Assessing the usefulness of clostridia spores for evaluating sewage sludge hygienization

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Abstract

The capability of clostridia spores to act as pathogen indicators in sewage sludge treatment was investigated. Sulfite-reducing clostridia and *E. coli* levels were monitored during waste activated sludge pre-treatments (alkali and ultrasound) and its subsequent mesophilic anaerobic digestion. *E. coli* was maintained or reduced depending on treatment type and intensity. However, alkali pre-treatment (35.3 gNaOH/kg TS) by itself and alkali (157 gNaOH/kg TS) and ultrasound (27,000 kJ/kg TS) pre-treatments followed by anaerobic digestion provoked reproducible clostridia increases. Specifically, up to 2.7 $\log_{10}$ after 35.3 gNaOH/kg TS pre-treatment and up to 1.9 and 1.1 $\log_{10}$ after digesting the 157 gNaOH/kg TS and 27,000 kJ/kg TS pre-treated sludge, respectively. Having rejected the hypotheses of sporulation and floc dissipation, the most plausible explanation for these clostridia increases is re-growth. These results question the suitability of clostridia spores as indicators of sludge treatment and other biological treatments where clostridia may have a role.

Keywords

1. Introduction

Ever more solutions are being used worldwide to manage the sewage sludge produced in wastewater treatment plants (WWTPs), including biological, mechanical, thermal and chemical treatments, and a combination of these. Treatment processes convert sewage sludge into biosolids, a stabilized product suitable for beneficial use. However, due to the high levels of pathogens naturally present in sludge (Guzmán et al., 2007; Lepeuple

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et al., 2004), only some treatments (or more specifically, only some treatment conditions) guarantee the safe levels required for use of biosolids as fertilizer, the preferred management option. In this context, the use of indicator microorganisms is an excellent tool to evaluate the microbiological risk associated with these practices, since they can indicate the hygienization capacity of treatment processes. Current regulations and regulation proposals already include some pathogens (e.g. *Salmonella*, enteric viruses) and/or indicator microorganisms (e.g. *Escherichia coli*, fecal coliforms) to assess sludge microbiological quality (European Commission, 2000; The Council of European Communities, 1986; US Environmental Protection Agency, 2003). However, conventional fecal indicator bacteria such as *Escherichia coli* (*E. coli*) or *Enterococcus* are not suitable to determine the behavior of pathogens that are highly resistant to intensive treatment processes, such as protozoa oocysts or helminth ova. To overcome these limitations, the spores of sulfite-reducing clostridia (SSRC) have been proposed and used for years as alternative indicators in water treatment processes (Agulló-Barceló et al., 2013; Hill and Sobsey, 1998; Lepeuple et al., 2004; Marti et al., 2011; Payment and Franco, 1993). The use of SSRC as indicators in sludge has several advantages: (i) they are present in large numbers, (ii) they exhibit high resistance to intensive treatments, and (iii) ther are easy, rapid and standardized detection methods (Guzmán et al., 2007; Lepeuple et al., 2004; Warnes and Keevil, 2004). Nonetheless, they can exhibit excessive resistance to treatment (Lepeuple et al., 2004) and concentrations may even increase after treatment (Rainisalo et al., 2011; Ruiz-Hernando et al., 2014; Salsali et al., 2008). This latter is a major drawback and calls into question the suitability of clostridia spores as indicators in sewage sludge treatment, since concentration increases are not expected for the pathogens they are indicating, i.e., protozoa oocysts and helminth ova. The nature of these unexpected increases has not yet

been studied in depth and it still remains unknown whether they are due to reactivation, sporulation processes, re-growth or some other cause. Conversely, the reactivation of *E. coli* from a viable but non culturable state in response to certain treatments is well-known (Erkan and Sanin, 2013; Pascual-Benito et al., 2015).

Anaerobic digestion (AD) is one of the most common sewage sludge treatment processes at WWTPs, since it degrades organic matter, produces energy in the form of methane and kills pathogens. A mixture of primary sludge and waste activated sludge (WAS) is typically fed to WWTP anaerobic digesters. However, WAS has a relatively low biodegradability due to its microbial biomass and extracellular polymeric substances (EPSs) composition (Carrere et al., 2016; Ruiz-Hernando et al., 2015). The introduction of pre-treatments before AD can be used to improve WAS biodegradability. Moreover, these treatments may act on pathogens and may even be used as post-treatments after AD to achieve sludge hygienization (Astals et al., 2012).

