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**FINAL  
REPORT**

# Examination of Reactivation and Regrowth of Fecal Coliforms in Centrifuge Dewatered, Anaerobically Digested Sludges

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**EXAMINATION OF REACTIVATION AND  
REGROWTH OF FECAL COLIFORMS IN  
CENTRIFUGE DEWATERED,  
ANAEROBICALLY DIGESTED SLUDGES**

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***2006***



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## ABSTRACT AND BENEFITS

### Abstract:

Recent testing at several utilities has shown that counts of fecal coliforms (FCs) are very low or nondetectable after thermophilic anaerobic digestion. However, immediately after digested solids are dewatered using high-solids centrifuges, the counts increase by up to six orders of magnitude. An increase was also observed for mesophilic anaerobic digestion. The principal investigators of this project hypothesized that the bacteria entered a viable but non-culturable (VBNC) state during digestion. This meant that the bacteria would not grow on standard culturing media, and therefore standard culturing methods (SCMs) would underestimate the actual viable concentration of indicator organisms. However, the bacteria would still be viable and could be reactivated. Once reactivated, additional growth of these reactivated cells would contribute to added numbers of organisms measured. For this research, bacteria are defined in the VBNC state as simply being bacteria that are not cultured and therefore are not enumerated by SCMs. Furthermore, reactivation or resuscitation is defined as the change in culturability of the bacteria, with the bacteria changing from VBNC to culturable by SCMs.

To examine reactivation and the VBNC hypothesis, the project team examined if an inducer-like substance was being released and was acting as a signal for growth. A series of experiments was performed in which filter sterilized dewatering centrate obtained from a facility showing reactivation during centrifuge dewatering, was mixed with un-dewatered liquid digester effluent that previously had low or non-detectable FC counts. The raw sludges before and after thickening contained  $10^5$  FC/g DS, and after the thermophilic digester (TD) the FCs were below the detection limit. When the filter sterilized centrate was added to the TD sample, the FCs increased by at least two orders of magnitude, and addition of the centrate plus cationic polymer resulted in an increase of at least four orders of magnitude. Since the centrate was filter sterilized prior to addition to the TD sample, rather than through FC reseeding, this rapid increase in FC was likely due to induction by a signaling substance present and released into the centrate during the dewatering step. The induction suggested that the mechanical dewatering step released a chemical signal that subsequently resulted in FC becoming culturable.

Subsequent to this mechanistic study, a competitive polymerase chain reaction (cPCR) method was used to enumerate *E. coli* (an FC bacteria and indicator organism) in biosolids samples to verify the VBNC hypothesis. This method quantified *E. coli* based on the number of their DNA copies which is independent of their culturability. Seven full-scale facilities were sampled using both standard culturing methods and cPCR. The sampling sites included facilities with mesophilic digestion, thermophilic digestion, and temperature-phased anaerobic digestion (TPAD).

The results confirmed the VBNC hypothesis at four facilities: one thermophilic, two mesophilic, and one TPAD. The cPCR enumeration demonstrated that the *E. coli* counts were very similar before and after dewatering, but the *E. coli* were not completely enumerated by SCMs before dewatering. The results suggest that SCMs used to quantify FCs (which include *E. coli*) can produce false negative results when the bacteria are in the VBNC state.

Three of the facilities that were sampled—one with thermophilic digestion, one with TPAD, and one mesophilic—did not appear to have VBNC bacteria that could be resuscitated

after high solids centrifugation. The research was not able to clearly identify the specific conditions that caused some plants to have VBNC bacteria while others did not.

The research determined a two-step reaction. The dewatering process by high-solids centrifugation resulted in reactivation of the *E. coli*, which made them culturable again resulting in an increase in culturable counts. After reactivation, the indicator organisms could grow quickly and increased by several orders of magnitude (up to  $10^8$  cells/g DS) within several days of storage. It is this combination of events, a rapid reactivation followed by a slower regrowth that resulted in FC counts increasing to levels sometimes beyond regulatory target levels.

**Benefits:**

- ◆ Demonstrates that some wastewater treatment facilities that use anaerobic digestion can get a more accurate *E. coli* count by using a competitive polymerase chain reaction (cPCR) method based on *E. coli* DNA.
- ◆ Demonstrates that *E. coli* which become VBNC state after some anaerobic digestion processes can be culturable after centrifuge dewatering, leading further regrowth during storage.
- ◆ Explains results from utilities that have reported high concentrations of indicator organisms after dewatering despite low or non-detect counts just prior to dewatering.
- ◆ Implies a need for evaluating: current mesophilic, thermophilic, and temperature-phased anaerobic digestion processes; *E. coli* quantification methods; and processes and techniques for further fecal coliform destruction.

**Keywords:** *E. coli*, fecal coliforms, mesophilic, thermophilic, temperature-phased, anaerobic, digestion, viable but non-culturable (VBNC), indicator organisms, reactivation, resuscitation, competitive polymerase chain reaction (cPCR)

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## LIST OF ACRONYMS

AI	autoinducer
BSA	bovine serum albumin
CTAB	hexadecylmethylammonium bromide
cPCR	competitive polymerase chain reaction
DNA	deoxyribonucleic acid
DS	dry solid
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
ELFA	enzyme-linked fluorescent immunoassay
FC	fecal coliform
gadA/G	glutamate decarboxylase gene
HML	Hoosier's Microbiological Laboratory
HPC	heterotrophic plate count
LSD	least significant difference
MF	membrane filtration
MGD	million gallons per day
MPN	most probable number
PCR	polymerase chain reaction
RNA	ribonucleic acid
SCM	standard culturing method
SDS	sodium dodecyl sulfate
SM	standard methods
SRT	solids retention time
TBE	tris-borate-EDTA
TD	thermophilic digester
TE	tris-EDTA
TMC	total microbial counts
TPAD	temperature phased anaerobic digestion
VBNC	viable but non-culturable
VSR	volatile solids reduction



# EXECUTIVE SUMMARY

Recent testing at several utilities has shown that counts of fecal coliforms in dewatered solids are rapidly increasing after treatment at facilities using anaerobic digestion and high-solids centrifuges for dewatering. Immediately after centrifugation or solids conveyance, fecal coliform counts are rising from very low or nondetectable levels by up to six orders of magnitude. This increase was also observed for mesophilic anaerobic digestion.

The increase in coliform counts occurred rapidly over relatively short periods of time—often within 20 minutes. Under ideal conditions, these microbes take 20-30 minutes to double in population. As a result, the researchers suspected that some bacteria, including indicator organisms such as fecal coliforms, are entering a viable but non-culturable (VBNC) state during anaerobic digestion. Standard culturing methods (SCMs) are unable to measure bacteria when they are in VBNC state, and so significantly underestimate their concentrations. The reason for the bacteria becoming non-culturable during digestion is currently unknown. Several possibilities include the presence of an inhibitor or transition into a survival state that reduces their culturability. Once exposed to high solids centrifuges used for dewatering, however, the bacteria can become culturable again, and are then enumerated by the standard culturing methods. The process by which the bacteria become culturable is often called resuscitation or reactivation. For this research, bacteria are defined in the VBNC state as simply being bacteria that are not cultured and therefore are not enumerated by SCMs. Furthermore, reactivation or resuscitation is defined as the change in culturability of the bacteria, with the bacteria changing from VBNC to culturable by SCMs.

Researchers first conducted mechanistic tests at one plant to examine the VBNC and reactivation hypothesis and subsequently a verification study at seven full-scale facilities to determine whether or not bacteria are in the VBNC state after anaerobic digestion and are reactivated during high-solids centrifugation. The sampling sites included facilities with mesophilic digestion, thermophilic digestion, and temperature-phased anaerobic digestion (TPAD).

The results confirmed the reactivation hypothesis at four facilities: one thermophilic, two mesophilic, and one TPAD. Three of the facilities that were sampled—one with thermophilic digestion, one with TPAD, and one mesophilic—did not appear to have VBNC bacteria that could be resuscitated after high solids centrifugation. The research was not able to clearly identify the specific conditions that caused some plants to have VBNC bacteria while others did not.

## **Testing the Reactivation, Regrowth Hypothesis**

To examine reactivation and the VBNC hypothesis, the project team examined if an inducer-like substance was being released and was acting as a signal for growth. A series of experiments was performed in which filter sterilized dewatering centrate obtained from a facility showing reactivation during centrifuge dewatering, was mixed with un-dewatered liquid digester effluent that previously had low or non-detectable FC counts. The raw sludges before and after thickening contained  $10^5$  FC/g DS, and after the thermophilic digester (TD) the FCs were below the detection limit. When the filter sterilized centrate was added to the TD sample, the FCs

increased by at least two orders of magnitude, and addition of the centrate plus cationic polymer resulted in an increase of at least four orders of magnitude. Since the centrate was filter sterilized prior to addition to the TD sample, rather than through FC reseeded, this rapid increase in FC was likely due to induction by a signaling substance present and released into the centrate during the dewatering step. These mechanistic tests suggested that some agent was released during mechanical dewatering that reactivated the fecal coliform and *E. coli*, which allowed them to be enumerated by standard culturing methods. This agent could include nutrients, substrate, or autoinducers.

Subsequent to this mechanistic study, seven sites were field-sampled, multiple times. The sites included facilities with mesophilic, thermophilic, and temperature-phased anaerobic digestion (TPAD). Researchers analyzed the samples using standard culturing methods for fecal coliforms, *E. coli* and heterotrophic plate counts. In addition, a competitive polymerase chain reaction (cPCR) method was used to enumerate *E. coli* based on the numbers of copies of their DNA in the samples. The cPCR method did not rely on the bacteria being culturable, therefore cPCR could enumerate *E. coli* that were viable but non-culturable as well as *E. coli* that were culturable.

Results from the sampling showed *E. coli* in the VBNC state during digestion at four of the facilities (one thermophilic, one TPAD, and two mesophilic), and were therefore not enumerated correctly by the standard culturing method in samples after digestion. Interestingly, for these cases, the *E. coli* counts measured by cPCR before and after dewatering often were equivalent, but the counts by standard culturing methods showed lower numbers prior to dewatering and higher numbers after dewatering. These results support the mechanistic study and the hypothesis that bacteria can become VBNC during digestion, and that they then can be reactivated during certain dewatering processes.

After dewatering, results for *E. coli* numbers using the cPCR and standard culturing method were in agreement, suggesting that most of the *E. coli* after dewatering were culturable. Using the cPCR method, most facilities had about  $10^5$  to  $10^6$  *E. coli*/g DS before and after dewatering. After the dewatering induced reactivation, additional regrowth was observed. The *E. coli* counts increased to approximately  $10^8$  cells/g DS within several days of cake storage. Thus, the conditions for rapid growth exist in the cake after centrifuge dewatering.

Three of the plants that were sampled did not appear to have VBNC bacteria that could be resuscitated after high solids centrifugation. This included one plant with thermophilic digestion, one plant with TPAD, and one mesophilic plant. The research was not able to clearly identify the conditions that caused some plants to have VBNC bacteria while others did not. The results did suggest that thermophilic digestion using completely mixed reactors in series was able to adequately destroy fecal coliforms, suggesting reactor hydraulics are an important component of digestion.

The mechanisms by which the VBNC are resuscitated are not yet well understood. A second phase of this research is focused on these issues as well as on developing an understanding of how processes achieve reduction in VBNC coliforms and minimize resuscitation.

### **Current Research Findings and Next Steps**

One important conclusion of this work is that standard culturing methods currently in use can significantly underestimate the numbers of indicator organisms in biosolids samples if these

organisms are in a VBNC state. The results do provide some insight into possible mitigation strategies. For example, the multi-stage thermophilic process was able to completely destroy the fecal coliform and *E. coli*, suggesting that reactors in series or in more general terms, reactor hydraulics, may be an important component in attaining desired reductions of indicator organisms. In addition, there may be some simple chemical additions, such as low-dose lime addition to the cake, which could be used to control subsequent regrowth.

Longer-term storage also could be a strategy to reduce culturable fecal coliforms to desired levels. Although standard culturing methods underestimated VBNC fecal coliforms and *E. coli* in digester effluents, the cPCR results generally showed that the *E. coli* densities after mesophilic digestion were able to meet Class B biosolids as defined by the U.S. EPA, assuming the *E. coli* were the major fecal coliform in the samples. However, subsequent to reactivation, centrifuge dewatering led to additional regrowth of these organisms. The regrowth during storage increased these numbers by several orders of magnitude to exceed U.S. EPA targets at times.

Significant additional research is needed to better understand how and why bacteria enter the VBNC state. Research is needed to better establish the relationship between design parameters such as SRT and reactor hydraulics and the actual destruction of indicator organisms. In addition, the development of future design criteria for digestion needs to incorporate analysis of indicator organisms in the VBNC state to fully understand the relationship among time, temperature, and microbial destruction. This research is partly being undertaken in a second phase of WERF supported research.



## CHAPTER 1.0

# INTRODUCTION, HYPOTHESES, AND OBJECTIVES

## 1.1 Introduction

Recent reports indicate that several wastewater treatment agencies that use anaerobic digestion and high-solids centrifuges for dewatering have experienced large increases in fecal coliforms (FCs) immediately after centrifugation and/or solids conveyance (Iranpour et al., 2003, Cheung et al., 2003, Monteleone et al., 2004, Erdal et al., 2004, Qi et al., 2004). Suggestions are that this phenomenon is a result of regrowth of the indicator organisms (Qi et al., 2004) or of floc disintegration during dewatering that allows the indicator organisms to be better cultured compared with before dewatering (Monteleone et al., 2004 and Cheung et al., 2003).

An analysis of this phenomenon suggests this increase in FC is too great to be accounted for by FC regrowth in most cases, because even under ideal conditions, the doubling time for these bacteria is 20-30 minutes. The typical residence time in a dewatering centrifuge is about 10 to 25 minutes, so orders of magnitude increases should not occur. In addition, regarding the floc breakup hypothesis, it seems flocs actually are aggregated during dewatering rather than broken apart. This suggests other mechanisms may explain these regrowth findings. The hypotheses developed for this research to explain high FC after dewatering are provided below.

## 1.2 Hypotheses

The researchers developed several key hypotheses to explain the high concentration of indicator bacteria in biosolids cake despite very low concentration being found in the liquid digest just prior to dewatering.

### 1.2.1 Hypothesis 1 – Bacteria are Viable but Nonculturable

A number of researchers have reported results supporting the hypothesis that under certain conditions, bacterial cells can be viable but nonculturable (VBNC) (Oliver, 1995, Rahman et al., 1996, Reissbrodt et al., 2000 and 2002). When exposed to stress, bacteria are not culturable by standard culturing methods (SCMs) with *in vitro* tests, but the bacteria remain viable. Often, the cells can be induced to grow in the presence of certain growth promoters or enriched media, and this process is called “resuscitation” or “reactivation” (Rockabrand et al., 1999, Makino et al., 2000, Reissbrodt et al., 2002).

Bacteria that have been found in the VBNC state include many gram-negative enteric pathogens of concern in biosolids such as strains of *E. coli* (including O157:H7), *Enterococcus faecalis*, *Shigella dysenteriae*, *Vibrio cholerae*, and *Salmonella sp.*

An interesting question is whether the bacteria are VBNC purposefully as a survival mechanism or whether they are simply sub-lethally injured such that they can no longer grow using the standard, selective culturing media for their enumeration. Numerous researchers in the 1970s and 1980s have shown that under stress that can cause sub-lethal injury, some bacteria

become non-culturable when growth is attempted using selective media and especially selective media specified in standard culturing methods. But if the media are amended or non-selective, culturability is greatly enhanced (Lin et al., 1976, Martin et al., 1976, Green et al., 1977, Stuart et al., 1977, LeChavallier et al., 1982, McFeters et al., 1982, McDonald et al., 1983, McFeters et al., 1986).

The sensitivity to selective media has been attributed to peroxide concentrations, deoxycholate, or more non-specific stress associated with selective media (Barry et al., 1956, Martin et al., 1976, McFeters et al., 1982). For example, Martin et al. (1976) demonstrated that addition of catalase (which can degrade hydrogen peroxide) to selective media increased the culturability of several different bacteria. Similarly, other quenchers of hydrogen peroxide such as pyruvate have been shown to increase recoveries of injured bacteria in both selective and non-selective media (McDonald et al., 1983, Mizunoe et al., 1999).

Researchers also have shown that changes in the media formulation or cultivation temperature regimes also can improve recoveries (Lin, 1976, Stuart et al., 1977, Green et al., 1977, LeChavallier et al., 1982, McFeters et al., 1982). For example, LeChavallier et al. (1982) developed a medium, termed m-T7, that recovered about three times more coliforms from drinking water compared with the standard m-Endo agar. McFeters et al. (1986) reported that the use of m-T7 agar increased the recovery of coliforms by a factor of eight to 38 times the results from m-Endo agar.

These researchers clearly show that the standard methods that use selective media for enumerating certain types of bacteria (such as coliforms) can significantly underestimate the concentration present in samples. Since recovery can occur with the use of enhanced culturing conditions, these injured bacteria can still be considered viable. Development of a selective media that can enumerate all coliforms remains a challenge today.

The VBNC theory leans more toward the concept that bacteria enter this state purposefully as a survival mechanism rather than as a response to sub-lethal injury that reduces their capability to grow on selective media. Most of the evidence for a VBNC state is similar to that for the sub-lethal injury state. During exposure to stress, the bacteria become non-culturable on typical media, but analysis using non-culturing-dependent methods reveals the viable counts are relatively constant or remain significantly greater than the culturable counts (Colwell et al., 1985, Byrd et al., 1991, Pommepuy et al., 1996, Whitesides and Oliver, 1997, Lleo et al., 1998, Mizunoe et al., 1999, Grey and Steck, 2001, Adams et al., 2003, Chaveerach et al., 2003).

