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Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering

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FC, fecal coliform
VBNC, viable but non-culturable
CFU, colony-forming units
MPN, most probable number
DS, dry solids

ABSTRACT

Recent literature has reported that high concentrations of indicator bacteria such as fecal coliforms (FCs) were measured in anaerobically digested sludges immediately after dewatering even though low concentrations were measured prior to dewatering. This research hypothesized that the indicator bacteria can enter a non-culturable state during digestion, and are reactivated during centrifuge dewatering. Reactivation is defined as restoration of culturability. To examine this hypothesis, a quantitative polymerase chain reaction (qPCR) method was developed to enumerate *Escherichia coli*, a member of the FC group, during different phases of digestion and dewatering. For thermophilic digestion, the density of *E. coli* measured by qPCR could be five orders of magnitude greater than the density measured by standard culturing methods (SCMs), which is indicative of non-culturable bacteria. For mesophilic digestion, qPCR enumerated up to about one order of magnitude more *E. coli* than the SCMs. After centrifuge dewatering, the non-culturable organisms could be reactivated such that they are enumerated by SCMs, and the conditions in the cake allowed rapid growth of FCs and *E. coli* during cake storage.

1. Introduction

During the last few years, researchers have reported relatively low concentrations of fecal coliforms (FCs) after anaerobic digestion; however, immediately after mechanical dewatering, relatively high concentrations of FCs were measured (Iranpour et al., 2003; Cheung et al., 2003; Monteleone et al., 2004; Erdal et al., 2003, 2004; Qi et al., 2004). For example,

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Iranpour et al. (2003) reported results from different field trials using thermophilic digestion. They found that in trials with continuous flow digestion at 57.7 °C as well as a two stage batch digestion at 54 °C, the FCs after digestion were generally below 1000 most probable number (MPN) per gram of dry solids (DSs) with average values near 10^2 MPN/g DS, which meets the United States Environmental Protection Agency (USEPA) requirements for Class A biosolids (USEPA, 1999). However, after dewatering using a full-scale high solids centrifuge and placement in a silo, the FCs were 10^6 MPN/g DS, a dramatic increase of greater than three orders of magnitude. Similarly, Erdal et al. (2003) reported that after mesophilic anaerobic digestion, the FC densities were about 5.6×10^4 colony-forming units (CFU) per gram DS. However, after high solids centrifugation and further conveyance, the FC densities in the cake were 6.5×10^5 cfu/g DS. Storage of this cake for one day resulted in a further increase to 1.5×10^7 cfu/g DS. Similar results after high solids centrifugation have been reported in other studies (Cheung et al., 2003; Qi et al., 2004; Monteleone et al., 2004).

Several different reasons for the large increase in FC density after dewatering have been presented. For example, Qi et al. (2004) suggested regrowth of the FCs was a possible mechanism to explain this phenomena. Alternatively, Cheung et al. (2003) reported that the difference in enumeration between the liquid and the cake was likely due to sample matrix effects. Similarly, Monteleone et al. (2004) suggested that the shear experienced by the solids during high solids centrifugation improved the "release" of the bacteria from the floc matrix which increased the numbers that could be cultured compared with before dewatering. Iranpour et al. (2003) reported regrowth or that contamination of the biosolids with FC could explain the high counts measured after dewatering and storage.

Several potential problems exist with these possible explanations. For example, for regrowth to occur, a significant time is needed to increase the counts by several orders of magnitude. Since the doubling time under ideal conditions for *E. coli* is around 20 min (about the retention time in high solids centrifuges), the large increase in FCs or *E. coli* cannot be explained by regrowth alone. The release of *E. coli* or other FCs from the floc during centrifugation also seems unlikely since during conditioning and dewatering, coagulants such as cationic polymer are added, which aggregate the floc, and allow for the formation of cake. In addition, during the preparation of cake samples for *E. coli* and/or FC analysis, samples are typically diluted in water and homogenized by processes such as blending or put through a stomacher, which should destroy most flocs and release bacteria. In addition, it is not clear that microbial growth would not occur, even if the bacteria are present within a floc matrix.

The authors of this paper propose an alternative hypothesis to explain these results: that the bacteria can enter a non-culturable state during digestion. This renders the bacteria non-culturable using standard culturing methods (SCMs) even though they are present and considered viable. As a result, these SCMs fail to enumerate the viable bacteria present after digestion. During the dewatering process, the bacteria are reactivated or resuscitated, rendering them culturable again. One possible reason that the bacteria become non-culturable is that they enter what is called a "viable but non-culturable" (VBNC) state. A number of different bacteria have been shown to enter a VBNC state such as *E. coli*, *Salmonella sp.*, *Enterococcus faecalis*, *Shigella dysenteriae*, *Vibrio cholerae*, *and Helicobacter pylori* (Byrd et al., 1991; Reissbrodt et al., 2002; Adams et al., 2003; Mizunoe et al., 1999; Gupte et al., 2003). Although there is some controversy in the literature regarding the VBNC state, most of the evidence seems to support this phenomena.

