PREDOMINANCE OF *BACILLUS ANTHRACIS* IN THE BIOLOGICAL POPULATION OF AN ACTIVATED SLUDGE REACTOR

By

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Civil and Environmental Engineering
in the Department of Civil and Environmental Engineering

Mississippi State, Mississippi

April 2011

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Date of Degree: April 29, 2011

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Title of Study: PREDOMINANCE OF *BACILLUS ANTHRACIS* IN THE BIOLOGICAL POPULATION OF AN ACTIVATED SLUDGE REACTOR

Pages in Study: 72

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The effects of heavy metals, endocrine disruptors, and other contaminants on activated sludge and wastewater treatment operations have been studied extensively. However, the potential for infiltration of bacterial species not native to activated sludge has not been extensively examined. This project investigated the effect of the contamination of activated sludge wastewater treatment systems by *Bacillus anthracis*. From a pure culture of *Bacillus megaterium* inoculations of two bench-scale, sequencing batch reactors with different solids retention times was studied. Microbial enumeration testing was initially done on the mixed liquor alone, but to further detail the *Bacillus* path through the reactor, further testing was done to examine the supernatant. The data indicated that little effect would be caused from *Bacillus* inoculation of activated sludge reactors as population diversity returned to normal within a period of time equal to approximately one quarter of the solids retention time.

ACKNOWLEDGEMENTS

The author expresses his sincere thanks to the opportunity, guidance and support that was vital to the completion of this thesis. Firstly, appreciation is extended to Dr. Dennis D. Truax, Department Head and Thesis Director, for his constant availability and aid through the course of the research and writing of this work. Appreciation is extended to Dr. Benjamin S. Magbanua for his role in my environmental education and hands on instruction in the testing vital to this research. Thanks are also extended to Dr. Jairo N. Diaz-Ramirez and Dr. James L. Martin for their roles as committee member and graduate coordinator, respectively, in the review and approval of this work. Finally, great appreciated is extended to the Mississippi State University Department of Civil and Environmental Engineering for the financial support of both this project and graduate education of the author.

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CHAPTER I

INTRODUCTION

The effects of heavy metals, endocrine disruptors, and other contaminants on activated sludge and wastewater treatment operations have been studied extensively and rightly so. These contaminants can cause changes in efficiency of operations resulting in degradation of receiving environments as well as hazards to operators and adjacent residents. However, the introduction of bacterial species not native to the activated sludge has not been extensively examined. Changes in species diversity in the activated sludge matrix could have the potential to change process efficiencies, produce species in the effluent resistant to subsequent treatment processes, and contaminate the waste sludge in a way making it unsuitable for land disposal.

This research set out to investigate the effects of an outside pathogenic bacteria species, specifically *Bacillus anthracis*, on the bacterial population diversity within the activated sludge. More specifically, we wished to determine if this species could become prevalent or even dominant within an activated sludge community if introduced in large amounts. If such a change could be effected, operational efficacy of the system could be compromised and a hazard to operators and the environment could be created.

Bacillus was chosen as a design species for this research for multiple reasons. This genus of bacteria is naturally occurring in activated sludge, and therefore, the

environmental conditions and nutrients in a wastewater treatment application are sufficient to support these organisms (Bitton, 1999). *Bacillus anthracis* is the specific bacteria of concern due to the damaging nature of the endospore ingestion and subsequent germination that can cause acute disease in humans and animals. These can be naturally occurring in soils and through the food chain become concentrated in animals, especially those that have died. Hospital wastes have also been found to be a source of these bacteria. A more recent concern is that of bioterrorism and *Bacillus anthracis* has been indicated by the US government as an agent of this type of attack (Counce, et al., 2008) If this were to contaminate a wastewater, the fate of these bacteria is of concern due to the possible risks to the treatment facility operations, operators and downstream contamination. Research of previous publications by use of SciFinder Scholar, Academic Search Premier and Google Scholar was done to investigate the growth kinetics of these bacteria in an aquatic environment, but the results were highly limited and not applicable to the study. Therefore the research to be done examines the situational instance of a slug of the bacteria being introduced and monitoring of the behavior within the reactor.

CHAPTER II

LITERATURE REVIEW

Biological Diversity in Activated Sludge

The makeup of activated sludge is such that treatment is highly efficient, and organisms within the population are well adapted to the substrates available. In activated sludge applications the microorganisms are maintained in suspension by constant mixing and aeration. During this process their biological activities of growth and maintenance utilize the organic carbon and other nutrients found within the wastewater, thus providing the desired treatment of biochemical oxygen demand removal. The dominating process of the uptake of carbon is primarily to do heterotrophic bacteria being the dominant species within the activated sludge (Nazaroff & Alvarez-Cohen, 2001). While applications of activated sludge can be specialized to remove carbon, phosphorus, or nitrogen, this research is focused on a typical application of organic carbon removal (Kampfer, Erhart, Beimfohr, Bohringer, Wagner, & Amann, 1996). A typical activated sludge consists of a highly diverse population of microorganisms dominated by bacterial genus *Pseudomonas*. However, there are a number of other species present in this biological matrix including *Bacillus* which occur naturally. A typical distribution of the bacteria in a carbon removing activated sludge can be found in Table 1. While these

values may vary dependent on operation parameters, season and regional characteristics, this tabulation demonstrate the biological diversity of activated sludge (Bitton, 1999).

Table 1

Distribution of Aerobic Heterotrophic Bacteria in Standard Activated Sludge (Bitton, 1999)

Genus or Group	Percentage of Total Isolates
Comamonas-Pseudomonas	50.0
Flavobacterium-Cytophaga	13.5
Paracoccus	11.5
Alcaligenes	5.8
Bacillus	1.9
Aeromonas	1.9
Unidentified (gram-negative rods)	1.9
Pseudomonas (fluorescent)	1.9
Micrococcus	1.9
Coryneform	5.8
Anthrobacter	1.9
Aureobacterium-Microbacterium	1.9

Bacillus anthracis

The genus of *Bacillus* are characterized by being aerobic, Gram positive, endospore-forming, rod shaped bacteria. Generally these bacteria are capable of utilizing a variety of substrates and some capable of facultative anaerobic survival. When stressed, due to lack of substrate availability or harsh environmental conditions, the *Bacilli* are able to enter into a dormant state. In entering this state the bacteria sporulates to form protective endospores which can survive the lack of favorable conditions for an

extended period of time up to multiple decades (Gardener, 2001). The endospore is capable of surviving drying, heat, UV light, gamma radiation and some disinfectants (Dixon, Meselson, Guillemin, & Hanna, 1999). Upon conditions returning to favorable conditions, the bacteria are then able to return to an active or vegetative state (Gardener, 2001).

Bacillus anthracis is typically found in soil and posses all of the characteristics of a typical *Bacillus* species. It is specifically of concern because of the acute illness caused by an infection from the bacteria. Infection begins with the intake of the *Bacillus anthracis* endospores through ingestion, inhalation or contact with an open wound. The endospore is completely dormant as it does not divide or metabolize at any detectable level. Inside the body the bacteria germinate and enter the active stage (vegetative) during which time they begin to spread. There is no evident immune response to the *Bacillus anthracis or* the subsequent release of the toxin anthrax, causing death (Dixon, Meselson, Guillemin, & Hanna, 1999).

A variety of typical sources exist for contact and infection with *Bacillus anthracis,* but the most common is from occupational hazards associated with contact with animals or animal products. This type of infection is called Cutaneous Anthrax and is associated with skin lesions and sores that are not generally fatal even without medical treatment. Gastrointestinal Anthrax is of higher concern as the infection can be fatal; however, these infections are less common. The common source of the gastrointestinal infection is through the consumption of meat contaminated with the endospore. The other infection source of concern is through inhalation. While rare, the infection

typically is attributed to working with contaminated animals and their hides. Once airborne the spore size is ideal for inhalation and absorption into the lungs and is typically a fatal infection. (Dixon, Meselson, Guillemin, & Hanna, 1999)

Although the typical source of *Bacillus anthracis* being from animals is not of primary concern to the contamination of a wastewater facility, conditions could exist where this source could be problematic. Runoff from feedlots and grazing areas has a potential for entering sewerage systems. Sewer systems are often a home of rodents and provide adequate substrate for *Bacillus,* a concentration of the cells in a dead carcass and eventual contamination could occur. Liquid wastes from hospitals is generally discharged into municipal treatment systems with little pretreatment. In the treatment of the patients it was decided that person to person contact would not cause infection. However, the instruments and dressings involved in treatment were viewed as possible contamination sources (Swartz, 2001). Improper treatment of these contaminated items could potentially lead to a contamination of the wastewater stream during disposal.

Finally, there is also a potential that intentional introduction of the organism will result from the actions of individuals wishing to harm the populous by releasing the organism into the environment. After the use of anthrax as an agent of bioterrorism in 2001, the Center for Disease Control and Prevention (CDC) was forced to further investigate this species. *Bacillus anthracis* endospores are ideal for transport and release through a variety of methods. These are in turn readily taken into the human body and can cause acute illness and even death (Dixon, Meselson, Guillemin, & Hanna, 1999). Identified as a Category A agent, the threat of this form of contamination was brought to

the forefront. Category A agents are those that pose the greatest threat to the public, may have a widespread effect, and require extensive planning for prevention (Center for Disease Control and Prevention, 2003). Although the application of this terrorism method takes skill, most especially in the application of aerosol attacks, the threat is valid. A variety of situations could occur where this type of contamination of a wastewater facility could occur; be it accidental or desired release or cleanup efforts after a release.

Selection of Surrogate *Bacillus* Species

In order to complete the research, it was decided a surrogate species should be utilized to model the behavior of the *Bacillus anthracis*. In this way we could minimize the human health and environmental risks that would be associated with using the target species. As previously discussed the characteristics of *Bacillus anthracis* include being an aerobic, Gram positive, endospore-forming, rod-shaped bacterium. When conditions are favorable for growth, these bacteria will grow and divide as all other bacteria do.

However, when conditions are not favorable an endospore is formed to protect the bacteria. This sporulation allows for the bacteria to survive for many years of unfavorable conditions, making it especially troublesome as a contaminant. These can exist in soil, water or be air-born until finding a host where nutrients are sufficient. During this spore stage the *Bacillus* can survive boiling, freezing and some disinfectants (including alcohol). Upon the condition changes, the spore becomes active and begins to grow and multiply, causing the acute illness mentioned earlier. Such survival techniques

and long life spans make these of special concern from all sources and have lead to their appeal in terrorist applications (Gardener, 2001). It is this almost unique sporulation behavior that limits the selection of a surrogate to another *Bacillus* species.

Of the various species within this genus, *Bacillus megaterium* is also an aerobic, Gram positive, endospore-forming, rod-shaped bacterium. *Megaterium* has also been found to be a facultative anaerobe that can utilize nitrate for respiration. This species of *Bacillus* is considered a representative species of the genus and thus is often used as a surrogate species (Vary, 2007). As an inhabitant of many different environments this species is ideally suited for life in an aquatic environment. Studies performed on *Bacillus megaterium* indicate that the species can survive in fresh, saline and distilled water, furthering the favorable nature of this species' application to the study. However, this study did not include relevant information pertaining to the kinetics of the species in this environment (Counce, et al., 2008). While generally not toxic like the *Bacillus anthracis* release, this species also releases products from the cell which indicate further similarities between the species. In a genome study of *Bacillus megaterium* it was indicated that this species was of the same class of *Bacillus* as *Bacillus anthracis* (Vary, 2007).

Another genetic study identified *Bacillus megaterium* as a suitable surrogate for *Bacillus anthracis* in the study of the sporulation of *Bacillus anthracis* (Greenberg, Busch, Keim, & Wagner, 2010). While some small differences were indicated in the genetics of the *megaterium* species, it was considered suitable where available for studies not involving the genetic mutations of the species (Greenberg, Busch, Keim, & Wagner, 2010). The study also identified some differences in the nature of the endospore formed

by the bacteria. However, these concerns deal with the genetic mutations that occur in sporulation and do not apply to the survival of the *Bacillus* in stressed conditions. Although the study indicated *Bacillus thuringiensis* as the ideal surrogate in the genetic study of the spores, *Bacillus megaterium* was of the top three choices (Greenberg, Busch, Keim, & Wagner, 2010). Rejection of *megaterium* as a surrogate for the study mentioned was based on the application of the study and the necessary biosafety level II (BSL-2) treatment of the species for the specific nature of the work being done. The applicability of the *Bacillus megaterium* to aquatic environments, classification as a BSL-1 organism and similarities to *Bacillus anthracis* led to the selection of this species as the surrogate of choice for this research.

Another factor in the selection of a surrogate is the non floc forming nature of the *Bacillus* species. *Bacillus anthracis* do not form filaments that aid in the formation of flocculated particles (Greenberg, Busch, Keim, & Wagner, 2010). Thus, the surrogate must not form these filaments either or the results from the testing would not be appropriate for the modeling of the *anthracis* species. In a study aiming to isolate the floc forming bacteria species found in activated sludge, three species of *Bacillus* were found to occur and were examined. Both *Bacillus cereus and Bacillus lentus* were found to form filaments within a 48 hour period (McKinney & Weichlein, 1953). *Bacillus megaterium* was also found in the activated sludges studied, but this species was not found to form the filaments that aid in flocculation occurring in the other species (McKinney & Weichlein, 1953). Another study in fact found *Bacillus megaterium* to form some very light filaments (Bergersen, 1954). However, these filaments were only

seen when the bacteria was grown in blood based media as a response to nutritional stimulation that is not seen in other conditions (Bergersen, 1954). As the media used in this research is based on typical wastewater requiring carbon removal, it is not expected that this mutation would occur. Therefore, in all areas of concern *Bacillus megaterium* is a suitable surrogate for the research to be performed.

Biomass Physical Properties in Activated Sludge

Activated sludge posses certain physical properties based on the operational characteristics which lend information towards processes that are undergone within the sludge. One such characteristic is the solids concentration which is directly related to the solids retention time (SRT). This parameter may also be referred to as the sludge age or mean cell residence time (Θ_c). For our purposes and for consistency, this operational parameter will be referred to as solids retention time or SRT.

With increased solids retention time the mixed liquor suspended solids (MLSS) also increases. This in turn reduces the food to microorganism (F/M) ratio. The activated sludge kinetics will favor biological growth and sludge production when the biological population is dominated by younger bacteria. This is compared to activated sludge consisting predominately of older organisms which result in processes that favor the use of nutrients for cell maintenance as a much lower rate of sludge production (Nazaroff & Alvarez-Cohen, 2001). The effective age of the microorganisms in the activated sludge is characterized and controlled by the SRT.