As with the sub-lethal injury results, VBNC bacteria can be resuscitated under specific conditions such as using enhanced media and/or temperature shifts during culturing (Whitesides and Oliver, 1997, Mizunoe et al., 1999, Reissbrodt et al., 2000, Haro-Kudo, 2000, Grey and Steck, 2001, Reissbrodt et al., 2002, Gupte et al., 2003). Researchers also have attributed physiological changes that are observed in bacteria after exposure to stress and entrance into the VBNC state as evidence of a survival mechanism (Baker et al., 1983, Tholozan et al., 1999, Signoretto et al., 2000, Heim et al., 2002). For example, based on proteome analysis, Heim et al. (2002) concluded that the VBNC state for *Enterococcus faecalis* was a defined physiological stage that the bacteria would enter as a response to stress.

It seems critical that, to support the survival mechanism theory, the physiological changes must enhance survival during exposure to different stressors. This has been shown to be true in some cases (Berlin et al., 1999, Signoretto et al., 2000, Heim et al., 2002).

The potential viability and virulence of the organisms during and after they achieve a VBNC state also is important to understand. The VBNC theory posits that, by definition, the bacteria are viable and therefore are capable of causing infection. The ability of the microbes to be resuscitated is generally considered a direct indication of viability, and the ability of pathogens to retain virulence factors in the VBNC state has been demonstrated (Rahman et al., 1996, Chaiyanan, et al., 2001). It is thought that the bacteria that are VBNC remain viable and also can cause infection *in vivo*, even when they are nonculturable by standard methods (Makino et al., 2000, Rahman et al., 1996). This hypothesis has been debated in the literature; however, some evidence supports the concept that VBNC bacteria can cause infection *in vivo* (Colwell et al., 1985, Colwell et al., 1996, Pommepuy et al., 1996, Bogosian et al., 1998, Cappelier et al., 1999, Chaveerach et al., 2003). In contrast to these results, several researchers have reported that VBNC bacteria are not capable of causing infection (Caro et al, 1999, and Kolling and Matthews, 2001). Much additional research is needed to better understand the potential virulence of VBNC bacteria, and the public health significance of this phenomenon.

Very little research has been performed to study VBNC bacteria in biosolids. The researchers have proposed that indicator bacteria, such as fecal coliforms, and potential pathogens in anaerobic digesters are exposed to prolonged stress such as low substrate and nutrient concentrations, which results in the cells becoming VBNC. As a result, standard culturing methods are unable to enumerate these microbes adequately in the liquid digester effluent. The EPA regulations stipulate that Standard Method 9221E or 9222D be used to quantify fecal coliform (APHA, 1998) and these methods are not able to resuscitate VBNC fecal coliforms. However, the researchers posit, non-culturable cells remain viable with potential for resuscitation or reactivation.

For this research, bacteria are defined in the VBNC state as simply being bacteria that are not cultured and therefore are not enumerated by SCMs. This is a broad definition, which would include both sub-lethally injured bacteria and VBNC bacteria. The intent of this project was not to distinguish between these reasons for non-culturability, but to understand the cause of high fecal coliforms counts in dewatered biosolids where, prior to dewatering, the counts were significantly lower.

### **1.2.2 Hypothesis 2 – Dewatering Equipment Creates Conditions that Result in Bacteria Becoming Culturable (Reactivation)**

The researchers proposed that after passing through some types of dewatering equipment, such as a high-solids centrifuge, environmental conditions change that result in an increase in the numbers of bacteria that are culturable. The conditions may include increased substrate availability, changes in iron availability, or release of certain growth auto-inducers.

All of these factors have been shown to result in increased culturability of bacteria that were not previously culturable (Kolling and Matthews, 2001; Reissbrodt et al., 2000 and 2002). This would explain results that showed high concentrations of bacteria in cake samples even though very low concentrations were measured in the liquid samples just prior to dewatering. This resuscitation of VBNC cells would account for the large increase in a relatively short period. For this research, resuscitation is defined as the change in culturability of the bacteria, with the bacteria changing from VBNC to culturable by SCMs. The term reactivation is used interchangeably with resuscitation in this report.

### **1.2.3 Hypothesis 3 – The Presence of Substrate Supports Additional Growth After Reactivation**

After centrifugation, cells become culturable because of the presence of some induction factor, as discussed above, which would increase culturable numbers. In addition, growth of these reactivated cells also would contribute to greater numbers of organisms measured. Growth will occur as long as sufficient substrate is available.

Recent research has shown that biosolids cake has a significant quantity of bio-available substrate, such as protein and carbohydrates, which did not appear to be available until after centrifugation (Higgins et al., 2005). This substrate would be available for growth of bacteria and would increase FC counts further. In addition, high-solids centrifuges have been shown to decrease methanogenic activity, which would decrease competition for substrate and further support the growth of bacteria (Higgins et al., 2005).

### **1.3 Research Objectives and Scope**

The objectives of this research were to prove or disprove these hypotheses. To achieve these objectives, a systematic sampling of full-scale treatment processes was performed and indicator organisms were quantified using standard culturing techniques. In addition, molecular methods were used to enumerate indicator organisms without relying on SCMs, but instead would be based on the presence of their DNA. Several mechanistic studies were performed to investigate these hypotheses and better understand these phenomena. Finally, several experiments also were performed to examine the growth of indicator organism after resuscitation during cake storage.

## CHAPTER 2.0

# METHODS AND MATERIALS

## 2.1 Overview

Seven full-scale treatment processes were sampled, and several of these sites were sampled multiple times. The sites included two facilities with thermophilic digestion, three facilities with mesophilic digestion, and two facilities with temperature phased anaerobic digestion (TPAD). The TPAD process uses thermophilic digestion followed by mesophilic digestion. A summary of the facilities sampled and the sampling dates is provided in Table 2-1. Four of the facilities were sampled multiple times.

The sampling typically included influent and effluent from the digester, and the dewatered cake. After collection, samples were split and shipped under appropriate conditions to the appropriate labs. Samples were analyzed using SCMs to quantify FCs, *E. coli* and heterotropic plate counts (HPCs), and for most sites, the samples were analyzed for the presence of *E. coli* 0157:H7 and *Salmonella* by Hoosier Microbiological Laboratory, Inc. (HML). Epifluorescence was used to quantify total microbial counts (TMCs). In addition, samples were analyzed by the Bucknell University Environmental Engineering and Science Lab using molecular methods to enumerate *E. coli*.

At several sites, an initial screening was performed to evaluate the site and in some cases to test different analytical methods under development. As a result, not all tests were performed for each sampling event and each location. The detailed methods and method development activities are provided in the following sections.

**Table 2-1. Summary of Sampling Locations and Dates.**

Field Site	Digestion Type	Sampling Dates
TPAD - 1	TPAD	December 16, 2003 January 26, 2004 March 22, 2004 November 17, 2004
TPAD -2	TPAD	April 12, 2004
Thermo-1	Thermophilic – Four- Stage	January 27, 2004 May 15, 2004
Thermo-2	Thermophilic Single-Stage	January 27, 2004 May 15, 2004
Meso-1	Mesophilic – Single Stage	June 15, 2004 September 20, 2004
Meso-2	Mesophilic – Single Stage	May 15, 2004
Meso-3	Mesophilic – Single Stage	October 26, 2004

## 2.2 Microbial Quantification Methods

### 2.2.1 Sample Collection

Biosolids samples were collected aseptically in sterile containers. Samples generally included digester influent and effluent as well as the dewatered cake sample. It should be noted that the digester effluent was the feed to the high-solids centrifuge. Samples to be analyzed using standard culturing techniques were immediately put on ice in a cooler, and transported by overnight shipping for testing to begin within 24 hours of collection. The samples to be used for molecular methods were placed in dry ice and then shipped using overnight shipping.

### 2.2.2 Enumeration of FC, *E. coli*, and HPC by Culturing Methods

**Sample processing.** Samples were processed according to Standard Methods (1998). A Waring blender was used for processing the samples. The blender containers were 500 mL Nalgene blender vessels with lids, and they were sterilized by autoclaving for 15 minutes at 15 psi. Thirty grams of the sample were aseptically weighed and added to the blender containers containing 270 mL of sterile phosphate buffered water according to Standard Method 9050C (SM9050C). Samples were blended for two minutes at 18,000 rpm.

**Fecal coliform analyses.** The FC (MF) analyses were conducted according to SM9222D using m-FC media incubated at  $44.5 \pm 0.2^\circ\text{C}$  for 24 hours. The FC MPN procedure (SM9221E) also was used for enumerating fecal coliform.

***E. coli* analysis.** *E. coli* were enumerated using the procedures described in SM9221F.

**Heterotrophic plate count.** The heterotrophic (culturable) bacterial count was conducted in accordance with pour plate method (SM9215B) using standard methods agar incubated for 48 + 3 hours at  $35 \pm 0.5^\circ\text{C}$ .

**Salmonella.** The *Salmonella* analysis was conducted according to EPA/625/R-92/013 method.

***Escherichia coli* 0157:H7 and Salmonella.** These were confirmed with enzyme-linked fluorescent immunoassays (ELFAs) by the mini vidas system and the analysis was performed to determine simply a presence or absence of the organisms..

### 2.2.3 Molecular Methods for DNA and *E. coli* Quantification

#### 2.2.3.1 Overview

A quantitative polymerase chain reaction (PCR) method was used to quantify bacteria that were VBNC, and therefore were not quantifiable by standard culturing methods. Quantitative PCR can enumerate specific bacteria by measuring the number of copies of their DNAs in the sample. Specific bacteria can be enumerated by targeting a DNA sequence that is specific to the bacteria that is to be quantified.

For this research, *E. coli* were chosen as the target bacteria since they generally compose a significant proportion of the microbes measured in the FC test and they have been shown to enter the VBNC state. The DNA sequence that was targeted was for the gene that encodes the *E.*

*coli*-specific enzyme, glutamate decarboxylase (*gadA/B*) (Grant et al., 2001, McDaniels et al., 1996)

In general, the sampling procedure entailed collecting at least three to five replicates of the appropriate samples, such as digester influent and effluent, and cake, and processing the sample according to the following steps:

1. Extract and purify the total DNA in the five replicate samples
2. Quantify the total amounts of DNA in the replicates
3. Enumerate *E. coli* using competitive PCR (cPCR)
4. Compare the results to culturing methods

By using these methods, the bacteria can be quantified without relying on culturing techniques and on whether the bacteria are actually culturable.

The following sections describe the development of the specific molecular methods for these analyses and results from some preliminary tests to evaluate the method. It should be noted that during the initial phase of the research, these molecular methods were in the development stage, and changed during the first six months until the best methods for each analysis had been developed. Several of the full-scale sampling events were performed to test the analytical protocols under development during this period, and some of the results are presented in report.

#### **2.2.3.2 Quantification through Competitive Polymerase Chain Reaction (cPCR)**

The *gadA/B* gene, a gene encoding glutamate decarboxylase, is used as the indicator for *E. coli* enumeration, which consequently indicates the abundance of coliforms in the biosolids. The *gadA/B* gene was chosen over the other commonly used *uidA* gene (coding for  $\beta$ -D-glucuronidase) for its better specificity and sensitivity (McDaniels et al., 1996). Two DNA quantification methods, DNA/DNA hybridization and competitive polymerase chain reaction, were evaluated, and it was determined that competitive PCR yields more sensitive results than direct hybridization. Therefore, *E. coli* enumeration through quantification of the *gadA/B* gene using competitive PCR was adopted.

**Total DNA Extraction.** Previously, a commercial DNA extraction kit, FastDNA<sup>®</sup> Spin Kit for Soil from QBIogene (Carlsbad, CA), which was reported to have high DNA recovery for complex environmental samples, was used for DNA extraction in this research. However, recent literature indicated extremely low recovery of DNA using the kit even though it was among the best in the industry. Therefore, several DNA recovery tests were performed with dewatered biosolids as the target material, and unsatisfactory results were observed. Since the main goal of the method is to quantify the amount of *E. coli* in biosolids, a good recovery of DNA from the sample is crucial to achieving quality results. As a result the researchers developed a modified DNA extraction method, which provided approximately 80% of DNA recovery from biosolids for use in this research.

Exactly 100 mg of wet dewatered biosolids was weighed directly into a Lysing Matrix E tube (QBIogene, Carlsbad, CA), and 750  $\mu$ L of lysis buffer (100 mM Tris-HCl, 100 mM Sodium EDTA, 1.5 M NaCl, and 1% hexadecylmethylammonium bromide (CTAB), pH 8) was added. For liquid sludge, samples were first centrifuged at 14,000  $xg$  for 5 minutes, and 200 mg of the wet pellet, which has similar dry weight to the 100 mg wet dewatered biosolids, were used as the starting material. Bacterial cells then were homogenized with a FastPrep<sup>®</sup> Instrument at 5.5 speed for 30 seconds. Five  $\mu$ L of 20 mg/mL protease K was added and the sample mixture

was incubated at 55°C for 30 minutes, then 200 µL of 20% SDS was added and incubated at 65°C for 2 hours during which the samples were completely mixed every 30 minutes.

After incubation, the samples were centrifuged at 14,000 *xg* for 10 minutes and the supernatant was placed in a clean 5 mL microtube. The remaining pellets then were re-extracted twice with 500 µL of lysis buffer, homogenized, incubated at 65°C for 10 minutes, and centrifuged. All supernatants of the three serial extractions were combined and subjected to further purification.

DNA was purified through two phenol/chloroform/isoamyl alcohol extractions, followed by one chloroform extraction. Approximately 0.6 volumes of isopropanol was then added and DNA was precipitated overnight at 4°C. The precipitated DNA was pelleted by centrifuging at 16,000 *xg*, 4°C, for 10 minutes, and followed by two 70% alcohol washes, air drying, and re-dissolving in 100 µL of TE or DNase and RNase free water. If necessary, the DNA extract was further purified using the DNeasy Tissue Kit to remove humic substances following the instruction manual (Qiagen, Valencia, CA). All extracted DNA was stored at -80°C to prevent degradation until analyses were performed.

Recoveries of the current DNA extraction method were tested for both centrifuge feed and dewatered cake samples by spiking a known amount of calf thymus DNA. The commercial DNA extraction kit, FastDNA spin kit for soil (Qbiogen), also was evaluated based on the recommended protocol, with minor modifications to enhance DNA extraction efficiency, including longer DNA binding time, using a pH 8 elution buffer, and double elution under 50°C.

The results showed 78.4% and 84.3% recoveries for dewatered cake and centrifuge feed samples, respectively, with the modified extraction protocol. Among the extracted DNA, 70% was recovered during the first cell lysis and extraction, while 20% and 10% were recovered from the second and third extraction, respectively. As a comparison, 41.5% and 24.1% recoveries for dewatered cake and centrifuge feed were obtained with the commercial kit.

A quick agarose gel run of the extracted genomic DNA showed that the DNA in all three fractions of extract retained their integrity. In addition, cPCR was performed to quantify *E. coli* in the three fractions and results indicated that the third fraction contained less than 1% of the overall recovered *E. coli*, which suggested three extractions were enough to extract the majority of *E. coli* DNA. The results indicated a higher and similar level of DNA extraction for both centrifuge feed and dewatered cake by using the newly modified extraction method, which was suitable for this research.

The low DNA recovery of the unmodified commercial kit was probably due to the failure of complete cell lysis with only one lysis cycle, and to low DNA binding efficiency by the DNA binding matrix. A plate count of resuspended cell pellets after extraction was performed and a significant amount of microbial growth still existed with the commercial kit. The modified method, with three series of lysis, did not show any microbial activity.

The commercial kit uses a DNA-binding matrix to selectively bind soluble DNA for purification. However, the sample DNA could exceed the binding capacity of the matrix if a large amount of DNA is extracted (which is typical for biosolids samples). Biosolids samples, both centrifuge feed and dewatered cake, contained literally all biological cells, and therefore high DNA concentrations were expected. Hence, another explanation for the low recovery of the commercial kit is the failure of complete DNA binding.

This analysis of recoveries validated the conclusion that the modified DNA extraction method (with approximately 80% recovery) was a better choice for this research project.

**Total DNA Quantification.** A fluorescence-based DNA quantification method using PicoGreen<sup>®</sup> dsDNA Quantitation Reagent (Molecular Probe, Eugene, OR) was used for total DNA quantification (Ahn et al., 1996). Equal amounts of the PicoGreen reagent and sample were mixed and the fluorescence response was measured in a Turner TBS-380 Mini-Fluorometer (Turner BioSystems Inc, Sunnyvale, CA). The DNA concentration was determined by comparison to a known Calf Thymus DNA standard (Sigma-Aldrich Co, St. Louis, MO).

**Target DNA selection.** PCR amplification with the primers produces a 670 bp fragment of the *gadA/B* gene (307-976). *E. coli* (ATCC<sup>®</sup> 11229) was first cultured in nutrient broth and total DNA was extracted using the aforementioned method, followed by PCR amplification of the *gadA/B* gene in the following mixture: 0.5  $\mu$ M primers, 1X buffer, 2  $\mu$ M MgCl<sub>2</sub>, 0.8 mM dNTPs, 0.1% BSA, 1 unit of Taq polymerase, and approximately 5 to 10 ng DNA template. The primers used included a forward sequence (*gadA/BF*) 5' ACC TGC GTT GCG TAA ATA 3', and a reverse sequence (*gadA/BR*) 5' GGG CGG GAG AAG TTG ATG 3' (McDaniels et al., 1996).

The PCR was run on a Flexigene thermocycler (Techne Inc, Princeton, NJ) and a touch-down PCR program was chosen to remove non-specific bands while providing the best sensitivity. The thermal cycle program included an initial denaturation at 95°C for 5 minutes followed by one cycle each of denaturation at 94°C for 1 minute, 65°C with 0.5°C decrease per cycle for 45 seconds, and extension at 72°C for 40 seconds. The cycles were increased to two each when annealing temperatures reached 60°C, and were completed with 30 additional cycles when annealing temperatures reached 57°C. A final extension at 72°C for 15 minutes was included at the end of cycles before holding at 4°C. PCR products are checked on 1% agarose gel with a DNA ladder to ensure correct amplification.