The bacteria that enter the VBNC state often do so after exposure to environmental stress such as nutrient or substrate deprivation, metals, chlorine, salinity, and low temperatures (Mizunoe et al., 1999; Makino et al., 2000; Grey and Steck, 2001; Rockabrand et al., 1999; Lisle et al., 1998). The conditions present during digestion could impart stress that induces the bacteria to enter a VBNC state. These stresses could include low substrate and nutrient concentrations. In addition, for thermophilic digestion, the higher temperatures may also contribute, since higher temperatures have been shown to increase the entrance of certain bacteria into the VBNC state (Adams et al., 2003).

Although the cells enter the VBNC state, they are still considered viable by definition. There has been conflicting evidence in the literature whether the VBNC bacteria can cause infection, but a number of studies have shown that VBNC organisms are capable of causing infection in vivo both in humans and mice (Colwell et al., 1985, 1996; Pommepuy et al., 1996; Cappelier et al., 1999; Chaveerach et al., 2003). In addition, the cells can typically be induced to grow on media in the presence of certain growth promoters or enrichments, and this process is called "resuscitation" or "reactivation" (Lleò et al., 1998; Rockabrand et al., 1999; Makino et al., 2000; Reissbrodt et al., 2000, 2002). The ability of the microbes to be resuscitated is also another indicator of their viability.

The second hypothesis of this research is that dewatering, especially centrifugation, "resuscitates" the VBNC bacteria allowing them to grow and be enumerated using SCMs. The term 'resuscitation' has been used in the microbiology field to describe the transition from non-culturable to culturable. This term has been used interchangeably with "reactivation".

The implications of these hypotheses are significant since much of the design parameters that have been developed to achieve required pathogen or indicator organism destruction are based on using SCMs. Therefore, if these hypotheses are correct, the SCMs may significantly underestimate the actual viable counts of these bacteria.

2. Objectives

The objectives of this research were to examine the following two proposed hypotheses developed to explain field results that found high concentrations of FC and/or *E. coli* after dewatering despite low counts immediately before dewatering:

(1) During digestion, FC and/or *E. coli* can enter a non-culturable state, and are therefore not correctly enumerated by SCMs;
(2) Non-culturable FC and/or *E. coli* can be reactivated during dewatering such that they are able to be enumerated by SCMs.

3. Methods and materials

3.1. General approach

In order to investigate if bacteria enter a non-culturable state, an alternative method was needed to enumerate bacteria that does not rely on their culturability, since, they are by definition not culturable by SCMs. For example, many researchers use methods such as acridine orange direct count, microscopy or Coulter counter type methods to enumerate non-culturable bacteria. However, these researchers are typically working with pure cultures making this method feasible (Mizunoe et al., 1999; Heim et al., 2002; Byrd et al., 1991). In digested samples from wastewater treatment facilities, the heterogeneous mixture of different organisms makes these non-microbe specific staining or cell count methods impractical to enumerate FC and/or *E. coli*. The use of molecular tools provides an approach to enumerate bacteria without relying on culturing techniques. For example, quantitative polymerase chain reaction (qPCR) can be used to enumerate bacteria based on the number of DNA copies present in the sample. In order to do this, a target bacteria needs to be selected, and then a target gene or DNA sequence is needed that is specific to that bacteria. For the purpose of this research, *E. coli* were chosen as the target bacteria, since they are a member of the FC group, and are often a significant proportion of the total FC population. In this research, a specific qPCR method called competitive PCR (cPCR) was used to enumerate *E. coli*, and the target gene was the glutamate decarboxylase (gadA/B) gene which has been shown to be specific for *E. coli* (Grant et al., 2001; McDaniels et al., 1996). In addition to the cPCR method, FC and *E. coli* were also enumerated using standard culturing techniques for comparison.