The expected solids concentration can be analyzed further by the use of typical values used in the design of activated sludge systems. Equations and typical values from "Environmental Biotechnology: Principles and Applications" were used to calculate expected values for the mixed liquor volatile suspended solids, mixed liquor suspended solids and active biomass fraction for typical 20-day SRT and 5-day SRT reactors (Rittmann & McCarty, 2001). The typical values and equations utilized can be found below (Rittmann & McCarty, 2001):

Typical Values
True Yield, Y = 0.45 mg VSS_a/mg BOD_L
Max Specific Rate Substrate Utilization, q = 20 mg BOD_L/mg VSS_a-day
Biodegradable Fraction of Active Biomass, f_d = 0.8
Endogenous-Decay Coefficient, b = 0.15/days
Concentration Giving ½ Max Rate, K = 10 mg/L

Equations Utilized

Net Yield, $Y_n = Y\frac{1+(1-f_d)b\Theta_x}{1+b\Theta_x}$

Concentration Rate Limiting Substrate, $S = K\frac{1+b\Theta_x}{Yq\Theta_x-(1+b\Theta_x)}$

Concentration Active Biomass, $X_a = \frac{Y(S°-S)}{1+b\Theta_x}\frac{\Theta_x}{\Theta}$

Concentration Volatile Biomass, $X_v = \frac{\Theta_x}{\Theta}\left[\frac{1+(1-f_d)b\Theta_x}{1+b\Theta_x}Y(S°-S)\right]$

where system dependent values are defined as,
Θ_x = Solids Retention Time (days)
Θ = Hydraulic Retention Time (days)
S° = Influent Substrate Concentration (mg BOD_L/L)

Using the typical values and equations listed the expected values were calculated. These are based on the assumption that the chemical oxygen demand to biochemical oxygen demand ratio (COD:BOD) in the substrate are equal. This assumption is based on the stoichiometrical COD of 720 mg COD/L and the fact that all of the substrate

materials are known to be completely degradable. The data from the calculations is presented in Table 2. The values presented are in the high range of expected values found in the text utilized for the equations and typical values but do indicate the expected level of solids (Rittmann & McCarty, 2001). The net yield indicates the growth dominated nature of the 5-day SRT reactor versus the maintenance dominated 20-day SRT reactor as it produces more solids per gram of biochemical oxygen demand consumed. It can also be seen that the active biomass is much higher in the 5-day SRT reactor than the 20-day SRT reactor as is typically expected.

Table 2

Typical Solids Values for Activated Sludge

	20-day SRT	**5-day SRT**
Y_n (g VSS/g BOD)	0.18	0.30
S (mg/L)	0.23	0.40
X_a (mg VSS/L)	3238	1850
X_v (mg VSS/L)	5182	2128

The efficiency of the flocculation of biomass is also greatly affected by the solids retention time. As SRT increases, the availability of nutrients decreases with the increased solids concentration. It has been found that this is the most likely cause for the trend in the nature of flocculated particles in the activated sludge. At higher SRTs the flocculated particles are found to be larger, more concentrated and of a more stable

structure. These particles settle more efficiently and compact better than particles in lower SRT operations (Liao, Droppo, Leppard, & Liss, 2006). It was also seen that the higher SRT applications experienced a lower concentration of solids in the effluent that those of low SRT. This trend is associated with better floc formation and sweeping mechanisms during the settling of the particles (Liao, Droppo, Leppard, & Liss, 2006).

Bacillus Isolation Cycle

As discussed earlier, *Bacillus* bacteria are capable of surviving harsh environmental conditions through the formation of protective endospores. This survival technique is useful in studying *Bacillus* in a diverse system like activated sludge. Through a method of Pasteurization (referred to as a "kill cycle") the sample is placed into a hot water bath for a period of 20 minutes at 60 °C to destroy any cells that are not capable of forming endospores (non-*Bacillus* species). Previously a temperature of 80 °C for a period of 10 minutes had been used, but the losses of *Bacillus* at this time and temperature were significant (Francis, Lockley, Sartory, & Watkins, 2001). After this process it can be assumed that all of the living cells found in the sample are *Bacillus,* and thus, the genus has been isolated for enumeration. In this research, the initial use of the prescribed time and temperature were not creating an efficient kill of the non-*Bacillus* species. This was believed to be due to the sample size of 25 mL contained in a somewhat thick plastic of a centrifugal tube. Therefore, the time was increased to 30 minutes to assure the prescribed temperature was reached through the entire sample.

Disinfection

In the situation that it is found that *Bacillus* are the exit the wastewater treatment plant, it is necessary to address the current disinfection techniques and their effectiveness. The following information is a summary of the current disinfection techniques as applied to *Bacillus* and their spores. According to the United States Environmental Protection Agency there are three acceptable methods of disinfection: chlorine, ozone and ultraviolet light (US EPA, 1999). In wastewater treatment applications the design disinfection is based on the inactivation of *E. coli* (Bitton, 1999). In all applications the dosage level for inactivation is far less than that required to be effective on *Bacillus* spores (Counce, et al., 2008). Therefore, the effluent disinfection of this treatment application is far below the required level to manage a contamination of *Bacillus anthracis*. In the case that this contamination did become a viable threat, the disinfection process would need to be reevaluated.

Another primary concern of a discharge of *Bacillus anthracis* would be entering into surface waters that were also used as a drinking water source. However, the level of inactivation in these applications is based upon the inactivation of *Giardia cysts*. In studies *Bacillus* and their endospores have been found to be at least as susceptible to inactivation as the design level in applications of free chlorine (Counce, et al., 2008). In the applications of ozone, chlorine dioxide and chloramines disinfection techniques, the results were not as favorable. Disinfection studies using ozone and chlorine dioxide have not been performed on *Bacillus anthracis.* However, information collected from studies on spores of other species, though somewhat limited, may be correlated to *Bacillus*

anthracis. These studies indicate that the spores were not as susceptible as the *Giardia cysts* and would require an increased dose of each treatment method to provide the needed disinfection (Counce, et al., 2008). The data in applications of other disinfection methods like ultraviolet light, heat and other chemicals are limited as they are not commonly applied in drinking and wastewater disinfection.

CHAPTER III

MATERIALS AND METHODS

This section details the materials and methods used in the scope of work. In the subsequent sections the design and operations parameters of the reactors will be discussed. Sections on the solids and microbial enumeration methods are also included to indicate the sources of procedures and specific details utilized.

Reactor Design and Components

To provide a controlled environment in which to monitor activated sludge and bacterial growth, a 4-Liter bench scale batch reactor was utilized. Two reactors of identical design with the only difference factor being the volume of sludge wasted per cycle which provided the different solids retention times (5-day and 20-day SRT). This model of reactor simulates a combination of a completely mixed and plug flow reactor similar to the nature of an actual wastewater treatment plant. In field applications, flow enters and individual particles are theoretically retained the same period of time each. While the large scale system is completely mixed in application, inspection of the overall processes can be modeled by plug flow reactor with results very similar to the completely mixed system. Each of the reactors was initially seeded with four (4) Liters from the

Starkville Wastewater Treatment Plant in Starkville, MS. In this way, the bench-scale reactors were able to rapidly establish bacterial colonies representative of an actual wastewater facility. However, it should be noted that the bacterial makeup of this seed sludge is based upon the temperature and nutrients found entering the facility of origin. However, when used in the bench reactors the sludge is held at constant temperature, aeration rate and nutrient loading while being fed a different substrate. Thus the seed source should be of little consequence in the analysis of the final results.

In the wastewater treatment process, the influent initially enters the aeration basin of activated sludge which has been simulated in the lab by a feed, designed to provide for a wide range of bacterial growth. The feed and sludge are continuously aerated used diffused air and mixed by a shaft mixer over a period of five hours; again simulating actual processes of aeration and mixing. After the 5 hour period the mixing and aeration were shut off, and a volume of the sludge was wasted in each reactor, dependent upon the solids retention time. This was done in order to simulate the sludge wastage in the clarifier. After settling for an hour the supernatant was decanted down to a total volume of 2 Liters, providing a hydraulic retention time of 12 hours and again simulating the process of the overflow of supernatant in a clarifier. Upon decanting, the 6 hour cycle was repeated for a total of four cycles per day.

As stated previously, the only difference between the two reactors was the solids retention time. These were selected as 5 and 20-days in order to yield data over a range of standard operation solids retention time. While these rates differ dependent upon the

facility in order to attain different treatment goals, their overall efficiencies are similar (Bitton, 1999).

In these reactors ChronTrol digital timers with switches were utilized to provide the switching on and off of pumps, mixers and aerators as needed. Pumping was done using variable speed peristaltic pumps which were tested to provide exact volumes needed in each process. Mixing was provided using variable speed Barnstead shaft mixers, set such to provide turbulence and mixing throughout the reactor. The reactor containers utilized provided the 4 L volume required and were constructed with a baffled design to prevent vortex and assure complete mixing. Aerators were of the aquarium type with a fine bubble air diffuser to provide for maximum surface area between bubbles and liquid for increased gas transfer (DO above 2 mg/L). Temperature remained constant at room temperature (23 ± 2 ^{o}C). Feed containers remained sealed with air filters to prevent bacterial contamination of the feed solution.

Feed Composition

Feed design was based on stoichiometrical requirements of a typical bacterial composition found in activated sludge processes and was designed to mimic a municipal wastewater. A synthetic wastewater is ideal for the purposes of this research, as the characteristics of the sludge are being studied and must be held constant. This must be done in order to assure that the only variable affecting the sludge is the introduction of the *Bacillus* inoculations.

Feed design was based upon the feed design found in work done by Arslan-Alaton and Balcioglu in "Biodegradability Assessment of Ozonated Raw and Biotreated Pharmaceutical Wastewater". Table 3 provides the makeup of the feed and the concentrations represent those supplied to the reactors. In design of the feed and dilution water, a ratio of 1:5 feed to dilution was used (yielding 400mL of feed and 1600mL of dilution water per cycle). This was primarily based on the containers available for use in the research and to provide that feed and dilution would supply the reactors for about 4 days per makeup. The feed utilized distilled water while the dilution water utilized tap water that was aerated prior to introduction to the system. This aeration was done in order to remove the residual chlorine found in the system and to allow any additional nutrients found in the water to remain for use. As stated earlier, after autoclaving at 121C the feed bottles remained sealed with an air filter sufficient for stopping contamination by air born bacteria. Dilution water was left open to the air to allow for any additional chlorine removal through the air/water interface. The dilution water also contained the Potassium Phosphate, as this compound can decompose when autoclaved and adding sterilely was not practical.

Table 3

Reactor Feed Composition (Arslan-Alaton & Balcioglu, 2002)

Component	Concentration (mg/L)
Biogenic Substrates	
Peptone	321.43
Glucose	119.05
Sodium Acetate	285.71
Inorganic Nutrients	
$(NH_4)_2SO_4$	142.86
$MgSO_4$	4.76
$FeCl_3$-$6H_2O$	1.19
$CaCl_2$-$2H_2O$	9.52
KH_2PO_4	71.43

Solids Testing

In order to monitor the stability and health of the reactors total and volatile solids were tested. Maintenance of a constant level of solids indicates the stability of the reactor's SRT, which is a primary factor in maintaining bacterial levels. While the composition of the solids cannot be determined from this method, their concentrations are a good indicator of reactor health. Testing was done in accordance with ASTM 2540 D for total and volatile suspended solids. Under this method, dish and filter are to be prepared in accordance with the details of the standard. These are weighed after preparation for a weight prior to any solids being filtered. A representative sample of sufficient size is selected based on the criteria that the sample size should be such that filter is not overloaded, will yield between 2.5 and 200 mg of dried residue, and the volume is to pass the filter in less than 10 minutes when under vacuum.

Once selected the sample is filtered and dried at 103 ^{o}C for a period of 1 hour. This is placed in a desiccator to equilibrate temperature while preventing the uptake of moisture from the air which can cause errors in the data. These are again weighed and the change in weight indicates the total suspended solids in the given sample volume. Samples are then placed in a muffle furnace at 550 ^{o}C for a period of 15 minutes to burn off any organic matter, after which the desiccating and weighing procedure are repeated. The change in weight from the total suspended solids yields the volatile and non-volatile fractions of the volume tested (Clescerl, Greenberg, & Eaton, ASTM 2540 - Solids, 1999).

In this research duplicates for each sample were run to provide confirmation of results. Samples included a mixed liquor sample and a supernatant sample from each reactor. This aids in monitoring the reactor through the overall suspended solids content in the mixed liquor as well as the volatile fraction indicating the state of the reactor (steady, increasing, decreasing). The solids content in the supernatant aid in monitoring the settleability of the sludge, which can be indicative of problems in the reactor if settling is not occurring. Data from testing can be found in the results section of this paper.

Settleability

Upon completion of the initial research it was theorized that *Bacillus* was primarily being removed from the reactor during the effluent removal process. While the rates of hydraulic retention time were identical between for the two reactors a difference

in sludge settleability was observed between the two reactors. By examination of the sludge in the reactors during settling the 20-day SRT reactor was settling notably faster and more efficiently than the 5-day SRT reactor. This behavior was theorized to be attributed to the nature of the flocculated particles and in turn, their settleability. Thus the need to investigate the settleability of the floc in the reactors was determined. This information is to provide grounds for the settleability testing and the details of these theories and accompanying results will be discussed further in subsequent sections.

In accordance with ASTM 2540 F for the examination of settleable solids from the Standard Methods for testing of activated sludge, 1000 mL of sludge were placed into a 1000-mL graduated cylinder. The volume occupied by the sludge is then measured by volume at times of 10, 20, 30, 45 and 60 minutes. These values are then plotted to obtain a curve representing the rate of settling (Clescerl, Greenberg, & Eaton, ASTM 2540 - Solids, 1999). Although a simple test, the data is able to indicate the nature of the flocculated particles in the sludge. Results from the testing will be presented in the results section of the document.

Microbial Enumeration

Bacterial enumeration was the primary focus of the research and where the most difficulties were found in determining reliably accurate results. Initially the method of membrane filtration was utilized. Upon discovering the method was not providing the expected results, some alterations to the method were tried. After again getting

inconsistent results the method was shifted to the most probable number test. The details of each are discussed in the two subsequent sections.

Membrane Filtration

The procedure utilized was based upon the basic Standard Methods procedure ATSM 9222 (Clescerl L. S., 1999) with alterations to the media and sample preparation specific to *Bacillus* enumeration (Francis, Lockley, Sartory, & Watkins, 2001). Under this method all apparatus are sterilized by use of a 70% ethanol solution. A 10 mL volume of diluted sample (1:100 dilution) is filtered and filter membrane is placed in a Petri dish on a pad saturated with 2 mL of media. This was then incubated at 37 °C for 24 hours and checked for growth. Growth was again checked for after 48 hours to see if any changes occurred due to extended incubation. All work was done aseptically to assure no outside contamination occurred. The media initially used was Difco Nutrient Broth, prepared to the stipulations of the manufacturer which state adding 8 g/L of broth to distilled and deionized (DI) water. This is thoroughly stirred and allowed to boil. After preparation, the media was then autoclaved prior to storage and usage.

A modification of the media was also prepared that contained a small amount of Bromothymol Blue dye solution, which was added aseptically to the media prior to adding to the pad. This dye has been found to provide sufficient coloration of *Bacillus* to aid in enumeration (Francis, Lockley, Sartory, & Watkins, 2001). However, this dye has also been found to be inhibitory to some strains of bacteria. The research done used what

is found to be an acceptable level of Bromothymol blue dye at 0.005% (w/v) (Francis, Lockley, Sartory, & Watkins, 2001).

