using a tabletop mini-centrifuge (14,000 *xg* for 5 minutes) prior to filter sterilization using a 0.22 µm sterile filter. A 1 mL aliquot of *E. coli* supernatant was added to sterile LTB media aseptically.

5.4.1.4 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

5.4.2 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

5.5 Results and Discussion

Sludge samples from TPAD-1 were cultured in LTB presumptive media with 50 µM C6-HSL, 50 µM 3-oxo-C8-HSL, or 50 µM norepinephrine. TPAD-1 sludge samples were prepared using both the cell washing technique and without. The culturing data for both washed and unwashed cells is presented as Figure 5–1.

The non-amended LTB media served as the control for the experiment. A control was prepared with both washed and unwashed cells so that the resuscitation of VNC bacteria could be evaluated. Unfortunatley, after 48 hours (2 days) of incubation, none of the sludge samples cultured in enhanced (50 µM C6-HSL, 50 µM 3-oxo-C8-HSL, or 50 µM norepinephrine) and traditional LTB media for both washed and unwashed cells produced a change color. This lack of color change in the presumptive media is an indication for the absence of TC (the non-detect value was 6 MPN/g DS). However, after continuing the incubation for 6 days, LTB media did produce a color change which indicated the presence of TC. According to EPA Method 1680, the color change in LTB media for TC detection is supposed to occur within 48 ± 3 hours of incubation. Therefore, the usability of the data produced in this experiment should be qualified since the incubation time was longer than EPA Method 1680 allows.

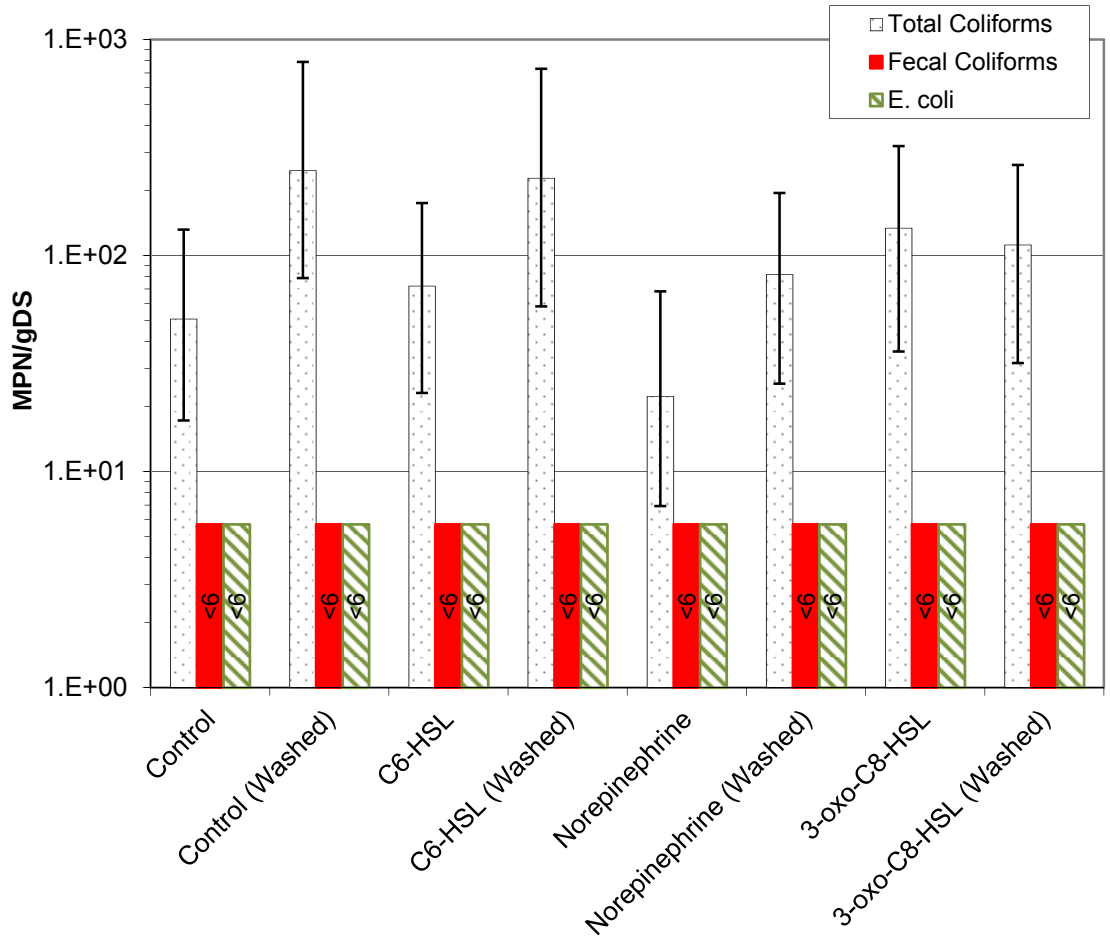

Figure 5–1: Thermally treated sludge (TPAD-1) prepared with and without a cell washing technique and cultured on autoinducer supplemented media (Error bars represent upper and lower 95% confidence intervals)

As depicted in Figure 5–1, the results for the presence of TC after 6 days of incubation in traditional and enhanced presumptive media (i.e., C6-HSL, 3-oxo-C8-HSL, and norepinephrine amendments) did not reveal differences in the density of TC when compared to the different media enhancements (as determined at the 95% confidence interval). Also, the TC densities for washed and unwashed cultures in each media enhancement were not significantly different (as determined at the 95% confidence interval). The TC densities for each of the presumptive media types were approximately 100 MPN/g DS. Confirmational phase sampling did not yield FC or *E.*

coli detections for either the traditional or enhanced media and for both washed and unwashed cells (the non-detect value was 6 MPN/g DS).

The observation that TC density did not increase after performing the cell washing technique may indicate that inhibitory substances were not removed and that resuscitation of VNC bacteria did not occur. However, in previous investigations at TPAD-1, the resuscitation of TC, FC, and *E. coli* occurs regularly after HSC dewatering as discussed in Section 2.5.1. Sludge samples were observed to have non-dectect densities of TC, FC, and *E. coli*, which are similar to the 48 hour incubation results from this experiment. Cake samples collected after HSC dewatering contained detectable densities of FC and *E. coli* above the Class A biosolids limit. Unfortunately, no cake samples were cultured as a part of this experiment with autoinducer addition and cell washing techniques. Nonetheless, the reactivation of non-culturable FC and *E. coli* in TPAD-1 sludge samples after HSC dewatering is a regular occurrence.

5.6 Research Significance

As described in literature, the addition of autoinducers to bacteria cultures may resuscitate non-culturable bacteria (Valle et al., 2004). However, in this experiment, the autoinducers C6-HSL, 3-oxo-C8-HSL, and norepinephrine that were supplemented to the presumptive LTB media were unable to promote TC, FC and *E. coli* resuscitation. The autoinducers were added to nutrient media in an effort to stimulate metabolic activity thus promoting bacteria population growth. Because the addition of autoinducers did not affect the TC, FC, or *E. coli* density, the addition of autoinducers alone does not appear to be sufficient to promote the resuscitation of VNC bacteria.

This experiment also tested whether a cell washing technique, described by Grey et al. (2001), could be used to resuscitate *E. coli* in a mixed culture. Although not statistically different at the 95% confidence interval, the TC density of washed cells was numerically greater than the TC

density of unwashed cells, typically 0.5 orders of magnitude greater. In this experiment, the resuscitation of potential VNC bacteria with the use of the cell washing technique was not able to reproduce the 2 order of magnitude increase described Grey et al. (2001) when compared to the unwashed cell cultures. The cell washing technique was not able to resuscitate FC and *E. coli* densities above the detection threshold, which indicates that cell washing alone may not be able to promote cellular resuscitation.

5.7 Conclusions

Data collected during the quorum sensing experiment indicated the following observations:

- The inclusion of autoinducer compounds into presumptive culture media did not promote the resuscitation of thermally treated FC and *E. coli*.

- Cell washing did not have an observable change in the culturability of TC, which was also observed in Chapter 6.

- The delayed culturability of TC may indicate that both the cell washing technique and autoinducer addition produced additional stresses on cells which diminished culturability.

Based on the experimental findings, the addition of autoinducer molecules to culturing media did not promote the resuscitation of FC and *E. coli*. Additionally, the cell washing technique was not effective in promoting the resuscitation of FC and *E. coli*. Because DNA data was not collected using qPCR at TPAD-1, the anticipated *E. coli* density is unknown. Additional research should be performed to determine if the addition of C6-HSL, 3-oxo-C8-HSL and norepinephrine promoted the non-culturable state or if the biosolids samples lacked detectable *E. coli*. A more rigorous experiment that evaluates *E. coli* density by SCM and qPCR should be performed to expand the data at TPAD-1.

Chapter 6: Investigation of Cell Washing, Adsorption Techniques, and *E. coli* Cell-Free Supernatant Addition on the Detoxification of Inhibitory Substances and the Resuscitation of VNC Bacteria

6.1 Introduction

One possible mechanism that could explain the increases in *E. coli* after centrifuge dewatering is the removal of inhibitory compounds such as quorum sensing molecules or some other unidentified substance. Quorum sensing molecules are produced by bacteria to relay information to other bacteria about environmental stresses and nutrient availability. Traditional culturing methods, which are selective for a single group of bacteria, may not effectively enumerate target bacterial populations because wastewater samples contain a mixture of bacteria, a mixture of chemical signaling molecules, and antagonistic growth factors as well as inhibitors in solutions.

For *E. coli*, the specific autoinducer used to provide cell-to-cell signaling has not been identified (Weichart et al., 2001; Reading et al., 2006). Nevertheless, cell-to-cell signaling molecules have been identified for other bacterial species which may be recognized by *E. coli* (Reading et al., 2006; Houdt et al., 2006; Yao et al., 2006; Weichart et al., 2001). The referenced literature indicated that signaling molecules can both promote cellular growth or restrict it. These signaling molecules have been identified to be extracellular and to diffuse freely through aqueous media. Because growth in aqueous media is common, Weichart et al. (2001) performed research to determine if cell-free supernatant extracts from bacterial cultures could promote resuscitation. Weichart et al. (2001) identified that cell-free supernatant from *E. coli* cultures both induced certain *E. coli* genes and inhibited growth in *E. coli* and other microorganism populations. This mixed observation did not conclusively determine if cell-free supernatants were able to promote the resuscitation of non-culturable bacteria.

Additional research was conducted to evaluate if cell-free supernatant collected from growth phase bacterial cultures could promote the resuscitation of VNC bacteria (Panutdaporn et al., 2006; Kaprelyants et al., 1994; Weichart et al., 2001). This research was important because the culturability of bacteria is assumed to be related to the presence of quorum sensing molecules. Growth phase bacterial cultures are assumed to not be controlled or inhibited by quorum sensing molecules since the bacterial population continuously increases until a population threshold is reached, the environment becomes toxic from waste products, or nutrients become scarce. Experimentation by Panutdaporn et al. (2006) identified that VNC bacteria can be effectively resuscitated when cultured with cell-free supernatants generated from growth phase bacterial cultures. The specification of growth phase cell-free supernatants appeared to be a significant criterion for promoting resuscitation.

Wastewater samples typically contain stationary phase bacterial populations. Weichart et al. (2001) observed that quorum sensing molecules from bacteria in the stationary phase have an inhibitory effect on cellular growth. These growth antagonists are likely present in wastewater biosolids. During the dewatering of biosolids by HSC, inhibitory substances could be removed which result in the resuscitation of VNC bacteria. This could explain the SI phenomena observed at full-scale thermophilic treatment processes.

6.2 Research Needs

Research needs to be performed to determine if antagonistic growth factors exist in wastewater biosolids that promote the non-culturable state and thus cause SCMs to undercount FC and *E. coli* populations. To determine if quorum sensing molecules or other antagonistic growth factors have a toxicity effect on bacterial growth, thus promoting the VNC state, detoxification techniques need to be investigated to evaluate if toxicants can be removed from wastewater samples. In addition to investigating the removal of toxicants, the addition *E. coli* growth phase cell-free

supernatant extract, which has promoted resuscitation in pure cultures, needs to be investigated in wastewater samples.

6.3 Research Objective

In this experiment, the culturability of bacteria was evaluated with two different presumptive media supplements and a cell washing technique to detoxify inhibitory agents. Each of the three experimental processes tested a different hypothesis. The two media supplements to be utilized were bentonite and cell-free exponential growth phase *E. coli* supernatant. Specifically, these compounds were added to presumptive phase media to evaluate their impact on the culturability of *E. coli*. The addition of cell-free exponential growth phase *E. coli* supernatant to presumptive media tested the hypothesis that growth phase quorum sensing molecules, other than those AHLs tested in Chapter 5, can resuscitate VNC bacteria. Bentonite was added to presumptive media to determine if inhibitory agents will sorb to the bentonite instead of being removed by cell washing, which was a proposed method for diluting inhibitory agents. This research hypothesizes that bentonite is effective in removing inhibitory agents from wastewater cultures which inhibit cellular growth. In addition to supplementing presumptive media with various constituents, the cell washing protocol, which was discussed in Chapter 5, was used to evaluate if toxins can be removed prior to presumptive phase culturing.