**Construction of Competitive DNA Fragment.** The competitor DNA fragment was constructed using a modified *gadA/BF* reverse primer and the identical *gadA/BR* primer based on McDaniels et al. (1996). The modified reverse primer, *cGAD-B*, developed in this project, had the following sequences attached to its 3' end: 5' GCA CTG ATC GAT TTC ACA 3'. Using this set of primers, a competitor DNA fragment of 544 bp with complementary sequences on both ends to the two original primers was constructed through a two-step PCR cycle. The composition of the PCR mixture was the same as described earlier except with primers *cGAD-B* and *gadA/BF*.

The PCR was run on a Flexigene thermocycler (Techne Inc, Princeton, NJ) with an initial denaturation at 95°C for 5 minutes followed by 15 cycles of denaturation at 94°C, annealing at 48°C, and extension at 70°C for 1 minute each. This program with low annealing temperature allowed production of the competitive DNA fragments, which served as templates for the following more stringent annealing temperatures: 25 cycles of denaturation at 94°C, annealing at 58°C, and extension at 72°C for 1 minute each, and a final extension at 72°C for 15 minutes. PCR products were checked on 1% agarose gel with a DNA ladder to ensure correct amplification. The amplified DNA fragments then were purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA). The purified competitive DNA fragments then were quantified, equivalent copies of DNA were estimated, and they were used for quantifying the DNA.

**DNA Quantification Through Competitive PCR Assay.** The competitive PCR incorporates an internal DNA standard in the PCR reaction to account for potential biases produced among different PCRs (Wang et al., 1989). Although PCRs have been used in identifying *E. coli* for their sensitivity and specificity (Rompre et al., 2002), it is not until recently that competitive PCRs were applied to enumerate *E. coli* in soil samples (Rose et al., 2003).

The PCR mixture was prepared in the same way as described for the 670 bp *gadA/B* gene fragment, except that 2  $\mu$ L of DNA template and an additional 2  $\mu$ L of serially diluted competitor DNA ranging from 5 to 50,000 copies were added. Both the 670 bp *gadA/B* fragment and the 544 bp competitor fragment were amplified at different levels depending on the amount of competitor fragment added. Since both target and competitor DNA compete for primer binding during PCR cycles, the more competitor DNA present, the less 670 bp fragment is produced. The unknown DNA target then can be determined when the PCR products of the two fragments are identical where they have identical starting template concentrations.

The same touch-down thermal program was used in the competitive PCR assay. The resulting PCR products were mixed with 5  $\mu$ L of loading buffer, and 5  $\mu$ L of the mixture was loaded in 1% agarose gel and run with 1x TBE buffer. The UVP imaging system (UVP Inc., Upland, CA) was used to obtain gel images, and band intensities were analyzed with the LabWorks Imaging and Analysis software. Figure 2-1 shows a typical cPCR assay profile with both 670 bp target and 544 bp competitive bands. DNA concentration was determined by plotting  $\log$  (competitive band intensity/target band intensity) against  $\log$  (number of competitive fragment copies). This ratio is in unity when its logarithm is zero (Figure 2-2). The abscissa at this point yields the estimated *E. coli* number.



Figure 2-1. Typical Competitive PCR Assay of *gadA/B* Gene.

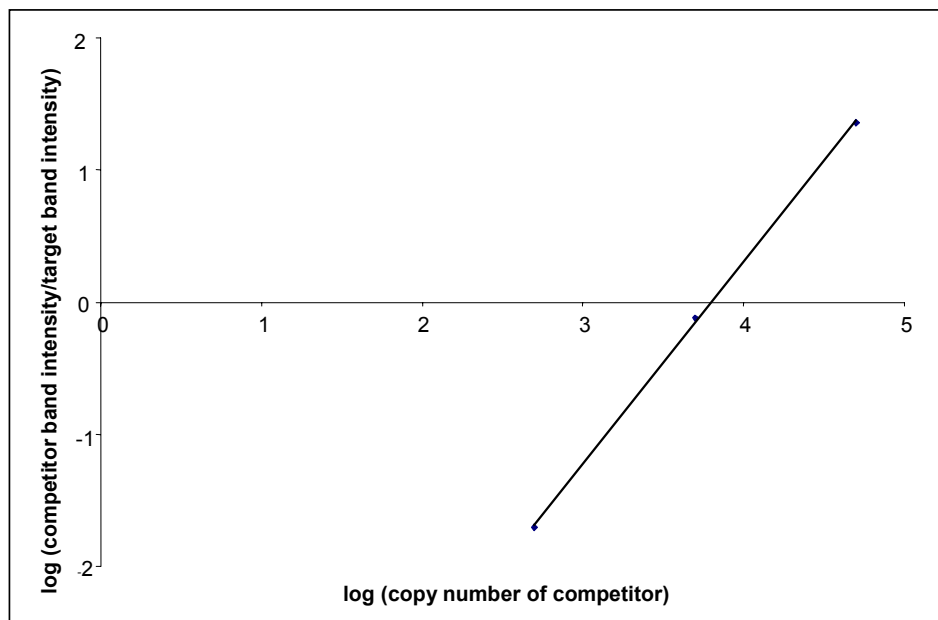


Figure 2-2. Calculation of Target DNA Copy Numbers Through Competitive PCR Band Intensities and Competitor Copy Number.

**Modification and Validation of the Competitive PCR Assay.** A further modification of the competitive PCR protocol was developed because of concern for potential biases of the method, as indicated by Raeymaekers (1993; 1995). There were two major biases that could occur: 1) differential amplification rates between target and competitor; 2) differential amplification rates for both target and competitor. Although both issues are rarely addressed in cPCR analysis, to ensure valid data is collected, it was critical to verify the potential problems, especially when a touchdown protocol was used in this research. Recent research also has adopted the competitive touchdown PCR to enumerate *E. coli* in soil samples. However, the theoretical slope, indicating constant amplification efficiency, was not obeyed (Rose et al., 2003).

Different amplification rates between the target and competitor causes the final number to shift by the factor of the rate differences. However, this problem can be easily verified and adjusted when running a known *E. coli* DNA sample against the same serially diluted competitors.

In previous work, the researchers had adjusted their values based on a cPCR run with the known *E. coli* DNA target. In addition, they had corrected this problem by employing a reverse competitive PCR method that used a fixed amount of competitors against a serially diluted known target DNA (Zachar et al., 1993), creating a standard curve for future unknown sample analysis. In this way, competitors only served as the intermediate conversion element between known *E. coli* standards and unknown samples. This method also provided an easier way to handle large routine cPCR analysis, in which no serially diluted sample runs were required as a standard curve was constructed.

Therefore, the protocol using reverse cPCR was used for this research and several fixed numbers of competitor fragments were evaluated to construct standard curves of all ranges. Standard curves with competitor fragments ranging from 40-40,000 copies were tested for this

purpose (Figure 2-3). All results showed linear relations with serially diluted known *E. coli* over a broad range, among which 40 and 405 copies provided the best detection sensitivity (down to seven copies).

The second problem, if it occurred, had no available method to correct the level of differences between the estimated number and the true value. However, the existence of this problem could be revealed by observing the slope of the standard curve (Raeymaekers, 1993; 1995). Based on the theoretical calculations of competitive PCR, the slope of the regression curve should be close to 1 (-1 for reverse cPCR) for non-differential amplification. That is to say, if the slope of the data was close to this theoretical value, there probably was no differential amplification.

Among all standard curves developed, competitor fragments with 40 and 405 copies showed slopes near negative one even when running with 50 ng of *P. putida* DNA serving as background DNA, and 405 copies provided low variability. For example, using 405 copies resulted in a slope of -1.0076 and a correlation coefficient of 0.98. Therefore, 405 copies of competitive fragments were chosen as the fixed standard for *E. coli* ranges from seven to 40,000 copies when loading 10 ng of template, or 7-119 when loading 50 ng of template. These two curves were sufficient for *E. coli* sample ranges that are typical for wastewater samples. The enumeration under high background DNA provided higher sensitivity of the protocol in which more DNA load was permitted in each PCR analysis.

The modified protocol also required the gel run to be performed with 5% TBE apolyacrylamide gel and with longer exposure time during imaging analysis to provide both accuracy and sensitivity.

PCR inhibitors, such as humic acids, co-extracted with DNA can inhibit or even terminate PCR reactions. Therefore, the existence of PCR inhibitors can underestimate enumerations or report false-negative data. Three sets of template samples were analyzed for potential PCR inhibitors: 1) biosolids DNA, 2) *E. coli* DNA, and 3) biosolids plus *E. coli* DNA. T-test comparison of “biosolids DNA” and “(biosolids plus *E. coli* DNA) – (*E. coli* DNA)” was used to evaluate the significance of PCR inhibitors. It was observed that when 50 ng of biosolids DNA was used as a template, no significant PCR inhibition was observed for digester feed, but a slight inhibition was observed for both centrifuge feed and dewatered cake. The increase of PCR inhibitors in centrifuge feed and cake samples was probably due to formation of humic substances during anaerobic digestion.

Typically, a DNA clean-up kit is used to further purify DNA and remove PCR inhibitors. However, because of the low recovery of the tested commercial clean-up kits, the dilution approach was used as an alternative. Dilution of DNA template reduces the amount of PCR inhibitors in a PCR reaction and can avoid their effect. The test has shown that when only 10 ng of biosolids DNA is used, no significant PCR inhibition is observed even for centrifuge feed and dewatered cake DNA. The drawback of using less template DNA is the increase in the detection limit.

In summary, the modified protocol included three replications of cPCR with each reaction containing 405 copies of competitive fragments and 50 ng or 10 ng of DNA template. The same touchdown thermocycle program was used for DNA amplification. Five  $\mu$ L of each PCR products was loaded on a 5% TBE polyacrylamide gel and electrophoresized at 100V for

60 minutes. Gels then were stained with SYBR green for 15 minutes, followed by imaging analysis with the UVP biosystem.

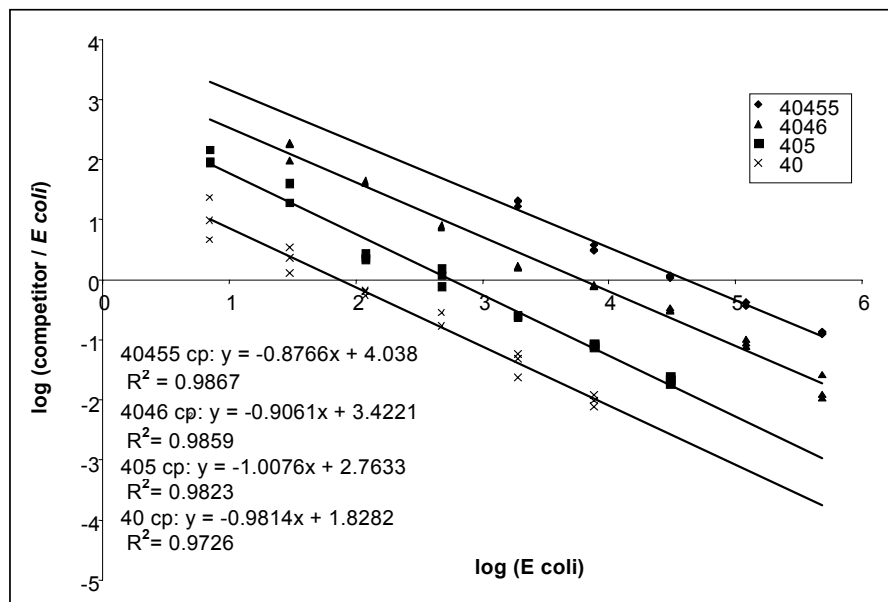


Figure 2-3. Standard Curves of Reverse Competitive PCR.

## 2.3 Methods for Mechanistic Studies

### 2.3.1 Procedure for Centrate and Polymer Testing of Biosolids

Several mechanistic studies were performed to examine whether the centrate or polymer could reactivate FC or *E. coli* in the VBNC state. In these experiments, 30 g of biosolids were aseptically weighed and placed in a sterile container. For centrate testing, 10 mL of filter sterilized centrate was added to the 30 g of sample in the sterile container. For polymer testing, 0.5 mL of polymer solution obtained from the facility was added to the 30 g of sample in the sterile container. The samples were incubated at room temperature for 24 hours, and sterile phosphate buffer was added to bring the sample volume to 270 mL. The samples then were blended on low speed for 2 minutes, and then analyzed for FC, *E. coli*, and other bacteria as described previously.

## 2.4 Storage Experiments

During the storage experiments, biosolids were stored at the given temperature in sealed sterile containers. The temperatures used during incubation included room temperature (22°C) and 37°C. At the prescribed time intervals, typically days 1, 2, 3, and 7, sub-samples of biosolids were aseptically removed from the container and processed with the different analytical methods as described previously.

## 2.5 Statistical Analysis of Data

Statistical analyses of the data were performed, including one-way ANOVA to evaluate differences among all three samples, and both LSD (least significant difference) and Tukey methods were used to further compare differences between each pair of samples at  $p=0.05$ . For

correct statistical analysis, all data were log-transformed to obtain constant variances and a test of homogeneity of variance was performed to verify this quality.

## CHAPTER 3.0

# RESULTS FROM FIELD SAMPLING

## 3.1 Field Testing

### 3.1.1 Overview

Seven sites were sampled, which included three facilities with mesophilic digestion, two facilities with thermophilic digestion, and two facilities with TPAD processes. Every facility that was sampled used high-solids centrifugation for dewatering. A summary of the different treatment facilities and an overview of the processes is provided in Table 3-1. Sites were named with a prefix that describes the digestion type and a number. These names are used throughout this document to denote each facility.

Table 3-1. Summary of Full-Scale Processes Sampled.

Field Site	Digestion Type	Digestion SRT (d) and temperature	Digestion VSR (%)	Cake Solids Content
TPAD - 1	TPAD	15 d at 58°C and 21 d at 37°C	60%	30%
TPAD -2	TPAD	8 d at 55°C and 20-25 d 43°C	40-45%	27-29%
Thermo-1	Thermophilic – Four-Stage	Total 22 d at 56°C	60-62%	30-32%
Thermo-2	Thermophilic – Single-Stage	15-20 d at 55°C	65%	35%
Meso-1	Mesophilic – Single Stage	21 d at 37°C	40-45%	21%
Meso-2	Mesophilic – Single Stage	30-35 d at 37°C	50-60%	22-24%
Meso-3	Mesophilic – Single Stage	22 d at 36°C	45-58%	30-33%

Several of the sites were sampled multiple times, and the results for each individual site are provided in the following sections. At the end of these sections, a summary of the site data is provided followed by the results from the mechanistic testing.

### 3.1.2 Results from Temperature-Phased Anaerobic Digestion Processes

#### 3.1.2.1 Results for TPAD-1

TPAD-1 had reported problems with high FCs in solids samples after dewatering, but low counts in solids samples prior to dewatering. This treatment process uses centrifuge thickening of primary and secondary sludges, and the thickener effluent undergoes thermophilic digestion followed by mesophilic digestion. After digestion, the solids are stored in a holding tank and the effluent from the holding tank is then dewatered. Dewatering is performed on a high-speed

centrifuge. The complete process flow diagram is shown in Figure 3-1. In addition, the specific process operation information is provided in Table 3-2.

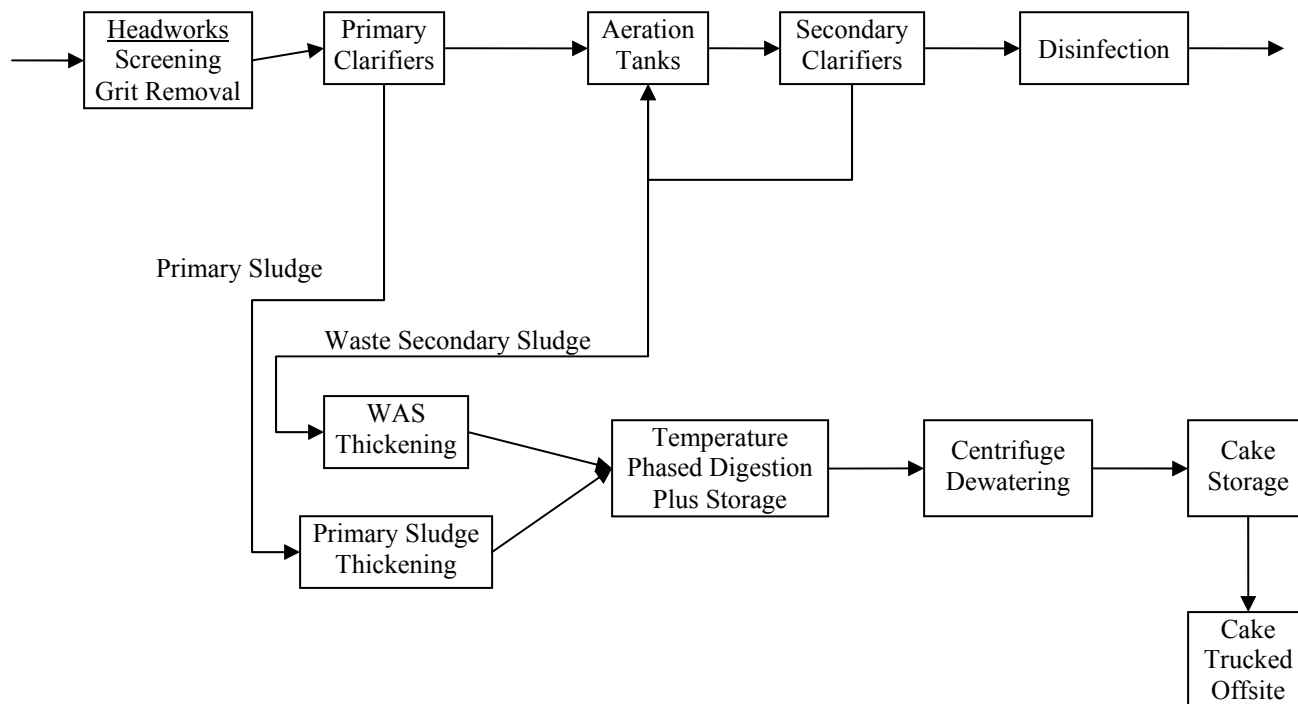


Figure 3-1. Process Flow Diagram for TPAD-1.