A "kill" cycle was also utilized in order to isolate *Bacillus* bacteria which are the primary focus of the research. These bacteria are endospore forming and this sporulation can be induced by a hot water bath of 60 Celsius for 20 minutes. As discussed previously, this sporulation protects the bacteria from damage and they can lie dormant for long periods of time. Under this procedure a 50 mL centrifugal tube containing sample is placed into the hot water bath for the prescribed time. This causes the liquid inside to reach the 60 degree Celsius temperature which is fatal to most bacteria found in activated sludges. For our purposes, we are assuming bacilli are the only species to survive to water bath and thus any growth after the "kill" is due to *Bacillus* (Francis, Lockley, Sartory, & Watkins, 2001).

However, after performing this membrane filtration method sufficient growth was not seen. Expected values were much larger than the colonies actually seen on the Petri dishes. Some modifications were made to the procedure to try to find the source of error. This included the use of a *Bacillus* specific growth media (which will be discussed later) and changes in the amount of media on the pads in the dishes. Despite these efforts, the expected growth could not be found through this method. Thus a more vigorous testing method was utilized which gives the results of this research presented next.

Most Probable Number (MPN)

This test method is called the most probable number (MPN) microtechnique which utilizes serial dilutions and positive growth tubes to determine the most probable number of microorganisms in a sample. The test was run in accordance with ASTM 9221 (Clescerl, Greenberg, & Eaton, ASTM 9221 Multiple Tube Fermentation Technique for Members of the Coliform Group, 1999) with slight alterations to the method for application to *Bacillus* enumeration (Magbanua, Poole, & Grady, 1998). All work was done in an aseptic environment with instruments sterilized by 70% ethanol solution or autoclaving as appropriate.

Results from the test are not actually an enumeration but a level of concentration of bacteria in a unit volume and associated limits and probabilities are calculated. The procedure was based upon that described in Estimation of the Competent Biomass Concentration for the "Degradation of Synthetic Organic Compounds in an Activated Sludge Culture Receiving a Multicomponent Feed" (Magbanua, Poole, & Grady, 1998). The procedure utilizes a microbial growth media called R2A media, the composition of which can be found in Table 4. This media is prepared using DI water and autoclaved prior to storage and use at 121 °C as prescribed for liquid sterilization. The procedure also requires a sterile 0.9% (weight/volume) saline solution to be prepared. Saline is also prepared using DI water and autoclaved prior to storage and use.

Table 4

Modified R2A Media Composition

Component	Concentration (mg/L)
Casamino Acids	500
Glucose	500
Protease Peptone #3	500
Soluble Starch	500
Yeast Extract	500
$MgSO_4$-$7H_2O$	50
K_2HPO_4	300
Sodium Pyruvate	300

Samples were treated similar to that of the membrane filtration method as far as utilization of a "kill" cycle and "non-kill" cycle samples. However, this procedure does call for an additional step of sonication prior for preparation. For the samples not undergoing the hot water bath, the sample is placed in a 50 mL centrifugal tube and sonicated using a probe-type sonicator at 30% power for eight 30 second periods with 30 second cooling periods between each. These were then vortexed to ensure a homogeneous sample. The same procedure is used for those in the water bath but it is done after the hot water bath.

Upon sample preparation serial dilutions were set up using 96 (12x8) microwell plates. These allow for 2 replicates of each test (side by side) on each plate with a blank column for each as a control. A starter plate is aseptically prepared by placing 180 µL of saline into the appropriate number of wells based on the number of samples to be tested with a multichannel pipette. The appropriate number of microwell plates are filled with 90 µL of R2A media in each well (each plate accommodates 2 replicates of the each test).

A 20 µL aliquot is then placed into 5 of the saline starter plate wells and mixed by drawing and dispensing the solution into the pipette 20 times, creating 5 wells with a 10 fold dilution. 10 µL are then drawn from each well (including the blank) with fresh pipette tips and transferred to the first row of the appropriate test plate, creating a 10^{-2} dilution. Again changing tips, this is mixed by the same draw and return method and 10 µL transferred to the next row (including the blank row). This procedure is repeated until reaching the end of the tray and then done again for each sample, ending in a 10^{-9} dilution in the final row. All work is done under a laboratory hood in a sterile environment. The blank column indicates if the test was compromised by any contamination. These were then incubated for 3 days at 37 °C and checked for positive wells based upon turbidity indicating growth (Magbanua, Poole, & Grady, 1998). This data can then be related to the most probable number of bacteria by use of charts or spreadsheet software. These results are presented later in the paper.

It is important to note that the results of the test are only a most probable number of culturable heterotrophs (CH) per 100 mL of liquid. The conditions of the test and media composition limit the test results to culturable heterotrophs (CH), and the results do not indicate the total number of bacteria within the sample. With these values there are associated probabilities and limits of expected values associated with the most probable number. While results found were consistent, the accuracy of the numbers determined is not as concrete as a more intensive counting method. However, what the method did provide was a reliable indication of trends in the bacterial counts.

For the purpose of this research the most probable number was determined from the use of spreadsheets programmed into Microsoft Excel. The preparation of the spreadsheet was done in accordance with the method described by Briones and Reichardt in "Estimate microbial population counts by 'most probable number' using Microsoft Excel." In this document the programming of the spreadsheet is described along with the utilization of the Solver Add-in for the simplification and flexibility of the data solution for a variety of dilution levels (Briones & Reichardt, 1999).

Inoculation Preparation

To do actual studies of the behavior of the dosed reactors, inoculations were done using concentrated doses of *Bacillus*. Pure cultures of *Bacillus* for inoculations were prepared from a preserved sample of pure *Bacillus megaterium* obtained from ATCC (Strain # 35985). The specifics of this species of *Bacillus* and its relation to *Bacillus anthracis* are discussed in a previous section. Media specific for growth of *Bacillus* and subsequent sporulation was prepared with the constituents found in Table 5. The media was prepared as others described in this paper have been which is briefly described as using DI water and autoclaving prior to storage and use. This media is a combination of three separate research ventures that have improved results of sporulating *Bacillus* upon one another. The original work being "Isolation and Characterization of Rifampin-Resistant and Streptolydigin-Resistant Mutants of *Bacillus subtilus* with Altered Sporulation Properties" which gives the basic composition of the media (Soneshein, Cami, Brevet, & Cote, 1974). "Acid-Soluble Spore Proteins of *Bacillus subtilus*" then

makes the improvement of doubling the nutrient broth concentration and adding glucose to the media (Johnson & Tipper, 1981). The final alteration is done by "Enhancing sporulation in *Bacillus subtilus* grown on medium containing glucose:ribose" which substitutes the glucose addition with D-ribose (Warriner & Waites, 1999). This compilation of research forms a media that is determined to be ideal for the growth and subsequent sporulation of *Bacillus*. Here is also found the procedure for preparing the pure samples of sporulated *Bacillus*.

Table 5

Modified *Bacillus subtilus* Sporulation Media Composition

Component	**Concentration (g/L)**	**Added Sterilely**	
Nutrient Broth	16	**Component**	**Concentration (M)**
D-Ribose	1	$Ca(NO_3)_2$	10^{-3}
KCl	1	$MnCl_2$	10^{-4}
$MgSO_4$-$7H_2O$	0.25	$FeSO_4$	10^{-6}

Under this inoculation procedure the preserved sample is added to 50 mL of the described media in a volumetric flask. Using a foam stopper to allow oxygen transfer, the volumetric flask was closed. This is then placed into an incubated rotary shaker at 37 °C and approximately 250 revolutions per minute. Growth was monitored visually and by use of changes in turbidity from an initial value. Upon reaching what could be considered exponential growth, a 2 mL sample was taken an added to a new 50 mL of media. This process was repeated until a substantial amount of pure growths were prepared. Samples were then centrifuged to form pellets. The excess liquid was removed and the pellet washed with sterile saline and the process repeated until the sample was

media free. The pellet was then transferred to a 1 mL vial with a small amount of saline and stored at 4 °C until ready to use (Warriner & Waites, 1999). To assure the samples were pure MPN tests were run on the samples utilizing a kill and non-kill run. As expected the two results were identical, indicating only *Bacillus* were present in the sample.

After the reactors had been run until equilibrium was reached, inoculations were performed to study the impacts of a slug of *Bacillus* being added to the reactor. The reactors also experienced some fluctuations due to the sensitivity of bioreactors and equipment errors. However, prior to inoculation these issues had been ironed out to assure reliable results. Inoculation was performed by the addition of two 1mL concentrated samples to each reactor. As these were *Bacillus* spores, at least 12 hrs were allowed for the *Bacillus* to acclimate before any microbial enumeration tests were performed. The enumeration results of the inoculants for use in completing the mass balance will be discussed later.

CHAPTER IV

RESULTS AND DISCUSSION

In this section the results of all of the testing performed are reported and discussed. As stated previously the research was initially done in an exploratory effort to begin the formation of theories and understanding of the processes taking place within the reactors. The initial testing studied solids in the mixed liquor and supernatant as a means of monitoring reactor health and stability. Microbial enumeration was performed on the mixed liquor and examined in terms of total bacteria and *Bacillus*. These results led to theories that involved the effluent which not studied under the microbial enumeration testing. The additional testing involved reseeding the reactors as they had been shut down. Once the reactors were again stable, testing was again done to monitor solids and microbial enumeration before and after inoculations as had been done previously. However, this was done with greater attention to detail as microbial enumeration was performed at decreased time intervals after inoculation. The detail of the study was also increased by performing the microbial enumeration of total bacteria and *Bacillus* in the supernatant. From this data the complete nature of the *Bacillus'* path through the reactor could be monitored. In the results section the data from the initial testing will be referred to as "Round 1" and those from the following testing as "Round 2" for clarity. In reference to the inoculations, the initial run utilized two inoculation runs

as did the subsequent testing. These inoculations will be labeled in sequential order of 1 through 4.

Solids

Solids testing were performed throughout the span of the research to observe the amount of suspended and volatile solids within the reactors. While this data is important in monitoring the stability of the reactor, it can also aid in making conclusions about the nature of the activity levels of the bacteria when coupled with the MPN testing results. Table 6 and Figures 1-4 present the data from the initial testing while Table 7 and Figures 5-8 present the data from the second round of testing. Initially it can be seen that results were variable and somewhat higher than expected and higher than the stabilized levels of solids. The initial high levels are due to the seed activated sludge which operate at a different SRT than the reactors and receive different levels of nutrients from the influent than the feed provides. The fluctuations in the initial stages are due to the sensitivity of bioreactors. Any fluctuations in wastage, feed, dilution or decanting can lead to variations in the sludge solids levels. As expected the solids in the 20-day SRT are higher than that of the 5-day SRT. This is expected but we also know that the level of active organisms is higher in the 5-day SRT than that of the 20-day SRT due to the F/M ratio (Bitton, 1999).

Table 6

Round 1 Solids Testing Data (all units in mg/L)

Date	20-day Mixed Liquor		5-day Mixed Liquor		20-day Supernatant		5-day Supernatant	
	TSS	VSS	TSS	VSS	TSS	VSS	TSS	VSS
5/13/2010	4800.0	4325.0	1137.5	1100.0	24.0	23.0	11.0	9.0
5/17/2020	5580.0	4935.0	980.0	915.0	15.0	10.5	76.0	72.5
5/24/2010	2440.0	2125.0	255.0	230.0	62.5	58.8	181.3	152.5
6/2/2010	4450.0	4050.0	1200.0	700.0	41.2	32.5	688.7	616.3
6/23/2010	1420.0	1305.0	805.0	775.0	47.0	41.0	28.0	26.0
6/30/2010	1555.0	1380.0	440.0	375.0	56.0	50.0	394.0	356.0
7/14/2010	2775.0	2415.0	870.0	790.0	87.1	78.3	57.0	47.0
7/21/2010	1560.0	1370.0	1105.0	960.0	75.0	62.0	69.0	57.0
8/16/2010	3830.0	3345.0	1690.0	1485.0	30.0	22.0	89.0	82.0
8/18/2010	3500.0	3020.0	1365.0	1240.0	29.0	25.0	70.0	66.0
8/24/2010	3425.0	2950.0	1530.0	1340.0	24.0	11.0	30.0	17.0
9/1/2010	3400.0	2950.0	1425.0	1335.0	24.0	11.0	27.0	17.0
9/7/2010	3290.0	2920.0	1140.0	995.0	33.0	29.0	20.0	13.0
9/16/2010	3035.0	2765.0	1330.0	1175.0	16.0	8.0	40.0	24.0
9/23/2010	2920.0	2615.0	1415.0	1115.0	19.0	7.0	24.0	17.0
9/30/2010	2977.5	2690.0	1372.5	1145.0	17.5	7.5	32.0	20.5
10/7/2010	3316.3	2975.0	1143.8	970.0	24.3	16.8	30.0	20.8
10/14/2010	3655.0	3260.0	915.0	795.0	31.0	26.0	28.0	21.0
10/21/2010	3665.0	3260.0	915.0	795.0	31.0	26.0	28.0	21.0
10/29/2010	1635.0	1395.0	900.0	810.0	89.0	73.0	84.0	74.0
11/2/2010	3030.0	2825.0	1225.0	1100.0	15.0	13.0	47.0	38.0
11/9/2010	3400.0	3135.0	1245.0	1150.0	13.0	7.0	49.0	39.0