6.4 Methods and Procedures

Class B, mesophically treated sludge was provided by the Los Angeles County Sanitation Districts (LACSD) Joint Water Pollution Control Plant (JWPCP). Samples were shipped on ice by overnight FedEx shipment. MPN analysis was performed on the sludge using both traditional and enhanced presumptive media as described herein. The method for detoxification of inhibitory substances is also discussed herein. Total percent solids analysis of the sludge and cake

samples was performed by Standard Method (SM) 2540B.

6.4.1 Multiple Tube Fermentation Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Sludge samples (30.0 ± 0.1 mL) and biosolid pellet (cake) samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). As described in Section 5.4.1.1, the biosolids pellet was generated by cell washing to remove inhibitory substances. Serial dilutions (1:10) were prepared for liquid and solid samples. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media (traditional and enhanced). Five replicate tubes were inoculated for each dilution. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive LTB media (traditional and enhanced), the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under UVB lighting.

6.4.1.1 Detoxification of Inhibitory Agents (Cell Washing)

Liquid sludge samples (30.0 ± 0.1 mL) were centrifuged at 14,000 *xg* for 5 minutes using a tabletop mini-centrifuge. The supernatant was removed, and the biosolids pellet was re-suspended to a total volume of 30.0 ± 0.1 mL with sterile PBS dilution water (1:10 dilution) using

a tabletop vortex mixer. The re-suspended pellet was then re-centrifuged at 14,000 *xg* for 5 minutes using a tabletop mini-centrifuge. The supernatant was removed, and the biosolids pellet was again re-suspended suspended to a total volume of 30.0 ± 0.1 mL with sterile PBS dilution water using a tabletop vortex mixer.

6.4.1.2 Traditional Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2006). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

6.4.1.3 Enhanced Presumptive Media

The traditional presumptive media, as described in Section 4.4.1.1, was augmented with combinations of bentonite and *E. coli* supernatant for use as the enhanced presumptive media. LTB presumptive media augmented with 5 g / 300 mL bentonite was sterilized in an autoclave at 121°C, 15 psi for 15 minutes.

E. coli supernatant additive was prepared by inoculating LTB media with *E. coli* (ATCC# 25922) and culturing at 35°C ± 0.5°C. *E. coli* growth phase was monitored by optical density measurements at 600 nm every 30 minutes until exponential growth phase was encountered. *E. coli* supernatant was generated from *E. coli* cultures prior to lag phase. Positive cultures were not transferred to EC-MUG media because a pure culture of *E. coli* was used initially. *E. coli* supernatant was generated using a tabletop mini-centrifuge (14,000 *xg* for 5 minutes) prior to filter sterilization using a 0.45 µm sterile filter. A 1 mL aliquot of *E. coli* supernatant was added to sterile LTB media aseptically.

6.4.1.4 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

6.4.2 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

6.5 Results and Discussion

Thermally treated sludge from LACSD was cultured in LTB presumptive media (control) and enhanced LTB presumptive media containing combinations of bentonite, and *E. coli* supernatant. The cell washing procedure was performed on sludge samples prior to culturing in LTB presumptive media and *E. coli* supernatant enhanced LTB presumptive media. Figure 6–1 presents the culturing results for this experiment.

The TC, FC, and *E. coli* density for the unwashed control sludge was 10^4 MPN/g DS. The washed sludge samples had a TC density (2×10^3 MPN/g DS) that was less than the unwashed control sludge. Confirmatory culturing for FC and *E. coli* was not performed for the washed sludge sample because washing negatively influenced TC density.

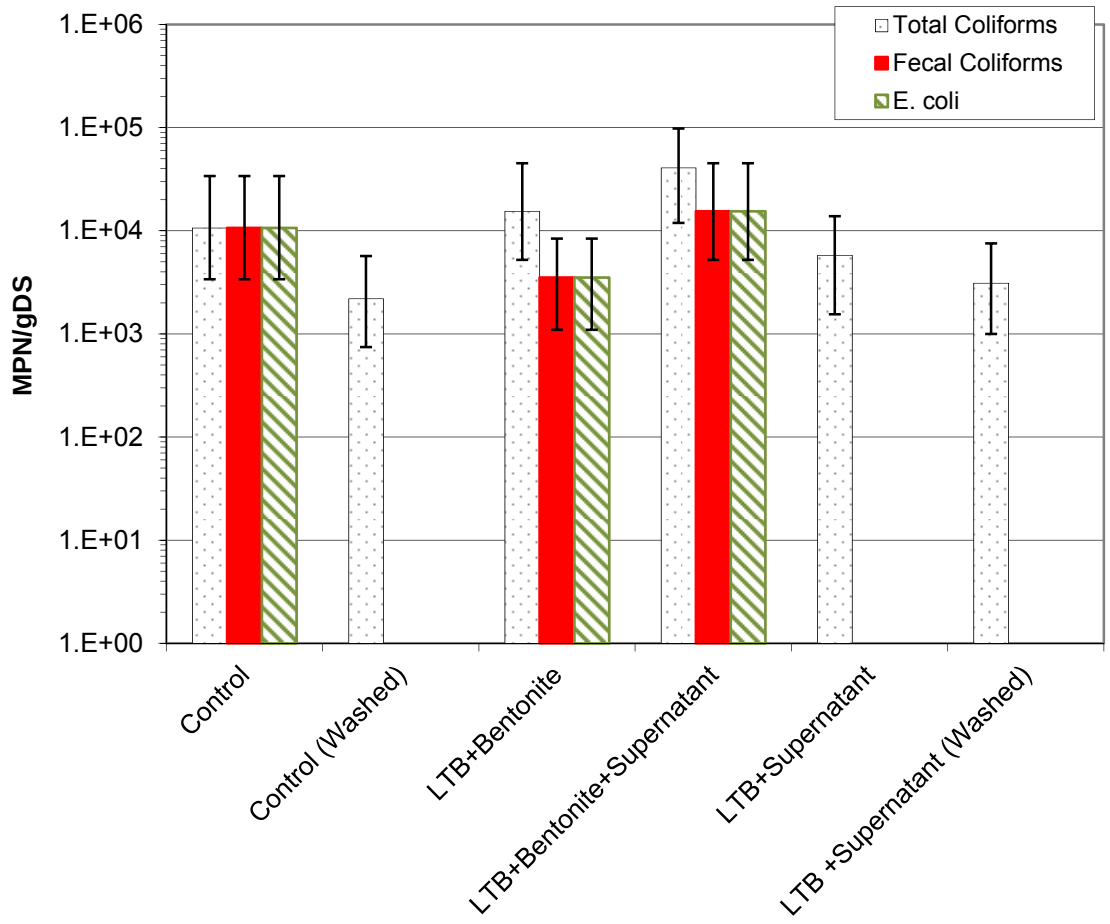

Figure 6–1: Bacterial culturability enhancements for Class B sludge samples from LACSD (Error bars represent upper and lower 95% confidence intervals)

Presumptive media supplemented with *E. coli* supernatant had a TC density of 6 x 10^3 MPN/g DS which was similar to the control sludge. Sludge samples that were cell washed and cultured on media supplemented with *E. coli* supernatant had a TC density of 3 x 10^3 MPN/g DS which was less than the TC density for the unwashed sludge. Neither the washed or unwashed samples cultured in *E. coli* supernatant supplemented presumptive media were transferred to EC-MUG media for the conformational phase since the TC density was less than the unwashed control.

Unwashed cells cultured in presumptive media supplemented with bentonite had TC densities

similar to the unwashed control sludge. The increase in TC density ranged from 2 x 10^4 MPN/g DS in media only supplemented with bentonite to 4 x 10^4 MPN/g DS in media supplemented with bentonite and *E. coli* supernatant. Confirmation samples indicated that the FC and *E. coli* density were similar to densities of the unwashed control sludge; the results were not statistically different at a 95% confidence interval. Based on these results, the use of bentonite does not appear to increase the culturability of bacteria in sludge samples. Therefore, the hypothesis that bentonite would sorb inhibitors within the sludge was not supported by these culture results.

The washing of cells also did not increase the TC density which would indicate possible bacteria resuscitation. Instead, cell washing resulted in lower TC density than the control sludge. The removal of toxins by cell washing may also stress cells thus decreasing their culturability. In addition to removing toxins, necessary quorum sensing molecules may have been removed which induce cell growth when nutrients are available and cell density is low.

6.6 Research Significance

Research presented by Weichart et al. (2001) and Panutdaporn et al. (2006) demonstrated that the addition of growth phase cell-free *E. coli* supernatant to liquid media effectively resuscitated *E. coli* cells. The selection of media enhancements that are capable of inducing resuscitation are an important approach toward creating a laboratory culturing methodology that accurately enumerates VNC bacteria. In this experiment, growth phase cell-free *E. coli* supernatant was added to liquid media that was inoculated with mixed culture wastewater samples. Contrary to the findings from Weichart et al. (2001) and Panutdaporn et al. (2006), the presumptive media enhanced with growth phase cell-free *E. coli* supernatant was incapable of inducing resuscitation and increasing the TC density when compared to the control media. Without an increase in TC density, the density of *E. coli* would also be less.

The removal of toxins and other antagonistic compounds in the wastewater sludge was also investigated in this experiment by supplementing bentonite to the presumptive media and by utilizing the cell washing technique discussed in Chapter 5. It was hypothesized that bentonite could be used to sorb the toxins and other antagonistic compounds in the sludge that could inhibit cellular growth. The cell washing technique was hypothesized to dilute the toxins and antagonistic compounds in the sludge that may inhibit resuscitation. Unfortunately, neither of these approaches were successful at inducing the resuscitation of *E. coli*. This experiment confirmed that finding in Chapter 5 that the cell washing technique was not a method that was capable of inducing resuscitation. Both the cell washing technique and bentonite addition techniques were tested in combination with the addition of growth phase cell-free *E. coli* supernatant. Each of these experimental trials resulted in TC densities that were comparable at the 95% confidence interval. Therefore, while the removal of toxins may be a necessary consideration for the promotion of bacterial resuscitation, without identifying the toxins and antagonistic growth factors affecting resuscitation, a specific method for removal cannot be determined.

6.7 Conclusions

Although conclusions from various journal articles have indicated that the culturability of *E. coli* can be enhanced by the detoxification of inhibitory agents (Weichart et al., 2001; Panutdaporn et al., 2006; Kaprelyants et al., 1994), the experiments performed with wastewater samples from LACSD did not yield different culturability values of TC when compared to the control. In this research, three techniques were utilized to detoxify cells from inhibitory agents: cell washing, exponential growth phase *E. coli* cell-free supernatant, and bentonite. Data collected during the detoxification of inhibitory substances experiment indicated the following observations:

- The cell washing technique utilized in this experiment did not affect the culturability of TC when compared to the control, which was consistent with the findings in Chapter 5. This result may indicate that the cell washing technique causes cellular stresses which sustained the VNC state.

- The exponential growth phase *E. coli* cell-free supernatant was not able to stimulate cellular growth. Suspected quorum sensing molecules may not be present in *E. coli* cell-free supernatant as hypothesized

- Bentonite was not effective in sorbing contaminants which may inhibit cellular growth.

Chapter 7: Effects of Shear Forces and Storage on the Sudden Increase and Regrowth of Fecal Coliforms and *E. coli* in Mesophilic Digested Biosolids

7.1 Introduction

Biosolids dewatering is an important component to any solids treatment system because of the cost associated with solids disposal. The major expenses associated with solids disposal are dewatering, hauling, and disposal. To minimize these expenses, solids treatment processes are designed to minimize the moisture content of wastewater solids. As discussed in Section 1.2, two of the most common dewatering technologies are belt filter presses and centrifuges, especially HSC, which are typically capable of producing 12-20% solids and 22-30% solids, respectively (Reynolds et al., 1996). Although dewatering technologies adequately remove moisture from biosolids, recent literature indicates that these same processes may be responsible for the SI and regrowth of bacteria (Cheung et al., 2003; Monteleone et al., 2004; Qi et al., 2004; Higgins et al., 2006). Shear effects have been cited as a possible reason for the regrowth and possibly SI of FC and *E. coli* (Cheung et al., 2003; Monteleone et al., 2004; Qi et al., 2004). The following provides a discussion about the experimental findings related to regrowth and shear from these researchers.