Table 3-2. Summary of Facility Operation Information for TPAD-1.

Process	Equipment	Chemicals	Operation	Notes
Primary Thickening	Centrifuge	— <sup>1</sup>	—	—
Secondary Thickening	Centrifuge	—	—	—
Thermophilic Anaerobic Digestion	Fixed cover	—	57-60°C 15+ day SRT,	—
Mesophilic Anaerobic Digestion	Fixed cover	—	20+ day SRT	60% VSR in TPAD system
Storage	—	—	—	Unmixed, unheated
Dewatering	—	—	30% solids	—

<sup>1</sup> A dash (—) means no data are available.

TPAD-1 has been sampled four times as part of this study; the sampling dates were:

- ◆ December 16, 2003
- ◆ January 26, 2004

- ◆ March 22, 2004
- ◆ November 17, 2004

The first two dates were sampled and the samples were analyzed using culturing and other techniques by HML to quantify FCs, TMC, and HPCs and to determine the presence of *E. coli* 0157:H7 and *Salmonella*. The third date combined these analyses with the molecular methods under development at Bucknell University (BU). The analyses were performed to establish the presence of VBNC bacteria and reactivation as well as to examine the mechanism. Results for the specific mechanistic studies are provided in Chapter 4.0. The results from testing at this facility on the three dates are provided below.

**TPAD-1, December 16, 2003 Sampling.** On this sampling date, the following samples were collected:

- ◆ Thickener influent
- ◆ Thickener effluent
- ◆ Thermophilic effluent
- ◆ Centrifuge cake
- ◆ Centrate from the centrifuge
- ◆ Polymer

A summary of results from this sampling event is provided in Table 3-3. After thermophilic digestion, the densities of FCs were below the detection limit and were reduced by at least five orders of magnitude during digestion, and the HPCs were reduced by two orders of magnitude compared with counts in the influent to the digester. However, sampling immediately after the centrifuges showed a significant increase in the FCs and HPCs, demonstrating that some type of reactivation of these microbes occurred as a result of the dewatering process. The total microbial counts (TMCs) by epifluorescence showed a modest increase in microbial counts compared to the FCs and HPCs results.

The researchers surmised that the reactivation was a result of released cellular components that signaled the microorganisms to grow. Data presented in Chapter 4.0 support this hypothesis.

**Table 3-3. Summary of Test Results for TPAD-1.**  
*December 16, 2003 Sample Date*

Sample	FC (cfu/g DS)	HPC (cfu/g DS)	TMC (cells/g DS)	<i>E. coli</i> 0157:H7
Thickener influent	$2.14 \times 10^5$	$1.30 \times 10^6$	$1.35 \times 10^8$	Negative
Thickener effluent	$3.44 \times 10^5$	$1.30 \times 10^6$	$1.67 \times 10^8$	Positive
Thermophilic Effluent	<7	$5.10 \times 10^4$	$1.03 \times 10^8$	Negative
Cake	$1.71 \times 10^5$	$4.00 \times 10^7$	$2.21 \times 10^8$	Negative

**TPAD-1, January 26, 2004 Sampling.** A second set of samples was collected from this facility on this date to test several methods being developed at Bucknell University, namely the

DNA and RNA extraction and quantification methods. At the same time, samples were collected and split for analysis by HML. On this date, the following samples were collected and analyzed:

- ◆ Thermophilic effluent
- ◆ Centrifuge cake
- ◆ Centrifuge centrate

The facility personnel collected the samples in sterile containers, and overnight-shipped one set to HML and one set to BU. Therefore, the samples had been stored on ice for 24 hours, which could have affected the DNA and RNA results. The results from the sampling are presented in Table 3-4.

This sampling event also showed significantly greater FC densities in the cake compared with those from the digester, as well as much greater HPC counts. As in the previous trial, the total microbial counts (TMC) did not increase nearly as much as the FCs and HPCs did. Therefore, the results suggested a reactivation of microbes that were measured by the FC and HPC methods, although they might not have been a significant portion of the total microbial population measured by the TMC method.

Interestingly, the total DNA decreased by a factor of approximately three. The reduction in DNA could have been from the 24-hour shipping time, when DNA-degrading enzymes would still be active and degrade the DNA that could have become available from cell lysis. Therefore, the results suggested that DNA extraction had to be done immediately after sampling collection, and the sample had to be processed to the point that it was stable and no degradation would occur. Additional testing showed that after the sample with the first DNA extraction reagents was processed initially, little degradation or loss of DNA was measured after 24 hours of storage at room temperature. As a result, the molecular testing protocol required on-site sample processing immediately after collection, followed by overnight shipping to the lab on dry ice for the remainder of the extraction, purification, and quantification procedures within 24 hours.

Despite these problems, it was interesting to note that if the FC, HPCs and TMCs were actually regrowing, a significant increase in DNA would be expected. However, if the microbes were reactivated, then the increase in DNA would not be expected to be as great.

**Table 3-4. Summary of Test Results for TPAD-1.**  
January 26, 2004 Sample Date

Sample	FC-MF (cfu/g DS)	HPC (cfu/g DS)	TMC (cells/g DS)	<i>E. coli</i> 0157:H7	Total DNA (µg/g DS)
Thermophilic Effluent	<5	3.90x10 <sup>4</sup>	5.40x10 <sup>7</sup>	Negative	525
Cake	1.15x10 <sup>5</sup>	2.40x10 <sup>7</sup>	6.21x10 <sup>7</sup>	Positive	172
Centrate	<90	<1	NT <sup>1</sup>	Negative	NT

<sup>1</sup> NT = not tested.

**TPAD-1, March 22, 2004 Sampling.** The third sampling event was aimed at comparing the quantification of *E. coli* using hybridization and competitive PCR (cPCR) with the results obtained using standard culturing techniques at HML. For this sampling, Dr. Yen-Chih Chen went to the site, and immediately after sample collections, processed them with the extraction

reagents to minimize degradation and loss of DNA. After processing, the samples were shipped to BU for the remainder of the analyses. As with the prior sampling event, a set of split samples also were sent to HML for analysis. The results from these tests are provided Table 3-5.

**Table 3-5. Summary of Test Results for TPAD-1.**  
January 26, 2004 Sample Date

Sample	FC (MPN/g DS)	HPC (cfu/g DS)	TMC (cells/g DS)	<i>E. coli</i> 0157:H7
Thermo Effluent	<13	5.40x10 <sup>4</sup>	5.40x10 <sup>7</sup>	Negative
Centrifuge Feed	<5.1	7.60x10 <sup>5</sup>	4.59x10 <sup>7</sup>	Negative
Cake	7.4	4.00x10 <sup>7</sup>	NT	Positive

<sup>1</sup> NT = not tested.

For this sampling event, no significant increase of FCs or *E. coli* was measured, contrary to previous testing results at this facility. The reason for this is unknown since this facility historically had had reactivation and all previous sampling results had shown increased FCs after dewatering. The hybridization results are shown in Figure 3-2, and this test also was unable to quantify any *E. coli*, as shown by a lack of “dots” on the hybridized membrane lanes with the samples in them. Similarly, the cPCR showed no measurable quantities of *E. coli* in the samples.

These results point to the conclusion that the numbers of FC and *E. coli* were actually low during this sampling time, although the reason for this is unknown. The sampling occurred on a Monday morning, and the centrifuge had to be turned on prior to sampling, so it was possible the storage of the sludge in the piping system may have played a role, although the centrifuge was run for one hour prior to sampling.

Despite the lack of increased FCs and *E. coli* after dewatering, some interesting results did emerge. For example, the HPC concentration increased significantly (by two orders of magnitude), suggesting a reactivation of these microbes, and the TMC remained basically constant before and after dewatering.

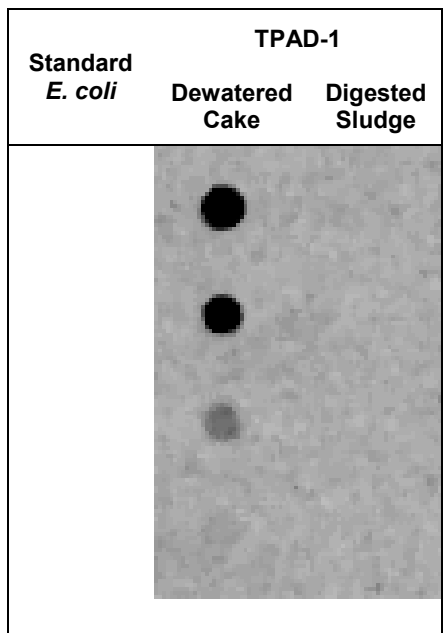


Figure 3-2. Dot-Blot Hybridization Results for Samples First Lane is *E. coli* Standard, Second Lane is TPAD-1 Dewatered Cake, and Lane 3 is the Digested Sludge From TPAD-1.

### 3.1.2.2 Results for TPAD-2

TPAD-2 also uses temperature-phased anaerobic digestion to stabilize both primary and secondary residuals as shown in Figure 3-3. A description of the operational parameters is provided in Table 3-6. TPAD-2 has a significant pulp and paper inflow into the facility, and the overall facility capacity is about 45 MGD. TPAD-2 was sampled on April 12, 2004 as discussed below.

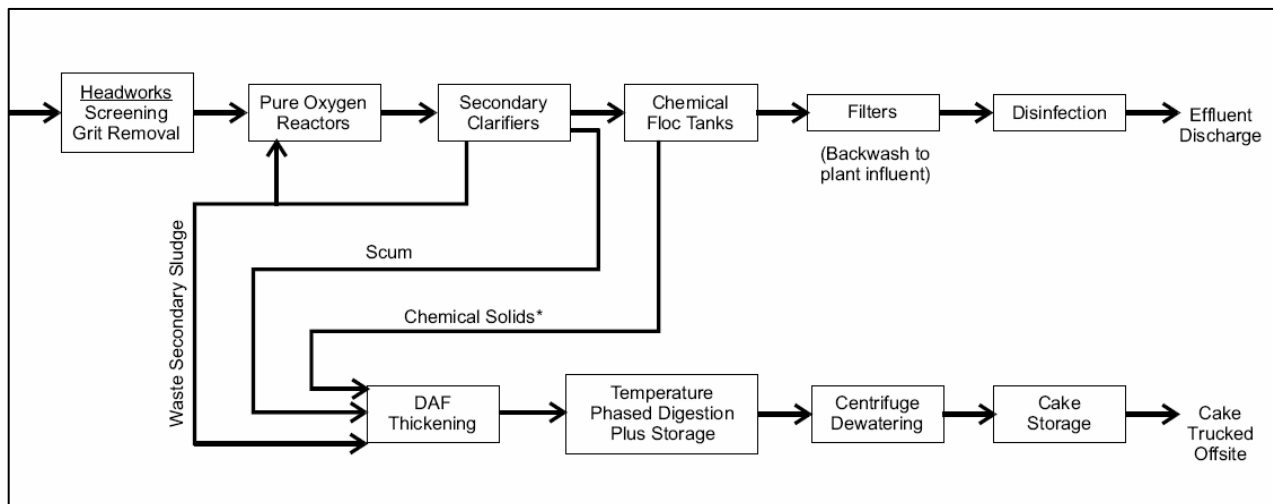


Figure 3-3. Process Flow Diagram for TPAD-2.

**Table 3-6. Summary of Facility Operation Information for TPAD-2.**

Process	Equipment	Chemicals	Operation	Notes
Secondary Thickening	DAF	Ciba Zetag 7692 4 g/kg polymer	5-6.5% solids	
1 <sup>st</sup> Stage of Thermophilic Anaerobic Digestion	Fixed cover	FeCl <sub>3</sub> added to digester	55°C 7-9 day SRT	
2 <sup>nd</sup> Stage of Mesophilic Anaerobic Digestion	Fixed cover		38-48°C 20-25 day SRT	40-45% VSR overall
Digested Biosolids Storage	Covered, pump mixed		2-3 day SRT	
Dewatering	High solids Sharples DS	Ciba Zetag 8818 12-20 g/kg	27-29% solids	
Cake Conveyance	Screw conveyor belt		10 meters 80 meters	

**TPAD-2 Sampling, April 12th, 2004.** TPAD-2 was sampled to assess the facility for potential reactivation of FCs. The results from the testing are provided in Table 3-7. Unlike the other field samples, the feed to the digester had very low (<19,000) FC and *E. coli* density, and also low counts in the feed and cake. The reason for this was unclear, but it may be related to the lack of primary sludge as well as the pure oxygen system and possibly the high percentage of industrial wastewater input to the facility. The results did show some reactivation of HPCs after dewatering, as noted by an increase of two orders of magnitude compared with centrifuge feed counts. Based on these findings, no cPCR was performed at this facility since the *E. coli* numbers were so low.

**Table 3-7. Summary of TPAD-2 Test Results.**

Sample	FC - HML (cfu/g DS)	<i>E. coli</i> - HML (cfu/g DS)	HPC - HML (cfu/g DS)	TMC (cells/g DS)
Digester Feed	<18,868	<18,868	1.09x10 <sup>9</sup>	1.92x10 <sup>8</sup>
Centrifuge Feed	<31,250	<31,250	4.30x10 <sup>4</sup>	1.08x10 <sup>8</sup>
Cake	<4,082	<4,082	5.00x10 <sup>6</sup>	1.73x10 <sup>8</sup>

A second set of samples was collected to determine if the centrifuge torque played a role in reactivation. In this trial, the centrifuge torque was set at 40, 50, 65, and 80%, and the optimum polymer dose was determined for each setting. At the optimum polymer dose, cake samples were obtained and analyzed for microbial counts. No significant differences were measured in any of the microbial count measures for the different torque values (data are not shown).

### 3.1.3 Results from Thermophilic Digestion Processes

#### 3.1.3.1 Results for Thermo-1

The process flow diagram for Thermo-1 is shown in Figure 3-4. The facility uses four stages of thermophilic digestion to achieve Class A biosolids. The specific operation parameters are provided in Table 3-8. This facility has a fairly high overall SRT at thermophilic temperatures (the highest of the study), with approximately 23 days.

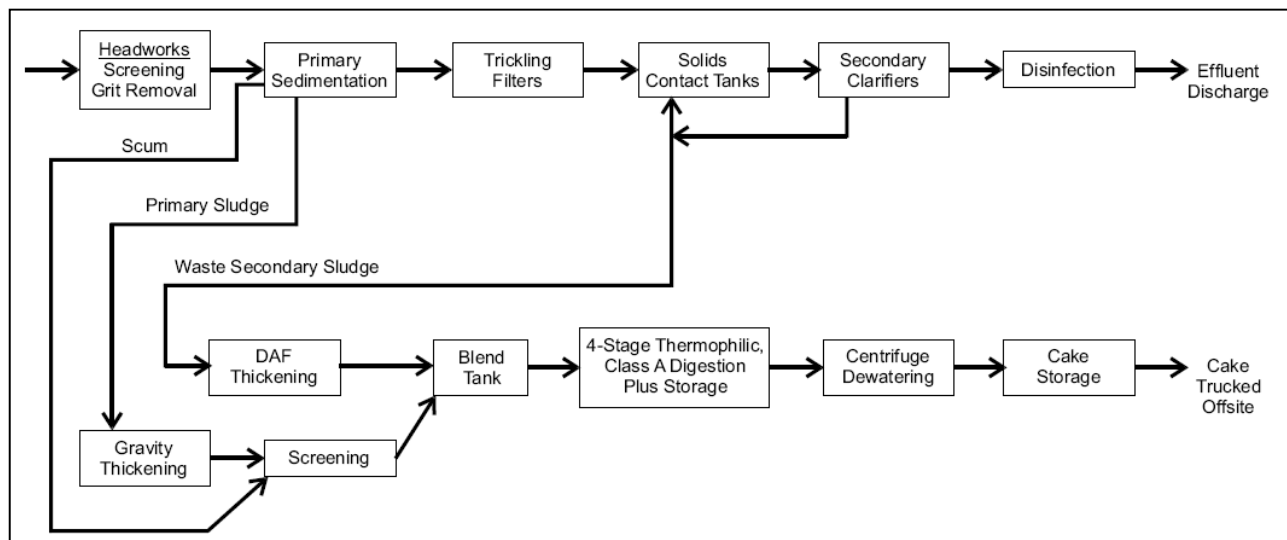


Figure 3-4. Process Flow Diagram for Thermo-1.

Table 3-8. Summary of Facility Operation Information for Thermo-1.

Process	Equipment	Chemicals	Operation	Notes
Primary Thickening	Gravity	None	4% solids	
Secondary Thickening	DAF	1 g/kg polymer	4.5% solids	
1 <sup>st</sup> Stage of Thermophilic Anaerobic Digestion	Fixed cover		56°C 16-19 day SRT,	
2 <sup>nd</sup> , 3 <sup>rd</sup> , 4 <sup>th</sup> Stage of Thermophilic Anaerobic Digestion	Fixed cover		56°C 1-2 day SRT for each (about 5 days total)	60-62% VSR overall
Digested Biosolids Storage			42-52°C ≈1 day SRT	Unmixed, Unheated
Dewatering	High-solids Sharples DSX 706	10 g/kg	30-32% solids	
Cake Conveyance	Screw conveyor and cake pumps		3 meters 30 meters	

Thermo-1 was sampled two times during the study. The first sampling was used to collect preliminary information and determine if reactivation might be occurring. The second sampling

was collected to quantify microbes with both standard culturing techniques and molecular methods.