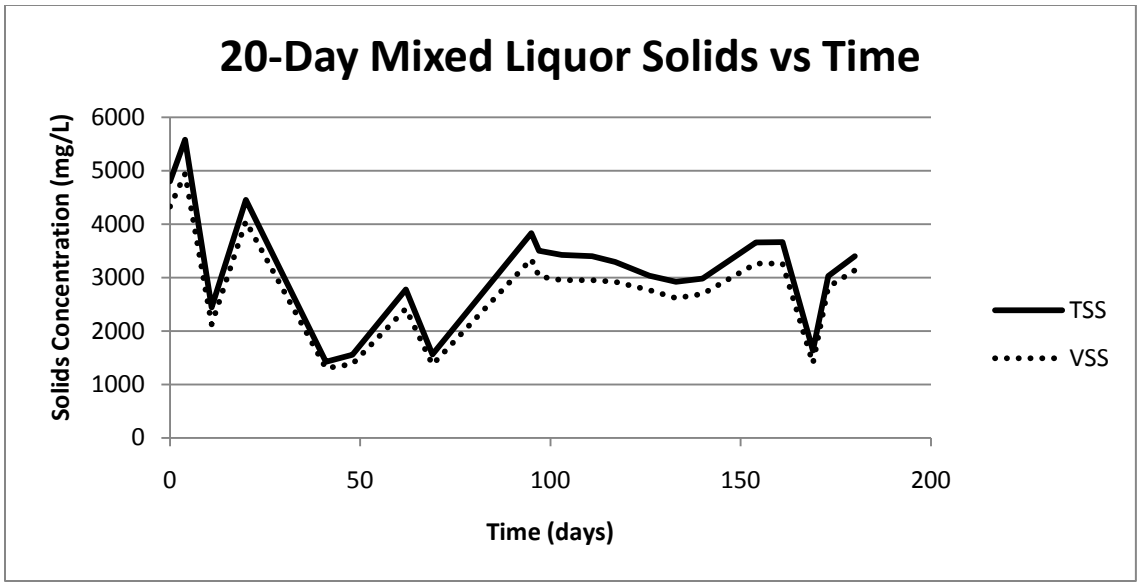

Figure 1

Scatter Plot of Round 1 - 20-day Mixed Liquor Solids versus Time

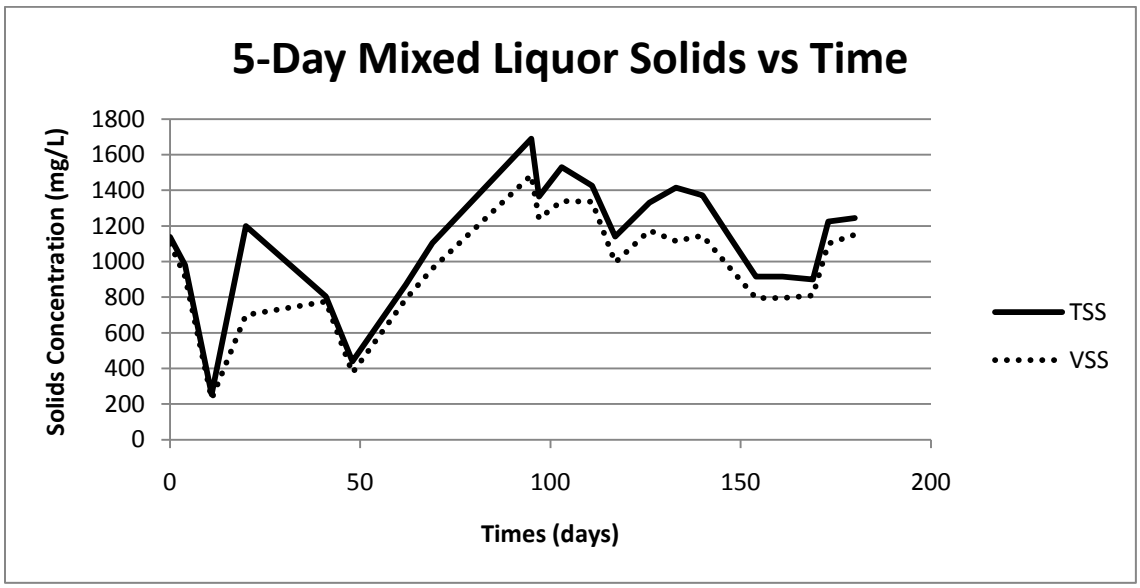

Figure 2

Scatter Plot of Round 1 - 5-day Mixed Liquor Solids versus Time

In Figures 1 and 2, the plots show some variation of the solids concentration between testing days. The initial variations were discussed previously. However, upon stabilization the variations reflected in the graph are not outside of the expected range of changes that can occur from day to day. There is one dip that occurs towards the end of the testing. This occurred due to a failure of the aerators of the reactors which led to some die off within the reactors. Once repaired, the solids rose back to normal levels as is illustrated in the plots.

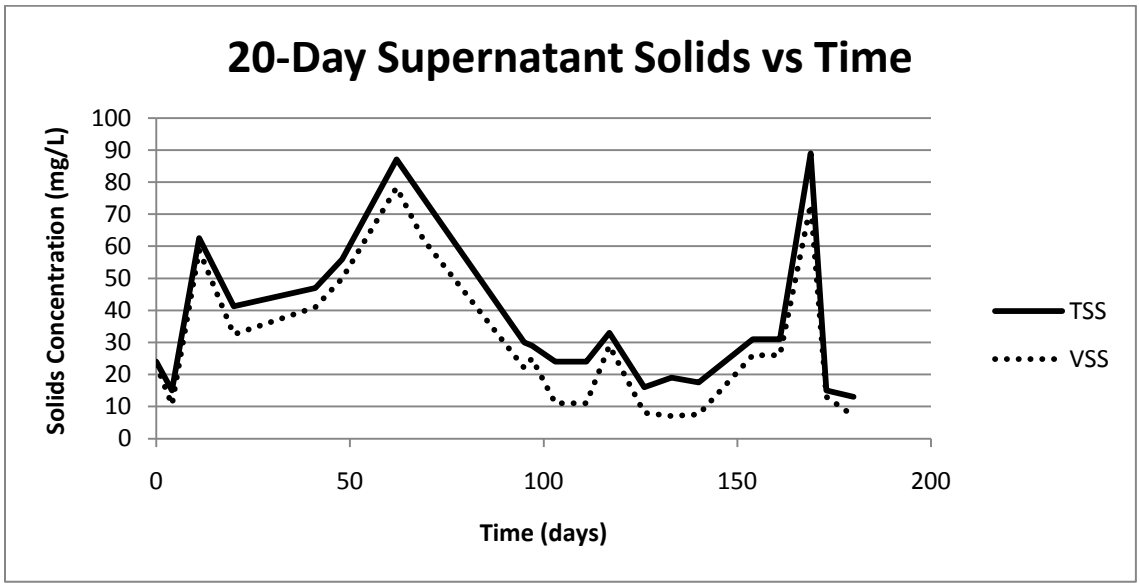

Figure 3

Scatter Plot of Round 1 - 20-day Supernatant Solids versus Time

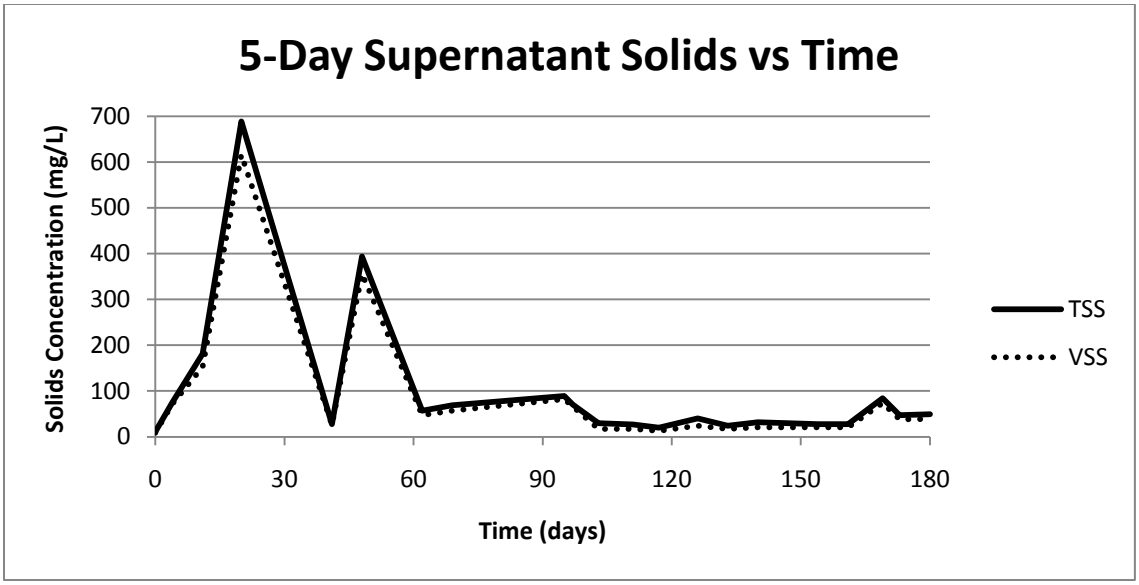

Figure 4

Scatter Plot of Round 1 - 5-day Supernatant Solids versus Time

Table 7

Round 2 Solids Testing Data (all units in mg/L)

Date	20-day Mixed Liquor		5-day Mixed Liquor		20-day Supernatant		5-day Supernatant	
	TSS	VSS	TSS	VSS	TSS	VSS	TSS	VSS
2/8/2011	1550.0	1395.0	1085.0	990.0	32.0	19.0	36.0	19.0
2/14/2011	1615.0	1430.0	985.0	885.0	34.0	21.0	35.0	21.0
2/22/2011	1525.0	1405.0	980.0	890.0	29.0	24.0	33.0	25.0
3/3/2011	1545.0	1375.0	1030.0	960.0	31.0	19.0	33.0	21.0