Ruiz-Hernando et al. (2014) assessed the hygienization capacity of ultrasound, low-temperature thermal and alkali treatments on WAS and their combination as mesophilic AD pre- or post-treatments. During these processes, bacterial and bacteriophage indicators were monitored (i.e., *E. coli*, somatic coliphages and SSRC). Although *E. coli* was the most sensitive microorganism, apparently being suitable to indicate enteric bacteria, alkali treatment increased *E. coli* levels under one of the specific conditions assayed (35.3 g NaOH/kg TS). Similarly, although SSRC were the most resistant microorganisms, apparently being suitable to indicate protozoa oocysts and helminth ova, unexpected results were obtained under several of the conditions assayed. Specifically, high increases in SSRC levels were observed (i) during alkali pre-

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treatment (35.3 g NaOH/kg TS), (ii) after mesophilic AD of alkali pre-treated WAS (157 g NaOH/kg TS) and (iii) after mesophilic AD of ultrasound pre-treated sludge (27,000 kJ/kg TS).

The aim of the present research was to further investigate the results obtained in the previous study (Ruiz-Hernando et al., 2014) and to conduct an in-depth assessment of the capacity of SSRC to act as indicators in sewage sludge treatments. To this end, all the treatments and conditions involved were repeated and carefully examined. The concentrations of *E. coli*, SSRC and total culturable cells of sulfite-reducing clostridia (TSRC) were closely monitored. Additionally, data for these indicators were obtained from raw wastewater, human feces and sludge samples.

2. Materials and methods

2.1. Sludge and wastewater origin

Sludge samples were taken from two municipal WWTPs (WWTP$_1$ and WWTP$_2$) in the Barcelona metropolitan area (Spain). WWTP$_1$ treats about 420,000 m³ wastewater/day and serves a population of 2,275,000 equivalent inhabitants. At WWTP$_1$, WAS is thickened by centrifugation after leaving the secondary tank. Afterwards, a mixture of 50 % primary sludge and 50 % WAS is subjected to mesophilic AD with a hydraulic retention time of 37-42 days. WWTP$_2$ has a treatment capacity of 64,000 m³ wastewater/day and serves a population of 385,000 equivalent inhabitants. At WWTP$_2$, a mixture of 60 % primary sludge and 40 % WAS is anaerobically digested under mesophilic conditions with a hydraulic retention time of 20-24 days. The WAS samples used in this study were collected from WWTP$_1$, whereas the digested sludge used as inoculum came from WWTP$_2$. The total solids (TS) content of the WAS samples was

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54.9 ± 11.6 g/L (mean ± standard deviation). The TS content of the inoculum sample was 23.1 g/L. In addition, sludge samples were taken from both WWTPs before and after AD, as well as raw wastewater. The TS and volatile solids were determined following the guidelines given by the standard methods 2540G (APHA, 2005).

2.2. Human feces origin

Two human feces samples were collected from healthy individuals to evaluate the sulfite-reducing clostridia and *E. coli* concentrations typically present in these types of sample. The TS content was calculated as described in Section 2.1 and was 275.7 ± 15.8 g/L (mean ± standard deviation).

2.3. Pre-treatment conditions

The pre-treatments studied in this research were ultrasound and alkali. The ultrasonic apparatus used was an HD2070 Sonopuls Ultrasonic Homogenizer equipped with a MS 73 titanium microtip probe (Bandelin, Berlin, Germany; 20 kHz). The beaker containing the samples was submerged in an ice bath to prevent an increase in sludge temperature due to the thermal effect of the cavitation phenomenon. The specific energies (E_S) applied were 27,000, 40,000 and 60,000 kJ/kg TS (named as 27,000-US, 40,000-US and 60,000-US, respectively). E_S were calculated as follows:

$$E_S = \frac{P \cdot t}{V \cdot TS} \tag{1}$$

where *P* is the ultrasonic power of the ultrasonic homogenizer (kW), *t* is the application time (s), *V* is the sample volume (L) and *TS* is the concentration of total solids (g/L).