**Thermo-1 Sampling Results, January 27, 2004.** Facility testing was conducted on this date to investigate if FC reactivation could be occurring and therefore if this site could warrant additional investigation. The sample collection included the digester effluent (feed to the centrifuge) and the dewatered cake samples. The samples were shipped to HML for FC, HPC and TMC analysis. The results from this trial are summarized in Table 3-9.

No increased FC in solids were measured at this site, although the HPC counts were higher in the cake compared with those from the digester. The TMC doubled in solids after dewatering compared with the digester values. The results from this testing did not show any measurable reactivation of fecal coliforms after dewatering, and possible a slight reactivation of HPCs. As a result, this facility was a good control site for analysis using molecular methods to quantify *E. coli*.

**Table 3-9. Summary of Results from Thermo-1.**  
January 27, 2004 Sample Date

Sample	FC (MPN/g DS)	HPC (cfu/g DS)	TMC (cells/g DS)
Thermophilic Effluent	<90	1.80x10 <sup>4</sup>	4.05x10 <sup>7</sup>
Cake	<3,236	4.50x10 <sup>5</sup>	9.18x10 <sup>7</sup>

**Thermo-1 Sampling Results, May 15, 2004.** A second sample set was taken on this date to better test the molecular methods for quantifying *E. coli* at a facility that did not have reactivation. The results from the testing are provided in Table 3-10. The culturing results showed a significant decrease in the FC and *E. coli* density after digestion, and no reactivation after dewatering. The cPCR results confirmed that the *E. coli* were, in fact, not present in significant quantities in the digester effluent, nor in the cake. The results suggested that this facility had effective digestion that significantly killed the FCs rather than have them survive in the VBNC state possibly be reactivated after dewatering.

**Table 3-10. Summary of Results for Thermo-1.**  
May 15, 2004 Sample Date

Sample	FC – HML (cfu/g DS)	<i>E. coli</i> – HML (cfu/g DS)	Avg. <i>E. coli</i> by cPCR (cells/g DS)	Std. Dev. <i>E. coli</i> by cPCR (cells/g DS)	HPC – HM (cfu/g DS)	TMC (cells/g DS)
Digester Feed	3.45x10 <sup>8</sup>	2.40x10 <sup>7</sup>	3.26x10 <sup>7</sup>	4.03x10 <sup>6</sup>	5.00x10 <sup>7</sup>	1.65x10 <sup>8</sup>
Centrifuge Feed	2.00x10 <sup>1</sup>	2.00x10 <sup>1</sup>	Non-detect	Non-detect	2.20x10 <sup>5</sup>	7.29x10 <sup>7</sup>
Cake	<1	<1	Non-detect	Non-detect	3.80x10 <sup>5</sup>	2.57x10 <sup>8</sup>

### 3.1.3.2 Results for Thermo-2

The process flow diagram for Thermo-2 is shown in Figure 3-5, and a summary of the operational parameters is provided in Table 3-11. This process does not have biological treatment of the liquid stream, and uses one-stage thermophilic digestion of primary sludge.

Thermo-2 was sampled two times. The first sampling on January 27, 2004 was used to screen for facilities with and without possible reactivation, using only standard culturing methods. A second sampling was performed on May 15, 2004, using both culturing and molecular techniques.

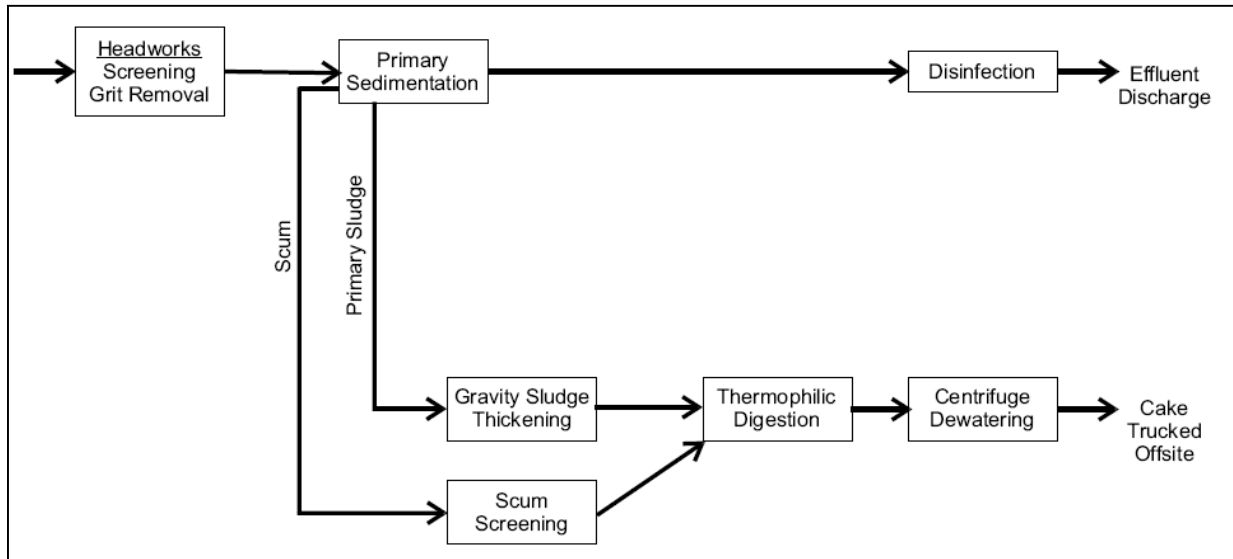


Figure 3-5. Process Flow Diagram for Thermo-2.

Table 3-11. Summary of Plant Operation Information for Thermo-2.

Process	Equipment	Chemicals	Operation	Notes
Primary Thickening	Gravity	None	4% solids	
Thermophilic Anaerobic Digestion	Fixed Cover		55 °C 15-20 day SRT 65% VSR	
Dewatering	High Solids Sharples DS 705		35% solids	
Cake Conveyance				No cake conveyance

**Thermo-2 Sampling Results, January 27, 2004.** Sampling at this site was conducted as a screening to identify facilities with a potential reactivation/regrowth problem. This facility also uses thermophilic digestion followed by high speed centrifugation. Samples were collected from the influent of the digester, the digester effluent, and after dewatering. The samples were analyzed by HML for FC, HPC, TMC, as well as for the presence of *E. coli* 0157:H7 and *Salmonella*. The results are summarized in Table 3-12.

The FC and HPC counts increased by three orders of magnitude after dewatering, although the TMC counts actually decreased after dewatering. Also, *E. coli* 0157:H7 was measured in all three samples. The digester effluent did not test positive for *Salmonella* but the dewatered cake did. These results suggest that reactivation was likely occurring at this site, and the microbes that were reactivating included FCs, HPCs, and *Salmonella*. As a result, a second sampling was performed to combine both molecular and culturing quantification of microbes as discussed below.

**Table 3-12. Summary of Test Results for Thermo-2.**  
January 27, 2004 Sample Date

Sample	FC (cfu/g DS)	HPC (cfu/g DS)	TMC (cells/g DS)	<i>E. coli</i> 0157:H7	<i>Salmonella</i>
Digester Influent	1.19x10 <sup>6</sup>	2.40x10 <sup>7</sup>	1.03x10 <sup>8</sup>	Positive	Positive
Digester Effluent	4.60x10 <sup>1</sup>	1.30x10 <sup>5</sup>	1.24x10 <sup>8</sup>	Positive	Negative
Cake	7.32x10 <sup>4</sup>	1.09x10 <sup>8</sup>	2.16x10 <sup>7</sup>	Positive	Positive

**Thermo-2 Sampling Results, May 15, 2004.** A second sample set was collected on this date to further investigate reactivation of VBNC indicator organisms at Thermo-2. A summary of the results is provided in Tables 3-13 and 3-14. The standard culturing methods found both FCs and *E. coli* were relatively high in the feed to digestion: approximately 10<sup>8</sup> and 10<sup>7</sup> cfu/g DS, respectively. But the counts were greatly reduced during digestion, and only 10<sup>1</sup> to 10<sup>2</sup> cfu/g DS were measured coming out of the digester. Immediately after centrifugation, the density increased by two to five orders of magnitude. For example, the FC measured by standard culturing methods increased from 10<sup>1</sup> to 10<sup>6</sup> cfu/g DS, a significant increase in a very short period of time.

Interestingly, the quantification of *E. coli* using cPCR showed that the counts of *E. coli* in the digester feed were very similar to the counts measured by standard culturing technique, both 10<sup>7</sup>. However, after digestion, the *E. coli* by cPCR was 10<sup>5</sup> cells/g DS versus 10<sup>1</sup> cfu/g DS for the culturing method. This strongly suggested that the *E. coli* were largely present after digestion, but were not culturable by the standard methods. Hence the data supported the hypothesis that digestion could cause the bacteria to enter the VBNC state.

In addition, immediately after centrifugation, the *E. coli* counts measured by culturing were 10<sup>4</sup> cfu/g DS. However, the cPCR method showed the same counts, 10<sup>5</sup> cells/g DS, as before dewatering. Therefore, dewatering reactivated a large portion of the *E. coli* such that they became culturable, and the overall numbers of the *E. coli* as measured by the presence of their DNA (cPCR method) did not change before or after dewatering. These results clearly supported the VBNC/reactivation hypothesis.

Also of great interest was that, according to the cPCR results, thermophilic digestion only reduced *E. coli* by two orders of magnitude, whereas the standard method culturing technique

suggested a reduction of six orders of magnitude. The results for HPC and TMC are shown in Table 3-14.

**Table 3-13. Summary of FC and *E. coli* Test Results for Thermo-2.**  
May 15, 2004 Sample Date

Sample	Avg. FC (cfu/g DS)	Std. Dev. FC (cfu/g DS)	Avg. <i>E. coli</i> (cfu/g DS)	Std. Dev. <i>E. coli</i> (cfu/g DS)	Avg. <i>E. coli</i> by cPCR (cells/g DS)	Std. Dev. <i>E. coli</i> by cPCR (cells/g DS)
Digester Feed	1.60x10 <sup>8</sup>	2.24x10 <sup>8</sup>	1.45x10 <sup>7</sup>	ID <sup>1</sup>	2.37x10 <sup>7</sup>	1.51x10 <sup>7</sup>
Centrifuge Feed	1.11x10 <sup>2</sup>	1.31x10 <sup>2</sup>	1.80x10 <sup>1</sup>	ID	6.80x10 <sup>5</sup>	2.75x10 <sup>5</sup>
Cake	2.94x10 <sup>6</sup>	4.94x10 <sup>6</sup>	2.08x10 <sup>4</sup>	2.11x10 <sup>4</sup>	2.20x10 <sup>5</sup>	8.49x10 <sup>4</sup>

<sup>1</sup> ID = insufficient data.

**Table 3-14. Summary of HPC and TMC Test Results for Thermo-2.**  
May 15, 2004 Sample Date

Sample	HPC (cfu/g DS)	TMC (cells/g DS)
Digester Feed	7.30x10 <sup>8</sup>	7.29x10 <sup>7</sup>
Centrifuge Feed	3.50x10 <sup>4</sup>	8.64x10 <sup>7</sup>
Cake	5.90x10 <sup>5</sup>	9.72x10 <sup>7</sup>

### 3.1.4 Results from Mesophilic Digestion Processes

#### 3.1.4.1 Results for Meso-1

Meso-1 had previously reported reactivation/regrowth of fecal coliforms and was therefore sampled, on June 15, 2004. The process flow diagram for Meso-1 is shown in Figure 3-6. The facility uses mesophilic, anaerobic digestion of primary and secondary sludges, with an average SRT of 21 days. A summary of the process operational parameters is provided in Table 3-15.

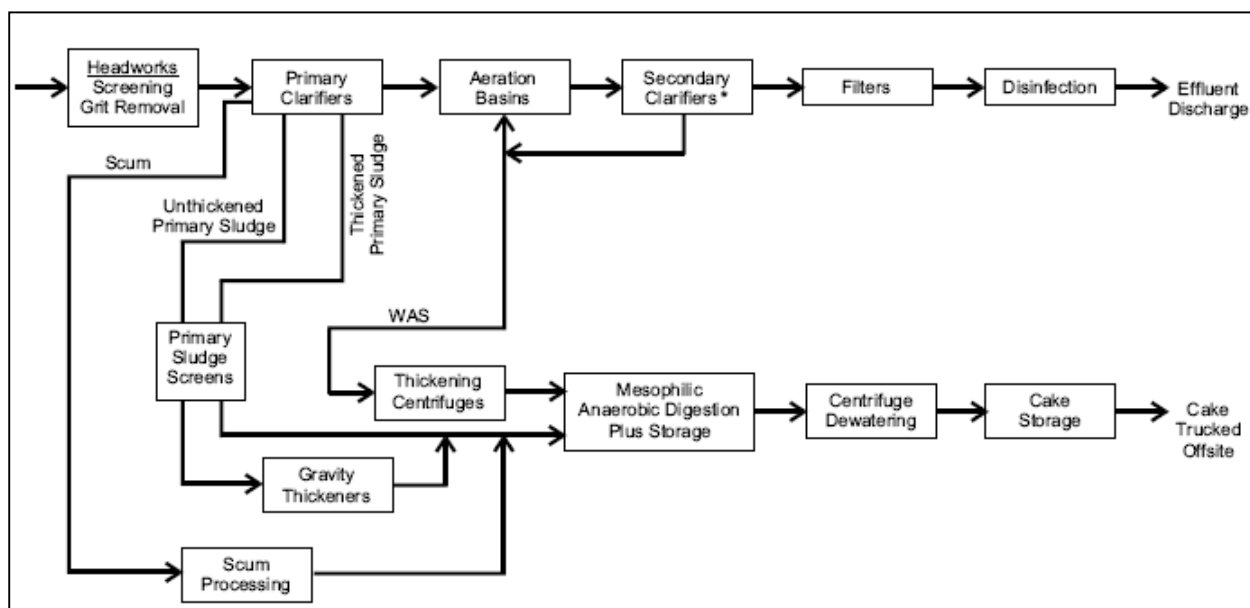


Figure 3-6. Process Flow Diagram for Meso-1.

Table 3-15. Summary of Facility Operation Information for Meso-1.

Process	Equipment	Chemicals	Operation	Notes
Primary Thickening	Gravity/clarifiers		4.5% solids	
Secondary Thickening	Sharples PM 95000 Centrifuge	Cytec Superfloc SD 2061	4.5% solids	
Anaerobic Digestion	Mesophilic, single stage		37°C 21 day SRT 40-45% VSR	
Storage	30-33°C		5 day SRT	No mixing
Dewatering	High Solids Sharples DS 906	Cytec Superfloc SD 2061 12 g/kg	21% solids	
Cake Conveyance	Screw		≈40 meters	3°C temp. increase during conveyance

**Meso-1 Sampling Results, June 15, 2004.** The samples collected from Meso-1 included the digester feed, the digester effluent/centrifuge feed, the cake immediately after dewatering, and the cake after lime addition. Meso-1 had been using lime addition to control reactivation of coliforms after dewatering. The results are summarized in Table 3-16. The results from the culturing method showed relatively high counts of FCs and *E. coli* in the digester feed, both  $10^7$ . However, after digestion, the counts were less than  $10^4$  cfu/g DS according to the culturing method. The actual value was not known since not enough dilutions were performed to determine the value. Immediately after dewatering, the cake had a significant increase in FCs and *E. coli*, up to  $10^6$  cfu/g DS, suggesting that a reactivation occurred.

The results, based on quantifying *E. coli* cells with cPCR, showed that the *E. coli* counts were very similar in the digester feed, the digester effluent, and immediately after dewatering. In

other words, the digester had little impact on reducing the *E. coli* counts. However, digestion caused the cells to enter the VBNC state, which rendered the cells non-culturable. Immediately after dewatering, the *E. coli* were reactivated, allowing them to be quantified accurately using the culturing method.

It was interesting to note that the cPCR results agreed well with the culturing method before digestion and after dewatering, the two cases where the cells were not VBNC. The cPCR method had much greater counts compared with those from the culture method for the sample collected after digestion. These results further supported the VBNC/reativation hypothesis, and suggested that standard methods are inadequate for accurately quantifying FCs when they are in the VBNC state.

**Table 3-16. Summary of Test Results for Meso-1.**  
June 15, 2004 Sample Date

Sample	FC - HML (cfu/g DS)	<i>E. coli</i> - HML (cfu/g DS)	Avg. <i>E. coli</i> by cPCR (cells/g DS)	Std. Dev. <i>E. coli</i> by cPCR (cells/g DS)	HPC - HML (cfu/g DS)	TMC (cells/g DS)
TPS	1.88x10 <sup>7</sup>	1.00x10 <sup>7</sup>	NDC <sup>1</sup>	NDC	1.59x10 <sup>8</sup>	2.51x10 <sup>8</sup>
TWAS	1.58x10 <sup>7</sup>	4.65x10 <sup>6</sup>	4.39x10 <sup>6</sup>	2.69x10 <sup>6</sup>	3.00x10 <sup>8</sup>	3.46x10 <sup>8</sup>
Centrifuge Feed	<45,454	<45,454	5.00x10 <sup>6</sup>	1.05x10 <sup>6</sup>	3.50x10 <sup>6</sup>	2.40x10 <sup>8</sup>
Cake	4.57x10 <sup>6</sup>	2.89x10 <sup>6</sup>	5.36x10 <sup>6</sup>	4.95x10 <sup>5</sup>	5.70x10 <sup>7</sup>	3.54x10 <sup>8</sup>
Cake + Lime Addition	NDC	2.09x10 <sup>4</sup>	4.21x10 <sup>6</sup>	NDC	NDC	NDC

<sup>1</sup> NDC = No data collected.

