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Inactivation of *Clostridium difficile* in Sewage Sludge by Anaerobic Thermophilic Digestion**Changyun Xu ¹, Hamidreza Salsali ² Scott Weese ³, Keith Warriner ^{1*}**¹ Department of Food Science, University of Guelph, Ontario, N1G 2W1, Canada² School of Engineering, University of Guelph, Ontario, N1G 2W1, Canada³ Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario, N1G 2W1, Canada

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Abstract

There has been an increase in community-associated *Clostridium difficile* infections (CDI) with biosolids derived from waste water treatment being identified as one potential source. The current study evaluated the efficacy of thermophilic digestion to decrease levels of *C. difficile* ribotype 078 associated with sewage sludge. Five isolates of *C. difficile* 078 were introduced (final density of 5 log CFU/g) into digested sludge and subjected to anaerobic digestion at mesophilic (36 or 42°C) or thermophilic (55°C) temperatures for up to 60 days. It was found that mesophilic digestion at 36°C did not result in significant reduction in *C. difficile* spore levels. In contrast performing thermophilic sludge digestion reduced endospore levels at a rate of 0.19 - 2.68 log CFU/Day depending on the strain tested. The mechanism of lethality was indirect by stimulating germination then inactivating the resultant vegetative cells. Acidification of sludge by addition of acetic acid (6 g/L) inhibited the germination of spores regardless of the sludge digestion temperature. In conclusion, thermophilic digestion can be applied to reduce *C. difficile* in biosolids thereby reducing the environmental burden of the enteric pathogen.

Keywords: *Clostridium difficile*, wastewater treatment, Community Associated Infections, sewage, anaerobic digester, thermophilic, endospores

Introduction

Clostridium difficile remains a highly significant pathogen and is a leading cause of hospital- or antimicrobial-associated diarrhea (Marina *et al.* 2009, Warny *et al.* 2005). The classic route for infection is through administration of antibiotics to treat a primary infection that results in disruption of the microbiome thereby enabling drug-resistant *C. difficile* to proliferate and

produce toxins (Evans and Safdar 2015). Clinical/hospital settings represent the most significant risk due to the high prevalence of the pathogen along with susceptible hosts (Evans and Safdar 2015). Yet, in recent years there has been a significant increase in community-acquired infections (CA-CDI) with persons not receiving antibiotics or having contact with a clinical setting (Kuntz *et al.* 2011). Although the mortality rate of CA-CDI is a fraction of hospital acquired infections it can lead to long-term complications such as recurrent diarrhea (Delate *et al.* 2015; Furuya-Kanamori *et al.* 2015).

Ribotype 027 is commonly implicated with hospital CDI which is in contrast to CA-CDI that is caused by a broader range of ribotypes with 078 being the most significant (Khanna *et al.* 2012). The sources of *C. difficile* implicated in community-associated CDI have been proposed as person-to-person, foodborne, zoonotic and acquired from the environment (Xu *et al.* 2014; Bauer and Kuijper 2015). In a previous report it was demonstrated that *C. difficile* endospores could survive wastewater treatment (encompassing mesophilic sludge digestion) and be recovered in the effluent discharged into streams and the biosolids applied to land (Xu *et al.* 2014). Therefore, wastewater treatment represents a continuous source of *C. difficile* into the environment.

The survival of *C. difficile* during wastewater treatment may not be unexpected given the intrinsic resistance of endospores to heat, acids and chemical sanitizers, amongst other antimicrobials (Nerandzic and Donskey 2010). However, endospores lose stress resistance when induced to germinate to form susceptible vegetative cells thereby facilitating inactivation by milder processes. This strategy has been proposed for controlling *C. difficile* associated with foods and inert surfaces (Nerandzic and Donskey 2010; Nerandzic and Donskey 2013).

In the course of wastewater treatment there is typically an anaerobic digestion process whereby organics that constitute the sludge are degraded to acids then finally methane. Although in