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The alkali pre-treatment was conducted at room temperature (approximately 25 °C) by adding different doses of NaOH with a contact time of 24 h. Samples were subsequently neutralized with $HCl_{35\%}$ to reach a pH range of 6.5-7.5. The concentrations studied were 35.3 and 157 g NaOH/kg TS (named 35.3-NaOH and 157-NaOH, respectively).

Another alkali pre-treatment was conducted using a glycine buffer to isolate the effect of pH (named GLY). The glycine buffer (0.25 M, pH 10.5) was prepared and 10 g of the WAS samples was diluted in a 1:10 (W/V) ratio with this buffer. Samples were then periodically acidified through the addition of $HCl_{35\%}$ during the 24 h treatment, with the aim of reproducing the pH drop in the 35.3-NaOH pre-treatment. Untreated sludge was monitored and used as control.

2.4. Mesophilic anaerobic digestion

AD was evaluated through biomethane potential (BMP) tests carried out under mesophilic conditions using batch tests. The procedure was the same as that described in a previous study (Ruiz-Hernando et al., 2014). Briefly, the BMP tests were performed in 115 mL serum bottles sealed with a PTFE/butyl septum, which was fixed by an aluminum crimp cap. The bottles were filled with 50 mL of inoculum and 8.7 mL of WAS sample (untreated or treated), which met an inoculum to substrate ratio of 2 in volatile solid basis considering the untreated WAS volatile solid value (52.7 g VS/L). Before sealing the bottles, all digesters were flushed with nitrogen for 1 minute (3 L/min). Finally, digesters were placed in a water bath at 37 ± 1 °C. The bottles were manually mixed by swirling twice a day. Digestion lasted 35 days and samples were taken at the beginning, after 15 days and at the end of the treatment.

2.5. Detection and enumeration of *E. coli* and sulfite-reducing clostridia

Five to ten grams of sludge was mixed in a 1:10 (W/V) ratio with phosphate buffered saline (PBS) pH 7.2, homogenized with a wrist action shaker at 900 osc/min for 30 min at room temperature and then centrifuged at 300 ×g for 3 min at 4 ºC. The resulting supernatant was used to analyze the *E. coli* and sulfite-reducing clostridia present in the sample. For *E. coli*, samples were cultured in Chromocult agar plates (Merck, Germany) supplemented with *E. coli*/coliform-selective supplement (Merck, Germany) using the pour plate procedure. Plates were incubated at 44 ºC overnight, and dark-blue/purple *E. coli* colonies were counted. For sulfite-reducing clostridia, TSRC were directly tested from the supernatant, while SSRC were tested after subjecting the supernatant to a thermal shock of 80 ºC for 10 min. Clostridia were cultured by mass inoculation in *Clostridium perfringens* selective agar (Scharlab, Spain), followed by incubation in anaerobic conditions at 44 ºC overnight. The typical black spherical colonies were counted as clostridia. According to the manufacturer's instructions, about 90 % of the colonies can be attributed to *C. perfringens*. Since no confirmation of results was conducted, the colonies obtained were counted as sulfite-reducing clostridia.

2.6. Experimental design

The initial stage of the study aimed to assess the impact of different pre-treatments on microbial indicators. First, the pre-treatment conditions from Ruiz-Hernando et al. (2014) that produced unexpected results by themselves or in combination with AD, were repeated to confirm data. They were 35.3-NaOH, 157-NaOH and 27,000-US. Second, 35.3-NaOH was studied in greater depth and a complementary alkali strategy, GLY, was applied to isolate the effect of pH. Later on, two more ultrasound pre-

treatment conditions were tested, i.e., 40,000-US and 60,000-US. In a second stage, untreated and pre-treated WAS samples were subjected to mesophilic AD to confirm Ruiz-Hernando et al. (2014) results. In the final stage, the study was complemented with (i) data from the WWTPs: raw wastewater and sewage sludge before and after mesophilic AD and (ii) data from human feces. All samples assayed in this study were tested in duplicate and the means and standard deviations were calculated. Fresh samples were collected for each experiment.