3.2. Sampling overview

Samples were collected from six full-scale digestion facilities across North America. All the facilities employed anaerobic digestion, two used thermophilic digestion, three used mesophilic digestion, and one plant utilized temperature phased anaerobic digestion (TPAD). TPAD processes utilize thermophilic digestion followed by mesophilic digestion in series. Samples were collected before and after digestion, as well as after centrifuge dewatering. Samples collected for enumeration by SCMs were immediately placed on regular ice, packaged in an insulated container/cooler and shipped overnight to a lab for analysis according to procedures recommended by the USEPA (1999). Samples collected for enumeration by cPCR were processed immediately on-site to prevent loss of DNA as explained below, and then packaged in an insulated container with dry ice, and shipped overnight to the Bucknell University Environmental Engineering and Science Laboratory. To ensure statistical reliability, between three and five replicates were collected for the majority of the samples. Where appropriate, the mean and standard deviations are presented for the data collected.

In addition to the *E. coli* and FC enumeration, the total solids concentrations were measured for each sample. The operational parameters were also collected from each plant to examine possible parameters that may impact FC densities.

3.3. Enumeration of fecal coliform and E. coli using culturing methods

The FC membrane filtration (MF) analyses were conducted according to Standard Method (SM) 9222D using m-FC media incubated at 44.5+0.2 °C for 24 h (APHA, 1998). The FC MPN procedure (SM9221E) also was used for enumerating FC. *E. coli* were enumerated using the procedures described in SM9221F (APHA, 1998).

3.4. Enumeration of E. coli using molecular methods

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

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

By using these methods, the bacteria can be quantified without relying on culturing techniques and on whether the bacteria are actually culturable. A detailed description of the development and verification of the DNA extraction method as well as the cPCR method is provided in Chen et al. (2006).

3.4.1. DNA extraction

The DNA extraction protocol was modified from the method developed by Gabor et al. (2003). In brief, 750 μL of lysis buffer was added to each 100 mg of biosolids and sheared in the Lysing Matrix E tube with the FastPrep® Instrument (QBIOgene, Carlsbad, CA) at 5.5 speed for 30 s. Five μL of 20 mg/mL protease K was added for protein digestion at 55 °C for 30 min, followed by the addition of 200 μL of 20% sodium dodecyl sulfate (SDS) for another 2 h of incubation at 65 °C with complete mixing every 30 min. Supernatants were collected after centrifugation at 14,000*g* for 10 min, and the pellets were re-extracted twice with 500 μL of lysis buffer with incubations at 65 °C for 10 min each. The extracted DNA solution was purified through two phenol/chloroform/isoamyl alcohol extractions, followed by one chloroform extraction. DNA was precipitated overnight at 4 °C with 0.7 volumes of isopropanol, washed twice with 70% alcohol, and re-dissolved in 200 μL of TE buffer. If necessary, the DNA was further purified using the Promega Wizard® Genomic DNA Purification Kit (Madison, WI) to remove humic substances based on the instruction manual. All extracted DNA was stored at −80 °C until just before use.

3.4.2. Total DNA quantification

A fluorescence-based DNA quantification method using PicoGreen® dsDNA Quantitation Reagent (Molecular Probe, Eugene, OR) was used for total DNA quantification (Ahn et al.,

1996). Equal amounts of the PicoGreen reagent and sample were mixed and the fluorescence response was measured in a Turner TBS-380 Mini-Fluorometer (Turner BioSystems Inc, Sunnyvale, CA). The DNA concentration was determined by comparison to a known Calf Thymus DNA standard (Sigma-Aldrich Co, St. Louis, MO).

3.4.3. *Reverse touchdown competitive PCR*

A reverse touchdown competitive PCR protocol was used to quantify *E. coli* densities in biosolids as explained by Chen et al. (2006). In brief, a DNA competitor with sequences complementary to primers gadA/BF and gadA/BR (McDaniels et al., 1996) was constructed through internal deletion. A fixed concentration of the competitor DNA was amplified with the unknown sample DNA using a touchdown thermal cycle program. *E. coli* density was determined by comparing DNA densities of the amplified target and competitor DNA on a polyacrylamide gel against a known standard curve. Triplicate PCR reactions were conducted for each sample analysis.

3.4.4. *Statistical analysis*

One way ANOVA and Tukey's pairwise comparisons were used for statistical analysis. For correct statistical analysis, all data are log transformed in order to obtain constant variances since FC and *E. coli* measurements are typically represented by a log-normal distribution.

4. Results and discussion

4.1. *Overview*

Six different digestion processes were sampled, and they included two plants with thermophilic anaerobic digestion, one plant with temperature phased anaerobic digestion (TPAD), and three with mesophilic anaerobic digestion. All of the digestion processes treated a combination of primary and secondary sludges, except Thermo-1 which treated only primary sludge. In addition, all of the treatment processes utilized high solids centrifuges for dewatering. A summary of the operational parameters for the different treatment plants sampled as part of this study is provided in Table 1. The results from the different digestion processes are described in the following sections.