principle the conditions would have been conducive to *C. difficile* germination and outgrowth there were no changes in levels suggesting the pathogen remains in the dormant state (Xu *et al.*, 2014). The result indicated that whilst suitable germinating agents were present (bile salts, free amino acids such as glycine) the spores remained dormant. One potential reason for lack of *C. difficile* germination could be attributed to the absence of thermal shock that makes spores receptive to germinating agents (Paredes-Sabja *et al.* 2014). Anaerobic sludge digestion is performed at 36 – 42°C which is insufficient to activate spore germination with temperatures >50°C normally required (Nerandzic and Donskey 2010; Nerandzic and Donskey 2013). The majority of wastewater anaerobic digesters operate in the mesophilic temperature range (36 – 42°C) although a complementary process uses higher temperatures in the thermophilic region (50 – 60°C). The main advantage of performing thermophilic anaerobic digestion is that the Hydraulic Retention Time (HRT) can be reduced to 10 days due to an accelerated process (Salsali *et al.* 2008). The elevated temperature also enhances enteric pathogen reduction, including *C. perfringens*, *Salmonella* and *Escherichia coli* O157:H7 (Salsali *et al.* 2006; Salsali *et al.* 2008). With regards to *C. perfringens*, it was reported that thermophilic sludge digestion (55°C) in the presence of 6 g/L acetic acid at pH 4.5 decreased levels of the pathogen by 4 log CFU (Salsali *et al.* 2008). Although not studied, it was thought the enhanced efficacy of thermophilic sludge digestion was through the stimulation of spore germination followed by inactivation of the vegetative cells in the presence of acetic acid (Salsali *et al.* 2008). The disadvantage of thermophilic digestion is the added energy requirement, although the potential of reducing *C. difficile* levels can be viewed as advantageous in terms of reducing the environmental burden of the pathogen. Therefore, the objective of the following study was to determine the inactivation of *C. difficile* in sludge as a function of temperature, acidity and time.

In addition, the mode of *C. difficile* inactivation was studied with respect to direct spore inactivation or via an indirect means, in which the vegetative cells following germination are inactivated.

Materials and methods

***C. difficile* isolates, spore preparation and sampling method**

Five *C. difficile* ribotype 078 isolates used in the study were derived from biosolids, river sediments or primary sewage sludge (Table 1). Endospores were produced from each isolate of *C. difficile* by inoculating into 50 mL BHI broth that was subsequently incubated anaerobically for 7 days at 37°C. The spores were harvested by centrifugation at 4500 ×g for 10 min then re-suspended in 5 mL of 50% ethanol and incubated at room temperature for 1 h. The spores were again harvested by centrifugation (4500 ×g, 10 min) before washing twice in sterile distilled water. The pellet was suspended in 5 mL sterile distilled water and a dilution series was prepared and aliquots (0.1 mL) plated onto *C. difficile* moxalactam norfloxacin (CDMN) plates and incubated anaerobically at 37°C for 24 h.

Fate of *C. difficile* during anaerobic digestion in primary digested sludge

Survival of *C. difficile* during simulated anaerobic digestion was determined at different temperatures (36, 42, or 55°C) in freshly digested sludge (sludge derived from the primary anaerobic digester) with or without added acetic acid (6.0 g/L). Volumes (500 mL) of fresh digested sludge collected from a wastewater treatment plant (WWTP) with a measured solids content varying between 0.7 – 2.6% w/v and ammonia 579 – 668 mg/L over the study period

(data provided by the waste water plant). The collected sludge was inoculated with endospores of the test *C. difficile* to a final concentration of 5 log CFU/mL. Each spore type was tested individually as opposed to being combined into a cocktail. The inoculated sample was then dispensed in 50-mL lots into sterile centrifuge tubes. The sludge samples were then incubated at 36°C (60 days), 42°C (53 days) or 55°C (25 days). Sampling was carried out during the digestion at the time of 6 h, 24 h, 48 h, 5 d, 10 d, 15 d, and 25 d, or extended to 60 d. Aliquots (1 mL) of sludge sample were withdrawn periodically during the digestion and a dilution series prepared and plated onto CDMN plates that were incubated anaerobically at 37°C for 24 h.

Mode-of-inactivation of *C. difficile* endospores during anaerobic digestion

The pH of digested sludge was adjusted from pH 7.2 to 5.0 or 6.0 using 6 M HCl. Endospores of the five *C. difficile* ribotype 078 isolates were separately inoculated into 200 mL of sludge to a final concentration of 5 log CFU/mL. Each of the mixtures were then dispensed into 50 mL centrifuge tubes and digested at 55°C for 6 days (pH 5.0), 48 h (pH 6.0) or 48 h (pH 7.2). Sludge samples (1 mL) were withdrawn at 6 h, 12 h, 24 h, and 48 h, or extended to 6 d and a dilution series prepared in saline, heat shock applied (70°C for 10 min), then plated onto CDMN. As a control, the above experiments were performed with the digested sludge being substituted with 50 mL CDMN broth without antibiotics, adjusted to pH 7.4, 5.0 or 4.5. An additional control was the test endospores of the *C. difficile* isolates incubated at 55°C for 7 days with samples being withdrawn at Day 0, 2 and 7.