The addition of lime to the cake is used to prevent reactivation and regrowth of the FCs. As shown in Table 3-16, the addition of low dosages of lime reduced the culturable *E. coli* from approximately 3 million to about 20,000 cfu/g DS. However, the *E. coli* measured by cPCR remained relatively constant after lime addition, see Table 3-16. This could suggest that the *E. coli* were put back into a VBNC state and could become non-culturable after lime addition rather than being killed. However, additional research would need to confirm this. For example, it is possible the *E. coli* were killed, yet their DNA had not yet degraded. A possible experiment would be to monitor *E. coli* during longer term storage after lime addition using both culturing and cPCR quantification.

**Meso-1 Sampling Results, September 20, 2004.** Meso-1 was sampled again on this date, and the results for this sampling event are shown in Table 3-17. These results are similar to those of the previous sampling, although the digester effluent or centrifuge feed had slightly greater counts of FC and *E. coli* by the SCM method, and the cake had lower counts. Replicate analysis for SCM and cPCR was performed for statistical analysis. Unfortunately, the FC data for the centrifuge feed only had one readable value for the culturing method, thus no standard deviation could be calculated for this sample. A graph comparing the SCM and cPCR results is shown in Figure 3-7. The results show a similar profile indicative of bacteria entering the VBNC state after digestion, followed by reactivation during dewatering.

**Table 3-17. Summary of FC and *E. coli* Enumeration by SCM and cPCR.**  
September 20, 2004 Sample Date

Sample	Avg. FC - HML (cfu/g DS)	Std. Dev. FC - HML (cfu/g DS)	Avg. <i>E. coli</i> - HML (cfu/g DS)	Std. Dev. <i>E. coli</i> - HML (cfu/g DS)	Avg. <i>E. coli</i> by cPCR (cells/g DS)	Std. Dev. <i>E. coli</i> by cPCR (cells/g DS)
Digester Feed	2.59x10 <sup>7</sup>	2.30x10 <sup>7</sup>	1.92x10 <sup>7</sup>	3.09x10 <sup>7</sup>	7.65x10 <sup>6</sup>	1.94x10 <sup>6</sup>
Centrifuge Feed	6.44x10 <sup>4</sup>	ID <sup>1</sup>	4.46x10 <sup>4</sup>	1.87x10 <sup>4</sup>	4.42x10 <sup>5</sup>	2.00x10 <sup>5</sup>
Cake	1.24x10 <sup>6</sup>	5.41x10 <sup>5</sup>	4.55x10 <sup>5</sup>	2.74x10 <sup>5</sup>	1.44x10 <sup>5</sup>	3.84x10 <sup>4</sup>

1 ID = insufficient data to calculate

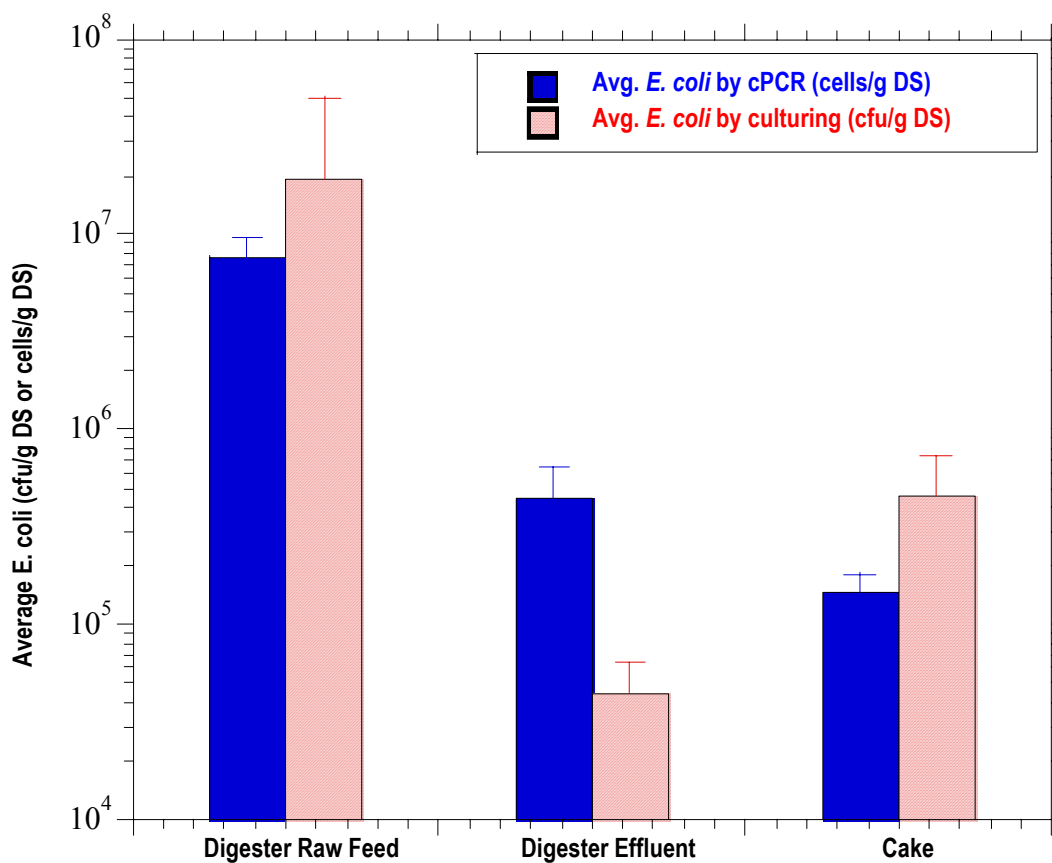


Figure 3-7. Comparison of *E. coli* Enumeration Results for SCM and cPCR at Meso-1.

### 3.1.4.2 Results for Meso-2

The facility at Meso-2 has a treatment capacity of 18 MGD, and 95% of the flow is municipal. The solids treatment consists of a single-stage mesophilic anaerobic digester followed by dewatering on a high solids centrifuge. A schematic of the process flow diagram is provided in Figure 3-8. The typical operational parameters are summarized in Table 3-18.

Meso-2 was sampled on May 15, 2004, and the testing including both standard culturing methods and cPCR.

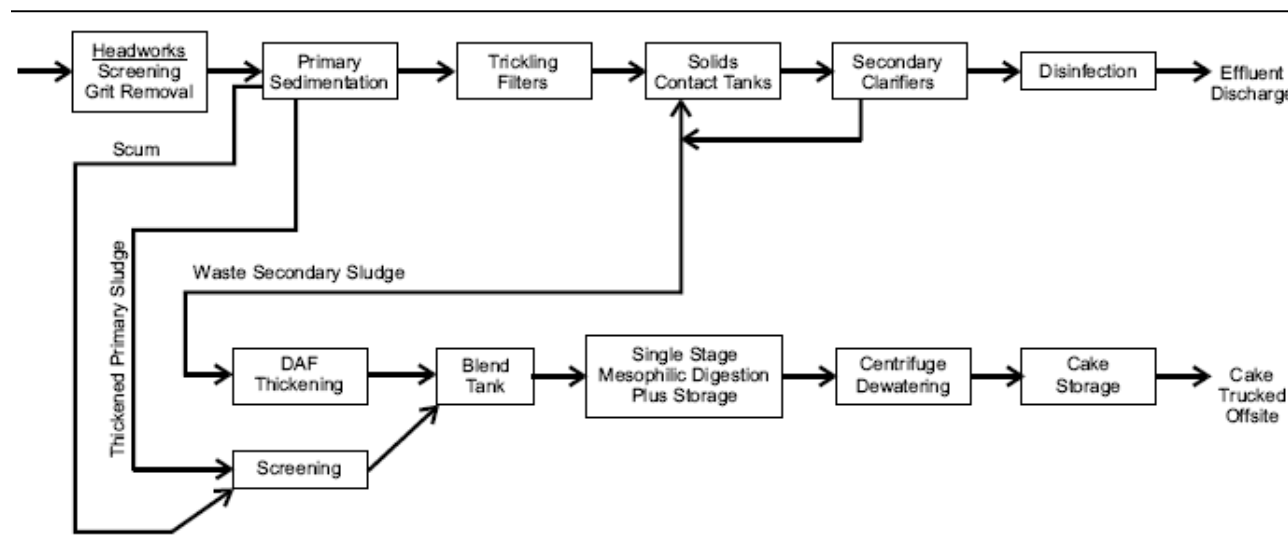


Figure 3-8. Process Flow Diagram for Meso-2.

Table 3-18. Summary of Plant Operation Information for Meso-2.

Process	Equipment	Chemicals	Operation
Primary Thickening	Clarifiers		3.5-4% solids
Secondary Thickening	DAF	Polymer 1 g/kg	3.5-4% solids
Anaerobic Digestion	Mesophilic, single stage		37-38 °C 30-35 day SRT 50-60% VSR
Storage	Pump mixed		≈ 1 day SRT
Dewatering	Humbolt/Bird?	Polymer 10-12 g/kg	22-24% solids
Cake Conveyance	Cake Pumps		≈25-30 meters

**Meso-2 Sampling Results, May 15, 2004.** The microbial quantification results from sampling at Meso-2 are summarized in Table 3-19. The samples included the digester feed, digester effluent/centrifuge feed, and dewatered cake. Interestingly, the digestion process at this plant reduced the FCs and *E. coli* densities by two orders of magnitude, and the bacteria did not appear to enter the VBNC state during digestion. For example, the *E. coli* counts by both the culturing and cPCR were approximately equivalent in the digester feed, the centrifuge feed, and the cake. Therefore, no reactivation could occur since the cells did not enter the VBNC state. These results were interesting in that the plant may provide insight into the factors that cause cells to enter or not enter the VBNC state.

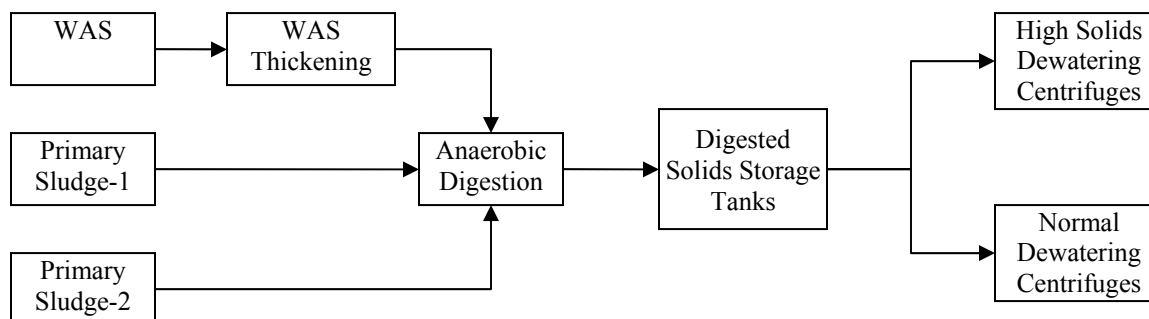
**Table 3-19. Summary of Test Results for Meso-2.**  
May 15, 2004 Sample Date

Sample	FC – HML (cfu/g DS)	<i>E. coli</i> – HML (cfu/g DS)	<i>E. coli</i> – BU (cfu/g DS)	<i>E. coli</i> by cPCR (cells/g DS)	HPC – HML (cfu/g DS)	TMC (cells/g DS)
Digester Feed	5.41x10 <sup>7</sup>	1.62x10 <sup>7</sup>	NT <sup>1</sup>	1.89x10 <sup>7</sup>	5.90x10 <sup>8</sup>	9.18x10 <sup>7</sup>
Centrifuge Feed	2.35x10 <sup>5</sup>	4.12x10 <sup>5</sup>	NT	1.60x10 <sup>5</sup>	9.00x10 <sup>5</sup>	1.51x10 <sup>8</sup>
Cake	8.00x10 <sup>5</sup>	4.44x10 <sup>4</sup>	4.76x10 <sup>5</sup>	7.26x10 <sup>5</sup>	1.13x10 <sup>7</sup>	4.35x10 <sup>8</sup>

<sup>1</sup> NT = not tested.

### 3.1.4.3 Results for Meso-3

Meso-3 uses a conventional secondary activated sludge process, and mixed primary and secondary solids are digested by mesophilic digestion. The typical flow of the facility is 190 MGD. The digested solids are dewatered on a combination of high- and medium-solids centrifuges. A schematic of the facility is shown in Figure 3-9, and a summary of operational parameters are provided in Table 3-20. This site was sampled one time on October 26, 2004.



**Figure 3-9. Process Flow Diagram for Meso-3.**

**Table 3-20. Summary of Facility Operation Information for Meso-2.**

Process	Equipment	Chemicals	Operation
Primary Thickening	Clarifiers		3.5-4% solids
Secondary Thickening			4-5% solids
Anaerobic Digestion	Mesophilic, single stage		
Storage	No mixing		≈ 1 day SRT
Dewatering	Humbolt and Bird Centrifuges	Polymer 10-12 g/kg	24-33% solids
Cake Conveyance	Belt conveyor		≈30-40 meters

**Meso-3 Sampling Results, October 26, 2004.** Samples were obtained from the high-solids centrifuge and were analyzed using both the standard culturing methods and cPCR. The

results from this testing are provided in Table 3-21. All three samples also tested positive for *E. coli* O157:H7, and only the digester feed tested positive for *Salmonella*.

**Table 3-21. Summary of Test Results for Meso-3.**  
October 26, 2004 Sample Date

Sample	Avg. FC – HML (cfu/g DS)	Std. Dev. FC – HML (cfu/g DS)	Avg. <i>E. coli</i> – HML (cfu/g DS)	Std. Dev. <i>E. coli</i> – HML (cfu/g DS)	Avg. <i>E. coli</i> by cPCR (cells/g DS)	Std. Dev. <i>E. coli</i> by cPCR (cells/g DS)
Digester Feed	5.85x10 <sup>7</sup>	4.93x10 <sup>7</sup>	2.18x10 <sup>7</sup>	8.87x10 <sup>6</sup>	3.01x10 <sup>7</sup>	1.13x10 <sup>6</sup>
Centrifuge Feed	4.76x10 <sup>4</sup>	ID <sup>1</sup>	<47619	ID	2.54x10 <sup>5</sup>	5.67x10 <sup>4</sup>
Cake	2.24x10 <sup>5</sup>	1.83x10 <sup>5</sup>	1.76x10 <sup>5</sup>	7.68x10 <sup>4</sup>	3.45x10 <sup>5</sup>	2.12x10 <sup>5</sup>

<sup>1</sup> ID = insufficient data to calculate.

For comparison, the results for *E. coli* measured by cPCR and SCMs are presented in Figure 3-10. As shown in the graph, the cPCR and SCM numbers agreed well for the digester feed samples, and no statistical difference was measured ( $p < 0.05$ ). However, the cPCR results were significantly higher than the SCM results for the digester effluent sample. It should be noted that the *E. coli* counts using the SCM were below the dilution values used, and were reported as less than 47,619. For graphing, a value of 20,000 cfu/g DS was used for this data point.

The results indicated that cells were in the VBNC state, and therefore, the SCM produced results that were lower than the total number of *E. coli* present. After centrifugation, the *E. coli* numbers increased by at least two orders of magnitude according to the SCM, but remained basically constant according to the cPCR results. No statistical difference was calculated between the digester effluent and the cake *E. coli* numbers for the cPCR results, which suggested that cells were present in the same concentrations before and after dewatering. However the SCM did not measure the majority of the cells after digestion since they were in the VBNC state. Interestingly, the SCM and cPCR results agreed much better in the cake samples. These results supported the hypothesis that the bacteria entered the VBNC state during digestion and were then reactivated during dewatering.

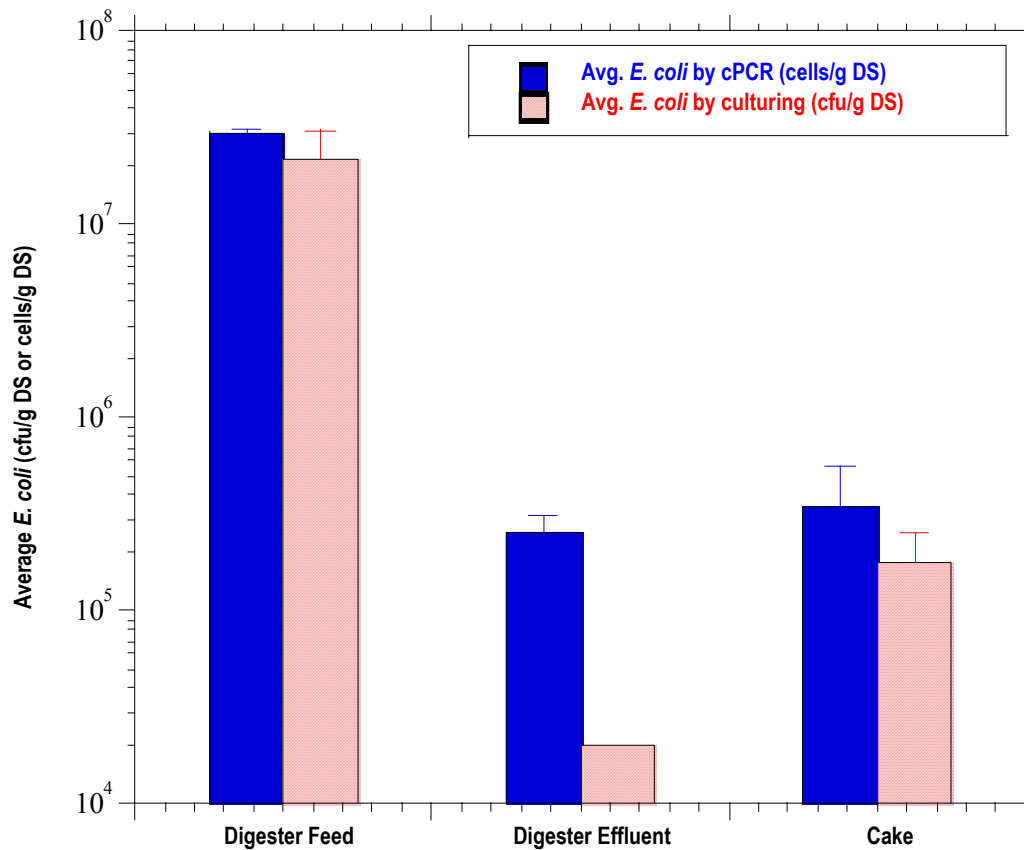


Figure 3-10. Comparison of *E. coli* Enumeration Results for SCM and cPCR at Meso-3.