4.2. *Thermophilic digestion*

Two thermophilic anaerobic digestion processes were sampled. Several of the important operational parameters are summarized in Table 1. During the sampling events for both sites, samples were collected from the digester influent, digester effluent as well as the cake samples immediately after dewatering.

Thermo-1 utilized single stage digestion with an SRT of 15–20 days, and temperature of 55 °C. The concentration of FC in Thermo-1 for each sampling location is shown in Fig. 1. The FC density measured in the influent to the digester was approximately 10^8 cfu/g DS which is typical for many digester influents sampled in the study. The effluent from the digester had less than 10^2 cfu/g DS which is a six log reduction in FC. However, immediately after centrifugation, the density of FC was 10^6 cfu/g DS, an increase of four orders of magnitude in a relatively short period of time. The results are similar to those reported by others, in that the concentration of FC were much greater immediately after dewatering compared to just prior to dewatering (Cheung et al., 2003; Monteleone et al., 2004).

The density of *E. coli* measured in each sample by the SCM and cPCR methods is shown in Fig. 2. The SCM results for measuring *E. coli* were similar to the FC results. The influent

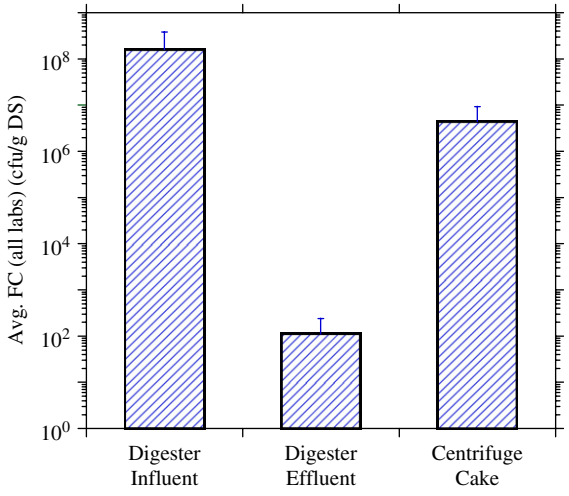

Fig. 1 – Average FC densities measured in samples taken from Thermo-1 (*Note*: error bars represent one standard deviation).

Table 1 – Summary of operational parameters for the different processes sampled

Field site	Digestion type	Digestion SRT (d) and temperature	Digestion VSR (%)	Cake solids content (%)
TPAD-1	TPAD	15 d at 58 °C and 21 d at 37 °C	60	30
Thermo-1	Thermophilic—single-stage	15–20 d at 55 °C	65	35
Thermo-2	Thermophilic—four-stage	Total 22 d at 56 °C	60–62	30–32
Meso-1	Mesophilic—single stage	21 d at 37 °C	40–45	21
Meso-2	Mesophilic—single stage	30–35 d at 37 °C	50–60	22–24
Meso-3	Mesophilic—single stage	22 d at 36 °C	45–58	30–33

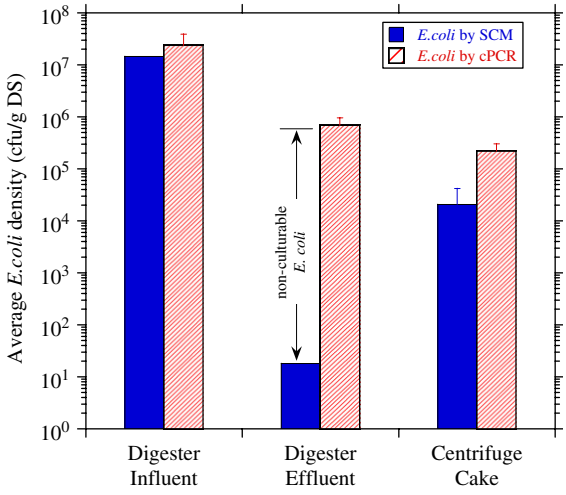

Fig. 2 – *E. coli* density measured by standard culturing methods and cPCR from samples collected at Thermo-1.

concentration was approximately 10^7 cfu/g DS and the effluent was 10^1 cfu/g DS, while the cake sample had approximately 10^4 cfu/g DS. Both the FC and *E. coli* showed a dramatic increase in density in the cake sample compared to the digester effluent. Interestingly, the *E. coli* density measured by the cPCR method agreed well for the digester influent; however, the cPCR results were much greater compared to the SCM for the digester effluent. For example, cPCR measured approximately 7×10^5 *E. coli* per gram of DSs in the digester effluent, compared to 2×10^1 cfu/g DS measured by the SCM, a difference of more than four orders of magnitude. The cPCR method enumerated 2×10^5 *E. coli* per g DS and the SCM measured 2×10^4 cfu/g DS in cake samples collected immediately after centrifugation.