3. Results and discussion

3.1. Impact of pre-treatments on indicators

The unexpected clostridia increases obtained in Ruiz-Hernando et al. (2014) may suggest a sporulation process in response to adverse conditions. In this scenario, vegetative clostridia cells would have transformed into the resistant form (spores) thus increasing the number of SSRC detected. To test this hypothesis, the pre-treatments involved were repeated and TSRC were monitored together with SSRC.

As shown in Fig. 1, it was found an increase of 2.73 $\log_{10}$ (CFU/g TS) in TSRC levels and 1.78 $\log_{10}$ in SSRC levels for 35.3-NaOH as compared with untreated sludge. The pH at the beginning of this pre-treatment reached 10.3, however, the buffer capacity of the sludge itself steadily reduced the pH to 7.0 after 24 h. Consequently, it was not necessary to add HCl$_{35\%}$ for neutralization. Contrariwise, for 157-NaOH, where the pH rose to more than 12 and it maintained for 24 h, both clostridia populations were similarly reduced, i.e., 1.61 $\log_{10}$ for TSRC and 1.63 $\log_{10}$ for SSRC. A smaller effect was observed for 27,000-US, with increases of 0.33 and 0.28 $\log_{10}$ for TSRC and SSRC respectively, in comparison with the untreated sludge. These results were similar to

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those obtained in the previous study, confirming their reproducibility for the pre-treatment conditions assayed. Furthermore, it is important to highlight the simultaneous increase in both spores and total cultivable cells, suggesting that the effect of sporulation alone is insufficient to explain the increase in clostridia levels.

Due to the notable increases observed in clostridia (TSRC and SSRC) after 35.3-NaOH pre-treatment in Ruiz-Hernando et al. (2014) and in the present study, this pre-treatment was repeated and further analyzed by monitoring pH and microbial concentrations during the 24 h of treatment contact time. Results are presented in Fig. 2. For the untreated WAS, the pH varied between 6.67 and 6.37 and sulfite-reducing clostridia levels were maintained. Specifically, mean values of 6.12 ± 0.16 and 6.09 ± 0.17 $\log_{10}$ (CFU/g TS) were obtained for TSRC and SSRC, respectively. For the alkali pre-treated sample, the initial pH value was 10.4, which decreased to 6.92 after 24 h. Regarding *E. coli*, no effect was observed this time after treating the sample and concentrations were maintained from 5.72 ± 0.05 to 5.79 ± 0.02 $\log_{10}$ (CFU/g TS) for the untreated sample and from 5.80 ± 0.01 to 5.96 ± 0.01 for the 35.3-NaOH sample. Thus, it appeared that the increase observed for this bacterium in the previous study (Ruiz-Hernando et al., 2014) was not a reproducible outcome. Contrarily, a considerable increase in sulfite-reducing clostridia levels was observed for the 35.3-NaOH sample, resulting in 1.29 $\log_{10}$ for TSRC and 0.77 $\log_{10}$ for SSRC. These increases took place during the mid to late phase of the treatment. Under optimal nutrient, temperature and pH conditions, reported doubling times for some *Clostridium* species are below 10 minutes (Labbe and Huang, 1995; McClane, 2003). In addition, a previous study has shown that alkali treatment of WAS induces carbohydrate and protein solubilization together with volatile fatty acids formation (Chen et al., 2007). Thus, 35.3-NaOH may have provided

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clostridia with these products during the initial phases of the pre-treatment and clostridia could have used them to grow during the mid to late phase, when the pH was favorable. Contrarily, the higher pH during 157-NaOH may have prevented clostridia re-growth under these pre-treatment conditions and the pre-treatment was so intense that reduced its concentrations (Fig. 1). However, re-growth may not be the only explanation for this increase in clostridia levels. It is widely known that colony forming unit (CFU) counts increase when cell flocs in a sample are dissipated. These flocs, which are naturally found in the sludge matrix, contain bacterial aggregates that are counted as a single CFU; thus masking the real cultivable bacteria numbers. Since floc dissipation may occur due to pH changes and sonication, the hypothesis of floc dissipation was launched.