The *C. difficile* counts recovered from samples were converted to log values then statistically analyzed by ANOVA using a $P < 0.05$ to highlight significant difference (IBM SPSS Statistics 22).

Results

Effect of sludge digestion temperature on *C. difficile* endospore levels

The effect of temperature on the viability of five isolates of *C. difficile* ribotype 078 was determined during sludge digestion (Figure 1). At 36°C there was no significant ($P > 0.05$) change in spore numbers over the 60-day digestion period. When sludge digestion was performed at 42°C differences between the *C. difficile* isolates were observed (Figure 1A). Isolate B28 endospore levels decreased significantly ($P < 0.05$) following a 10-day delay then decreased by 4 log CFU in the remaining 43-day period. The levels of the other four isolates did not change significantly ($P > 0.05$) over the 53-day sludge digestion period (Figure 1A; Table 2).

All five isolates of ribotype 078 were significantly reduced when sludge digestion was performed at 55°C (Figure 1B; Table 2). Here, endospores B28 began to decrease after a 5 h lag period and reached the level of detection (1 log CFU/mL) by 48 h. Endospores of the remaining four isolate types that were more tolerant in that a lag period of 48 h was observed before levels started to progressively decrease over a 23-day period (Figure 1B). The rate of inactivation following the initial lag period was significantly higher ($P < 0.05$) for B28 endospores compared to the other strains tested and when digestion was performed at 42°C (Table 2).

Effect of pH and acetic acid during sludge digestion on *C. difficile* endospore levels

Sludge was collected from the wastewater plant and supplemented with 6.0 g/L acetic acid that reduced the pH from 7.2 to 5.2. Anaerobic digestion was carried out at 36°C, 42°C or 55°C for 25 days. There was no significant change ($P > 0.05$) in endospore levels when sludge digestion was performed at 36 or 42°C. However, at 55°C digestion temperature, levels of B28 spores decreased by 0.11 log CFU/Day following a 5 day lag period. The levels of endospores

belonging to the other four isolates did not significantly decrease ($P > 0.05$) over the 25-day period compared to the original spore density at Day 1 (Figure 2). The results would suggest that the presence of acetic acid or acidic pH, inhibits or delays endospore germination compared to when pH neutral sludge was digested.

To assess the effect of acid type further trials were performed with the sludge pH prior to digestion being adjusted using HCl instead of acetic acid. At 36°C there was no significant ($P > 0.05$) reduction in endospore numbers irrespective of starting pH (results not shown). When sludge digestion was performed at 55°C a pH effect on endospore levels were observed (Figure 3). At pH 5.0 there was a 3.9 ± 0.2 log CFU reduction of isolate B28 endospores over a 24 h period (Figure 3A). In contrast there was no significant ($P > 0.05$) decrease in endospore numbers of the other four *C. difficile* isolates tested (Figure 3A). The rate of B28 spore inactivation at pH 6.0 was 5.29 ± 0.24 log CFU/Day that was significantly higher ($P < 0.05$) than the rate measured at pH 5.0 (3.54 ± 0.32 log CFU/Day). In addition, at pH 6.0 there was a decrease in R3 and R15 endospores (originally isolated from river sediments) following a 24 h lag period with a significantly ($P < 0.05$) lower decrease of GP2 and GP80 spores (isolated from primary sludge) under the same anaerobic digestion conditions (Figure 3B).

Mode-of-inactivation of *C. difficile* endospore inactivation at 55°C digestion temperature

The observed decrease in endospore levels when sludge was digested at 55°C could be attributed to direct or indirect inactivation. That is the direct thermal inactivation of the spore or indirectly by loss of viability of vegetative cells following germination.

To assess the effect of temperature and pH on *C. difficile* germination a series of trials were performed using CDMN broth in the absence of background microbiota (Figure 4). Here, samples were withdrawn over a 50-h period then plated before and following heat shock at 70°C

for 10 min to inactivate vegetative cells (Figure 4). On this occasion the spore types tested were B28, GP2 and R3 because the latter two had the same response as GP80 and R15, respectively. At pH 7.4 endospores of all three isolate types germinated within 6 h at 55°C with no outgrowth occurring upon prolonged incubation (Figure 4A). At pH 5.0 (Figure 4B) or 4.5 (Figure 4C) there was a decrease in B28 endospores over 24 h with levels of GP2 and R3 decreasing less than 0.7 log CFU/mL; however, when endospores of the different isolates were incubated in sterile water at 55°C, *C. difficile* spore numbers remain stable for GP2 and R3 over a 7-day period. However, a decrease of 2.5 log CFU/mL reduction in B28 spores was observed over the same time period (Figure 4C).