The results from Thermo-1 support the non-culturable theory hypothesized in this study. The *E. coli* densities measured by the cPCR before and after dewatering were not statistically different ($p < 0.05$), suggesting that the *E. coli* numbers were the same before and after dewatering. The relatively low counts of *E. coli* measured after digestion by the SCM indicates much of the bacteria were in a non-culturable state. If the difference between the cPCR and SCM numbers could be considered as non-culturable bacteria, this would be approximately 10^5 *E. coli* per gram of DSs that are not enumerated by the culturing method. After dewatering, the majority of these non-culturable *E. coli* are resuscitated or reactivated and are then able to grow on the standard culturing media. The mechanism for reactivation is not known, but several possibilities exist as discussed below.

Thermo-2 utilizes four completely mixed reactors in series with an overall SRT of approximately 22 days and temperature of 56 °C. The influent to the digestion process had *E. coli* densities of 2.4×10^7 cfu/g DS and 3.3×10^7 cells/g DS for the SCM and cPCR method, respectively. Interestingly, the effluent from the Thermo-2 digestion had less than 2 cfu/g DS and the cake had <1 cfu/g DS as measured by the SCM, and the cPCR results were all negative. Therefore, this digestion process appeared better able to destroy both FC and *E. coli*, and eliminate non-culturable organisms such that reactivation did not occur after centrifugation. It is possible that the multi-stage process which better simulates plug flow is needed for better destruction of these organisms.

4.3. *Mesophilic digestion results*

Two mesophilic plants were sampled, both with centrifuge dewatering. Mesophilic Plant 1 (Meso-1) had an SRT of 30–35 days at a temperature of 37 °C. The FC and *E. coli* measured by SCMs as well as the *E. coli* density measured by cPCR are shown in Fig. 3. The FC and *E. coli* are approximately 10^7 cfu/g DS in the digester influent, and less than 5×10^4 cfu/g DS in the effluent measured by the SCM. It should be noted that the FC and *E. coli* densities were measured to be less than 48,000 cfu/g DS and a value of 2×10^4 cfu/g DS was used for plotting purposes. The cPCR *E. coli* results were similar to the SCM results for the digester influent, although for the digester effluent, the cPCR method enumerated at least one order of magnitude more *E. coli* than the SCM. Immediately after dewatering, the *E. coli* density measured by the SCM was equivalent to the cPCR results prior to dewatering, suggesting a reactivation of the bacteria during dewatering.

Very similar results were found for Mesophilic Plant 2 (Meso-2), with a difference of about one order of magnitude between cPCR and SCM results, as shown in Fig. 4. The cPCR results were slightly lower in the cake sample, and this is thought to be due to a decrease in DNA extraction efficiency from this cake sample or potentially the presence of PCR inhibitors.

The results from the two mesophilic digestion processes suggest that the mesophilic digestion process does not produce as many non-culturing indicator bacteria compared to the single-stage thermophilic processes. Additional sampling of other plants along with the results presented here shows that typically around one order of magnitude difference exists between SCM and qPCR results for mesophilic

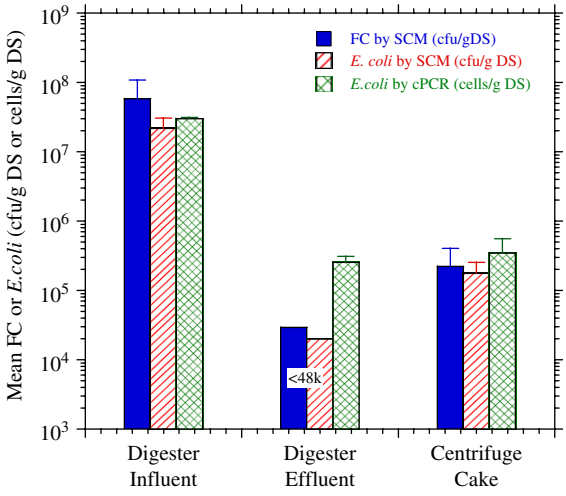

Fig. 3 – FC and *E. coli* densities measured in Meso-1 process by both SCMs and cPCR, error bars represent one standard deviation.