Cheung et al. (2003) identified that the *E. coli* density in mesophilically digested sludge increased after HSC. This experiment was significant because feed and dewatered samples were analyzed in triplicate for statistical verification. The increase between the *E. coli* density in the feed and dewatered cake was 2.17 $\log_{10}$ units. Additionally, laboratory homogenization techniques were investigated to determine if the shear effects could be replicated in the laboratory by shaking with glass beads at 300 rotations per minute (rpm) for 5 minutes, sonification at 50 kilohertz (kHz) for

2 minutes, and stomaching (a kneading procedure) at 230 rpm for 2 or 4 minutes. Of these homogenization techniques, only increasing the stomaching time from 2 minutes to 4 minutes increased *E. coli* densities in feed solutions by 0.5 $\log_{10}$ units. The conclusions presented by Cheung et al. (2003) indicated that shear effects promote the SI of *E. coli* in full-scale HSC, but these shear forces could not be effectively reproduced in the laboratory using standard homogenization techniques.

Monteleone et al. (2004) conducted experiments with mesophilically digested biosolids from treatment plants utilizing belt filter press technology and HSC. The four treatment plants utilizing HSC dewatering technology yielded increases in *E. coli* density in dewatered cake compared to the feed. The utility that dewatered biosolids with a belt filter press measured decreases in *E. coli* density in dewatered cake compared to the feed. Since *E. coli* densities only increased at sites with HSC dewatering, the SI phenomena is suspected to be related to the shear forces induced during centrifugation. Subsequent research by Monteleone et al. (2004) observed that batch laboratory centrifugation over a range of 500 to 2,500 x*g* did not promote the SI phenomena. Similar to Cheung et al. (2003), the Monteleone et al. (2004) experiment was unable to replicate the shear force effects in the laboratory using a laboratory-scale centrifuge.

In another experiment examining mesophilic digested biosolids at four treatment plants, Qi et al. (2004) investigated the changes in FC density before and after different dewatering technologies. One plant operated a belt filter press, low solids centrifugation (LSC), and HSC in parallel which allowed for comparative analysis of different dewatering technologies with the same feed. Increases in FC density were observed immediately after LSC and HSC and additional regrowth of FC was observed after 24 hours of storage. No increase in FC density was observed after belt filter press dewatering either immediately or after 24 hours of storage. At a second plant, pre- and post-centrifugation samples were collected from a mesophilically digested biosolids over a period of 8 days during one month. Increases in FC density were observed on seven of the eight

days; of these increases, three were statistically significant above the 95% confidence interval compared to the feed. The experiment conducted at the second plant was repeated at another facility utilizing centrifuge dewatering of anaerobically digested, polymer-conditioned biosolids. The cake samples at this third plant had FC densities that were less than that of the feed, although the decrease was not statistically significant. Lastly, a fourth plant that generated LSC and HSC cakes from two different mesophilically digested biosolids reported statistically higher FC density than the feed. Samples at the fourth plant were also stored prior to culture analysis; these samples indicated that FC regrowth occurred. Based on the conclusions from Qi et al. (2004), SI and regrowth are a common observation for centrifuge dewatered sludges that are mesophilically digested.

Qi et. al (2004) also suggested that the intensive shearing forces associated with centrifugation may be a reason for the increased enumeration of FC. Qi et al. (2004) suggested that shearing forces were responsible for the breakup of flocs which would increase the surface area where bacteria could culture. Upon testing this hypothesis with a kitchen blender (maximum speed for 3 minutes at room temperature [25°C]), Qi et al. noted that the blended and unblended samples contained the same concentration of FC. Microscopy of the blended and unblended samples identified that the blending process produced smaller floc sizes. Nevertheless, the blending of digester feed sludge did not result in higher FC enumeration compared to unblended feed sludge (Qi et al., 2004). Therefore, similar to Cheung et al. (2003) and Monteleone et al. (2004), laboratory methods of shear could not replicate what happens during centrifuge dewatering.

A recent study published by Flemming et al. (2009) collected culture data from six utilities in Ontario, Canada to evaluate the risk associated with various pathogens. Each wastewater treatment plant operated mesophilic anaerobic digesters that utilized either centrifuge dewatering technologies, belt filter presses, or a combination of both. The FC and *E. coli* data collected by SCM in this experiment is important because the sample population size was great enough to

perform a statistical analysis. Only 2 of the 4 utilities utilizing HSC dewatering were observed to have a statistically significant SI phenomenon ($p<0.05$), but this reactivation was not determined to be significant since the density increase was less than 1-log. At three of the utilities, regrowth was found to be a statically significant phenomenon ($p<0.05$) after 2-3 days of cake storage; two of these utilities utilized HSC dewatering technologies while the third utility utilized a combination of BFP and LSC dewatering. Since another utility utilizing BFP dewatering alone did not induce regrowth, it is assumed that the shear forces imparted to the sludge by LSC dewatering induced regrowth.

Shear during centrifuge dewatering likely occurs during two phases – the liquid and cake phases. The first is the shear that occurs during the nearly instantaneous acceleration of the sludge and polymer mix from 0 to up to 3000 rpm as it enters the centrifuge. The second type of shear is imparted to the cake directly due to the scrolling of the cake up the bowl while the cake is being pressed against the bowl under high pressure from the centrifugal forces. Additional shear is imparted to the cake as it is decelerated and extruded out of the centrifuge. The laboratory methods include shearing of both the liquid phase (sludge and polymer) as wells as the cake.

7.2 Research Needs

Research needs to be performed to determine alternate laboratory methods that are able to promote regrowth of VNC bacteria in sludges. We hypothesize that the shear imparted to the solids during centrifuge dewatering releases bioavailable material that supports regrowth. However, research have shown that laboratory methods including blending, stomaching, sonification, and lab centrifugation did not replicate the SI or regrowth phenomena during HSC dewatering. Additional laboratory techniques need to be investigated for pre-treatment of wastewater samples prior to SCM. Specifically, research needs to examine the centrifugal, screw conveyance, and dewatering forces present in HSC. The literature presented only examined

centrifugal, screw conveyance, and dewatering forces independently. Additional research is required to evaluate if centrifugation in combination with shear during dewatering promotes the resuscitation and regrowth of bacteria during SCM.

7.3 Research Objectives

The objectives of this experiment were to determine the effects that the laboratory shearing methods had on the regrowth phenomena from mesophilic treated sludge. More specifically, these experiments investigated:

- Using a novel laboratory shearing method to replicate the shear forces from HSC dewatering and screw conveyance present during full-scale centrifuge dewatering processes.

- Storage of cake samples after laboratory shearing and dewatering to identify if the laboratory method produces a comparable regrowth of indicator bacteria to full-scale HSC dewatering processes.

- Performing the novel laboratory shearing method on stored sludge samples to conclude if the regrowth phenomena occurs after indicator bacteria densities have decreased by 2-3 orders of magnitude.

To accomplish these objectives, a series of three experiments were performed. In the experiments, mesophilic digested sludge was collected from the Meso-2 and Meso-3 plants and was dewatered in a laboratory technique hypothesized to mimic full-scale HSC. The laboratory produced cake samples were compared to full-scale cake samples. The full-scale sludge and cake and laboratory dewatered cake were then incubated for up to 13 days. The culturability of TC, FC, and *E. coli* were determined daily to observe whether the SI or regrowth phenomena

occurred. The final experiment investigated whether sludge with diminished FC and *E. coli* concentrations, which occurred after 6 days of incubation, could be sheared and dewatered to promote SI or regrowth. This research hypothesized that increasing amounts of shear force caused greater amounts of regrowth through the release of biodegradable materials. The individual experimental designs for these three experiments are discussed in the following sections.

7.3.1 Regrowth Experiment with Meso-2 Samples (Event 1)

Cake and sludge was provided by Meso-2 and received on June 21, 2006. Sludge samples were dewatered according to the laboratory shear/dewatering technique for comparison to control cake samples. Increasing amounts of laboratory shearing was used to determine the consequence of shear forces on regrowth.

7.3.2 Regrowth Experiment with Meso-3 Samples (Event 2)

Sludge was provided by Meso-3 and received on June 27, 2006. Event 2 utilized sludge from a different wastewater treatment plant to determine if regrowth effects hypothesized to occur from the laboratory shear/dewatering technique could be replicated using another sludge source. Laboratory shearing was performed at increasing amounts to determine the consequence of shear forces on resuscitation. Unfortunately, laboratory generated cake could not be compared to full-scale treatment plant cake samples (no cake samples were provided from Meso-3).

7.3.3 Regrowth Experiment with Meso-2 Samples (Event 3)

Cake and sludge was provided by Meso-2 and received on July 19, 2006. Similar to Event 1, Event 3 utilized sludge from the same wastewater treatment plant to determine if resuscitation and regrowth effects hypothesized to occur from the laboratory shear/dewatering technique could be replicated. However, in this case the sludge was allowed to incubate at 35°C for 6 days prior

to performing laboratory dewatering. After 6 days, the FC and *E. coli* densities had decreased to less than 10^2 MPN/g DS. The aged sludge was then utilized to determine if resuscitation effects hypothesized to occur from the laboratory shear/dewatering technique could be used to resuscitate non-culturable bacteria. Increasing levels of laboratory shearing was imparted to the cake to determine the consequence of shear forces on regrowth.

7.4 Methods and Procedures

Class B sludge and cake were provided by the Meso-2 and Meso-3 plants. Samples were shipped on ice by overnight FedEx shipment and were stored at 4°C prior to the start of the experiment which commenced on the same day as sample receipt. MPN analysis was performed on the sludge and cake using traditional presumptive media as described herein. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B.

7.4.1 Laboratory Dewatering and Shearing

A laboratory method was developed to simulate the shear in a high solids centrifuge. Homogenized sludge was conditioned using a high shear mixing with the plant-specific polymer provided by each wastewater treatment facility. The optimum polymer dose (OPD) was determined before conditioning the bulk of the sludge, as described in Section 7.4.1.2. Once conditioned, the sludge was batch centrifuged at 3,000 x*g* for 10 minutes at 20ºC, as described in Section 7.4.1.3. The dewatered pellets, which were now considered to be the cake, were then combined and sheared using a sterilized Kitchen-Aid® Food Grinder on high power, as described in Section 7.4.1.4. Additional shearing was performed by grinding the cake multiple times with the Kitchen-Aid® Food Grinder. The amount of shearing was denoted by the number of passes through the grinder. Bacterial quantification of the grinded, dewatered cake was assessed using MPN analysis as discussed in Section 7.4.2.

7.4.1.1 Biosolids Incubation

Biosolids were incubated at 35ºC for 14 days to observe bacterial growth patterns. Incubation occurred in a thermostatically controlled room without sample agitation.

7.4.1.2 Conditioning

The sludge was conditioned using cationic polymer provided by the plant. One liter of solids was placed in a baffled reactor, and polymer was added to the solution. The sludge was mixed at a predefined mixing intensity and time, to achieve a given energy input or shear, the Gt value, where “G” is the velocity gradient in reciprocal seconds and “t” is the time of mixing in seconds. The typical Gt value used for these experiments was 100,000 which has been shown to be an equivalent shear associated with high solids centrifugation (Higgins et al., 2006b; Murthy et al., 2004). After mixing, the capillary suction time (CST) was measured. This process was repeated with varying polymer dosages until the OPD was obtained, as measured by the polymer dose that produces the minimum CST.

7.4.1.3 Dewatering

The conditioned sludge was placed in sterile 250 mL Nalgene bottles and dewatered using a laboratory centrifuge at 3000 x*g*, for 10 minutes. After centrifuging, the supernatant was removed, and the cake was combined from the different tubes. Typically, about 200-300 g of wet cake were generated, with solids contents between 17 - 20%. This cake was then pressed using a laboratory scale belt filter press (Phipps and Bird). In order to maintain sterility during the dewatering procedure, the pellet was placed inside sterile absorbent pads while a pressure of 250 pound-foot was exerted on the pellet for 2 minutes to achieve cake solids in the range of 25-30% which is typical for full-scale centrifuges.

7.4.1.4 Processing

The wet cake samples were processed to simulate the high shear experienced in the full-scale centrifuge due to scrolling of the cake in the centrifuge. The cake was processed through a meat-grinder apparatus that pushes the cake forward using a scroll-conveyor, followed by extrusion through a small opening at the end of the conveyor. This meat grinder is an attachment on a KitchenAid® mixer. All equipment was sterilized by autoclaving or boiling for 15 minutes prior to use in the HSC process. Different amounts of shear were achieved by passing the cake through the grinder multiple times, either 5 or 10 passes through the grinder. These laboratory generated cakes are labeled as Bucknell University (BU) 0 Pass Cake (control without grinding), BU 5 Pass Cake, and BU 10 Pass cake. After processing, the cake samples were analyzed for FC and E. coli as described in Section 7.4.2.

7.4.2 Multiple Tube Fermentation Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Sludge samples (30.0 ± 0.1 mL) and cake samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). Serial dilutions (1:10) were prepared for liquid and solid samples. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media (traditional and enhanced). Five replicate tubes were inoculated for each dilution. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive LTB media (traditional and enhanced), the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG

media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under UVB lighting.