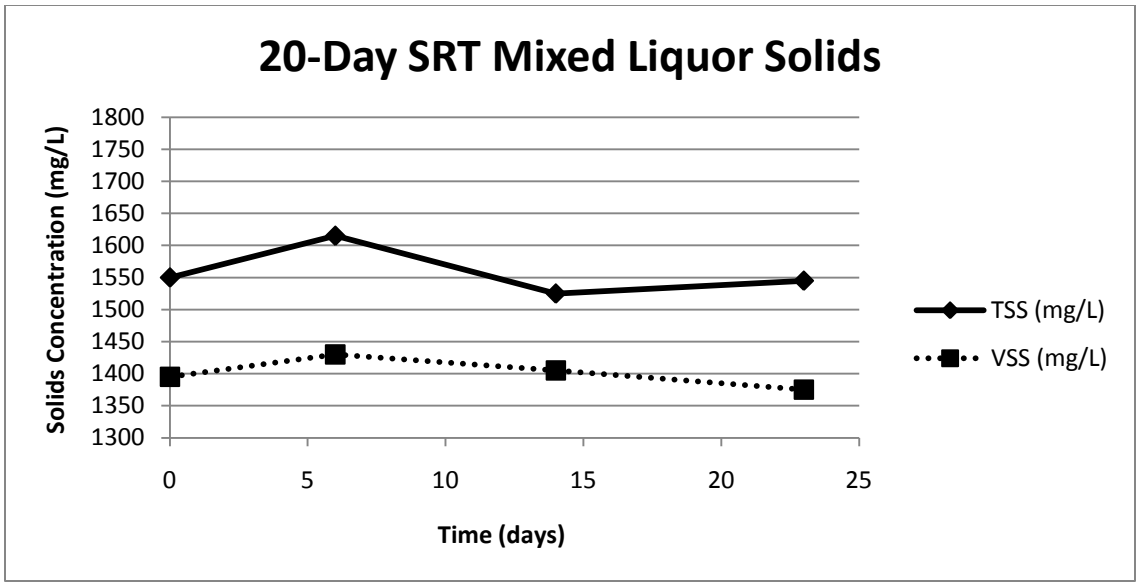

Figure 5

Scatter Plot of Round 2 - 20-day Mixed Liquor Solids versus Time

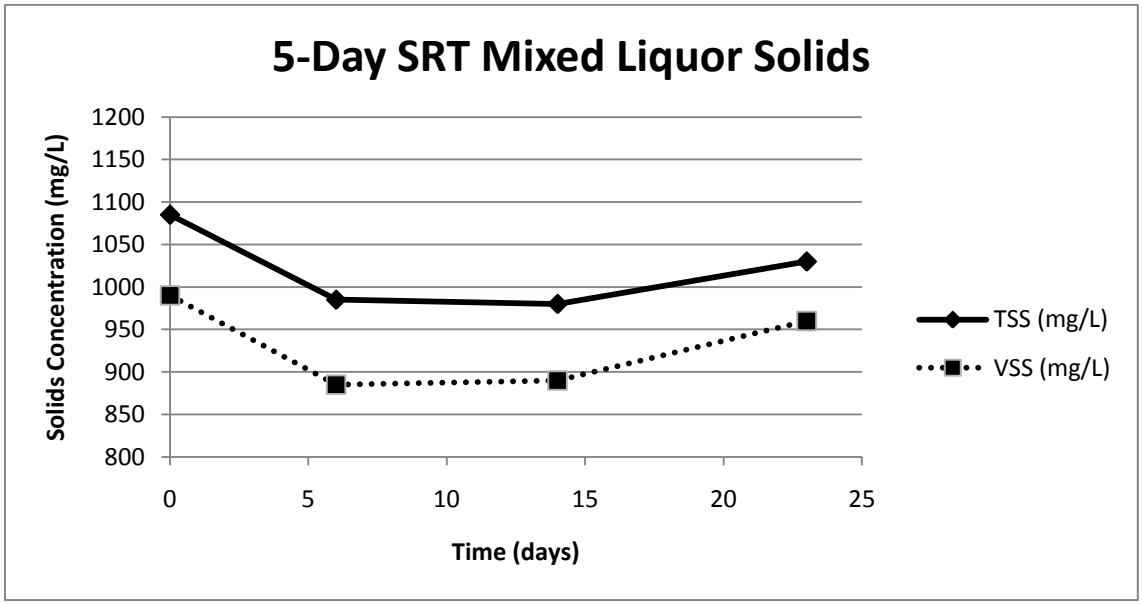

Figure 6

Scatter Plot of Round 2 - 5-day Mixed Liquor Solids versus Time

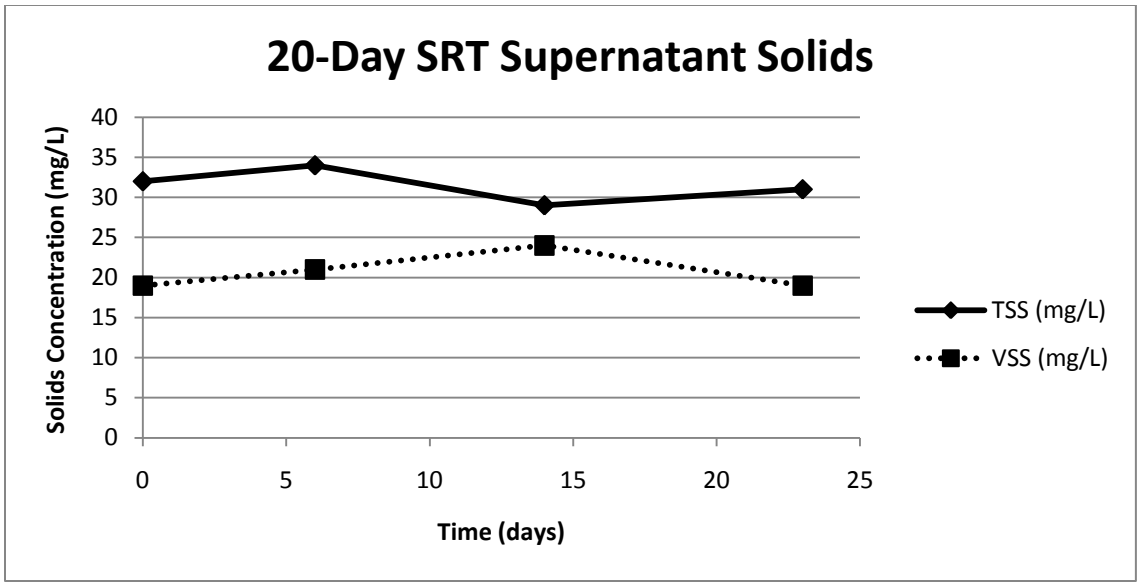

Figure 7

Scatter Plot of Round 2 - 20-day Supernatant Solids versus Time

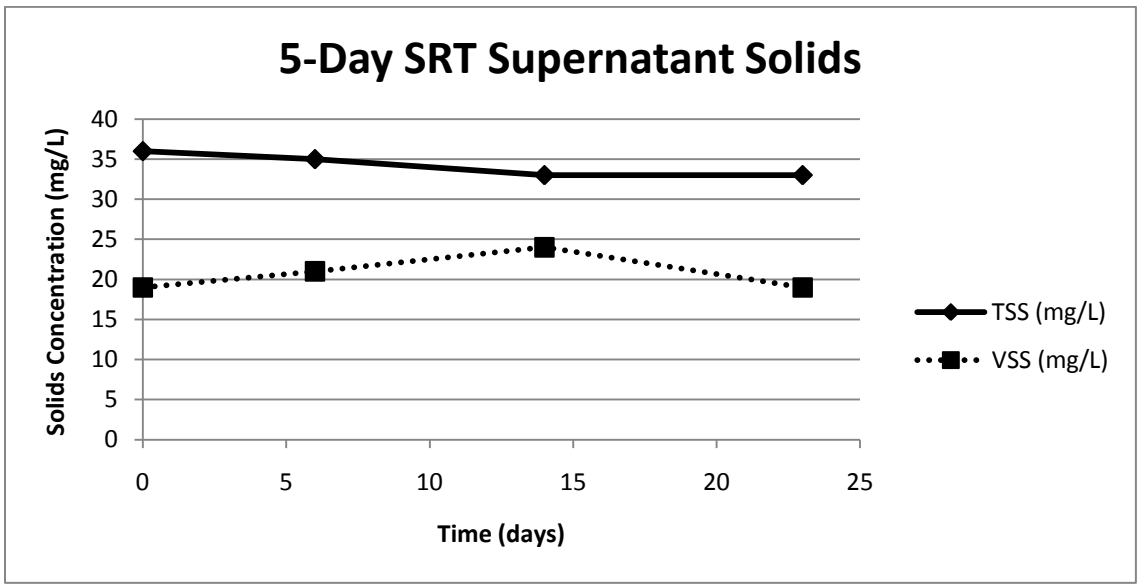

Figure 8

Scatter Plot of Round 2 - 5-day Supernatant Solids versus Time

Examination of the data for the second run of testing shows an unexpected trend in the solids concentration in the 20-day SRT reactor. As discussed in the literature review, these values are expected to be substantially higher than the values found in the second round of testing. While still higher than the values of the 5-day SRT reactor, the difference is not as great as is expected. This discrepancy was not noticed during the course of the testing and thus went without repair. The hypothesized reasons for the shift are due to error in the feed or aeration. Errors in the feed could be from bacterial contamination that utilized part of the substrate prior to introduction to the reactor or from human error in preparation. However, as both reactors were fed from the same source and the 5-day SRT reactor was close to expected values, the primary suspected source is the rate of aeration due to restricted flow through the air diffuser. Without proper aeration the solids concentration would be much lower than expected as the electron donor (oxygen) would limit the substrate utilization and thus the bacterial growth. As the reactors were not monitored for the dissolved oxygen content this error would not be noticed by visual inspection except in extreme cases. Also without the dissolved oxygen monitoring this source of error can only be hypothesized.

However, the graphs of solids content do show the stability of the reactors as the small variations seen are anticipated as the reactor fluctuates up and down slightly. There were none of the large variations seen like those in the initial testing as the issues with consistent operation seen previously had been fixed. This testing began after approximately one month of the reactor operating and adjusting from the seed activated

sludge. As can be seen from the data the reactors were considered stable at the beginning of testing and through the course of the research work.

In the second round of testing the solids were also tested for settleability. The data is presented in Table 8 and Figure 9 below. As can be seen from the data, the 20-day SRT reactor had a much higher settling rate. Coupled with the suspended solids testing information, the final sludge level also gives information about the settleability of the sludge. Although the 20-day SRT reactor has a higher solids concentration, the final sludge volume of 95 mL versus 280 mL in the 5-day SRT reactor shows better formed floc in the 20-day reactor. Table 8 also presents the sludge volume index (SVI) for the settling time of one hour that was used in the reactors. This calculation is performed by dividing the settled sludge volume by the mixed liquor suspended solids multiplied by the volume of the sample (Rittmann & McCarty, 2001). SVI values less than 100 mL/g indicate a well settling sludge and values greater than 150 mL/g indicate possible bulking and poor settling (Rittmann & McCarty, 2001). As can be seen from the data, the 20-day SRT reactor SVI indicates the sludge is well settling at a value of approximately 60 mL/g. However, the 5-day SRT reactor SVI indicates settling issues with a high value of 275 mL/g.

Table 8

Data from settleability testing

Time (min)	Sludge Volume Occupied	
	5-Day SRT	20-Day SRT
0	1000	1000
10	770	110
20	510	100
30	410	95
45	340	95
60	280	95
SVI (mL/g)	275	61

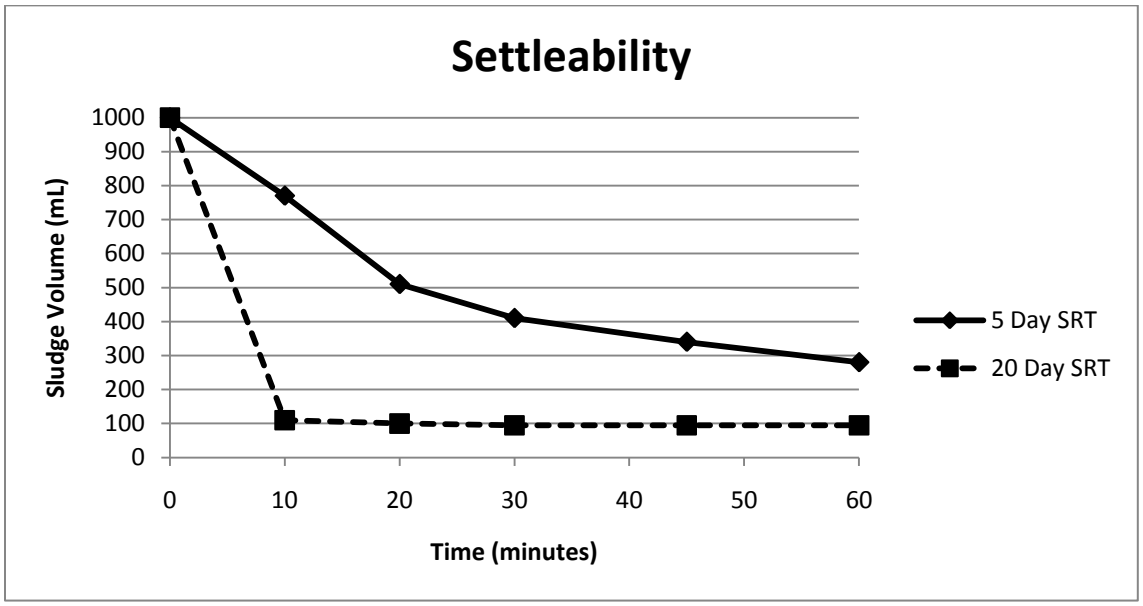

Figure 9

Scatter plot of sludge volume versus time from settleability testing

Microbial Enumeration

In this section the results of the initial MPN testing can be found. MPN was run prior to any inoculation to gain an understanding of the reactor composition. However, as this method is highly sensitive to any error by human mistake and sonicator issues, the initial results were somewhat variable. These bacterial count numbers were a great deal higher than that found just prior to inoculation testing, but does indicate the presence of *Bacillus* prior to any inoculation. While the percentages of *Bacillus* presented below are higher than that of what is referred to as a typical activated sludge in Table 1, it is hypothesized that this is either due to the nature of the local municipalities operations or factors influenced by the feed composition. The sludge seed source receives sewage from Mississippi State University which has a large amount of agricultural and cattle farming activities. As previously mentioned, a large source of *Bacillus* is from activities associated with farming of this type as the cattle ingest the bacteria regularly (Dixon, Meselson, Guillemin, & Hanna, 1999). Thus, these activities could be a possible source of the increased *Bacillus* population within the reactors. The seed facility also goes through very low flow periods due to the primary source of waste being the university. During these times the SRT is driven up to maintain the activated sludge which could also contribute to the *Bacillus* population numbers.

Alterations were also made to the "kill" cycle to find the source of such high *Bacillus* counts. This was to increase the time from 20 to 30 minutes, ensuring that all non-*Bacillus* species would be killed in the sample. The alteration of the method

produced closer to expected results and thus was continued to be utilized through the course of the inoculation testing.

The variability from day to day found while testing is also somewhat expected. At the point of the running of these tests, the reactors were in their initial stages of conversion from seeding to new sludge. Biological reactors are also highly sensitive to changes and equipment issues were still being ironed out as far as wastage, decanting, feed and dilution level controls. Again this testing was only used to track the nature of the bench scale reactors and is not used in the inoculation analysis. In order to give a somewhat more reliable set of results, outliers were eliminated and the median, average and standard deviation are presented. As can be seen from the standard deviations, the consistency of the results still left much to be desired. However, as research progressed the numbers became much more consistent from week to week and were considered stable prior to beginning inoculations. Table 9 presents the data from the testing described. In this table CH describes the total number of culturable heterotrophs, and this abbreviation will be used throughout the results section.

Table 9

Round 1 MPN data prior to inoculation

Under Standard Conditions w/o Inoculation		
Organism	**20-day SRT**	**5-day SRT**
CH/100 mL	14182	15665
***Bacillus*/100 mL**	3644	1263
% *Bacillus*	25.7	8.1

In Table 10 the data for the reactors in the second round of testing is presented. During this period the data was very consistent as the issues with reactor operations had been corrected in the initial testing. The percentage of *Bacillus* is somewhat closer to the expected values presented in Table 1. As discussed previously, the variation from the typical values could stem from a variety of reasons, but the important information gained from the data is the natural existence of *Bacillus* in the seed sludge. The data also includes the microbial enumeration data for the supernatant prior to the inoculations.

Table 10

Round 2 MPN data prior to inoculation

Under Standard Conditions w/o Inoculation		
Mixed Liquor		
Organism	**20-day SRT**	**5-day SRT**
CH/100 mL	55611	25766
***Bacillus*/100 mL**	3809	2625
% *Bacillus*	6.9	10.2
Supernatant		
Organism	**20-day SRT**	**5-day SRT**
CH/100 mL	1327	2787
***Bacillus*/100 mL**	247	488
% *Bacillus*	18.7	17.5

While the monitoring of the reactors prior to inoculations was important in observing the nature of the reactors, the true goal of the research was achieved after inoculations. During this process, about prior to inoculation a MPN test was run. This

was done in order to find the state of the reactors prior to inoculation as a reference point. At this point the reactors were considered very stable. After inoculation following the procedure discussed previously, the MPN test was run at 1, 3 and 7 days after to observe the changes in the reactor due to the inoculation. This initial inoculation data can be found in Tables 11 and 12 and Figures 11 and 12 below. The figures also feature a "PI Point" vertical line, indicating the point at which testing is representing the data after inoculation. As stated previously, MPN indicates a level of concentration of cells and results include an upper and lower control limit at the 95% confidence level. Thus, the resulting meaning that although a most probable number of organisms are reported, the number will fall within the presented range at the 95% confidence level. This data is also reported in this table and the subsequent tables describing the microbial enumeration results.

Table 11

20-day SRT Inoculation 1 MPN Data

20-Day SRT - Inoculation 1 Data (Species/100 mL)						
Time	**CH**			***Bacillus***		
	LCL, 95%	**MPN**	**UCL, 95%**	**LCL, 95%**	**MPN**	**UCL, 95%**
1 Week Prior	1272	3272	8412	896	2303	5920
1 Day After	3037	7809	20077	1900	4885	12561
3 Day After	3037	7809	20077	1900	4885	12561
7 Day After	1272	3272	8412	896	2303	5920

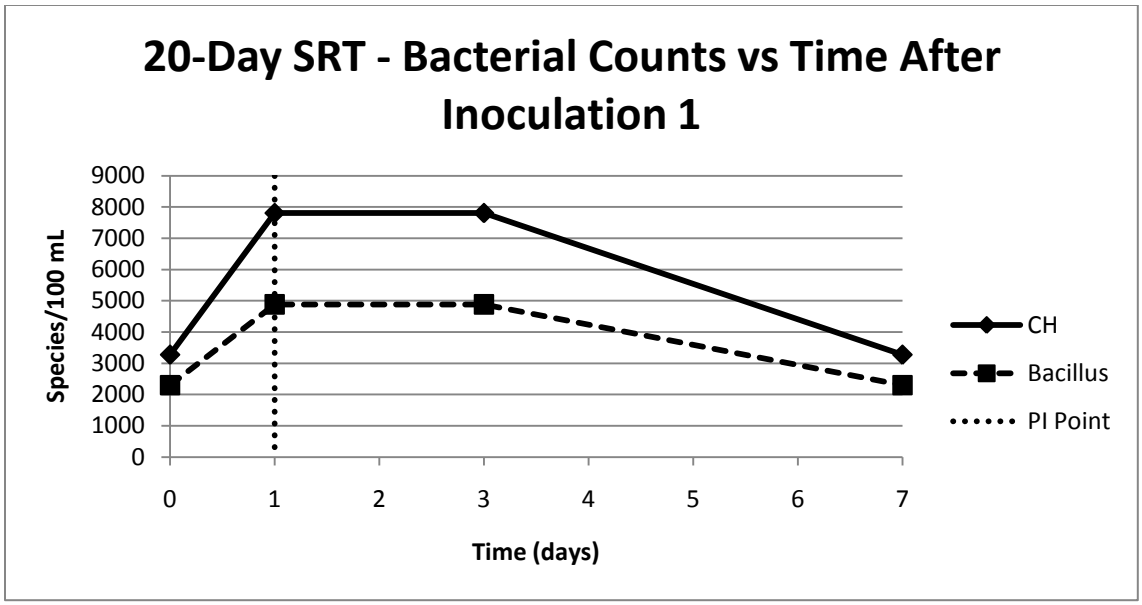

Figure 10

Scatter Plot of 20-day SRT Inoculation 1 MPN Data

Table 12

5-day SRT Inoculation 1 MPN Data

5-Day SRT - Inoculation 1 Data (Species/100 mL)						
Time	**CH**			***Bacillus***		
	LCL, 95%	**MPN**	**UCL, 95%**	**LCL, 95%**	**MPN**	**UCL, 95%**
1 Week Prior	8955	23025	59200	495	1273	3273
1 Day After	12726	32719	84126	896	2303	5920
3 Day After	8955	23025	59200	495	1273	3273
7 Day After	8955	23025	59200	495	1273	3273

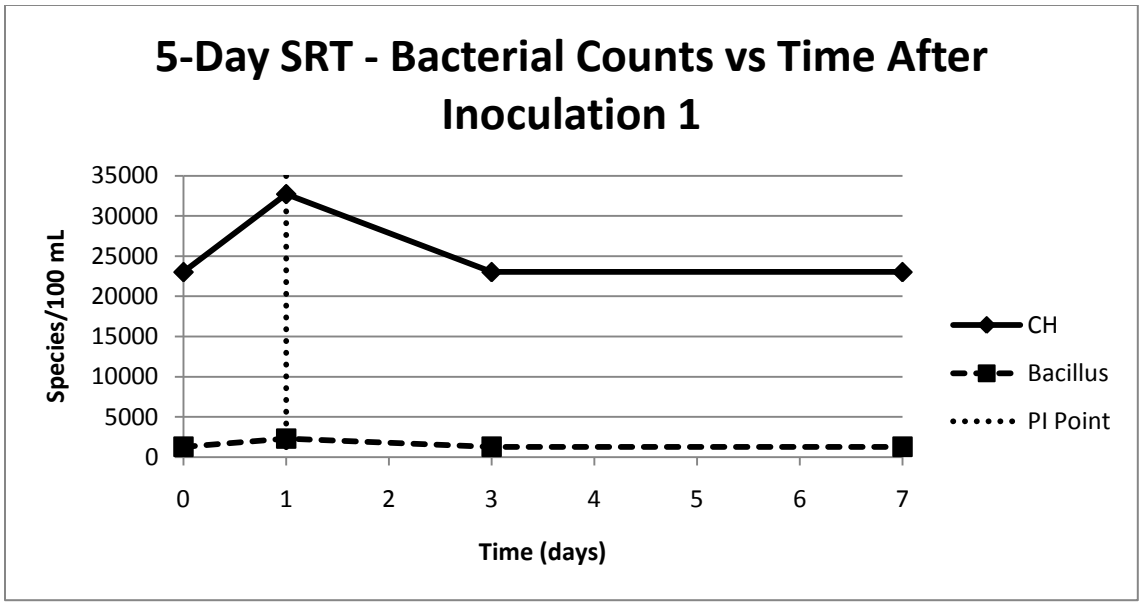

Figure 11

Scatter Plot of 5-day SRT Inoculation 1 MPN Data

As can be seen in Table 11 and Figure 10 for the 20-day SRT, the dosage causes an increase in the bacterial counts of both *Bacillus* and non *Bacillus* species. This level is maintained for approximately 2 days and then begins to decrease towards the original numbers. By the end of day 7 the data indicates that the reactor has returned to its original state found prior to the inoculation. In the 5-day SRT reactor nearly the same behavior is seen with the exception of the plateau. Under this SRT the bacterial counts actually spike and begin to decrease again rapidly. By the end of day 3 the reactor has returned to its original state and that level is maintained throughout the remained of the 7 day period. However, from looking at the data one can see that there are gaps within what might actually have occurred over the multiple day waiting periods between tests.