The effect of pH on SSRC was tested through an alkali treatment using a glycine buffer (GLY) instead of NaOH. As shown in Table 1, the pH curve for 35.3-NaOH was satisfactorily reproduced. However, in this case, no differences in clostridia levels were found when comparing the untreated and the GLY-treated samples. Specifically, SSRC concentrations varied from 7.01 ± 0.02 to 7.14 ± 0.03 $\log_{10}$ (CFU/g TS) for the untreated sample and from 6.87 ± 0.09 to 6.94 ± 0.04 $\log_{10}$ (CFU/g TS) for the GLY sample. Accordingly, the posited floc dissipation did not occur. From this experiment, it may be concluded that pH is not the causal agent of clostridia increases or, at least, it is not the only factor implicated. It is conceivable that the reagents and conditions used to cause this pH trend were also important factors. To determine the impact of sonication on CFU counts WAS samples were subjected to more intense ultrasound treatments, specifically to 40,000-US and 60,000-US. The aim was to increase treatment intensity to such an extent that it produced floc dissipation and a consequent increase in clostridia

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and *E. coli* counts (Foladori et al., 2007). The results are shown in Fig. 3. Neither the 40,000-US nor the 60,000-US treatment produced increases in TSRC or SSRC levels. Nevertheless, these treatments were sufficiently intense to produce *E. coli* death, resulting in decreases of 0.77 and 1.35 $\log_{10}$ for 40,000-US and 60,000-US, respectively.

3.2. Impact of mesophilic anaerobic digestion on indicators

To analyze sulfite-reducing clostridia (TSRC and SSRC) behavior during mesophilic AD a new WAS sample was pre-treated, i.e., 35.3-NaOH, 157-NaOH and 27,000-US. Then, 8.7 mL of untreated or pre-treated WAS were mixed with 50 mL of inoculum. The larger volume and the higher sulfite-reducing clostridia concentrations (7.07 ± 0.17 $\log_{10}$ of TSRC/g TS and 7.17 ± 0.04 $\log_{10}$ of SSRC/g TS) made inoculum the major contributor of sulfite-reducing clostridia in the digestion media (Fig. 4). This contribution can be observed comparing the untreated samples in Fig. 1 and Fig. 4. After 35 days of digestion, TSRC and SSRC levels in the untreated and 35.3-NaOH-treated samples remained similar to the beginning, while 157-NaOH and 27,000-US resulted in TSRC and SSRC increases. These increments in sulfite-reducing clostridia were already visible after 15 days and more remarkable after 35 days of digestion (Fig. 4). For 157-NaOH, TSRC concentrations increased by 1.97 $\log_{10}$ and SSRC by 1.45 $\log_{10}$, while for 27,000-US, TSRC concentrations increased by 1.16 $\log_{10}$ and SSRC by 0.49 $\log_{10}$. These results are similar to those obtained in Ruiz-Hernando et al. (2014) and confirm that the combination of some pre-treatments (or more specifically, some pre-treatment conditions) followed by digestion increase clostridia numbers. These increases in TSRC and SSRC concentrations cannot be linked to pH changes since pH remained constant (7.13 – 7.41) throughout the entire digestion process. The paragraphs

that follow consider a number of possible explanations for the unexpected sulfite-

reducing clostridia behavior, and conclude with a plausible hypothesis.

Pre-treatments kill sulfite-reducing clostridia competitors in WAS favoring them

numerically.

The sludge cell disruption and solubilization is a well-known phenomenon for most

WAS pre-treatments (Carrere et al., 2016). Therefore, it can be expected that pre-

treatments could have impacted on some WAS bacterial populations in a greater extent

than sulfite-reducing clostridia, favoring them numerically when mixed with inoculum.

This hypothesis implies that the contribution of WAS to the active biomass in a BMP

test is relevant. However, the inoculum contribution to clostridia abundance in the

digestion media was 1,000 and 10 times higher than the 157-NaOH and 27,000-US pre-

treated WAS contribution, respectively. Furthermore, this hypothesis cannot explain the

sulfite-reducing clostridia increases reported after digesting 27,000-US-treated WAS. In

Ruiz-Hernando et al. (2014) this pre-treatment condition did not kill SSRC, *E. coli* or

somatic coliphages; and therefore it is unlikely that it disrupted other bacteria.