7.4.2.1 Traditional Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2005). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

7.4.2.2 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

7.4.3 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

7.5 Results and Discussion

Sludge and cake samples from the Meso-2 and Meso-3 plants were collected according to the methods and procedures presented in Section 7.4. Sludge and cake samples were collected from the Meso-2 and Meso-3 plants in June and July 2006. Sludge was dewatered and sheared

(grinded) prior to incubation at 35°C. The data presented includes dewatered and sheared cake samples with 0 passes, 5 passes, and 10 passes through the Kitchen-Aide® grinder. The dewatered and sheared cake samples are also discussed in relation to the digested sludge and centrifuged cake from the facility.

7.5.1 Regrowth Experiment with Meso-2 Samples (Event 1)

Culturing results for sludge and cake samples collected from full-scale digester and HSC at Meso-2 are presented in Figure 7–1 for TC, Figure 7–2 for FC, and Figure 7–3 for *E. coli* density. The samples from the digester and the initial cake sample had TC, FC, and *E. coli* densities less than the Class B biosolids requirements. In addition, no SI was observed which is defined as the increase immediately after dewatering (cake versus sludge). This is consistent with previous testing that showed SI does not typically occur with mesophilic processes.

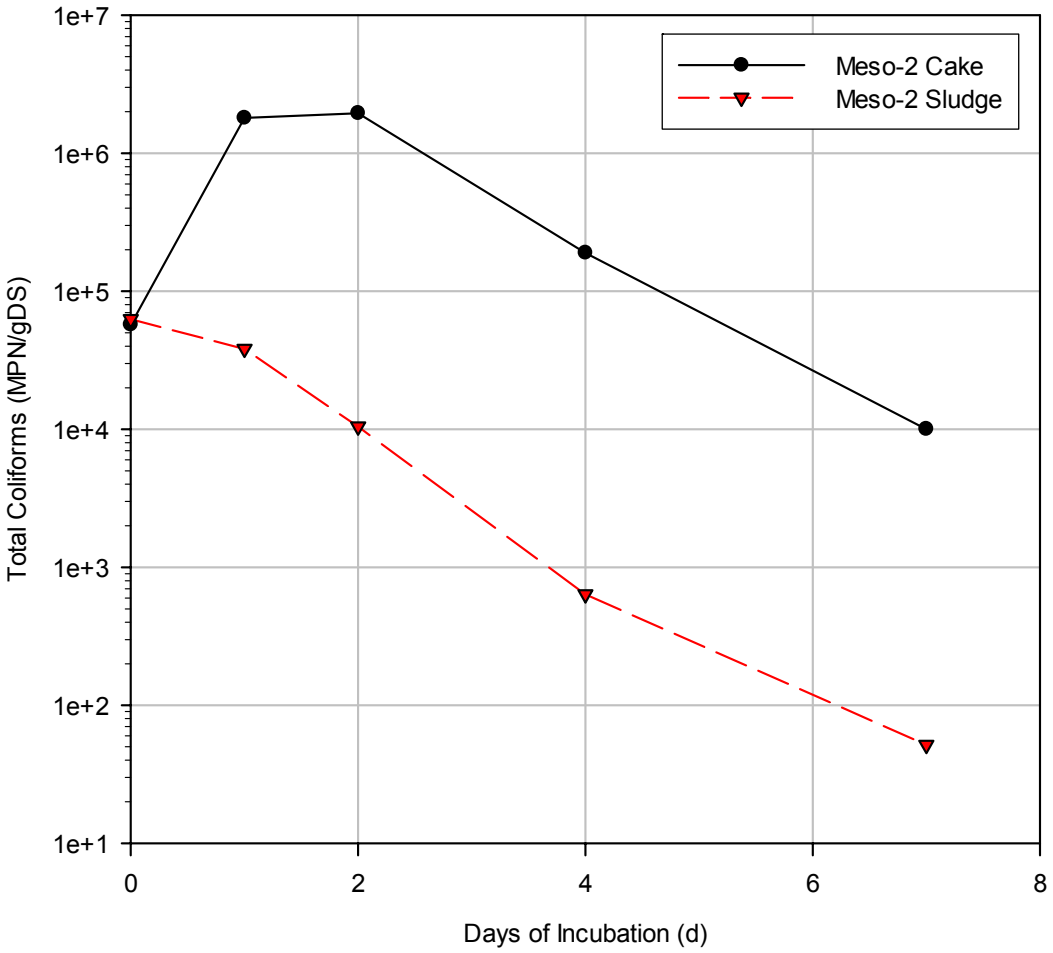

Figure 7–1: Total coliform results for Meso-2 cake and sludge samples (Event 1)

During cake storage, regrowth and die-off was observed. The TC densities in cake samples were initially observed at nearly 10^5 MPN/g DS and increased to 10^6 MPN/g DS within the first 48 hours before the population declined to 10^4 MPN/g DS on day 7. The Meso-2 cake samples had similar values for FC and *E. coli* density and showed a similar regrowth and die-off pattern.

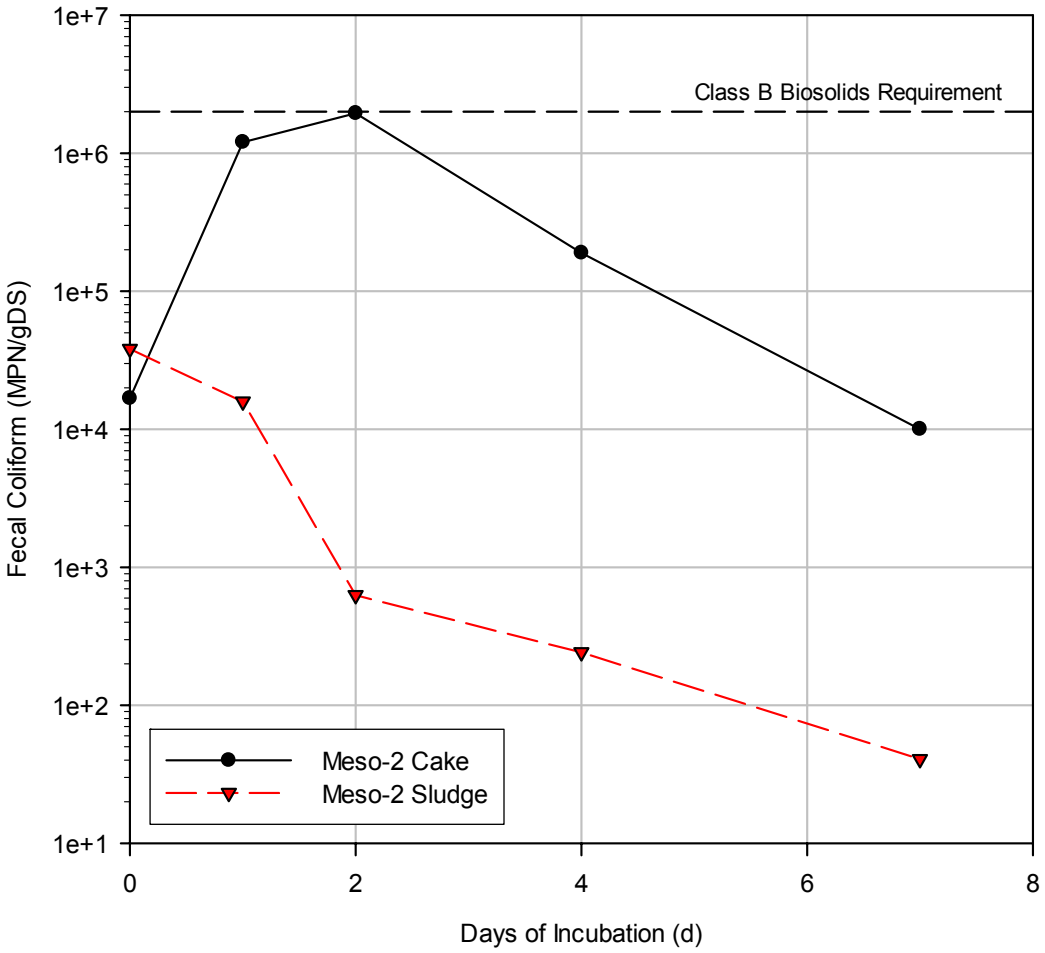

Figure 7–2: Fecal coliform results for Meso-2 cake and sludge samples (Event 1)

In comparison, the TC, FC, and *E. coli* densities in the Meso-2 liquid (undewatered) sludge continuously decreased during storage. During the 7 day sampling period, the TC density in the sludge sample decreased from nearly 10^5 to 10^1 MPN/g DS. The FC and *E. coli* densities in sludge samples were similar to the TC densities and showed a similar decrease during storage (see Figure 7–2 and Figure 7–3).

The regrowth curve of the cake fits the signature for FC and *E. coli* increases when biosolids are dewatered by HSC. First, the FC and *E. coli* densities in cake samples increase to a peak within 24 to 48 hours. Then, the FC and *E. coli* densities decrease during further storage.

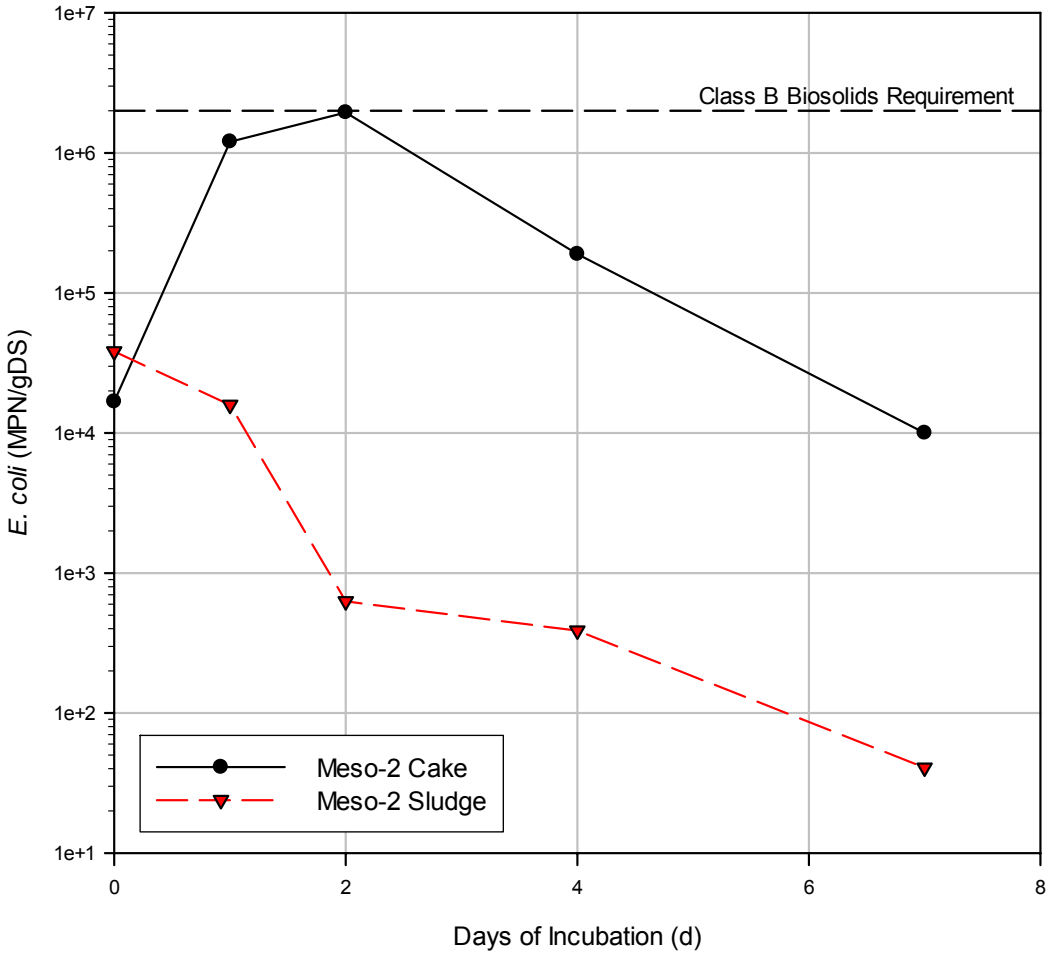

Figure 7–3: *E. coli* results for Meso-2 cake and sludge samples (Event 1)

In addition to sampling the Meso-2 sludge and cake samples directly, the liquid sludge was dewatered using the laboratory shearing method as discussed in Section 7.4.1. Culturing data results for the laboratory generated cake samples are presented in Figure 7–4 for *E. coli*. TC and

FC data has been omitted since the data trends and values are consistent with the data presented for *E. coli*. The *E. coli* results from the full-scale HSC cake (Meso-2 cake) are also shown on Figure 7–4 for comparison.