### 3.2 Discussion and Implications of Results

For this research, bacteria are defined in the VBNC state as simply being bacteria that are not cultured and therefore are not enumerated by SCMs. Furthermore, reactivation or resuscitation is defined as the change in culturability of the bacteria, with the bacteria changing from VBNC to culturable by SCMs. The results of this research support the hypotheses that indicator organisms can be VBNC after digestion, and that reactivation or resuscitation can occur after centrifuge dewatering. The cPCR method is able to quantify *E. coli* that are VBNC and are not enumerated by standard culturing techniques. For example, a plot of the data from Thermo-2 is shown in Figure 3-11. The culturing method shows a significant decrease (six orders of magnitude) in the *E. coli* density as a result of digestion. However, the cPCR results only show a decrease of two orders of magnitude. Immediately after dewatering, the culturing method enumerated  $10^4$  *E. coli*, and the cPCR method was still  $10^5$ , the same order of magnitude as before dewatering.

The results support the hypothesis that bacteria are VBNC after digestion, and therefore are not enumerated correctly by standard culturing techniques. This can result in a significant underestimation of the viable bacterial counts. The immediate conclusion from these results is that standard culturing techniques may be inadequate for enumerating bacteria after anaerobic digestion if they are VBNC.

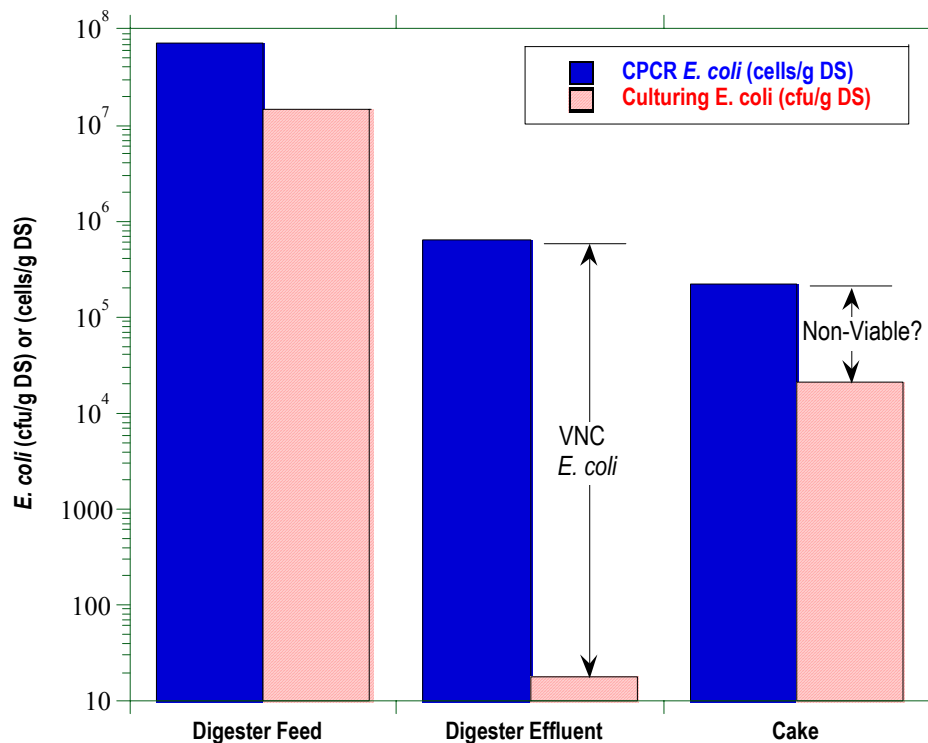


Figure 3-11. *E. coli* in Samples from Thermo-1 as Measured by cPCR and Standard Culturing Methods.

It is interesting to note that the facilities sampled included three mesophilic facilities, two TPAD facilities, and two thermophilic facilities, and at least one of each type exhibited reactivation, and one did not. The logical question was: Why did some facilities exhibit reactivation and others did not? A summary of the VBNC/reactivation results for each field site is presented in Table 3-22 along with some of the facility operation data to compare what factors may have contributed to reactivation. All the facilities used high-solids centrifuges for dewatering, though different models were used.

The data presented in Table 3-22 provide no clear-cut relationship between operational variables and the occurrence of VBNC bacteria. Comparing TPAD-1 and 2, which both used TPAD, TPAD-1 had reactivation and TPAD-2 did not. However, TPAD-2 did not have any significant amounts of FC entering digestion and it seemed to be a result of the very high industrial (pulp and paper) inflow into the facility. This made a comparison between the two facilities difficult.

Thermo-1 and Thermo-2 each used thermophilic digestion, but only Thermo-2 exhibited reactivation. The main difference between the two facilities was that Thermo-1 had a four-stage digestion process with an estimated SRT of 22 d, whereas Thermo-1 had single-stage digestion with estimated SRT between 15-20 d. Therefore, Thermo-1 had a longer SRT that may have resulted in better kill rates during digestion. In addition, use of a four-stage process better simulates a plug-flow reactor, which is much more efficient and less likely to exhibit short-circuiting compared with a single-stage completely mixed reactor. This, too, may have contributed to better *E. coli* destruction rates at Thermo-1.

An interesting study would be to collect effluent samples from each of the four stages from Thermo-1 to determine the *E. coli* destruction rate of each stage. The first stage may have been similar to Thermo-2. This study is planned for Phase 2 of the research.

Finally, Meso-1, 2 and 3 used mesophilic digestion. Meso 1 and 3 showed the patten for bacteria entering the VBNC state followed by reactivation after centrifuge dewatering. However, the digestion process of Meso-2 did not decrease the FCs and *E. coli*, and the *E. coli* did not enter the VBNC state during digestion. It was not clear why this occurred since the SRT for the digestion process was quite high.

**Table 3-22. Summary of Field Site Operation Data and VBNC/Reactivation Results.**

Field Site	Reactivation of <i>E. coli</i>	Digestion Type	Digestion SRT (d)	Digestion VSR (%)	Cake Solids	Comments
TPAD -1	Confirmed	TPAD	15 at 58°C and 21 d at 37°C	60%	30%	Longest overall SRT
TPAD-2	Negative	TPAD	8 d at 55°C and 20-25 d 43 °C	40-45%	27-29%	No FCs into digestion, Major industrial flow
Thermo-1	Negative	Thermophilic – four-stage	22 d at 56°C	60-62%	30-32%	Long SRT, 4-stages
Thermo-2	Confirmed	Thermophilic single-stage	15-20 d at 55°C	65%	35%	
Meso-1	Confirmed	Mesophilic – single-stage	21 d at 37°C	40-45%	21%	Two holding tanks after digestion
Meso-2	Negative	Mesophilic – single-stage	30-35 d at 37°C	50-60%	22-24%	No reactivation or VBNC
Meso-3	Confirmed	Mesophilic – single-stage	22 d at 36°C	45-58%	30-33%	Reactivation

The results seem to indicate that bacteria transition from a culturable state to a VBNC state during digestion, and extended digestion will kill the bacteria.

The data for average total DNA extracted from each sample at each facility are shown in Table 3-23. Although not shown in the table, the results are all from at least three replicates, and the relative standard deviations were all less than 5% of the mean value. Very similar trends were found for most facilities, and the total DNA values showed similar trends as those of the FCs and *E. coli*. The digester feeds had relatively high DNA concentrations, from 3,100 to 10,500 µg/g DS, and these values were reduced during digestion generally by 50% or more for most facilities. However, Meso-2 only had a reduction of about 17% during digestion, the lowest of all the facilities. Meso-2 also had the lowest reduction in *E. coli* counts because of digestion, and the *E. coli* did not enter the VBNC state, but remained culturable.

The VS reduction generally was less than the DNA reduction, suggesting that DNA was readily degraded in the digester, likely after release from dead microorganisms. The cake samples contained approximately equivalent DNA concentrations compared with those in the centrifuge feed, similar to the cPCR results for *E. coli*. The cPCR results for *E. coli* also are summarized in Table 3-24 for comparison. It is interesting to note that the cPCR results showed

that the mesophilic digestion facilities had *E. coli* counts that were generally close to the EPA Class B requirements of less than two million FCs. Although the measurement was for *E. coli*, the main group of FCs is typically *E. coli*.

**Table 3-23. Total DNA Concentrations in Sampling, Units in µg/g DS.**

Sample Location	TPAD-1	Thermo-1	Thermo-2	Meso-1 "9/04	Meso-1 6/04	Meso-2	Meso-3
Digester Feed	3609	4682	3113	10467	6366	5563	7525
Thermo	832	ND <sup>1</sup>	ND	ND	ND	ND	ND
Meso	626		ND	ND	ND	ND	ND
Centrifuge Feed	761	1444	1846	4804	2621	4590	3126
Cake	797	1404	983	4558	3181	3825	2785

<sup>1</sup>ND=no data

**Table 3-24. Summary of *E. coli* cPCR Results for Each Plant.**

Sample Location	Thermo-1	Thermo-2	Meso-1 6/04	Meso-1 9/04	Meso-2	Meso-3
Digester Feed	3.26x10 <sup>7</sup>	2.37x10 <sup>7</sup>	4.39x10 <sup>6</sup>	7.65x10 <sup>6</sup>	1.89x10 <sup>7</sup>	3.01x10 <sup>7</sup>
Centrifuge Feed	Non-detect	6.80x10 <sup>5</sup>	5.00x10 <sup>6</sup>	4.42x10 <sup>5</sup>	1.60x10 <sup>5</sup>	2.54x10 <sup>5</sup>
Cake	Non-detect	2.20x10 <sup>5</sup>	5.36x10 <sup>6</sup>	1.44x10 <sup>5</sup>	7.26x10 <sup>5</sup>	3.45x10 <sup>5</sup>

## CHAPTER 4.0

# MECHANISTIC AND REGROWTH RESULTS

### 4.1 Introduction

A series of mechanistic experiments was performed to support or refute the Hypotheses 2 and 3 discussed in Chapter 1.0. Hypothesis 2 theorized that some type of inducer is released during centrifuge dewatering that resuscitated bacteria, enabling them to become culturable. Hypothesis 3 stated that after resuscitation, conditions in the cake, such as nutrient and substrate availability, were favorable for growth. The results from experiments to examine these hypotheses are provided in the following sections.

### 4.2 Examining Centrate for Induction Properties/Autoinducer Activity

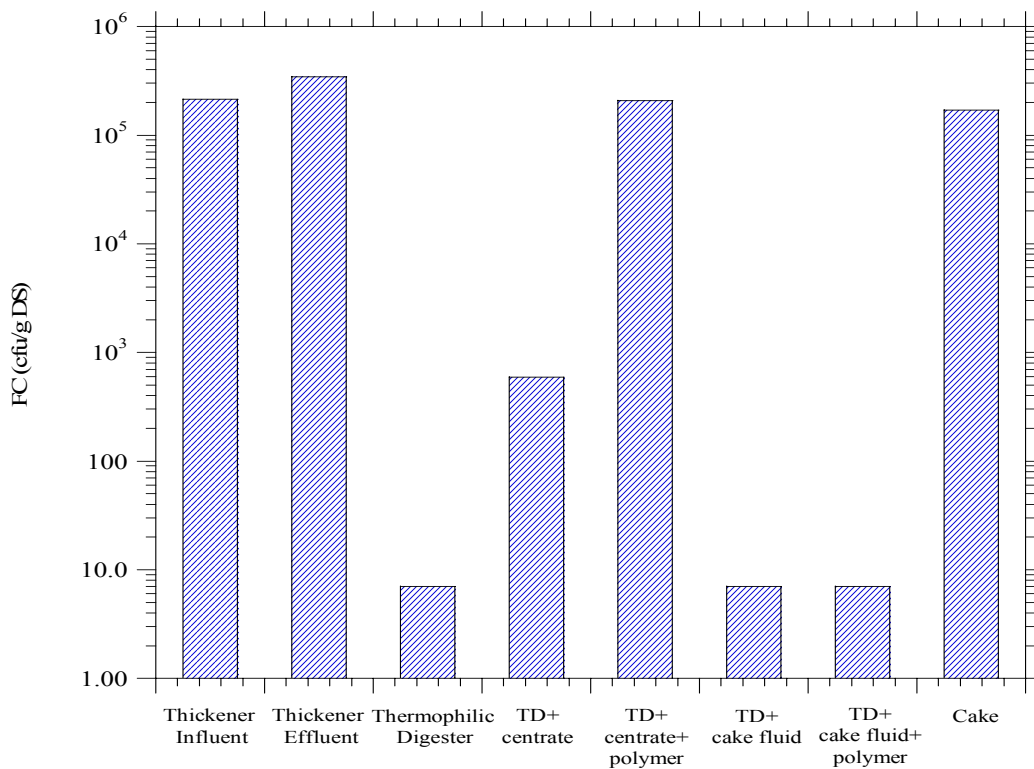
Based on the research hypothesis, it was believed that VBNC microbes could be reactivated given the proper signal. For example, research has shown that the presence of molecules termed “autoinducers” (AI) can be a signal for microbes to grow after being in the VBNC state. This signaling mechanism has been shown to occur for a number of different microbes including *E. coli* and *Salmonella*. The hypothesis in this research was that high solids centrifuges could cause the release of AIs into solution, thereby providing the signal for microbes to grow. After this induction, the microbes also could become culturable by standard techniques and they could also grow during cake storage. The resuscitating or inducing agent could also simply be the presence of some key nutrient or substrate that becomes available during centrifuge dewatering. In addition, it is possible that a change in environmental conditions such as redox could result in greater culturability.

To examine the possibility that an inducer-like substance was being released and was acting as a signal for growth, a series of experiments was performed in which the centrate from a facility showing reactivation was mixed with the un-dewatered digester effluent that previously had low or non-detectable FC counts. The results from the first set of experiments with TPAD-1 are shown in Figure 4-1.

The raw sludges before and after thickening contained  $10^5$  FC/g DS, and after the thermophilic digester (TD) the FCs were below the detection limit. When the centrate was added to the TD sample, the FCs increased by at least two orders of magnitude, and addition of the centrate plus the polymer resulted in an increase of at least four orders of magnitude. The centrate was filtered and sterilized prior to addition to the TD sample. The FC densities after centrate and polymer addition were the same order of magnitude as the counts measured in the cake. These results strongly suggest that the centrate contained a substance that could induce the microbes to become culturable. The polymer enhanced this effect.

It was not clear why the polymer enhanced the induction of the microbes, but additional testing is planned in Phase 2 to understand the impact of polymer on inducing the microbes to become culturable. The addition of the fluid extracted from the cake did not result in any

increased FC counts. This was perhaps because the inducers had already been taken up by the microbes and none was available for further reactivation of the microbes. Comparing the FC counts in the sample prior to digestion to that in the cake showed that they were approximately equal. This suggested that the digester did not kill the FCs, but instead rendered them VBNC.



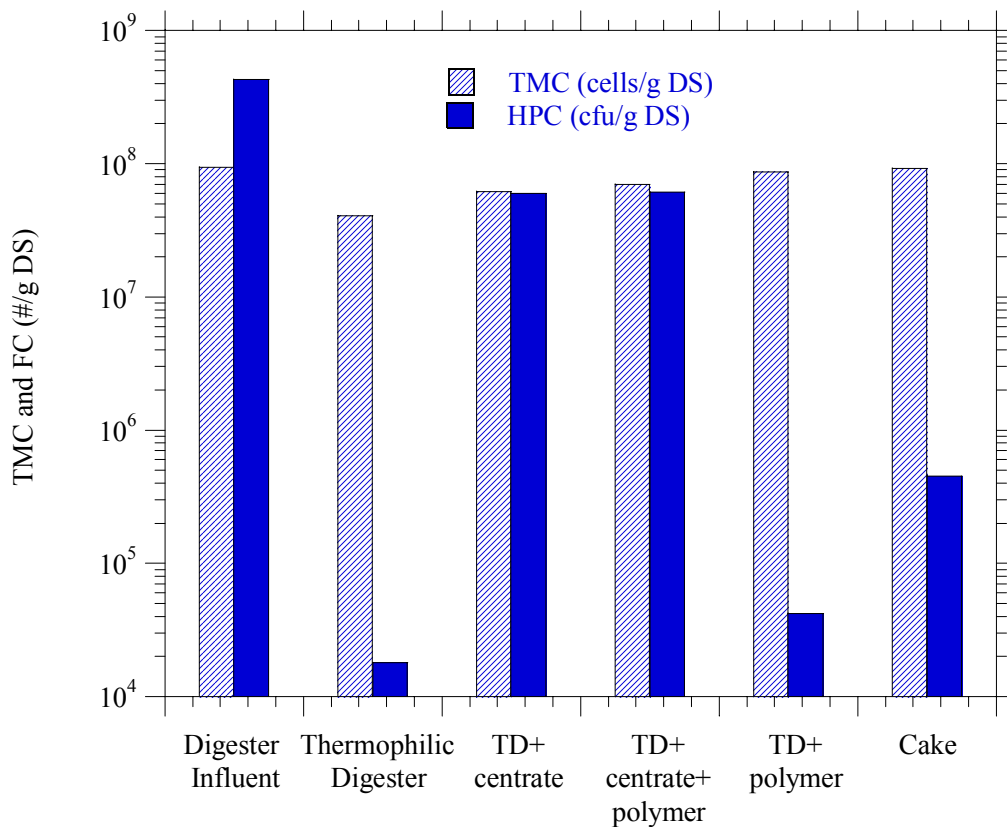
**Figure 4-1. FC Counts for Different Samples and Additions.**  
*TD = Thermophilic Digester*

A similar set of experiments was performed with the Thermo-1 samples. The digester samples were spiked with the centrate and/or polymer from TPAD-1 since it was believed that TPAD-1 had AI-type activity. The initial testing of Thermo-1 did not show any reactivation of FCs or *E. coli*, but HPC counts were much greater in the cake compared with those from the digester. Therefore, HPCs showed the ability to reactivate. The results from these experiments are shown in Figure 4-2.