Discussion

As observed in previous studies, there was no change in *C. difficile* levels in the course of mesophilic sludge digestion following a prolonged incubation period (Xu *et al.* 2014). However, when sludge digestion was performed at 55°C there was a significant reduction in spores of all the isolates tested. Collectively, the results of the study would suggest the mode-of-inactivation was indirect with respect to germination being induced then dying off of germinated cells. The theory is supported by the fact that spores incubated in sterile distilled water at 55°C in the absence of germinating agents did not decrease in levels indicating no direct spore inactivation. In addition, under acidic conditions spore germination was inhibited, the spore levels did not significantly change over a 25-day period despite being held at 55°C. The thermal resistance of *C. difficile* endospores has been reported with the conclusion that recommended cooking temperatures for meat (71-74°C) are insufficient to inactivate spores of the enteric pathogen (Rodriguez-Palacios *et al.* 2010; Rodriguez-Palacios and LeJeune 2011). Although no z values

have been published, taking the data from (Rodriguez-Palacios and LeJeune 2011) the estimated z for *C. difficile* suspended in a meat matrix was 14.7°C that equates to a $D_{55} > 300$ min. The endospores of *C. difficile* are thermally sensitive compared to *C. perfringens* that has a D_{120} of 0.48 min and z of 16.8 min (van Asselt and Zwietering 2006). Therefore, although more heat sensitive, *C. difficile* endospores would resist thermal inactivation when incubated at 55°C as observed in the current study.

From the isolates tested, B28 appeared to especially sensitive to a higher temperature for sludge digestion. Unlike the four other isolates tested, levels of B28 endospores decreased at digestion temperatures of 48°C. If inactivation is attributed to viability loss of germinating spores, it can be concluded that B28 required less heat activation and/or was more sensitive to germination agents present in the sludge. It has been previously reported that *C. difficile* isolates vary with respect to germinating rates, a phenotypic characteristic linked to sensitivity towards the germinating agent, taurocholate (Heeg et al. 2012; Paredes-Sabja *et al.* 2014). Therefore, it is possible that isolate B28 fell into the category of being hyper-sensitive to interaction with germinating agents. Yet, it was also noted the B28 spores decreased when suspended in sterile distilled water at 55°C which would suggest a degree of direct thermal inactivation for this isolate.

An interesting feature of germination observed in thermophilic sludge digestion was the delay (lag) in germination followed by a progressive decrease in spore levels. The results would suggest that endospores within a population germinate at different times over the digestion period. The result can be explained by superdormancy which has been commonly observed in different spore types produced by clostridia and bacilli (Rodriguez-Palacios and LeJeune 2011; Chen *et al.* 2014). The mechanisms of superdormancy remains to be fully elucidated but in terms of sludge digestion the result would underline the need to have sufficient HRT to achieve the

target level of *C. difficile* inactivation.

It was evident that the application of heat was a required step to induce germination in *C. difficile* spores. However, even with the application of heat shock and presence of germinating agents the acidification of the sludge or CDMN media resulted in an inhibition of the germination cascade. It has been hypothesized that the susceptibility of patients to CDI is through reduced acidity (HCl) that prevents inhibition of *C. difficile* germination by stomach acid (Nerandzic *et al.* 2009). In the current study both acetic acid (transiently encountered in sludge digestion) and HCl exhibited an inhibition of spore germination. Again, isolate B28 was the exception with spore levels decreasing under acidic pH although to a lesser extent when acetic acid was supplemented into the digest as compared to HCl. The results are in contrast with those observed for *C. perfringens* whereby the combination of volatile acids and thermophilic sludge digestion temperatures enhanced the lethality against spores compared to inactivation in neutral-pH sludge (Salsali *et al.* 2008). The pH of sludge is approximately pH 7.2 suggesting in practical terms the addition or endogenous production of volatile acids within a thermophilic digestion process would counter the ability to decrease spore levels.