Sulfite-reducing clostridia use soluble compounds and cell debris released during WAS

pre-treatments to grow during digestion.

The visualization of TSRC and SSRC levels increasing under favorable pH conditions

during the 35.3-NaOH pre-treatment (see Section 3.1) gave rise to the hypothesis that

sulfite-reducing clostridia from the 157-NaOH sample could have increased during

digestion as a result of the better pH conditions and the large amount of easily

biodegradable compounds. Recent studies using molecular techniques have revealed the

multifaceted role of *Clostridium* on AD, which can take part of most AD steps prior to methanogenesis (i.e., hydrolysis, acidogenesis, acetogenesis and acetate-oxidation) (Shah et al., 2014; Stiborova et al., 2015; Tian et al., 2015; Yuan et al., 2015). Interestingly, Jang et al. (2014) and Park et al. (2014) reported the growth of some groups of *Clostridium* after subjecting thermal and alkali pre-treated sludge to mesophilic AD. The presence of easily biodegradable compounds may have also favored sulfite-reducing clostridia re-growth during digestion of 27,000-US-treated WAS. The presence of easily biodegradable compounds should have also favored sulfite-reducing clostridia re-growth during sonication (27,000-US); however, this may have not been observed due to the short treatment time (7-8 minutes depending on sample TS). Nonetheless, this hypothesis cannot explain why in Ruiz-Hernando et al. (2014) sulfite-reducing clostridia did not grow after digesting low-temperature pre-treated WAS (80 °C and 15 min); sample that showed similar solubilization degrees than 157-NaOH and 27,000-US.

Pre-treatment derived inhibitory compounds promote sulfite-reducing clostridia re-growth over other bacteria.

The last hypothesis is related to the presence of inhibitory compounds in the digestion media from WAS pre-treatments, which could have favored sulfite-reducing clostridia re-growth over other anaerobic bacteria. In this scenario, the Na^+ introduced during the alkali pre-treatments or the inhibitory compounds generated during sonication (Peces et al., 2015) could have favored sulfite-reducing clostridia re-growth by limiting the growth of other bacteria. However, the addition of 157 g NaOH/g TS only represents an extra load of 0.74 g Na^+/L, giving a final digestion media concentration of 1 g Na^+/L when mixed with the inoculum. This Na^+ concentration is known as not inhibitory for

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acetoclastic methanogens and therefore, even less likely to be inhibitory for bacteria

(Astals et al., 2015). Indeed, in Ruiz-Hernando et al. (2014) no inhibition (rate and

extent) was observed during 157-NaOH and 27,000-US BMP testing. However, a

change in bacteria populations caused by the presence of inhibitors cannot be detected

by this technique. This hypothesis can explain why in Ruiz-Hernando et al. (2014) no

sulfite-reducing clostridia re-growth was observed after low-temperature pre-treated (80

°C and 15 min) WAS digestion; since this pre-treatment conditions do not generate

inhibitory compounds (Carrere et al., 2016; Carrère et al., 2010).

Overall, it is likely that sulfite-reducing clostridia increased during digestion as a

response to favorable conditions generated by the pre-treatments assayed. Although it is

not completely clear which are the dominating mechanisms behind TSRC and SSRC

increases, results seem to indicate that re-growth is the most plausible explanation.

However, reactivation from a viable but non culturable state cannot be completely

discarded.

3.3. Sulfite-reducing clostridia and *E. coli* behavior in the wastewater treatment process

TSRC, SSRC and *E. coli* levels found in human feces and during the wastewater

treatment process conducted at two full-scale WWTPs are shown in Table 2. The

findings obtained do not expect to be an exhaustive analysis of the microbial

concentrations present in the different samples studied, since the number of samples

was limited. However, they can be useful to reflect the general assumed behavior of

sulfite-reducing clostridia and *E. coli* during the wastewater treatment process. *E. coli* is

a non-conservative indicator with low resistance to treatment, whereas SSRC are

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conservative, capable of resisting high intensity treatments. *E. coli* concentrations in

fresh human feces greatly exceeded sulfite-reducing clostridia levels and similar results

were found in wastewater. As treatment progressed, clostridia concentrations were

maintained due to their higher resistance, whereas *E. coli* levels dropped. A calculation

of the SSRC/*E. coli* ratio clearly reflects this tendency. After AD, clostridia

concentrations in sludge were significantly higher than those for *E. coli*. Wéry et al.