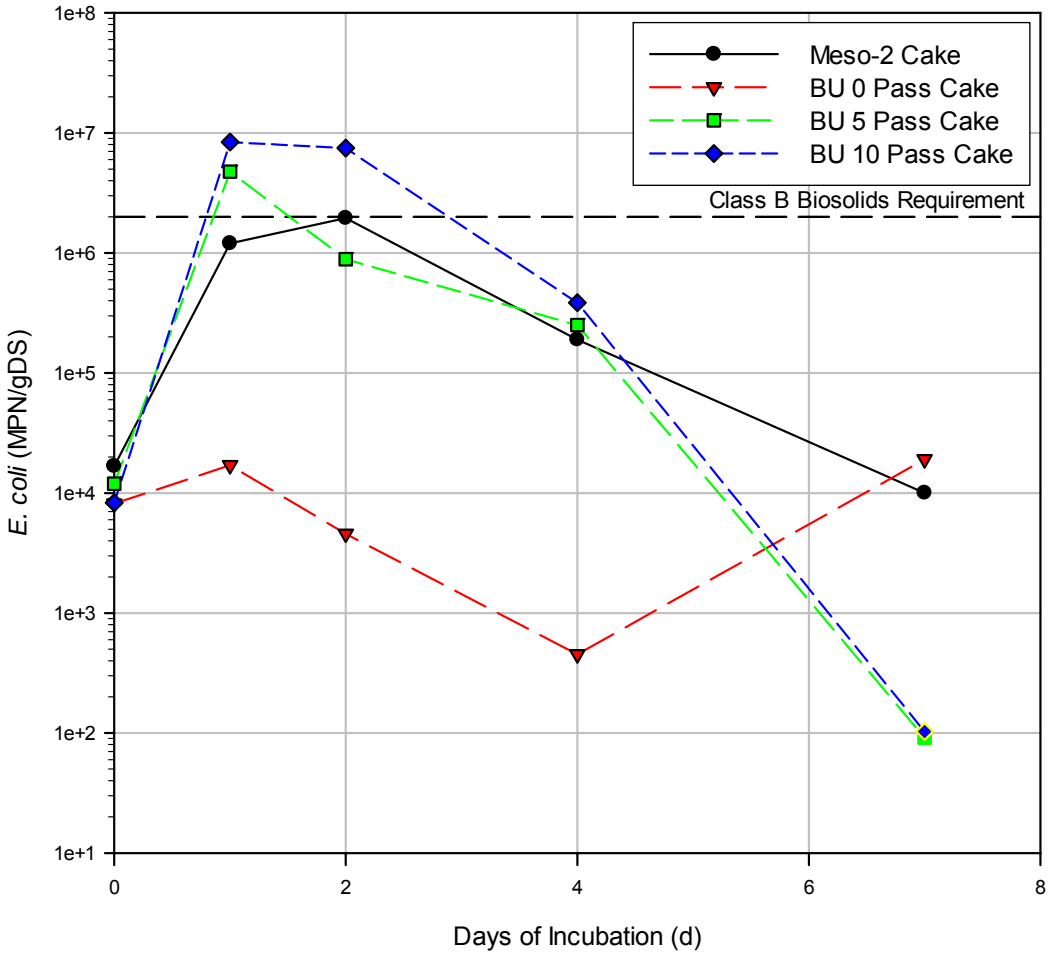

Figure 7–4: *E. coli* results for Meso-2 lab dewatering and shearing (Event 1)

The *E. coli* density immediately after laboratory shearing and dewatering (day 0) for the three laboratory generated cakes was comparable to the initial Meso-2 sludge sample. Therefore, the laboratory shearing technique does not appear to promote the SI phenomena. The BU 5 Pass

Cake and BU 10 Pass Cake closely follow the trend for the Meso-2 cake sample (Figure 7–4). The *E. coli* density in the BU 0 Pass Cake averaged 10^4 MPN/g DS and ranged between 10^2 to 10^4 MPN/g DS. The *E. coli* density for the BU 5 Pass Cake and BU 10 Pass Cake increased from 10^4 to 10^7 MPN/g DS within the first 48 hours. After peaking at 10^7 MPN/g DS, the *E. coli* density declined to 10^3 MPN/g DS on day 7. The *E. coli* density of the BU 5 Pass Cake and BU 10 Pass Cake were about an order of magnitude greater than the Meso-2 cake sample on days 1 and 2, which was also greater than the limit for Class B biosolids. Overall, the laboratory dewatering/shearing technique was able to produce a cake that had a similar regrowth pattern as the full-scale HSC cake.

Previous research had failed to develop a laboratory method to replicate the full-scale regrowth phenomena (Cheung et al., 2003; Monteleone et al., 2004; Qi et al., 2004). In this experiment, laboratory shear forces were introduced to Meso-2 sludge samples through the sludge conditioning and dewatering technique described in Section 7.4.1. Shear forces were measured by Gt value during conditioning and by the number of passes through a Kitchen Aide® Grinder. This laboratory procedure was utilized to generate laboratory dewatered cakes with various levels of shear. This research differed from previous efforts by including the shear effects of the conditioning process representing the sudden acceleration increase during centrifugation and of the grinding process representing the cake scrolling.

The BU 0 Pass Cake, which was the control sample for the sludge conditioning process, did not demonstrate the regrowth phenomena; although, the steady decline observed with sludges did not occur but rather the density remained relatively constant during the 7 days of storage. The BU 5 Pass and BU 10 Pass Cakes, which were generated by grinding the conditioned sludge to varying amounts, were observed to have *E. coli* density increases greater than the full-scale cake samples. This elevated regrowth of *E. coli* is a unique occurrence that has not been described previously in literature. Therefore the effects of this laboratory shearing procedure appear to

promote the regrowth of *E. coli* in a quantitative fashion that closely matches the full-scale cake produced from HSC. Specifically, it appears that the grinder shearing step, which mimics the scroll shear of the HSC, is key to the regrowth phenomena. Additionally, increasing amounts of grinding passes appears to be more effective in promoting the regrowth phenomena.

7.5.2 Regrowth Experiment with Meso-3 samples (Event 2)

A similar experiment was performed on a sample from the Meso-3 digester to confirm that sludges prepared with the laboratory shearing method developed regrowth of FC and *E. coli*. Culturing data collected during the Meso-3 lab dewatering and shearing experiment (June 28, 2006) is presented in Figure 7–5 for *E. coli*. TC and FC data has been omitted since the data trends and values are consistent with the data presented for *E. coli*. The storage of the Meso-3 sludge sample resulted in a decrease in the *E. coli* densities. Over the course of 7 days, the *E. coli* density in the sludge sample decreased from 10^4 to 10^2 MPN/g DS. This is similar to the results for Meso-2 presented in Section 7.5.1.

Three laboratory prepared cake samples, designated as BU 0 Pass Cake, BU 5 Pass Cake, and BU 10 Pass Cake, were generated by the dewatering process discussed in Section 7.4.1. From these three cake samples, two diverse data sets appear. The *E. coli* density for the BU 0 Pass Cake increased relatively slowly from 10^3 to 10^5 MPN/g DS over the 7 day period. The BU 0 Pass Cake prepared from Meso-3 liquid sludge had an order of magnitude increase in *E. coli* density after 7 days of storage (see Figure 7–5). The BU 0 Pass Cake prepared from Meso-2 liquid sludge did not have an increase in *E. coli* density after 7 days of storage (see Figure 7–4). SI for *E. coli* was not encountered on day 0. The BU 0 Pass Cake was not subjected to shearing through the Kitchen-Aide® grinder; however, it was exposed to considerable shear (Gt=100,000) during conditioning. This shear from the conditioning process likely contributes to the regrowth observed in the BU 0 Pass Cake.

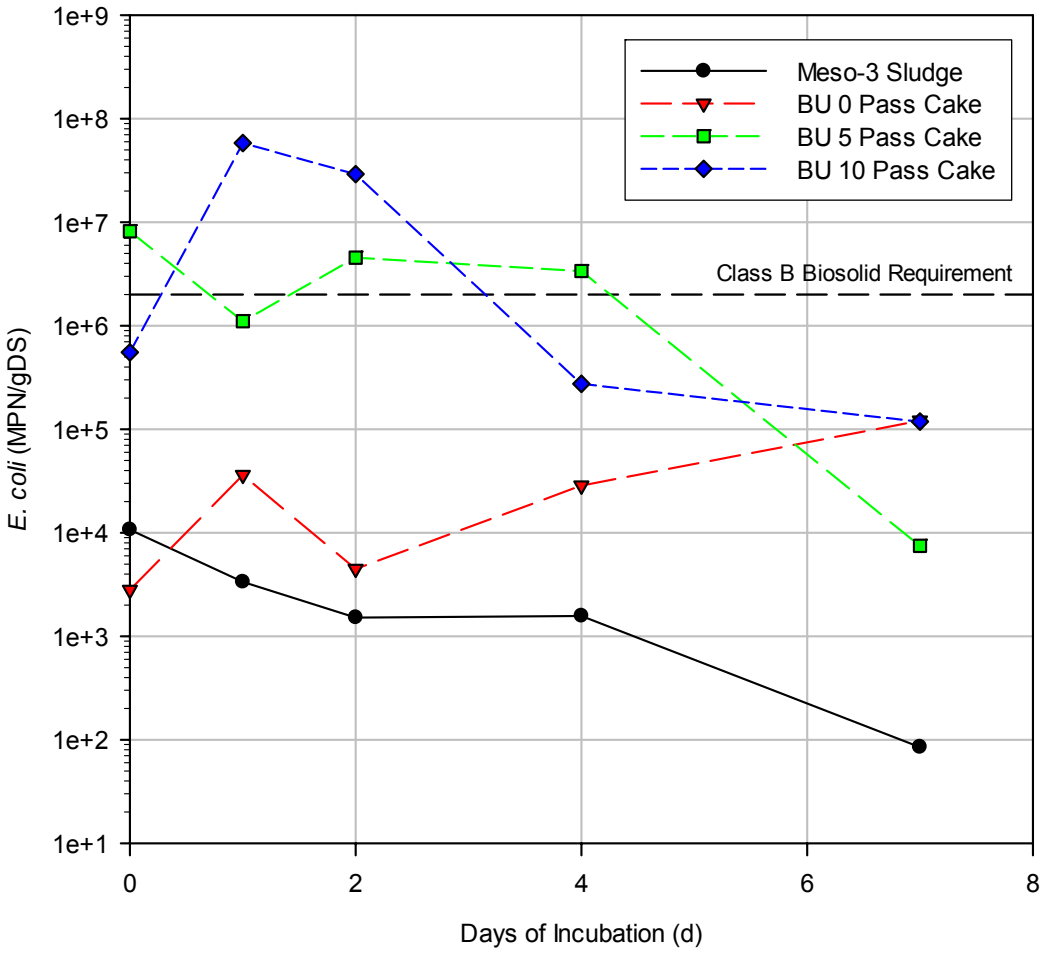

Figure 7–5: *E. coli* results for Meso-3 lab dewatering and shearing (Event 2)

The BU 5 Pass Cake and 10 Pass Cake had SI on day 0 where the *E. coli* densities are 2 to 3 orders of magnitude higher than the Meso-3 sludge indicating that SI occurs after the laboratory dewatering/shearing process. Based on previous data for Meso-3 sludge and cake samples, SI was not common at this utility which uses mesophilic anaerobic digestion prior to HSC dewatering. The *E. coli* density in the BU 5 Pass Cake was 10^7 MPN/g DS on day 0 remained relatively constant during the first 4 days of storage and then decreased to about 10^4 MPN/g DS

on day 7. In comparison, the BU 10 Pass Cake had 10^6 MPN/g DS on day 0 that increased to 10^8 MPN/g DS on day 1 and decreased after day 1 continuously to 10^5 MPN/g DS on day 7.

This experimental data supports the hypothesis that the dewatering process produces an increased density of *E. coli* by the regrowth phenomena. Additionally, this experiment data also identified that the SI phenomena occurred after laboratory shearing and dewatering which was not anticipated. The laboratory shearing technique discussed in Section 7.4.1 was able to produce comparable results from both Meso-2 and Meso-3 laboratory generated cakes. Increasing amounts of shear result in increased amounts of regrowth. Additionally, SI was observed from laboratory generated cake samples only from Meso-3 immediately after generation. One possible explanation for the increased regrowth with increasing amounts of shear was hypothesized by Hendrickson et al. (2004), who suggested that shearing forces were responsible for the breakup of flocs which would increase the surface area where bacteria could culture. However, Qi et al. (2004) concluded that the increased surface area caused by floc breakup did not result in increased culturability of bacteria. The release of substrate from within floc particles is an alternate explanation. This alternate hypothesis describes how substrates that fuel regrowth are released during floc breakup caused by shear forces. Higgins et al. (2006b) showed that HSC cake had pools of protein and polysaccharides that were bioavailable and degraded during cake storage. A subsequent experiment is discussed in Chapter 8 to further investigate the substrate release hypothesis.

7.5.3 Regrowth Experiment with Meso-2 Samples (Event 3)

A second sampling event with Meso-2 was performed to further investigate the laboratory shearing methods. Culturing data collected for the full-scale sludge and cake samples are presented in Figure 7–6 for *E. coli*.