As this was the initial inoculation the exact time periods were not known and this indicated the need for more vigorous testing.

It was noticed that more detail was needed to truly observe the changes in the quickly changing 5-day SRT reactor, thus another inoculation was run. However, this test experienced issues as the aeration apparatus failed during the run. After waiting a week to ensure the *Bacillus* introduced had passed, another inoculation run with more vigorous testing of 1, 2, 3, 5 and 7 days after inoculation was completed. The results of which can be seen in Table 13 and 14 and Figure 12 and 13 below.

Table 13

20-day SRT Inoculation 2 MPN Data

20-Day SRT - Inoculation 2 Data (Species/100 mL)						
Time	**CH**			***Bacillus***		
	LCL, 95%	**MPN**	**UCL, 95%**	**LCL, 95%**	**MPN**	**UCL, 95%**
1 Week Prior	1900	4885	12561	495	1273	3273
1 Day After	3037	7809	20077	896	2303	5920
2 Day After	3037	7809	20077	896	2303	5920
3 Day After	3037	7809	20077	896	2303	5920
5 Day After	1900	4885	12561	495	1273	3273
7 Day After	1900	4885	12561	495	1273	3273

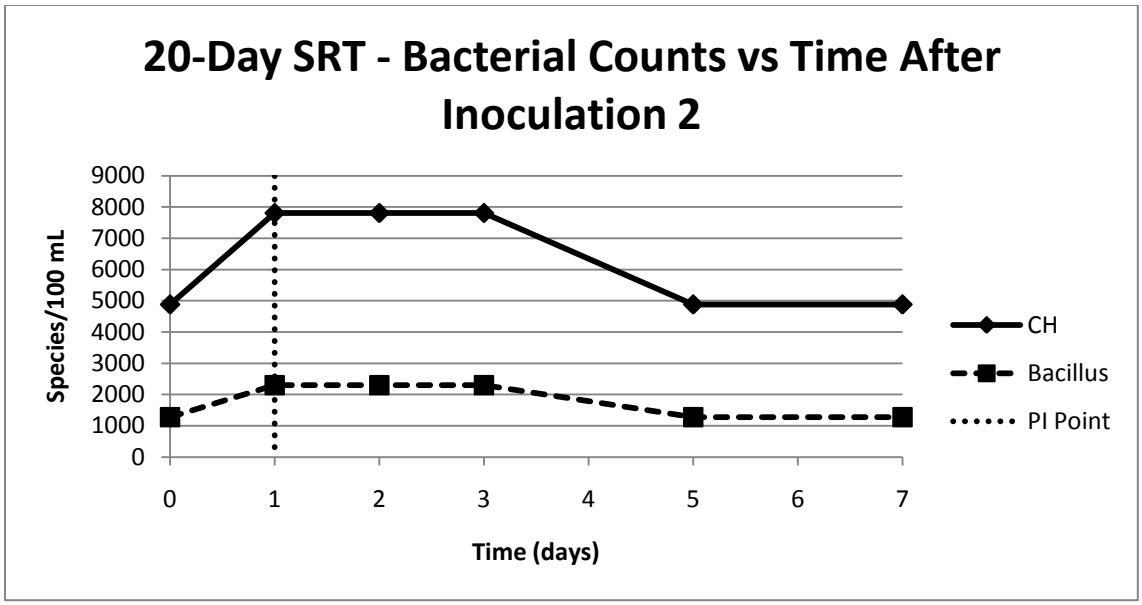

Figure 12

Scatter Plot of 20-day SRT Inoculation 2 MPN Data

Table 14

5-day SRT Inoculation 2 MPN Data

5-Day SRT - Inoculation 2 Data (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Week Prior	8956	23027	59204	304	781	2008
1 Day After	12726	32719	84126	495	1273	3273
2 Day After	8956	23027	59204	304	781	2008
3 Day After	8956	23027	59204	304	781	2008
5 Day After	8956	23027	59204	304	781	2008
7 Day After	8956	23027	59204	304	781	2008

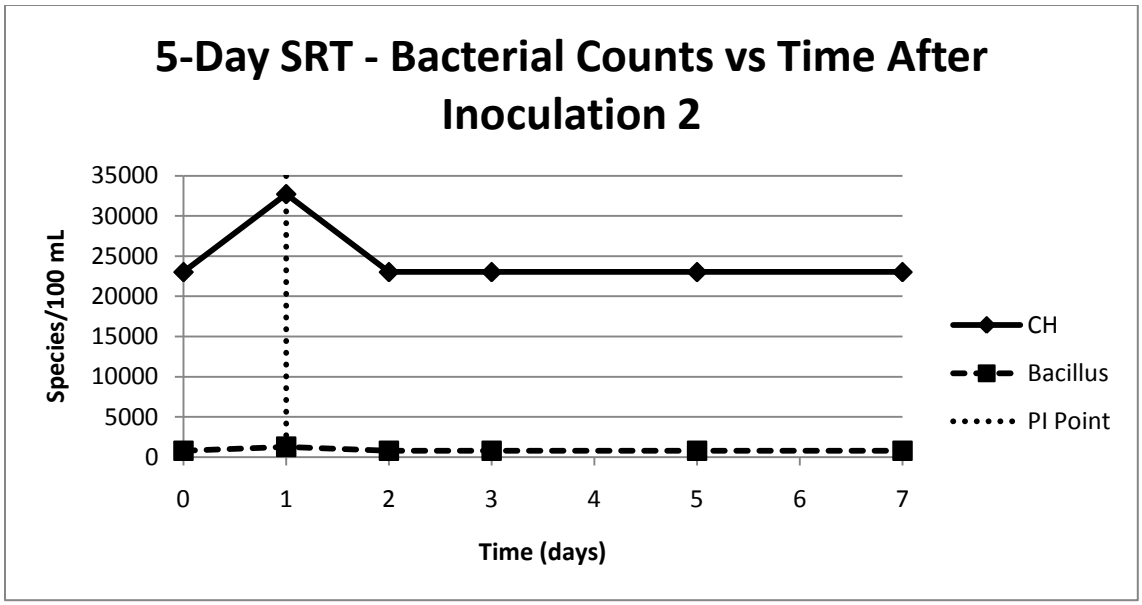

Figure 13

Scatter Plot of 5-day SRT Inoculation 2 MPN Data

As expected, the same general behavior was observed with slightly different bacterial numbers in the second inoculation run. These variations are not unexpected due to the sensitivity of biological processes and the limits of precision of the test. We also see that the need for additional testing days between the gaps previously used did yield additional information. In the 20-day SRT reactor test we see that the plateau in fact began to decrease return to the original state by day 5. After day 5 the reactor maintained the bacterial levels until the end of day 7, which indicated there was no need for further testing. In the 5-day SRT test even more information was gained from the additional testing. As this reactor is passing solids within 5-days, the changes are much more rapid. This can be seen in the data as the spike that occurred after 24 hours had in fact returned

to the original state by 48 hours after inoculation. In all cases, these trends of the data were seen for both the overall bacterial counts as well as the *Bacillus.*

An additional detail was tested in the second round of inoculations in order to show full representation of the *Bacillus* in the reactor. The supernatant was testing for bacterial and *Bacillus* numbers, as this is the theorized method of removal of *Bacillus* from the reactors. A MPN test also was run on the inoculant in order to determine how much *Bacillus* was being added to each reactor. This value was found to be approximately 100000 *Bacillus* cells being added per inoculation. The assumption that all inoculations are approximately of this value is being made, as all were prepared in the same manner. However, it is not practical to place this data into the graphical representations of the data. Although the value of the inoculation is known, the numbers presented from the MPN test represent bacterial counts per 100 mL of solution in the reactor. The inoculation is providing a slug of *Bacillus* to the reactors which begin to grow and multiply within the reactor. Placing the data into a plot would detract from the visualization of the trends being presented. The data for the inoculation runs are shown below in Tables 15-22 and Figures 14-21. These present the microbial enumeration of total bacteria and *Bacillus* for inoculation run 3 and 4 for both the mixed liquor and supernatant in the 20 and 5-day SRT reactors.

Table 15

20-day SRT ML Inoculation 3 MPN Data

20-Day SRT ML - Inoculation 3 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	12726	32719	84124	1900	4885	12561
1 Day After	19004	48862	125631	3037	7809	20077
2 Days After	19004	48862	125631	3037	7809	20077
2.5 Days After	19004	48862	125631	3037	7809	20077
3 Days After	19004	48862	125631	3037	7809	20077
3.5 Days After	19004	48862	125631	3037	7809	20077
4 Days After	12726	32719	84124	1900	4885	12561
4.5 Days After	12726	32719	84124	1900	4885	12561
5 Days After	12726	32719	84124	1900	4885	12561

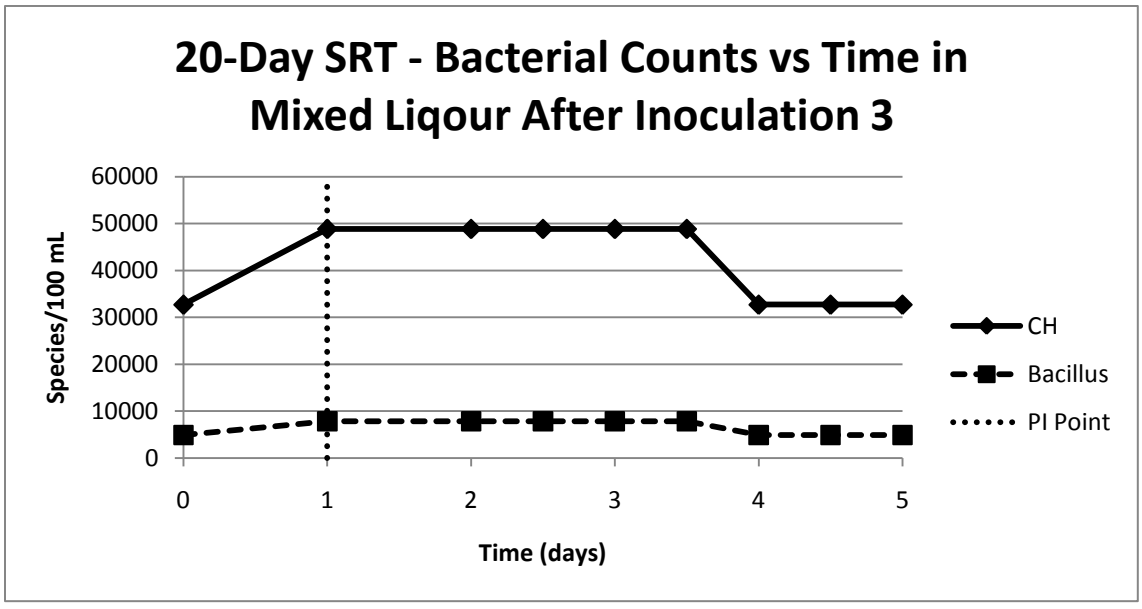

Figure 14

Scatter Plot of 20-day SRT ML Inoculation 3 MPN Data

Table 16

5-day SRT ML Inoculation 3 MPN Data

5-Day SRT ML - Inoculation 3 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	8383	21554	55418	1273	3272	8412
1 Day After	12726	32719	84124	1900	4885	12561
2 Days After	8383	21554	55418	1273	3272	8412
2.5 Days After	8383	21554	55418	1273	3272	8412
3 Days After	8383	21554	55418	1273	3272	8412
3.5 Days After	8383	21554	55418	1273	3272	8412
4 Days After	8383	21554	55418	1273	3272	8412
4.5 Days After	8383	21554	55418	1273	3272	8412
5 Days After	8383	21554	55418	1273	3272	8412