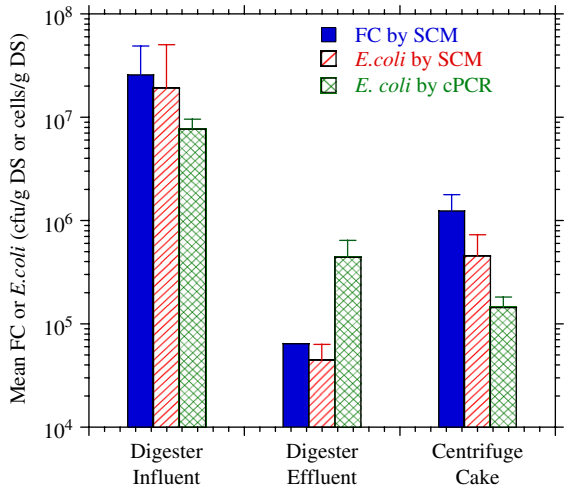

Fig. 4 – FC and *E. coli* measured by SCM and cPCR for Meso-2, error bars represent one standard deviation.

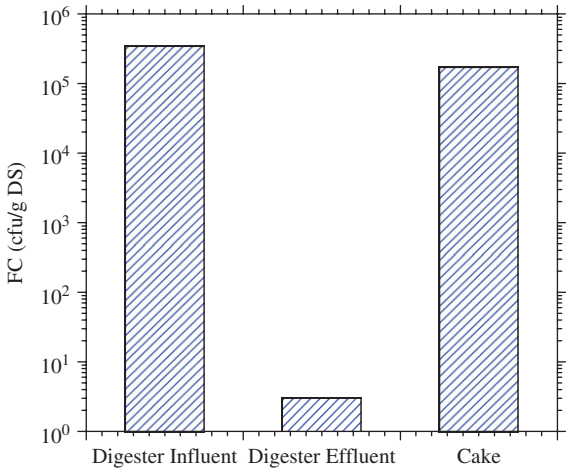

Fig. 6 – FC densities measured in TPAD-1 process.

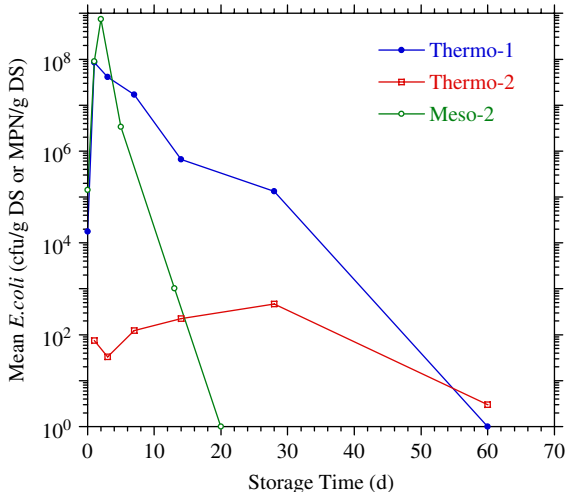

Fig. 5 – Regrowth of *E. coli* in cake samples during cake storage.

digestion. For thermophilic digestion, the differences have been measured to be up to 4 or 5 orders of magnitude.

4.4. *Growth after resuscitation*

After centrifugation, the non-culturing bacteria become culturable; and therefore can be enumerated by SCMs (the process called resuscitation or reactivation). In addition, rapid growth of both *E. coli* and FC was measured during the storage of the cake samples. For example, FC and *E. coli* measured during the storage of the cake samples at 35 °C is shown in Fig. 5. For both Meso-2 and Thermo-1, the *E. coli* densities increased in the first few days to between 10^8 and 10^9 cells/g DS, after reaching a peak, the densities decreased with further storage. The mesophilic cake decreased faster, reaching a non-detectable level after 20 days of storage. After 60 days of storage, the thermophilic cake had some detectable *E. coli* in the cake. The results suggest that growth conditions are favorable after dewatering, and that rapid growth of reactivated non-culturing organisms as well as culturable organisms in the case of the mesophilic sample can occur.

Interestingly, little regrowth was measured during storage of the cake for Thermo-2. This digestion process appears to have eliminated most of the *E. coli* and FC, and also created conditions in the cake which prevents regrowth. This may be due to competition from other organisms or lack of available substrate.