The addition of the centrate to the thermophilic digester (TD) sample increased the HPC counts by three orders of magnitude, but did not impact FCs. The polymer did not have any significant additional effect as was found for TPAD-1. As in the previous trial, the counts after centrate addition were close to the counts prior to digestion, suggesting the microbes were present and became reactivated. In addition, the total microbial counts (TMC) were approximately equivalent for all the samples, suggesting the microbes were present in all the samples at roughly equal concentrations. However, they were not culturable in the digester sample.

The results supported the hypothesis that inducer-type activity was being released during centrifugation, and this compound could induce the microbes to become culturable. In addition,

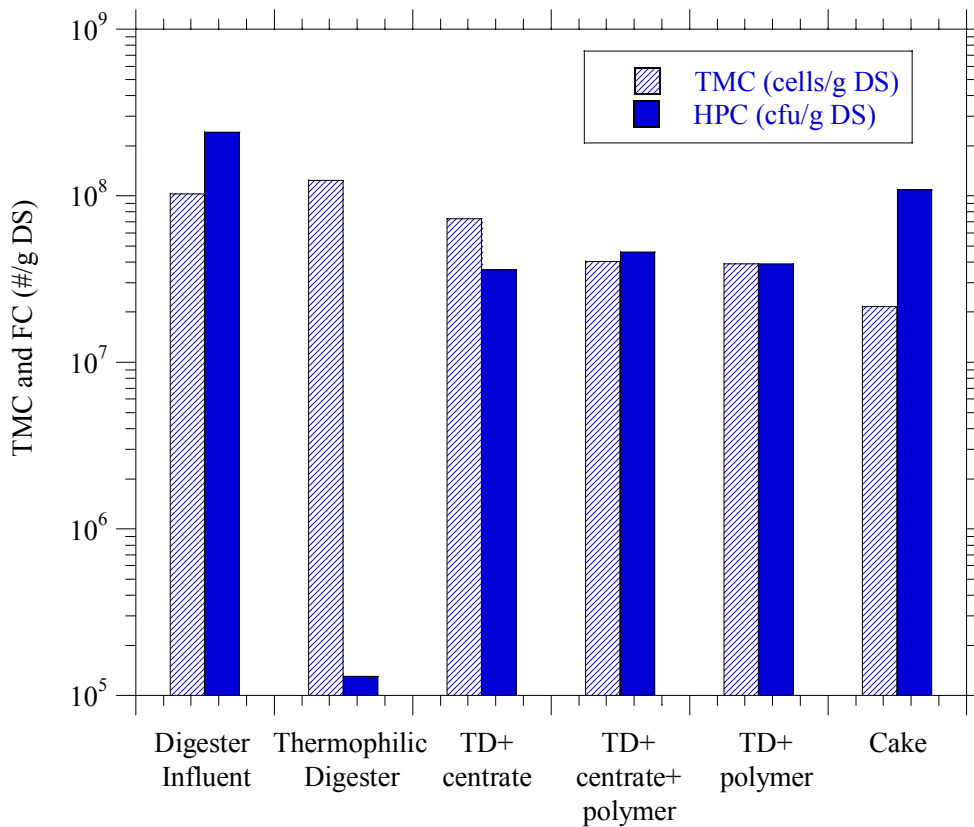
the results also supported the hypothesis that the microbes were viable but not culturable in the digester.



**Figure 4-2. Heterotrophic Plate Counts and Total Microbial Counts for Thermo-1 Digester Sample with Centrate and/or Polymer Addition.**

*TD = Thermophilic Digester*

The centrate addition study also was performed on Thermo-2 samples. The centrate and polymer from TPAD-1 was added to the thermophilic digester sample to determine if the centrate and/or polymer could induce the microbes to become culturable. The results from these tests are shown in Figure 4-3. In these tests, the FC counts did not increase due to centrate and/or polymer addition, but the HPC counts did. As with Thermo-1, the TMCs remained relatively constant, suggesting the microbes were present, but were not culturable in the digester sample. The addition of the centrate and/or the polymer resulted in the microbes becoming culturable.



**Figure 4-3. Heterotrophic Plate Counts and Total Microbial Counts for Thermo-2 Digester Sample with Centrate and/or Polymer Addition.**

*TD = Thermophilic Digester*

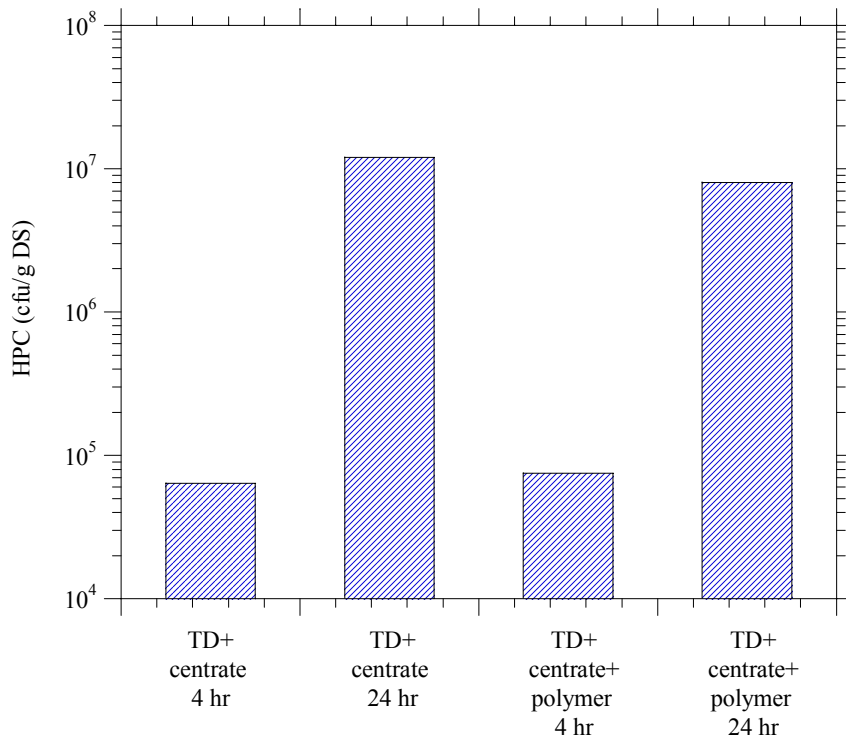
### 4.3 Impact of Storage Time on Reactivation

Several studies were performed to examine the impact of storage time on microbial densities and activity. For TPAD-1, the samples were analyzed after four and 24 hours of storage after the addition of centrate and/or polymer to the un-dewatered effluent from the thermophilic digester (TD). Only FCs and HPCs were measured in these tests. The HPC results from TPAD-1 are shown in Figure 4-4. The FC counts did not show reactivation in these experiments, so the data are not shown for clarity. Storage for 24 hours compared with four hours resulted in much greater HPCs. This could be caused by a regrowth during storage or by reactivation.

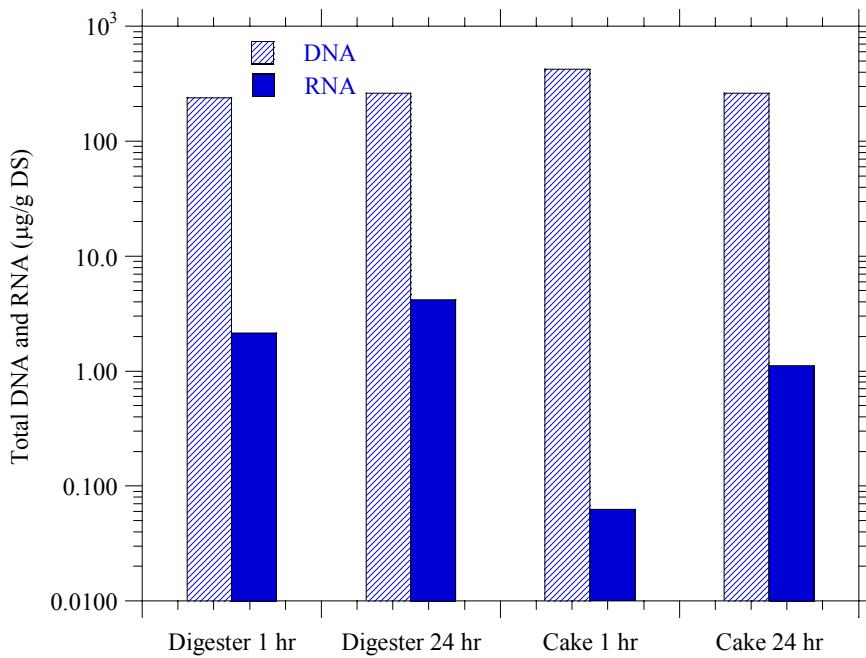
To investigate this further, a second experiment was performed (on a different date), in which the digester and cake samples were analyzed for total DNA and RNA. The results from this testing are shown in Figure 4-5. Interestingly, the total DNA in the digester and the cake remained fairly constant (within the same order of magnitude).

Immediately after dewatering, the RNA was significantly reduced when compared with that from the digester sample. This could be because of a loss of methanogenic activity as discussed previously. However, the RNA increased by two orders of magnitude during storage. It was likely that the total number of microbes remained relatively constant, but the increased RNA was likely caused by increased activity of the surviving and reactivated microbes. RNA is

produced by active microbes for the synthesis of proteins, and unlike DNA, many copies of RNA can be produced by microbes, therefore, increased RNA after storage was indicative of increased microbial activity.



**Figure 4-4. Effect of Storage time on HPC Counts for TPAD-1.**  
*TD = Thermophilic Digester*



**Figure 4-5. Effect of Storage Time Total DNA and RNA for TPAD-1.**

#### 4.4 Regrowth after Reactivation and VBNC during Cake Storage

The cake samples from Thermo-2 were stored, and during storage they were sampled for bacterial enumeration. The results from this storage experiment are shown in Figure 4-6. It should be noted that the cPCR results showed the centrifuge feed contained *E. coli* counts of  $6.35 \times 10^5$  cell/g DS (from Table 3-13), which was very similar to those of the cake sample at time zero. Also, the cPCR results matched well with the culturing method values for the cake.

The *E. coli* counts increased several orders of magnitude during the first one to two days of storage as measured by both the culturing methods and cPCR method, although some differences were measured between the two data sets from the two labs. After eight days of storage, the *E. coli* counts began to decrease to approximately  $4.62 \times 10^6$  cfu/g DS according to the culturing method. However, the cPCR method showed a relatively stable count not very different from the day-two sample, about  $4.62 \times 10^7$  cells/g DS.

This could be explained by two mechanisms. First, it was possible the *E. coli* began to enter the VBNC state due to a lack of substrate, and as a result they were still present as shown by the cPCR results, but they were becoming non-culturable. A second possibility was that the *E. coli* were in fact beginning to die, but their DNA remained intact for this period, resulting in an overestimate by the cPCR method.

Additional testing is needed to better understand the fate of *E. coli* during storage. If the mechanism was a re-entrance of *E. coli* into the VBNC state, is it possible then that they could again be reactivated? Also, at what point do *E. coli* actually die during storage? These questions will be investigated in the second phase of the research.

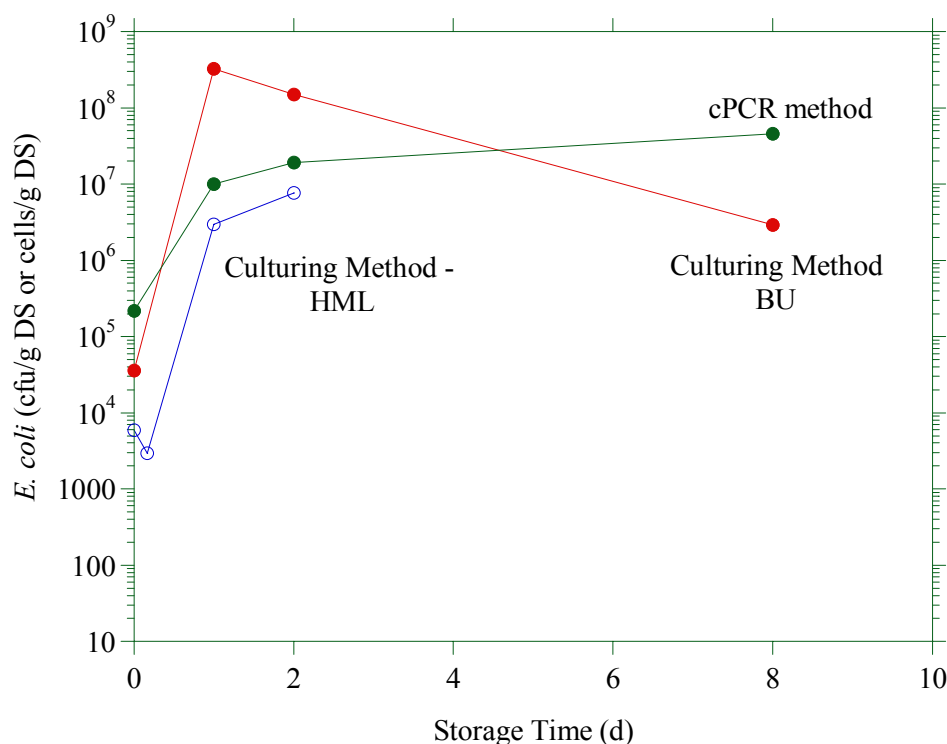


Figure 4-6. *E. coli* as Measured by Culturing Techniques by Two Labs and by cPCR During Storage.

The results, shown in Figure 4-6, also supported the validity of cPCR for quantifying *E. coli* and distinguishing between regrowth and reactivation. The graph shows proportional increases in both the cPCR counts and the counts from the culturing methods. This is indicative of regrowth. In contrast, after digestion when the ratio of the cPCR counts to culturing counts is high, this indicates the microbes are in the VBNC state. After dewatering, if this ratio decreases substantially, such that the cPCR and culture counts are approximately equivalent, this is indicative of reactivation.



## CHAPTER 5.0

# CONCLUSIONS AND RECOMMENDATIONS

### 5.1 Conclusions

Seven full-scale facilities were sampled to determine if indicator organisms could become VBNC after digestion and then be reactivated after high-solids centrifugation. A molecular method was developed to enumerate *E. coli* based on the number of copies of their DNA, and these results were compared with standard culturing methods. Based on these results, the following conclusions could be drawn:

- ◆ Solids from digestion facilities at four sites contained *E. coli* in viable but non-culturable state, and these bacteria were reactivated during dewatering. The digestion processes that resulted in VBNC bacteria included mesophilic, thermophilic, and TPAD processes.
- ◆ Digestion at three other field sites resulted in no apparent VBNC *E. coli*. The digestion process included mesophilic, thermophilic, and TPAD processes.
- ◆ No single operational parameter (such as digestion SRT or temperature) seemed to determine whether a process would cause VBNC conditions and reactivation, and additional research is needed to better understand the conditions that result in VBNC bacteria.
- ◆ Reactivation or resuscitation after centrifugation appears to occur through the release of some type of inducer compounds that renders the VBNC bacteria culturable.
- ◆ After reactivation, the indicator organisms were able to regrow rapidly and increase by three to four orders of magnitude during the first one to two days of storage.
- ◆ Quantitative PCR appears to be a good method to quantify VBNC bacteria since these bacteria were not enumerated by standard culturing techniques, but they could be enumerated by determining the number of DNA copies for the specific bacteria.
- ◆ Standard culturing methods have the potential to significantly underestimate the concentration of indicator organisms that are VBNC following anaerobic digestion.

The results do provide some insight into possible mitigation strategies. For example, the multi-stage thermophilic process was able to completely destroy the FC and *E. coli*, suggesting that reactors in series or in more general terms, reactor hydraulics, may be an important method to attain desired reductions of indicator organisms. In addition, there may be some simple chemical additions, such as low-dose lime addition to the cake, which could be used to control reactivation and regrowth. Longer-term storage also could be a strategy to reduce FCs to desired levels.

### 5.2 Recommendations

Additional research is needed to better understand how and why bacteria enter the VBNC state. Research is needed to better establish the relationship between design parameters such as

SRT and reactor hydraulics and the actual destruction of indicator organisms. In addition, the development of future design criteria for digestion needs to incorporate analysis of indicator organisms in the VBNC state to fully understand the relationship among time, temperature, and FC destruction rates. Otherwise, relying solely on standard culturing methods as done in the past may lead to designs that do not actually achieve the desired results.

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Anchorage Water & Wastewater Utility

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Phoenix Water Services Department  
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Delta Diablo Sanitation District  
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East Bay Municipal Utility District  
Eastern Municipal Water District  
El Dorado Irrigation District  
Fairfield-Suisun Sewer District  
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Las Virgenes Municipal Water District  
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Lodi, City of  
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Orange County Sanitation District  
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Santa Barbara, City of  
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District of Columbia Water & Sewer Authority

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JEA  
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Columbus Water Works  
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Greater Peoria Sanitary District  
Kankakee River Metropolitan Agency  
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### Louisiana

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