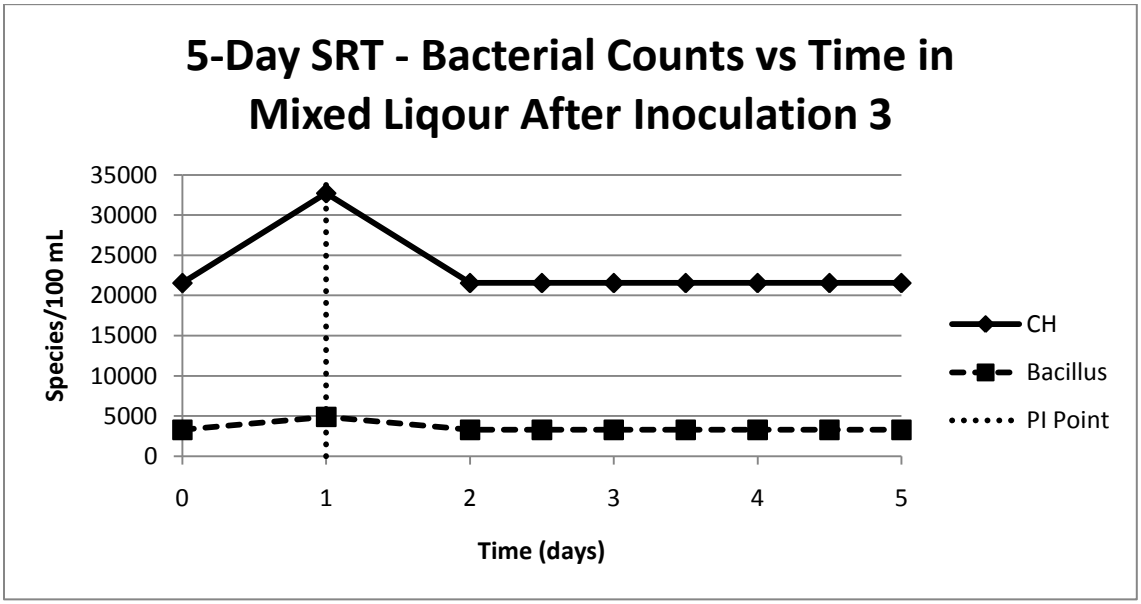

Figure 15

Scatter Plot of 5-day SRT ML Inoculation 3 MPN Data

Table 17

20-day SRT SN Inoculation 3 MPN Data

20-Day SRT SN - Inoculation 3 (Species/100 mL)						
Time	**CH**			***Bacillus***		
	LCL, 95%	**MPN**	**UCL, 95%**	**LCL, 95%**	**MPN**	**UCL, 95%**
1 Day Prior	495	1273	3273	66	168	433
1 Day After	838	2156	5542	304	781	2008
2 Days After	838	2156	5542	304	781	2008
2.5 Days After	838	2156	5542	304	781	2008
3 Days After	838	2156	5542	304	781	2008
3.5 Days After	838	2156	5542	304	781	2008
4 Days After	495	1273	3273	66	168	433
4.5 Days After	495	1273	3273	66	168	433
5 Days After	495	1273	3273	66	168	433

In the data and plots above the same trends in both reactors as seen in the initial runs of the research were again seen. The increased details of testing did reveal that the 20-day SRT was in fact clearing the *Bacillus* inoculation within 4 days instead of 5-days. While this does not change the trend the additional detail is informative. The two following tables and plots show the data from the testing of the supernatant. This testing indicated the same trends in the concentration of *Bacillus* in the supernatant as the mixed liquor. Although the magnitude of the concentrations was smaller in this portion of the reactor, the trend indicates that the *Bacillus* represent a large portion of the culturable heterotrophs in the supernatant. During the 4 cycles per day where the 2 liters are removed the bacteria here would also be removed, thus proving the effluent as a large portion of the removal path of the *Bacillus.*

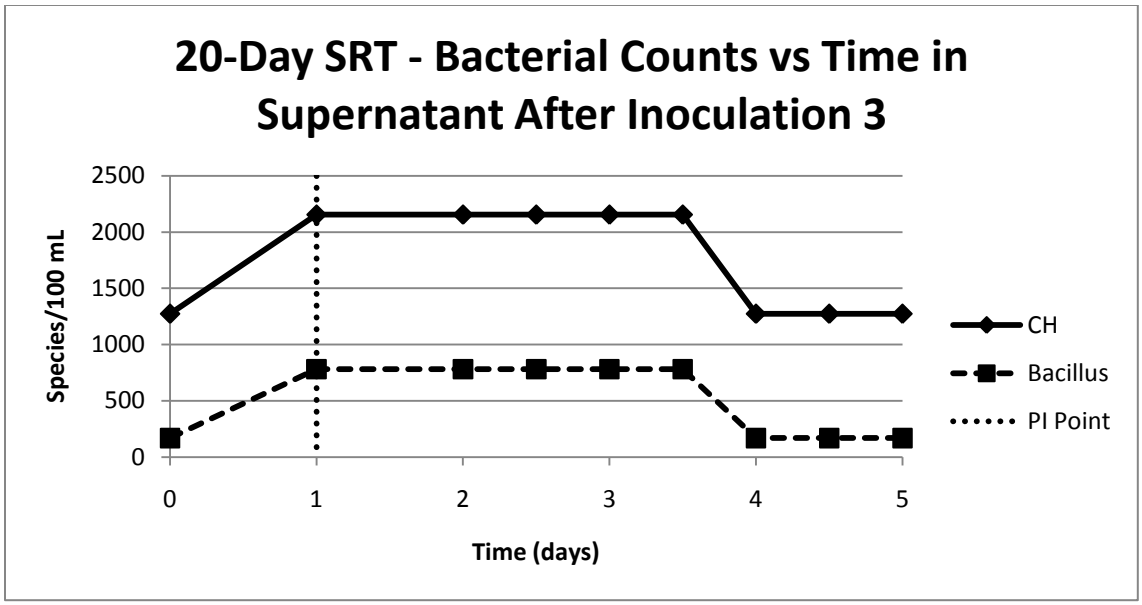

Figure 16

Scatter Plot of 20-day SRT SN Inoculation 3 MPN Data

Table 18

5-day SRT SN Inoculation 3 MPN Data

5-Day SRT SN - Inoculation 3 (Species/100 mL)						
Time	**CH**			***Bacillus***		
	LCL, 95%	**MPN**	**UCL, 95%**	**LCL, 95%**	**MPN**	**UCL, 95%**
1 Day Prior	896	2303	5920	190	489	1256
1 Day After	1273	3272	8413	495	1273	3273
2 Days After	896	2303	5920	190	489	1256
2.5 Days After	896	2303	5920	190	489	1256
3 Days After	896	2303	5920	190	489	1256
3.5 Days After	896	2303	5920	190	489	1256
4 Days After	896	2303	5920	190	489	1256
4.5 Days After	896	2303	5920	190	489	1256
5 Days After	896	2303	5920	190	489	1256

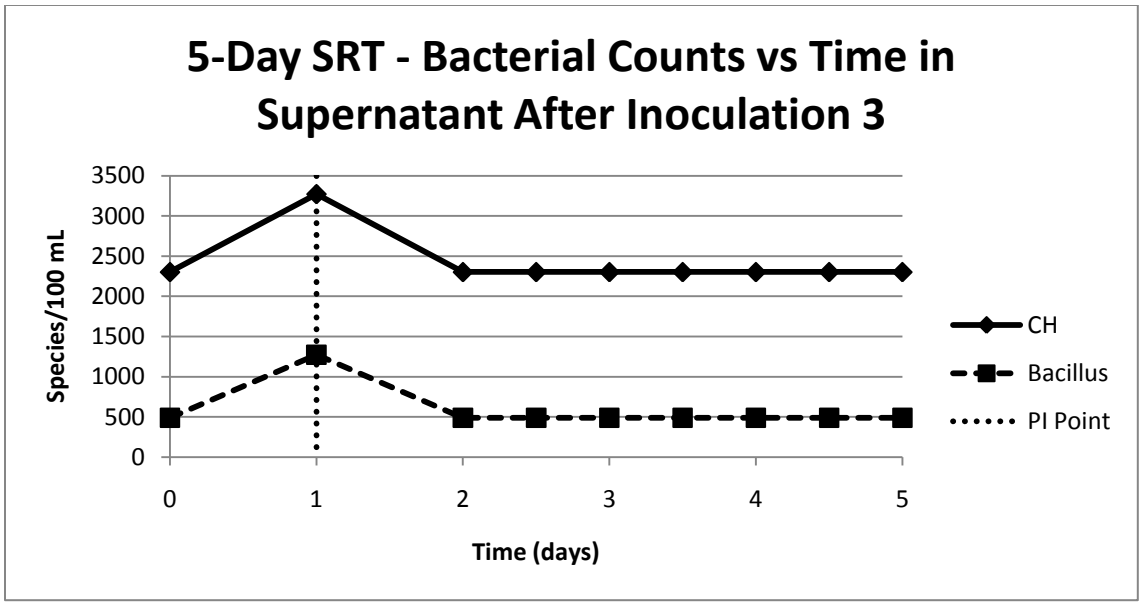

Figure 17

Scatter Plot of 5-day SRT SN Inoculation 3 MPN Data

In the subsequent tables and graphs the data for the fourth run of the inoculation are presented. These again indicate an identical trend in the concentrations of bacteria and *Bacillus* in the mixed liquor and supernatant in both reactors, as has been previously seen. One more detail was added in this testing with the microbial enumeration being tested at a time of 12 hours after inoculation. As can be seen in the data and graphs the value of bacteria and *Bacillus* are higher than that of the 24 hour post inoculation period. This indicates an exponential decrease in the concentration, especially in the 5-day SRT reactor. We again see the plateau of the bacterial counts in the 20-day SRT after the exponential decrease. The theorized causes of this trend will be discussed in the conclusions section of the report.

Table 19

20-day SRT ML Inoculation 4 MPN Data

20-Day SRT ML - Inoculation 4 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	12726	32719	84124	1900	4885	12561
0.5 Days After	30375	78099	200801	8956	23027	59206
1 Days After	19004	48862	125631	3037	7809	20077
2 Days After	19004	48862	125631	3037	7809	20077
2.5 Days After	19004	48862	125631	3037	7809	20077
3 Days After	19004	48862	125631	3037	7809	20077
3.5 Days After	19004	48862	125631	3037	7809	20077
4 Days After	12726	32719	84124	1900	4885	12561
4.5 Days After	12726	32719	84124	1900	4885	12561
5 Days After	12726	32719	84124	1900	4885	12561

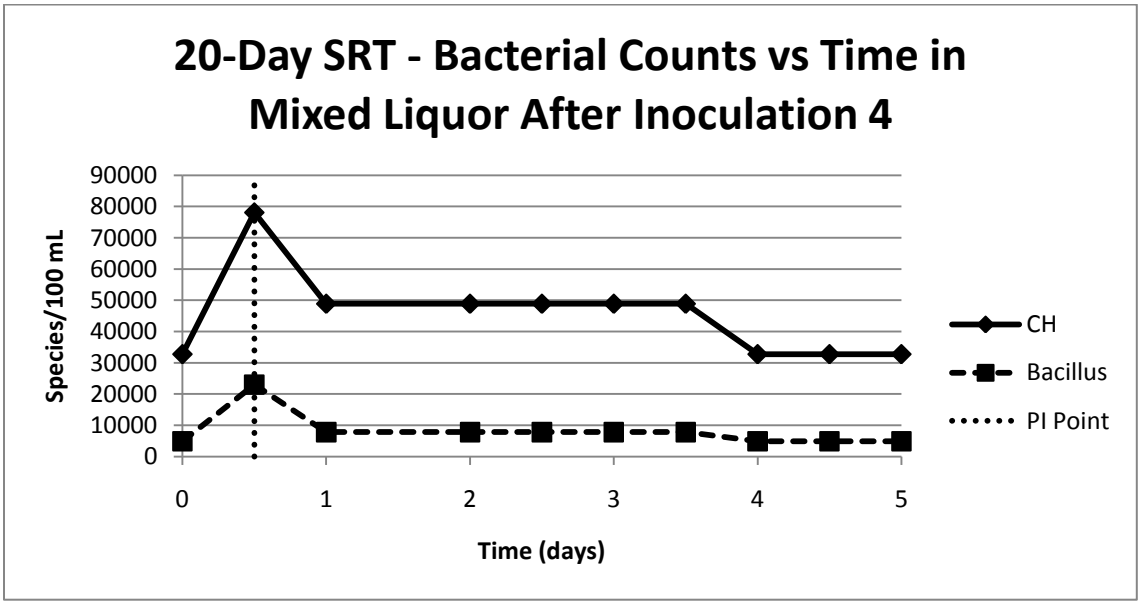

Figure 18

Scatter Plot of 20-day SRT ML Inoculation 4 MPN Data

Table 20

5-day SRT ML Inoculation 4 MPN Data

5-Day SRT ML - Inoculation 4 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	8383	21554	55418	1273	3272	8412
0.5 Days After	19003	48859	125622	4951	12731	32732
1 Days After	12726	32719	84124	1900	4885	12561
2 Days After	8383	21554	55418	1273	3272	8412
2.5 Days After	8383	21554	55418	1273	3272	8412
3 Days After	8383	21554	55418	1273	3272	8412
3.5 Days After	8383	21554	55418	1273	3272	8412
4 Days After	8383	21554	55418	1273	3272	8412
4.5 Days After	8383	21554	55418	1273	3272	8412
5 Days After	8383	21554	55418	1273	3272	8412

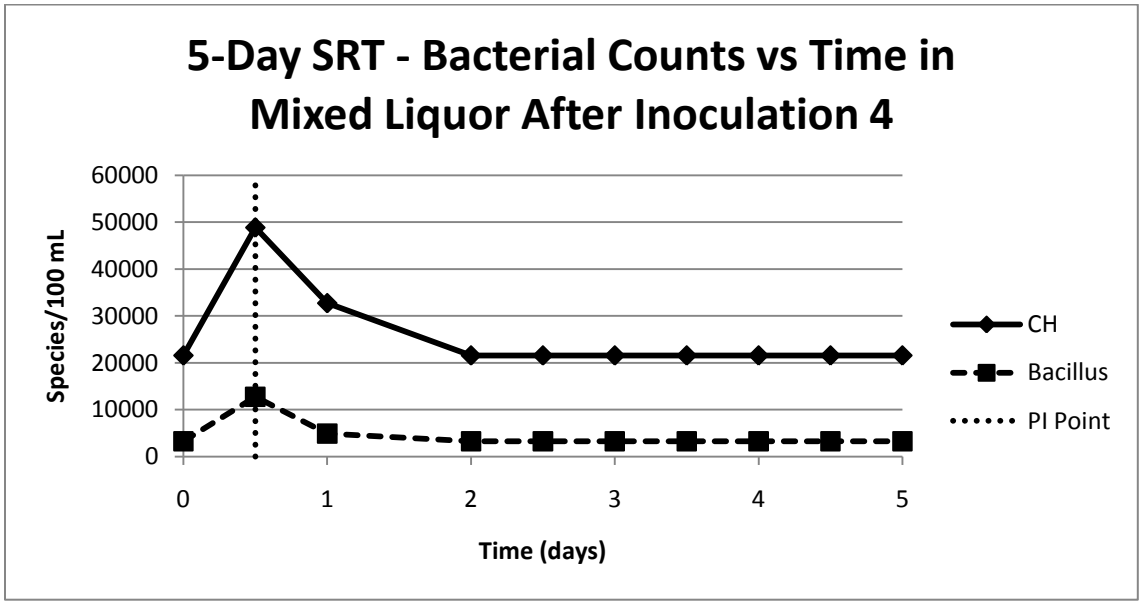

Figure 19

Scatter Plot of 5-day SRT ML Inoculation 4 MPN Data

Table 21

20-day SRT SN Inoculation 4 MPN Data

20-Day SRT SN - Inoculation 4 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	495	1273	3273	66	168	433
0.5 Days After	3038	7810	20080	1900	4886	12563
1 Days After	838	2156	5542	304	781	2008
2 Days After	838	2156	5542	304	781	2008
2.5 Days After	838	2156	5542	304	781	2008
3 Days After	838	2156	5542	304	781	2008
3.5 Days After	838	2156	5542	304	781	2008
4 Days After	495	1273	3273	66	168	433
4.5 Days After	495	1273	3273	66	168	433
5 Days After	495	1273	3273	66	168	433