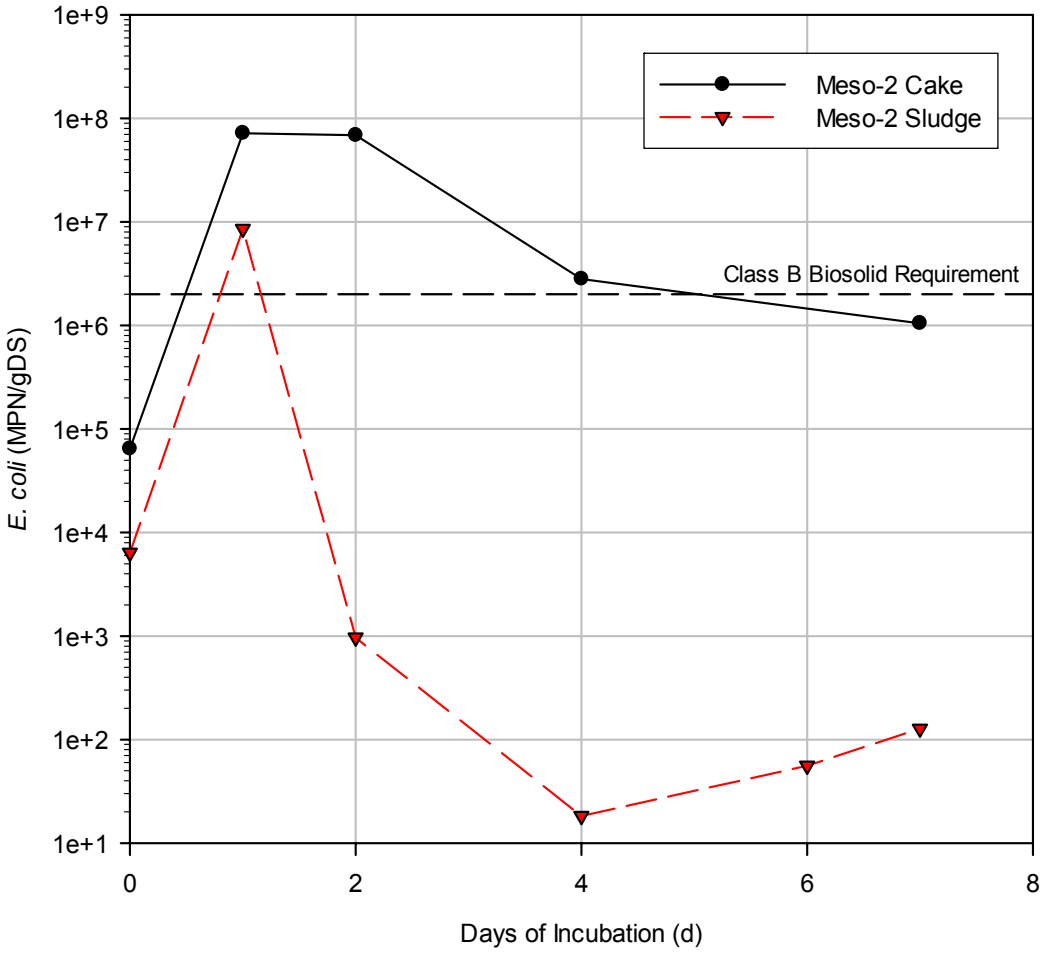

Figure 7–6: *E. coli* results for Meso-2 cake and sludge Samples (Event 3)

TC and FC data has been omitted since the data trends and values are consistent with the data presented for *E. coli*. The trends are similar to the first sampling event (see Section 7.5.1), where the *E. coli* density for full-scale sludge and cake samples were initially less than the Class B biosolids requirements (10^3 MPN/g DS and 10^4 MPN/g DS, respectively). However, after 24 hours of storage, the cake samples had *E. coli* densities greater than the Class B biosolids requirements (7 x 10^7 MPN/g DS). Additionally, sludge samples had *E. coli* densities that increased to nearly 10^7 MPN/g DS after 24 hours of storage. It is not known why the elevated *E.*

coli density is present on day 1. The first sampling event with Meso-2 sludge did not show this regrowth of *E. coli* in sludge samples. Finally, some SI was observed (1 order of magnitude) in the full-scale cake sample, which is defined as the increase immediately after dewatering (cake versus sludge). This is inconsistent with previous testing of Meso-2 biosolids which showed SI did not occur.

The Meso-2 laboratory dewatering and shearing experiment data is presented as Figure 7–7 for *E. coli* densities. TC and FC data has been omitted since the data trends and values are consistent with the data presented for *E. coli*. The *E. coli* density within 24 hours of storage for the BU 0 Pass Cake increased by about 3 orders of magnitude to 10^6 MPN/g DS, which was similar to the increase in the full-scale sludge *E. coli* density. The *E. coli* density in the BU 0 Pass Cake samples had densities that returned to day 0 levels after a one-day peak on day 1. The BU 5 Pass Cake and BU 10 Pass Cake increased by about 4 orders of magnitude to 10^7 MPN/g DS within the first 24 hours. The *E. coli* density then decreased steadily, although at different rates, until day 7. Therefore, the effect of the laboratory dewatering procedure, with a shearing process that mirrors high speed centrifugation, appears to produce cake samples that cause a regrowth in *E. coli* density. The laboratory shearing process was not effective in reproducing the SI observed between the full-scale sludge and cake samples on day 0.

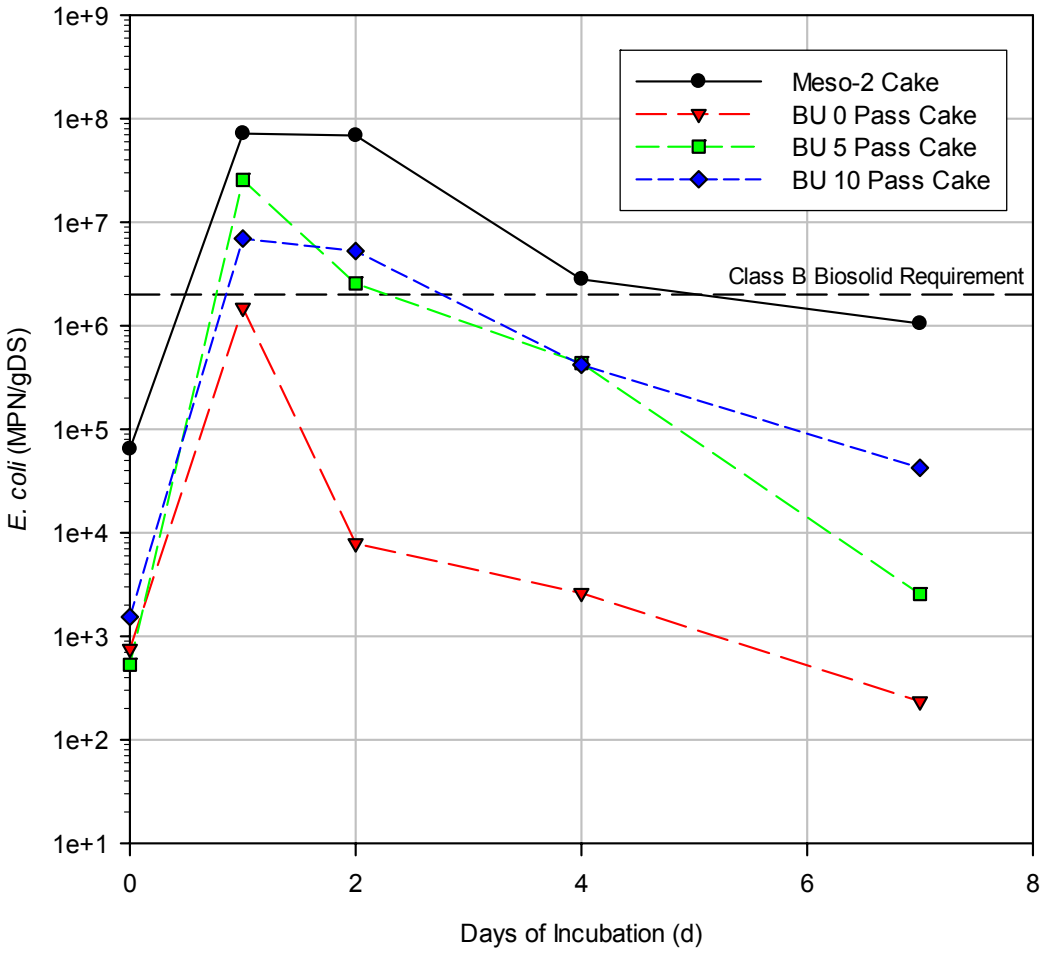

Figure 7–7: *E. coli* results for the Meso-2 lab dewatering and shearing samples (Event 3)

On day 6 of the experiment, a sample of the stored Meso-2 sludge was dewatered using the laboratory shearing process to determine if the regrowth of *E. coli* in stored sludge samples could be stimulated. The *E. coli* density for the laboratory dewatered cake that was prepared from the stored sludge is presented as Figure 7–8. The *E. coli* densities presented for Meso-2 Sludge and Cake are the same as presented in Figure 7–6 for days 0, 1, 2, 4, and 7.

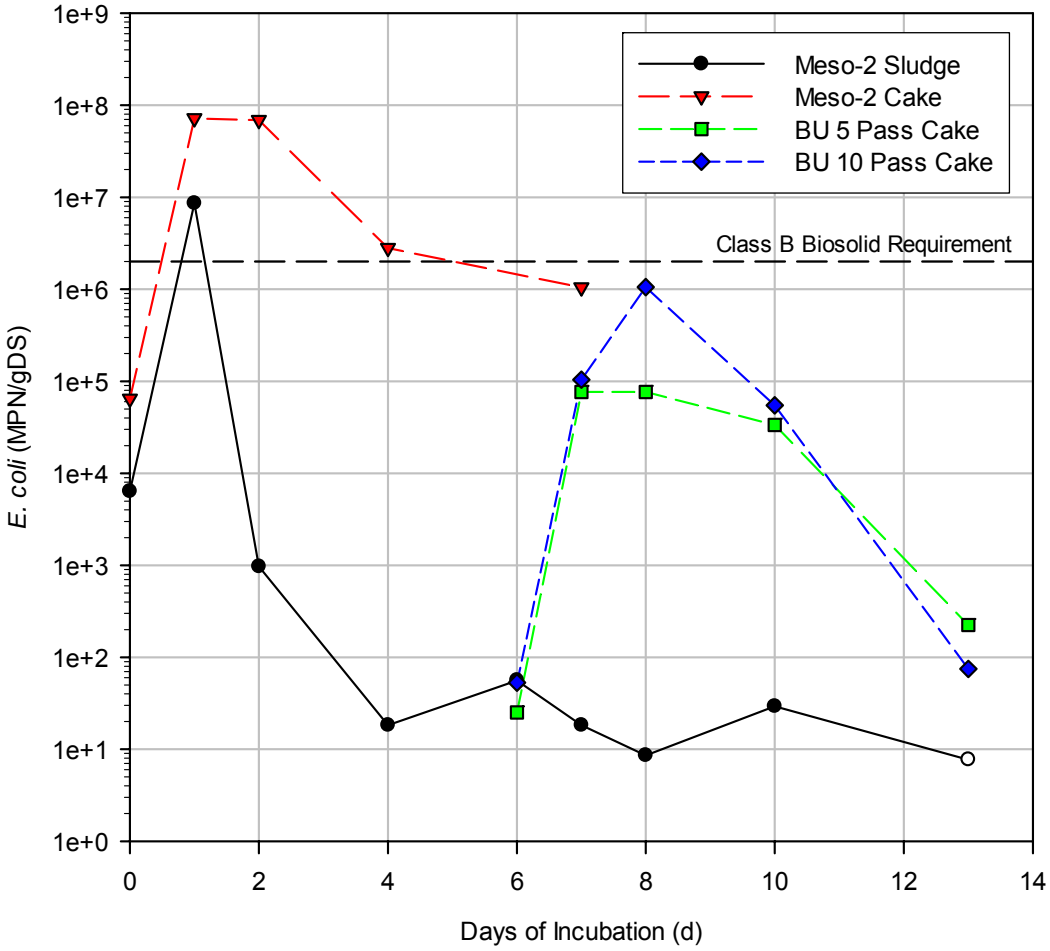

Figure 7–8 - *E. coli* results for the Meso-2 storage and lab dewatering and shearing samples (Event 3) (open symbols indicate non-detect value)

After the laboratory shearing and dewatering process, the *E. coli* densities in the cake increased by 3-4 orders of magnitude within 1-2 days of storage. *E. coli* densities from the BU 10 Pass Cake yielded the highest density of 10^6 MPN/g DS on day 8. Between day 10 and 13, the *E. coli* densities for the BU 5 Pass Cake and BU 10 Pass Cake decreased from 10^5 to 10^2 MPN/g DS, similar to the densities of the Meso-2 sludge prior to the laboratory dewatering process. These increases in *E. coli* density after the laboratory shearing and dewatering of stored sludge samples containing near non-detect *E. coli* densities indicates that the laboratory dewatering process is

effective in causing regrowth similar to full-scale HSC dewatering. This regrowth may be a consequence of bioavailable nutrients being released. Only regrowth was observed for the cake samples dewatered with the laboratory shearing process. The SI observed in the full-scale HSC dewatered cake was not replicated by the laboratory shearing and dewatering process with either the initial sludge (day 0) or the stored sludge (day 6).