The role of the microbiome in controlling the activity of *C. difficile* within the gastrointestinal tract has been well documented (Britton and Young 2014). In the current study it was noted that germination in CDMN broth at neutral pH, 55°C incubation temperature, occurred at a greater rate compared to primary sludge. This has been attributed to several factors including the preferential formation of chenodeoxycholate that retards germination (Heeg *et al.* 2012).

C. difficile is frequently encountered in biosolids produced by standard mesophilic sludge digestion thereby adding to the environmental burden of the enteric pathogen. Although a link between environmental *C. difficile* and CDI remains to be established the research has illustrated

that by using thermophilic sludge digestion the levels of *C. difficile* 078 can be significantly reduced. The inactivation of *C. difficile* via thermophilic sludge digestion is indirect with induction of germination followed by loss of viability. Thermophilic sludge digestion which has shorter HRT compared to mesophilic digestion can produce more biogas in a shorter time with less heat losses (Zupančič and Roš 2003), therefore potentially represents an intervention that can be applied in waste water treatments. However, it should be noted that the acidogenic phase of sludge digestion would be inhibited at thermophilic temperatures. Consequently, thermophilic digestion at 55°C should be considered a supplement to mesophilic treatments as opposed to an alternative. Nevertheless, thermophilic digestion should still be considered as a pathogen reduction step thereby reducing the environmental burden of pathogens such as *C. difficile*.

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Figures

Figure 1. Changes in *Clostridium difficile* ribotype 078 isolates (B28 ▲ R15 ■ R3 ● GP80 ♦ GP2 +) during digestion of digested sludge at 42°C (A) or 55°C (B). The stains were individually inoculated into digested sludge at 5 log cfu/ml with samples withdrawn periodically for plating onto CDMN agar. N is the *C. difficile* count at time t and N_0 the initial count at t=0.

Figure 2. Changes in *Clostridium difficile* ribotype 078 isolates (B28 ▲ R15 ■ R3 ● GP80 ♦ GP2 +) during digestion of digested sludge containing acetic acid (6g/l) at 55°C. The stains were individually inoculated into digested sludge at 5 log cfu/ml with samples withdrawn periodically for plating onto CDMN agar. N is the *C. difficile* count at time t and N_0 the initial count at t=0.

Figure 3. Changes in *Clostridium difficile* ribotype 078 isolates (B28 ▲ R15 ■ R3 ● GP80 ♦ GP2 +) during digestion of digested sludge at 55°C adjusted to pH 5.0 (A) or 6.0 (B) using HCl. The stains were individually inoculated into digested sludge at 5 log cfu/ml with samples withdrawn periodically for plating onto CDMN agar. N is the *C. difficile* count at time t and N_0

the initial count at $t=0$.

Figure 4. Germination of *Clostridium difficile* 078 isolates (B28 ▲ R3 ● GP2 +) in CDMN broth adjusted to pH 7.4 (A), 5 (B) or 4.5 (C) and held at 55°C. Endospores of the test isolate were inoculated into the broth culture and samples withdrawn periodically and a dilution series prepared. Aliquots (0.1 ml) were spread plated onto CDMN agar with the remainder of the sample being heat treated at 70°C for 10 min to inactivate vegetative cells. The counts displayed are those derived from the heat treated samples. N is the *C. difficile* count at time t and N_0 the initial count at $t=0$.

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Table1: *Clostridium difficile* ribotype 078 isolates used in the study.

078 isolates	Sources	Presence of Toxin Genes	Ribotypes	Toxinotypes	<i>tcd</i> c deletion	PFGE
B28	Biosolids	A+B+CDT+	078	V	39bp deletion	NAP 7
R3	River	A+B+CDT+	078	V	39bp deletion	NAP 8
R15	River	A+B+CDT+	078	V	39bp deletion	NAP 7
GP2	WWTP	A+B+CDT+	078	V	39bp deletion	NAP 7
GP80	WWTP	A+B+CDT+	078	V	39bp deletion	NAP 7

From Xu *et al.* (2014).

Draft

Table 2: Inactivate rate of *Clostridium difficile* endospores introduced into sludge and digested at different temperatures.

078 Ribotype		
Sludge Digestion Temperature / Inactivation Rate (log CFU/Day)		
Isolate	42°C	55°C
GP2	>0.003A	0.213±0.020A
GP80	>0.003A	0.214±0.025A
R3	>0.003A	0.200±0.032A
R15	>0.003A	0.188±0.051A
B28	0.075±0.013B	2.681±0.141B

Values with columns followed by the same capital letter are not significantly changed ($P > 0.05$)

Figure 1

Figure 3

Figure 4