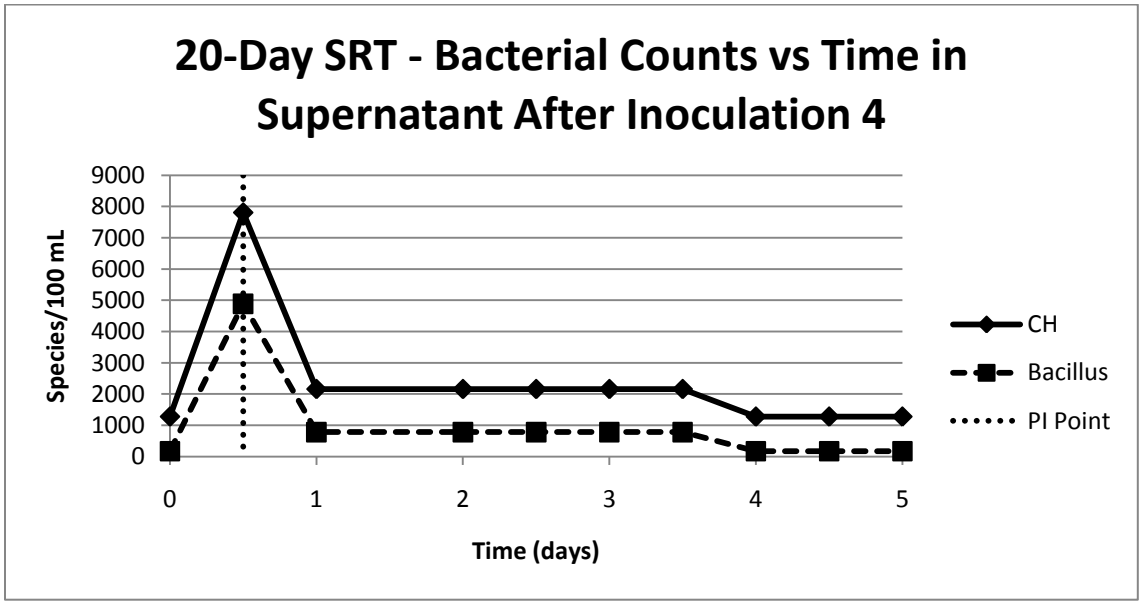

Figure 20

Scatter Plot of 20-day SRT SN Inoculation 4 MPN Data

Table 22

5-day SRT SN Inoculation 4 MPN Data

5-Day SRT SN - Inoculation 4 (Species/100 mL)						
Time	CH			*Bacillus*		
	LCL, 95%	MPN	UCL, 95%	LCL, 95%	MPN	UCL, 95%
1 Day Prior	896	2303	5920	190	489	1256
0.5 Days After	4952	12733	32738	3038	7810	20080
1 Days After	1273	3272	8413	495	1273	3273
2 Days After	896	2303	5920	190	489	1256
2.5 Days After	896	2303	5920	190	489	1256
3 Days After	896	2303	5920	190	489	1256
3.5 Days After	896	2303	5920	190	489	1256
4 Days After	896	2303	5920	190	489	1256
4.5 Days After	896	2303	5920	190	489	1256
5 Days After	896	2303	5920	190	489	1256

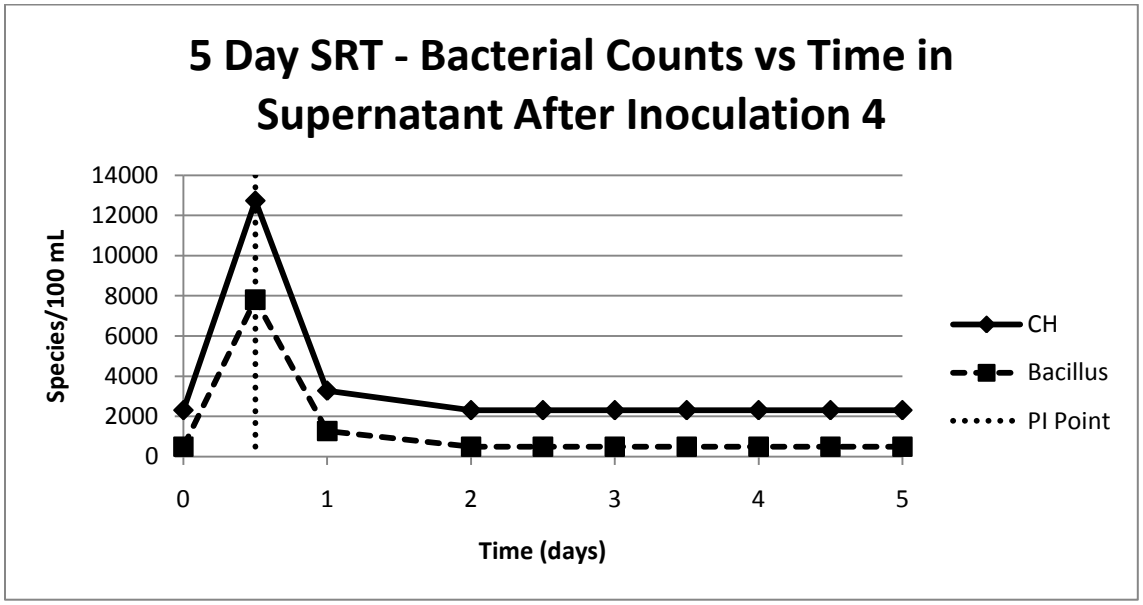

Figure 21

Scatter Plot of 5-day SRT SN Inoculation 4 MPN Data

With testing concluded some trends in the data are worthy of being noted to summarize all of the testing. In the 20-day SRT reactors the inoculation causes a spike in the microbial concentration in the reactor. This value exponentially decreases until reaching a plateau at a time of 24 hours post inoculation. The bacterial numbers then hold steady until a time of 4 days post inoculation after which the numbers again decrease back to the original state of the reactor by a time of 5-days. These trends are seen in every test of both the mixed liquor and supernatant. In the 5-day SRT reactors the inoculation also causes a spike in the microbial concentration in the reactor. These values exponentially decrease until returning to the normal operating conditions at a time of 2 days post inoculation. These trends were also reflected in all of the testing in both the supernatant and mixed liquor.

CHAPTER V

CONCLUSIONS

Some hypothesis must first be made to explain some variations within the data that were found during testing. One such inconsistency was the magnitude of change in the non-kill bacteria counts from the MPN testing. As can be seen in the data, these values shifted a greater magnitude than did that of the *Bacillus*. The most likely explanation of this is that not all *Bacillus* that were sampled survived the "kill" cycle. The fact that *Bacillus* can survive such temperatures does not mean that every organism will. It is also possible that upon returning to the vegetative state from an endospore, the *Bacillus* experienced some lag in their kinetics after the high stress environment. However, this does not change the resulting trends that were found in the data.

Another somewhat unexpected result was the higher levels of bacteria in the 5-day SRT than that of the 20-day SRT found when testing in the initial research. Looking at the solids in these reactors, one would expect to see a lower number of bacteria found in the 5-day SRT than that of the 20-day SRT. As discussed in previous sections, the activities of bacteria in such a low SRT are more active and the fraction of active organisms is greater than that found in a higher SRT sludge. In higher SRT reactors it has been found that decay dominates while growth dominates in lower SRTs (Bitton, 1999).

Our expectation is that this higher level of activity resulted in more positive results in the MPN testing. Meaning that even though the numbers of actual bacteria was higher in the 20-day SRT, the activity of the 5-day SRT led to a discrepancy between expected results and those actually found. For unknown reasons, this trend was not seen in the second round of the testing. However, identical trends in the shifts after inoculation were observed in both sets of data, thus the anomaly is not of large concern.

In regards to the goal of the research, the overall results between SRTs and multiple inoculation runs were consistent. After the introduction of a large number of pure *Bacillus,* the reactors returned to their normal operating state within 2 days for the 5-day SRT reactor and 4 days in the case of the 20-day SRT reactor. This indicates that the introduction of a non-native *Bacillus,* specifically *Bacillus anthracis,* should not become predominant species in the diverse microbial population contained within an activated sludge reactor. This also indicates that without additional efforts, the health of the biological system will not experience adverse effects and risks associated with the contamination are limited. These results were somewhat expected given the typical composition of an activated sludge wastewater treatment system. In particular, if *Bacillus* organisms were to become a dominant species in the biomass of such treatment systems, they would most likely already dominate the activated sludge prior to any inoculation. Their kinetics and reproduction rates simply are not high enough to compete with the other dominant species.

It is also relevant to comment on the means by which the *Bacillus* is exiting the reactors. The data indicate removal can be primarily credited to the decanting of the

supernatant and their leaving the system with the effluent with a smaller portion of the removal credited to the wastage process. The reasoning behind this conclusion is the speed at which the removal is occurring. If the removal were completely due to the solids removal through wastage, the retention time of the *Bacillus* would be closer to the overall solids retention time of the reactor. However, the organisms left the reactor operated at a 5-day SRT in about two days compared to being gone in four days for the 20-day SRT reactor. Thus, it was concluded that hydraulic retention time must be a large factor.

When considering a tracer test we do not expect the tracer to remain for the entirety of the solids retention time. Typically a tracer has passed the system within 3-5 multiples of the hydraulic retention time, which for these reactors was 12 hours (Nazaroff & Alvarez-Cohen, 2001). However, we do see a difference in the residence time of the *Bacillus* between the two SRTs tested. This indicates that SRT does play a role in the retention of these bacteria. It is also important to note that although the comparison to a conservative tracer is adequate in the visualization of the process, it is not an exact reflection of the process. The *Bacillus* introduced to the system undergoes growth and thus are not a conservative substance. When considering a mass balance of the system the microbial growth acts as an input term that cannot be ignored. A combination of the influence of this growth and the retention through other mechanisms result in the retention time of the *Bacillus* being different than that of a conservative tracer.

The mechanics of the retention process are not known but can be hypothesized to be retained due to sweep floc as discussed previously in the nature of flocculated particle

settling. We come to this conclusion because *Bacillus* are known to not be floc formers, yet are retained in the system. This indicates that the *Bacillus* are being captured in the sweep floc or else the bacteria would wash out completely as it remained suspended in the water. The testing of settleability and supernatant help prove this theory. In the settleability testing, the 20-day SRT reactor was seen to have more developed floc with a much greater settling velocity, as is expected in higher solids retention time reactors. These well flocculated particles are much more adept for sweeping particles along with them to the bottom during settling.

The supernatant testing performed during this study found higher levels of *Bacillus* in the supernatant of the 5-day SRT than that of the 20-day SRT. Coupled together, this data indicates that the sweep floc is aiding in the retention of *Bacillus* in the reactor. Therefore, the effluent is the primary carrier of the *Bacillus* from the reactor. However, it should be noted that this is due to the solids retention time of the reactors, as this is the controlling factor in the nature of the floc.

However, these results do raise other questions. The first question is what will happen to the *Bacillus* that is in fact washed through the system. Typical wastewater treatment systems require free chlorine levels and contact times great enough to remove fecal coliforms (Bitton, 1999). While chlorine has been found to be an effective means of treatment for the disinfection of waters containing *Bacillus* and their spores, the levels used in typical wastewater plants are not sufficient (Counce, et al., 2008). The disinfection goals need to be revisited and adjusted to prepare for a contamination like the one discussed. If reaching a drinking water facility water supply, *Giardia cysts* are

typically used as the design parameters for disinfection. These organisms have been found to be at least, if not more, resistant to disinfection by chlorine than *Bacillus anthracis* spores (Counce, et al., 2008). Thus we infer that disinfection aimed to remove *Giardia* cysts would be sufficient for removal of *Bacillus anthracis*. While there are many factors affecting the disinfection levels achievable, these would be assumed to generally be sufficient. The other factor to consider is that these bacteria do in fact exist naturally in the environment. While high levels would not be desired in the surface waters or soils where the effluent is discharged, it would not be of a large concern for small concentrations of these organisms to be discharged.

The other question raised is: are the *Bacillus* that are washing out the same ones as those introduced to the system from outside sources? The true answer to this question is unknown and cannot be reasonably concluded without DNA studies of the actual species of *Bacillus* that are in the reactor before and after inoculation. As we have discussed, the *Bacillus* genus acts nearly identical regardless of species. Under this knowledge any of these could exist within the activated sludge system. However, the diversity within the activated sludge is based on the influent wastewater. While a slug may cause some temporary shifts in the biological diversity, it is expected that the diversity would return to normal without any further contaminations.

CHAPTER VI

RECOMMENDATIONS

While the research completed yields conclusions about the effects of a contamination of *Bacillus anthracis* to an activated sludge wastewater treatment system, there are some limitations in the results. While the upper and lower limits of solids retention times have been utilized and intermediate values can be interpolated, only one hydraulic retention time has been tested. As the decanting of the effluent was determined to be the primary means of *Bacillus* removal, examination of other hydraulic retention times would be informative. However, based on expected changes to the nature of the biomass due to changes in the hydraulic retention time, hypotheses can be theorized for these changes.

As the hydraulic retention time tested (12 hours) is in the upper end of the typical values, it is most beneficial to examine a lower hydraulic retention time. When reducing the hydraulic retention time, the influent rate must in turn be increased to maintain a hydraulic level within the system. By increasing the influent rate the level of nutrients and thus the biomass concentration are increased as well. Theoretically this could increase the sweep floc mechanism theorized to be the retention method of the *Bacillus*. However, this increase would not be expected to be substantial in comparison to the increased effluent rate. As the solids retention time has been found to be the primary

factor in the floc structure (Liao, Droppo, Leppard, & Liss, 2006), the expectation would be for this to remain at or near the original retention level found in the testing. If there were no change in the amount of *Bacillus* retained by sweep floc, the *Bacillus* concentration in the effluent would remain the same. While some changes would be expected, the decrease of the *Bacillus* concentration in the effluent would not be expected reduce substantially. With all of these factors together the retention time of the *Bacillus* would theoretically decrease by a maximum factor of that equal to the factor the hydraulic retention time was reduced by. However, as the increased biomass concentration would be expected to have some influence it would not be expected for the reduction of retention rate to reach this maximum level.

If the hydraulic retention time were to be increased from the value tested, the opposite of the effects discussed would be expected. The influent and nutrient levels would be decreased and thus some decrease of the biomass concentration would be expected. This in turn would be expected to decrease the sweep floc mechanism slightly. However, these changes would be expected to be small in comparison to the changes in effluent rates as the solids retention time is the controlling factor in the floc structure. Again the effluent concentration of the *Bacillus* would not be expected to change substantially. Therefore, the expected change in retention time of the *Bacillus* would increase by a maximum factor equal to that of the change in the hydraulic retention time.

This research also pertains solely to activated sludge systems for carbonaceous material removal. While behaviors can be theorized, systems designed for nitrogen or phosphorus removal should also be investigated if concerns about *Bacillus anthracis*

contamination exist. These systems both utilize aerobic process as at least part of the treatment system. The *Bacillus anthracis* would be expected to survive the anaerobic process as well, either through endospore formation or facultative anaerobic processes.

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