7.6 Research Significance

The literature reviewed for this experiment described various laboratory methods to mimic the type of shear imparted to sludge during the HSC process. As discussed by Cheung et al. (2003), shaking, sonification, and stomaching were not able to recreate the SI and regrowth phenomena observed by full-scale HSC. The research presented by Monteleone et al. (2004) was not able to demonstrate that the centrifugal g-force imparted by laboratory-scale centrifugation could induce the SI and regrowth phenomena. Qi et al. (2004) was not able to promote SI and regrowth by imparting mechanical shear with kitchen blender to breakup flocs and create more surface area for bacterial growth.

This experimental procedure investigated two other types of shear imparted during dewatering: shear from sludge conditioning with polymer and shear from screw conveyance during the dewatering process. Sludge samples were conditioned with polymer by centrifugation for a set time to produce a Gt value of 100,000. The amount of polymer was determined based on finding the OPD based on minimum CST. The conditioned sludge samples were then dewatered after centrifugation using a laboratory-scale belt filter press. The dewatered cake samples were then ground with a Kitchen Aide® Grinder at a varying number of passes to mimic a screw conveyor.

The results from these experiments with Meso-2 and Meso-3 sludge indicate that, by imparting shear based on a measured Gt value and on the number of passes through a grinder, the SI and

regrowth phenomena were replicated in the laboratory. It is important to note that the shear from grinding is important for the replication of the SI and regrowth phenomena; without this shear, the SI and regrowth phenomena were not observed. This is an important finding because research performed to date has been unable to replicate the SI and regrowth phenomena in the laboratory. Additionally, increasing the number of times the cake was passed through the grinder produced higher densities of FC and *E. coli*. In many cases, the laboratory cakes generated by conditioning and grinding sludge were able to produce higher FC and *E. coli* densities when compared to the full-scale cakes.

The experiment using Meso-2 sludge also identified that the conditioning and grinding process could be used to induce regrowth of FC and *E. coli* in stored sludge samples. As described in Chapter 3, the culturability of *E. coli* decreased to non-detection levels after a week of storage while the concentration of DNA remained near constant for a month after storage. Having a laboratory method whereby regrowth can be induced is an important step toward enumerating the actual population of live cells.

7.7 Conclusions

The regrowth of FC and *E. coli* has been observed at utilities that use centrifuge dewatering of mesophilic sludge. In this experiment, samples collected from Meso-2 and Meso-3 were observed to have similar FC and *E. coli* regrowth curves during storage. Sludge samples from these utilities were observed to have generally decreasing FC and *E. coli* densities during storage experiments. Laboratory generated cake samples were prepared by introducing shear during laboratory conditioning and cake grinding. To impart laboratory shear, sludge samples were conditioned with polymer for a Gt of 100,000 and grinded with a Kitchen Aide® Grinder with a varying number of passes. These laboratory generated cake samples were capable of reproducing the regrowth phenomena, and to some degree the SI phenomenon, observed in the

Meso-2 and Meso-3 cake samples. The conclusions related to the full-scale, laboratory generated, and stored cake and sludge samples are discussed herein.

The following observations were made from the mesophilic sludge and HSC dewatered cake samples collected at full-scale utilities:

- HSC induces the regrowth phenomena in FC and *E. coli* with peak densities occurring within 24-48 hours of dewatering.

- During the storage of cake samples, the peak *E. coli* density was typically 2-3 orders of magnitude higher than the *E. coli* density of the digested sludge.

The following conclusions can be made from the experimental laboratory dewatering process:

- *E. coli* densities in laboratory dewatered cakes that included shear imparted to the liquid had *E. coli* densities that were typically 1-2 orders of magnitude higher than the full-scale HSC dewatered cake samples and 4-5 orders of magnitude higher than the full-scale sludge samples.

- Sludge samples that were only conditioned and dewatered had lower *E. coli* density trends than the sludge samples there were conditioned, dewatered, and sheared with a grinder.

- Conditioned sludge samples had a 1-2 orders of magnitude increase in *E. coli* density when compared to the full-scale sludge samples.

- The laboratory dewatering process was successful in producing regrowth of *E. coli* from sludge samples that were stored for 6 days.

Chapter 8: Investigating the Use of Coagulants to Reduce Regrowth at Full-Scale Wastewater Treatment Facilities

8.1 Introduction

Many researchers are investigating various aspects of wastewater treatment plant performance to improve design specifications and to reduce biosolids disposal cost, bacteria populations, and odor generation. Several researchers have identified that coagulant addition during sludge dewatering impacts both odor generation and bacteria population (Erdal et al., 2004; Higgins et al., 2006). Research performed by Erdal et al. (2004) identified that low-level lime dosing could control odor and prevent regrowth in FC populations. Erdal et al. (2004) also observed that lime dosing, which maintained a cake pH value below 11, successfully reduced odor generation. FC populations were found to decrease over time without the regrowth phenomena occurring when cake pH values were greater than 8.5 (Higgins et al. 2006; Erdal et al., 2004).

8.1.1 Bacteria Regrowth Controlled by Lime Coagulation

In the experiment by Erdal et al. (2004), mesophilic digested solids were conditioned with lime after HSC but prior to screw conveyance. Data indicated that liquid lime dosing that ranged from 3-9% was effective in controlling regrowth over a period of 35 days. FC densities in cake samples were found to decrease immediately with the 7% and 9% dosage rates to levels below the Class B biosolids requirement

Erdal et al. (2004) also investigated the effects on bacteria regrowth that occurred when both lime and ferric chloride were added prior to screw conveyance. Both lime and ferric chloride are common additives used to control odor and solids content. FC density data indicated that

regrowth occurred when low doses of lime (3%) and ferric chloride (2%) were utilized. Once lime doses were increased above 5%, bacterial regrowth did not occur. Although higher doses of lime were more effective in controlling FC density, the high doses of lime also caused increased odor.

From their research, Erdal et al. (2004) was capable of demonstrating that lime and ferric chloride coagulants were capable of eliminating the regrowth phenomena in wastewater samples after full-scale centrifuge dewatering processes. Their data identified that increases in the pH of biosolids had the greatest effect on minimizing bacteria regrowth.

8.1.2 Substrate Binding Promoted by Aluminum Sulfate Coagulation

One possible hypothesis that explains the observations from Erdal et al. (2004) was first discussed by Dental et al. (1987). In their research, Dentel et al. (1987) determined that aluminum sulfate (alum) salts were able to bind polysaccharides and proteins during simulated wastewater coagulation. This simulation was conducted by coagulating the proteinaceous extract from corn and the polysaccharides present in cellulose with alum at varying doses. Alum coagulation was determined to be effective in binding cellulose and proteins at both high and low alum doses.

Since dewatering processes commonly utilize coagulants, understanding how these chemicals interact with organics in solution is important when discussing the regrowth phenomena. Dentel et al. (1987) concluded that proteins and polysaccharides are bound by coagulants in simulated wastewater solutions. These proteins and polysaccharides are the nutrient sources required for bacterial growth. If these nutrients are bound by coagulants instead of being bioavailable, bacterial growth would be reduced. Erdal et al. (2004) identified that bacterial regrowth was diminished when coagulant addition was applied after the centrifuge dewatering processes. Therefore, the absence of the regrowth phenomena after centrifugal dewatering and coagulant addition as observed by Erdal et al. (2004) can possibly be explained in part by the coagulant

binding of nutrients discussed by Dentel et al. (1987); although, the higher pH from lime addition likely also contributed.

8.2 Research Needs

Coagulation with lime and ferric chloride has been shown to decrease regrowth in wastewater samples (Erdal et al., 2004). Although regrowth was controlled by lime coagulation and pH adjustment, the possibility that other coagulants are effective in controlling regrowth by different mechanisms is conceivable. As a coagulant, alum salts have already been shown to bind polysaccharides and proteins in simulated wastewater slurries (Dentel et al., 1987). One possible mechanism for controlling regrowth is the binding of nutrients required for bacterial growth to coagulants. No assessments have been conducted to determine if bound substrates, which are presumed not to be bioavailable, could restrict the regrowth phenomena by starving bacteria of needed nutrients for growth.

8.3 Research Objectives

This research investigates a method for inhibiting bacteria regrowth by binding nutrient sources in wastewater samples with coagulants during centrifuge dewatering at a full-scale treatment plant. Cake and sludge samples were sampled at Meso-2, an anaerobic, mesophilic wastewater treatment plant utilizing alum coagulation and HSC. Regrowth problems after HSC and cake storage have been described previously (Section 2.5.5). Various doses of alum were used during full-scale operation to generate cake samples for bacterial analysis by SCM. This experiment will investigate whether alum coagulation effectively binds substrates such that bacteria have less nutrients bioavailable which may inhibit regrowth. If the substrate binding hypothesis is true, alum addition should decrease the enumerations of FC and *E. coli* as cake solids are stored.

8.4 Methods and Procedures

Class B sludge and cake were sampled at Meso-2. Samples were transported on ice and were stored at 4°C prior to the start of the experiment which commenced within 24 hours of sample collection. MPN analysis was performed on the sludge and cake using traditional presumptive media as described herein. Total percent solids analysis of the sludge and cake samples was performed by SM 2540B.

8.4.1 Alum Amendment during High Solids Centrifugation

Meso-2 incorporated three doses of alum into cake produced by HSC. Once the HSC was operating at normal parameters, control cake and sludge samples were collected. Next in progressive succession, alum cakes containing 0.5%, 2%, and 4% were produced. Sufficient amounts of alum amended cake were generated so that cake exiting the screw conveyor was representative of the alum dose. Samples were collected from each alum dose trial.

8.4.2 Multiple Tube Fermentation Culturing Technique

Sample preparation, serial dilutions, and culturing methods were conducted in accordance with SM 9221B and EPA Method 1680. Sludge samples (30.0 ± 0.1 mL) and cake samples (30.0 ± 0.1 g) were homogenized with 270 mL sterile PBS dilution water (1:10 dilution) in a sterile blender for 2 minutes (EPA Method 1680). Serial dilutions (1:10) were prepared for liquid and solid samples. One milliliter of each serial dilution was aseptically transferred into 10 mL sterile LTB media (traditional and enhanced). Five replicate tubes were inoculated for each dilution. Triplicate analysis of each dilution set was performed for statistical analysis. Presumptive cultures were incubated in a 35°C ± 0.5°C water bath. At 24 ± 2 hours, presumptive cultures were swirled gently and examined for color change (purple to yellow indicating a positive

reaction). At 48 ± 3 hours, final assessment of color change was performed prior to transfer to the confirmation phase.

From the presumptive LTB media (traditional and enhanced), the three most dilute serial dilutions with positive detections were aseptically transferred from LTB broth into confirmatory EC-MUG media using a sterile wooden stick for FC and *E. coli* conformational culture analysis. Confirmatory cultures were incubated in a 44.5°C ± 0.2°C water bath for 24 ± 2 hours. FC detection was identified by gas buildup within the Durham tube. *E. coli* detection was ascertained by visual observation of EC-MUG media fluorescence under UVB lighting.

8.4.2.1 Traditional Presumptive Media

Quantification of TC was performed according to SM 9221B and EPA Method 1680 (Eaton, 1995; EPA, 2005). LTB (Difco, Sparks, MD) was supplemented with 0.01 g/L of bromocresol purple (Sigma Aldrich Co., St. Louis, MO) for colorimetric analysis of TC as described in SM 9221B.

8.4.2.2 Confirmatory Media

EPA Method 1680 was modified for FC and *E. coli* quantification using the proposed SM 9921F (Eaton, 1995). EC-MUG media (Difco), which contains the fluorogenic substrate MUG, was used in place of the EPA Method 1680 specified EC Media (Difco). A Durham tube was placed into the EC-MUG tubes to enumerate FC as described in SM 9921E (Eaton, 1995).

8.4.3 Most Probable Number Analysis

MPN statistics were calculated with the EPA Most Probable Number Calculator (EPA, 1996) with a 95% confidence level. MPN statistics were normalized for moisture content by converting the MPN/mL (wet weight) to MPN/g total solids (dry weight) using the percent total solids.