4.5. *Reactivation mechanisms*

The mechanism by which *E. coli* and FC are reactivated during dewatering is not clear. The results from this study as well as the literature suggest reactivation mainly occurs with centrifuge dewatering, and not belt filter press dewatering (Erdal et al., 2004). This could be a result of the greater solids content associated with centrifugation or possibly the shear associated with centrifuge dewatering. Shear may result in the release of growth factors such as autoinducers which resuscitate the bacteria into a culturable state. Reissbrodt et al. (2002) has shown that addition of autoinducers to the media for enumeration of VBNC bacteria resulted in a significant increase in the numbers that were enumerated. Similarly, other growth factors or signaling compounds may become present after dewatering that are able to resuscitate or reactivate the bacteria. If this hypothesis is correct, the growth factors may be present in the centrate. To test this hypothesis, centrate samples from a full-scale centrifuge dewatering a temperature phased anaerobic digested (TPAD-1) biosolids were added at different concentrations to the digester effluent. As shown in Fig. 6, this treatment plant has shown the pattern associated with the non-culturable phenomena with low concentrations prior to dewatering (or immediately after digestion) followed by high concentrations in the dewatered cake samples. Addition of filter sterilized centrate increased the culturable FC by about two orders of magnitude, whereas addition of centrate plus the polymer used for conditioning increased the FC enumeration by about

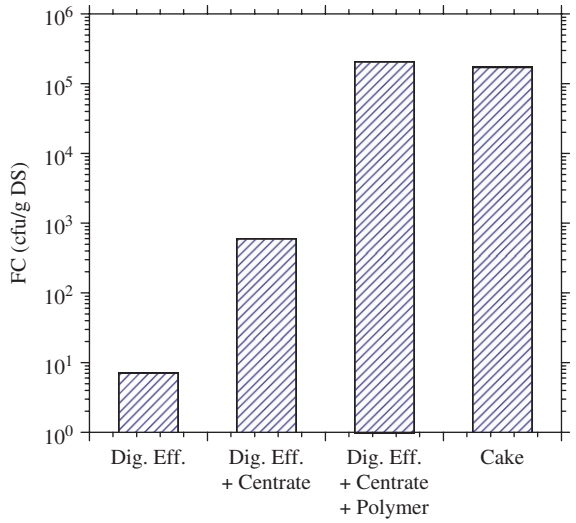

Fig. 7 – Impact of centrate and polymer on resuscitation of VBNC organisms (Dig. Eff. = digester effluent).

five orders of magnitude as shown in Fig. 7. The results suggest that components present in the centrate and/or polymer may resuscitate or reactivate the bacteria to make them culturable; and therefore, these inducing agents may be released during centrifuge dewatering. Repeat experiments on different months did not show the same reactivation with the centrate, suggesting these compounds may be unstable or other factors are important that are not currently understood. The role of polymer is not clear. Research has shown that polymers can be biodegradable, and therefore provide a substrate source (Dentel et al., 2000). However, research related to odors in centrifuged cakes has shown that large amounts of bioavailable protein and polysaccharide exist in the cake that could also provide substrate, so it would not appear that the cake is substrate limited (Higgins et al., 2006). Additional research is needed to better understand the mechanisms of reactivation, and current research is on-going to better understand the factors that affect reactivation of the bacteria, especially the role of autoinducers.

5. Implications of research

The results from this research potentially have significant implications. The destruction of indicator organisms is a key component to meeting product quality after treatment. Previous research that investigated pathogen destruction using culture based enumeration of indicator organisms such as FC or *E. coli*, may have underestimated the actual number of viable organisms present in the sample, especially for thermophilic digestion. As a result, design of digestion processes used to meet certain goals based on FC and/or E. coli, such as EU and US regulations, may not achieve these stated goals.

This research indicates that mesophilic digestion meets the goals of Class B biosolids as defined by the USEPA (1999), namely less than 2 million FC per gram of DSs. Proposed UK regulations would stipulate a 2 log reduction in *E. coli* and their densities must be less than 10^5 per gram DSs for "treated sludge" (Godfree and Farrell, 2005), and proposed EU regulations would require at least a 2 log reduction in *E. coli* for "conventional" treatment (Iranpour et al., 2004). The samples measured in this study by cPCR would put the digester effluent typically below these limits, since only up to one order of magnitude difference is measured between the culturable and non-culturable *E. coli* in most mesophilic plants. However, after centrifuge dewatering, conditions develop that encourage rapid growth of the FC, with concentrations peaking at 10^8 per gram DSs in just one to three days of storage. Therefore, centrifuge dewatering results in a destabilization of the microbial ecology, and provides conditions within the cake that encourage growth of FC and *E. coli*. However, continued storage beyond this peak concentration quickly reduces the FC and *E. coli* below the required limits. As a result, mesophilic digesters are meeting their desired goals for FC and *E. coli* destruction, however, centrifugation can result in the product quality not meeting desired goals, but only during the first few days of cake storage when peak concentrations of the indicators are measured. Continued storage leads to a decrease in the concentration below the Class B limits.

The results for some thermophilic digestion suggest that much greater densities of viable FC and *E. coli* are present than enumerated by SCMs. The difference can be attributed to non-culturable organisms, and this difference can be up to five orders of magnitude. Centrifuge dewatering results in reactivation of the indicator organisms, followed by rapid regrowth due to enhanced conditions in the cake. Therefore, based on SCM measurements from the effluent, thermophilic digestion may appear to meet Class A standards of less than 1000 FC per gram DSs, or the proposed EU "Enhanced" treatment of greater than 6 log reduction of *E. coli* and densities of less than 500 cfu/g DS. However, the results in this paper suggest much greater densities of FC and *E. coli* may be present than the density found by SCMs because they are in the non-culturable state, and the final product would not meet the Class A or "Enhanced" treatment criteria. It is also important to note that continued storage of the biosolids does result in a decrease in the culturable counts of *E. coli* and FC which may provide a simple solution to achieve the desired goals from an indicator organism perspective.

Similarly, it appears that a multi-stage thermophilic digestion process (Thermo-2) with relatively long SRT is able to achieve Class A or Enhanced treatment, eliminating non-culturable organisms that can be reactivated and grow during cake storage. It is not clear why this particular process is able to achieve the required destruction while other plants have not. The main difference is that Thermo-2 utilizes a reactors-in-series process configuration, while the other plants are all single stage digestion. Reactors in series help reduce short circuiting, and approach plug flow which may be one explanation for the better FC and *E. coli* destruction and lack of resuscitation and growth during cake storage. However, it should be stressed that the results from this research represent a relatively small sample size compared to the total population of anaerobic digesters, and additional research is needed to examine additional facilities and

provide a larger sample set. Additional research is also needed to better understand the impact of digestion process configuration on non-culturable organisms and reactivation and regrowth of organisms after centrifuge dewatering. This research is on-going. Finally, although the research undertaken in this study was aimed at supporting or refuting the non-culturable concept, other possible explanations may exist that could explain the data. For example, contamination of the centrifuge may result in the increased densities measured in the cake. Mass holdup on the walls of a scroll-type centrifuge could possibly contaminate the bulk of material moved through the centrifuge by the conveyor. Some regrowth could occur in this relatively stagnant mass. However, the interchange of solids between this mass and the solids in the moving stream is expected to be small and would not be likely to cause the huge increases in organism count observed.

It is not clear why the bacteria enter a non-culturable state. Several possibilities include: sublethal injury which reduces their ability to grow on selective media (McFeters et al., 1982); presence of growth inhibitors; and/or transition into a survival mode with changes in physiology that reduces culturability (Signoretto et al., 2000; Heim et al., 2002) associated with the VBNC state. In addition, the potential virulence of the non-culturable organisms after digestion is unknown, however, the ability to be resuscitated or reactivated suggests that the organisms are still viable. It is thought that VBNC bacteria remain viable and also can cause infection in vivo, even when they are non-culturable by standard methods (Makino et al., 2000; Rahman et al., 1996). This hypothesis has been debated in the literature; however, some evidence supports the concept that VBNC bacteria can cause infection in vivo (Colwell et al., 1996; Pommepuy et al., 1996; Cappelier et al., 1999; Chaveerach et al., 2003). In contrast to these results, several researchers have reported that VBNC bacteria are not capable of causing infection (Caro et al., 1999; Kolling and Matthews, 2001). Additional biosolids specific research is needed to more fully understand these implications. In addition, since a risk assessment approach has not been used for developing regulations, it is not clear what the potential public health implications might be of these indicator concentrations.

6. Conclusions

- During digestion, FC and *E. coli* can enter a non-culturable state in which they are not enumerated by standard culturing methods. As a result, the densities of these organisms can be significantly underestimated, especially for thermophilic processes. The reason for non-culturability is unknown, but could be due to sub-lethal injury or viable but non-culturable bacteria.
- For mesophilic digestion, FC and *E. coli* can be underestimated by up to one order of magnitude, but generally have low numbers of non-culturable *E. coli*. However, for thermophilic digestion, FC and/or *E. coli* can be underestimated by greater than five orders of magnitude.
- Centrifuge dewatering can reactivate the non-culturable organisms, which makes these organisms culturable again. This reactivation appears to be due to the release of some inducer-like compound or change in environmental conditions that stimulates growth.
- After reactivation, conditions in the cake are favorable for rapid growth of FC and *E. coli*, with peak densities of 10^8 cells per gram DSs measured within a few days of cake storage.
- One one process sampled had complete destruction of indicators, with no reactivation or regrowth after dewatering. The unique parameter associated with this process was that it utilized a multi-stage (reactors in series) thermophilic reactor configuration suggesting the potential importance of reactor configuration in pathogen destruction.

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