8.5 Results and Discussion

Meso-2 cake and sludge samples yielded the same starting concentration for *E. coli* density as determined by SCM (Figure 8–1). After one day of storage, *E. coli* density increased from 2 x 10^4 to 5 x 10^6 MPN/g DS, which was characteristic of the regrowth phenomena. Sludge data was not collected after initial sampling since Meso-2 sludge samples have demonstrated consistently a decline in FC and *E. coli* density with time (see Chapter 7). TC and FC data plots were not presented because of the consistency with *E. coli* density trends. On day 2, *E. coli* concentrations in Meso-2 cake samples peaked at 10^7 MPN/g DS. Between day 2 and day 7, *E. coli* density decreased to 10^5 MPN/g DS. As indicated by the Meso-2 cake data, Class B requirements were exceeded on day 1 through day 4 of storage.

E. coli densities for alum amended cake samples are presented in Figure 8–2. TC and FC data plots were not presented because of the consistency with *E. coli* density trends. Meso-2 cake samples with varying amounts of alum produced consistent starting concentrations, which were also consistent with the control cake. After 2 days of incubation, peak *E. coli* densities were observed, which is indicative of the regrowth phenomena. Cakes with 0.5% and 2% alum dosing produced *E. coli* densities of 5 x 10^7 MPN while the control and 4% alum dosed cakes yielded enumerations of 10^7 MPN/g DS. After day 2, the *E. coli* density in the control and 4% alum dosed cakes decreased below the Class B biosolids requirement. The *E. coli* density in the 0.5% alum dosed cake declined steadily but not below Class B biosolids requirements. Conversely, the *E. coli* density in the 2% alum dosed cakes maintained the same *E. coli* density as the peak concentration.

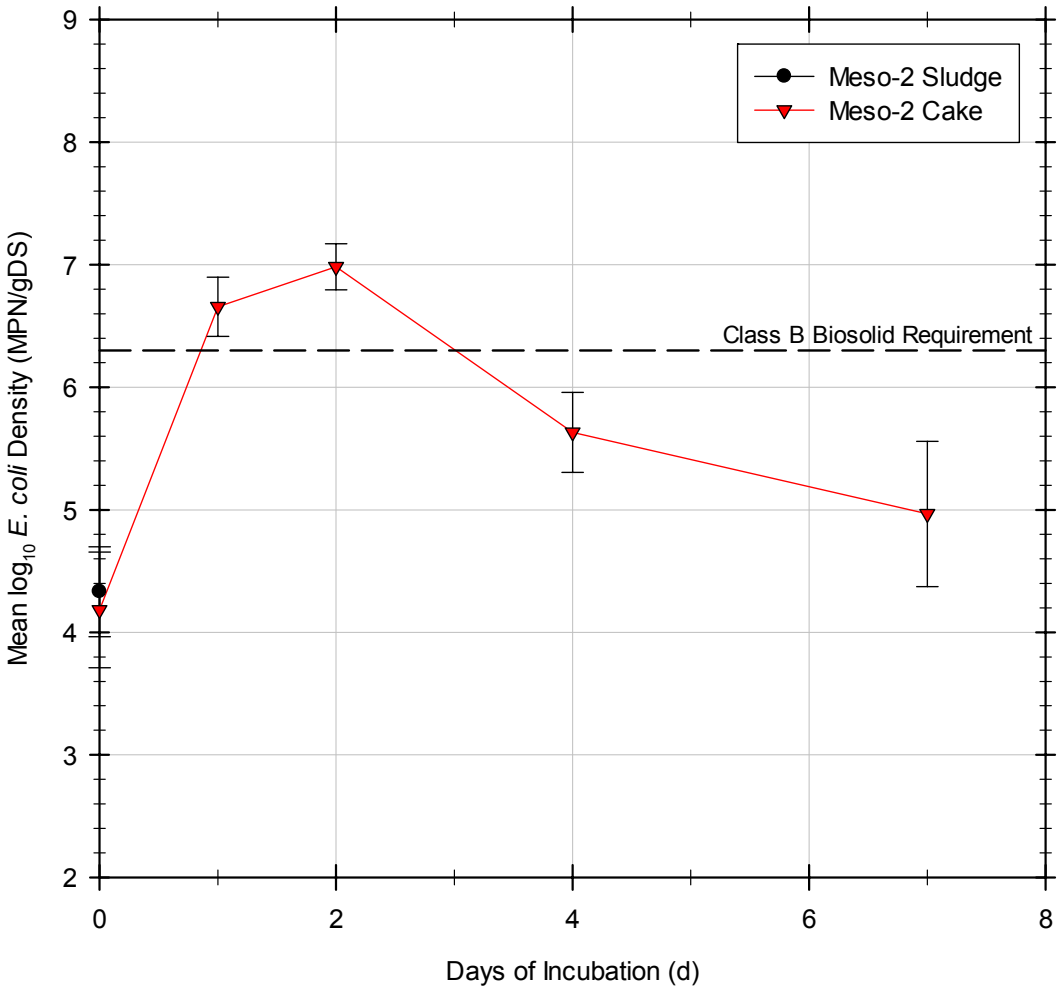

Figure 8–1: *E. coli* results for Meso-2 cake and sludge samples (Error bars represent one standard deviation)

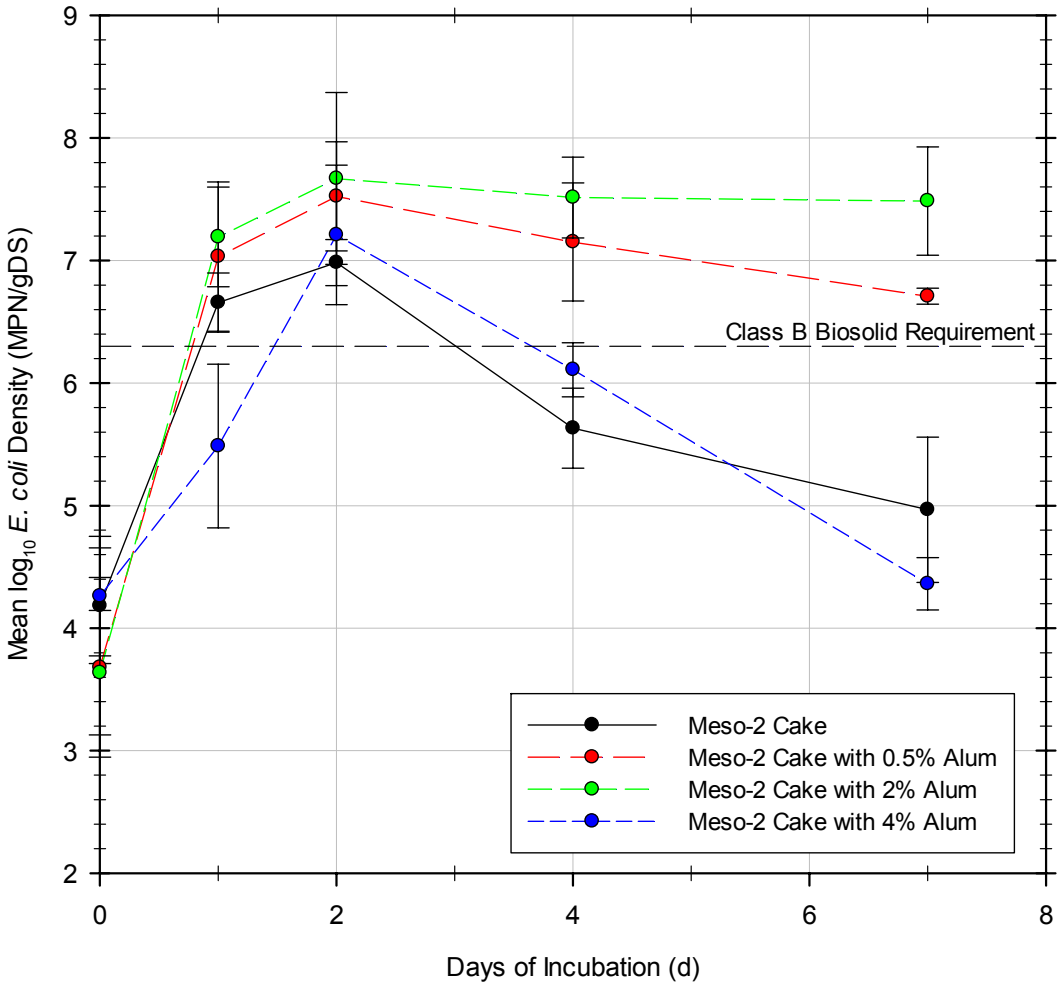

Figure 8–2: *E. coli* results for Meso-2 cake produced with varying amounts of alum (Error bars represent one standard deviation)

Although the 4% alum dose appeared to have little effect on regrowth when compared to the control, the 0.5% alum dose produced higher densities of *E. coli* indicative of regrowth. However, the elevated *E. coli* density in the 0.5% alum dosed cake was not sustained during storage. The 2% alum cake appeared to be the optimal dose since the *E. coli* density increased and was sustained until day 7 of storage.

8.6 Research Significance

As discussed by Erdal et al. (2004), the addition of low-level lime dosing after centrifuge dewatering was able to control *E. coli* density during storage. Based on the findings from Erdal et al. (2004), it was hypothesized that coagulants may bind nutrients in solution making them not bioavailable. For this experiment, alum was used during dewatering instead of lime during coagulation to determine if FC and *E. coli* densities could be controlled during storage or if regrowth would occur. The effect of adding alum was not comparable to low-level lime dosing because FC and *E. coli* regrowth occurred. This contradictory finding implies that not all coagulants have the same effect on the regrowth phenomena. The hypothesis that coagulants bind substrates thus starving bacteria of needed nutrients for regrowth appears to be more complex. Different coagulants may have yet unknown factors that inhibit or induce the regrowth phenomena. This experiment identified that alum coagulation increased the regrowth of FC and *E. coli*. From a standpoint of meeting Class B biosolids requirements, alum addition appears to promote the regrowth of FC or *E.* coli, making attainment of Class B requirements more difficult.

8.7 Conclusions

The addition of alum to a full-scale centrifugal dewatering process at Meso-2 appears to have a negative consequence for meeting the Class B biosolids requirements. From this experiment, the following conclusions can be made:

- Alum addition does not appear to prevent the regrowth phenomena through the binding of nutrient materials.

- Data illustrated that cake with a 2% alum dose promoted regrowth for 5 days as observed by sustained *E. coli* density.

- The 4% alum dose produced *E. coli* densities with regrowth and die-off trends similar to the non-amended cake.

This experiment does not provide conclusive evidence that substrate binding from alum coagulation is not a potential mechanism required to control bacterial resuscitation during centrifugal dewatering and biosolids storage. More research should be conducted to determine if substrate binding or some other unknown mechanism is occurring to promote regrowth.

Chapter 9: Conclusions

Conclusions concerning the investigations into enhanced media supplements, detoxification of inhibitory agents, the time and temperature relationship for meeting Class A biosolids requirements, laboratory shearing methods to promote regrowth are presented in brief:

- Analysis of the *E. coli* density by SCM and qPCR indicated that *E. coli* enumerations are significantly undercounted by SCM. When sampled by qPCR, the concentration of *E. coli* after anaerobic digestion remained 4 orders of magnitude higher than the non-detect levels identified by SCM.

- The SI phenomenon was most prevalent with biosolids generated by anaerobic thermophilic digesters and centrifuge dewatering processes.

- The regrowth phenomena was most frequently observed at utilities utilizing centrifuge dewatering processes after either thermophilic or mesophilic anaerobic digestion.

- Because of the SI and regrowth phenomena, biosolids produced according to the United States Environmental Protection Agency time and temperature requirements for Class A biosolids demonstrated up to a 5 order of magnitude increase in FC and *E. coli* concentrations after centrifuge dewatering.

- Culture media, supplemented with catalase, α-ketoglutaric acid, or sodium pyruvate to degrade peroxides in biosolids media, did not promoted resuscitation of VNC *E. coli*.

- Neither C6-HSL, 3-oxo-C8-HSL, or norepinephrine provided resuscitation effects for VNC *E. coli*.

- To remove inhibitory agents and toxicants, a cell washing technique was employed prior to performing SCM; however, cell washing may have increased cellular stresses since TC densities were diminished compared to control samples.

- The addition of bentonite and exponential growth phase *E. coli* cell-free supernatant to culturing media was not able to increase culturability of *E. coli* compared to the control.

- Fecal coliform and *E. coli* densities in laboratory prepared cake samples were both observed to be an order of magnitude higher than full-scale dewatered cakes.

- The laboratory-scale conditioning, dewatering, and shearing process was able to induce regrowth in FC and *E. coli* populations that resulted in cell densities in the laboratory generated cake samples that were 1-2 orders of magnitude higher than full-scale cake samples and 4-5 orders of magnitude higher than full-scale sludge samples.

- The addition of 2% aluminum sulfate to high solids centrifugation cake produced an increased regrowth of FC and *E.coli* that was sustained for 5 days *coli* during a full-scale experiment.

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