(2008) found a similar trend throughout wastewater treatment processes, including

composting.

As shown in Fig. 4, sulfite-reducing clostridia levels did not increase when the digesters

were fed with untreated WAS. Likewise, a study of AD routinely performed at the two

WWTPs (Table 2), where the sludge is not pre-treated to enhance digestion, revealed a

similar behavior. This agrees with previous studies (Bagge et al., 2005; Guzmán et al.,

2007; Sahlström et al., 2004) and suggests that sulfite-reducing clostridia and *E. coli*

populations are not generally increased by the effect of AD alone.

Taken together, the findings of this study confirm that some pre-treatments or

combinations of pre-treatments followed by AD led to increases in sulfite-reducing

clostridia from WAS. Further studies including the use of a molecular method, such as

qPCR, would clarify whether these increases are due to re-growth, reactivation or

simple increases in colony counts. Nevertheless, the hypotheses of sporulation and floc

dissipation have been rejected. Other authors have found similar increases in *C.*

perfringens or sulfite-reducing clostridia during storage of composted mixtures of

sludge and plant wastes (Rainisalo et al., 2011) or in digested sludge subjected to

different temperatures, volatile fatty acid concentrations and pHs (Salsali et al., 2008).

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In addition, recent studies (Shah et al., 2014; Stiborova et al., 2015; Tian et al., 2015; Yuan et al., 2015) have revealed the *Clostridium* multifaceted role in AD. All this information suggests that the use of SSRC or *C. perfringens* spores as indicators of protozoa oocysts and helminth ova may not be the most suitable choice for sewage sludge samples subjected to AD, since the indicator behavior does not reflect the pathogen behavior.

4. Conclusions

The capability of clostridia spores to act as pathogen indicators in sewage sludge treatment was examined. Sulfite-reducing clostridia (spores and culturable cells) and *E. coli* were monitored in waste activated sludge subjected to alkali and ultrasound pre-treatments and their combination with mesophilic anaerobic digestion. Some treatment conditions reduced *E. coli*. However, alkali pre-treatment (35.3-NaOH) and alkali and ultrasound pre-treatments (157-NaOH; 27,000-US) followed by anaerobic digestion repeatedly increased clostridia. Having rejected sporulation and floc dissipation, the most plausible explanation is re-growth. Considering the multifaceted role of *Clostridium* in anaerobic digestion, results question the suitability of clostridia spores as sludge treatment indicators.

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Figure captions

**Table 1.** pH measurements during GLY treatment in comparison with 35.3-NaOH.

Table 2. Sulfite-reducing clostridia and *E. coli* data for WWTP digestion, raw wastewater and human feces. Mean ± standard deviation. Data in $\log_{10}$ (CFU/g TS)[a] or $\log_{10}$ (CFU/mL)[b]. Analyses were performed twice.

Fig. 1. Effect of pre-treatments on sulfite-reducing clostridia concentrations. Error bars represent standard deviation.

Fig. 2. Variations in sulfite-reducing clostridia levels over 24 h in (a) untreated WAS, and (b) 35.3-NaOH treated WAS. Error bars represent standard deviation.

Fig. 3. Effect of high energy ultrasound treatments (40,000-US and 60,000-US) on sulfite-reducing clostridia and *E. coli* concentrations. Error bars represent standard deviation.

Fig. 4. Effect of digestion on sulfite-reducing clostridia concentrations after 0, 15 and 35 days: (a) Untreated, (b) 35.3-NaOH, (c) 157-NaOH, (d) 27,000-US. Error bars represent standard deviation.

Tables and Figures

Table 1
pH measurements during GLY treatment in comparison with 35.3-NaOH.

	Time (h)														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	24
Untreated	7.15	6.94	6.95	6.93	6.93	6.94	6.96	6.98	7.00	7.00	7.00	7.06	7.06	7.06	6.96
GLY	10.50	10.05	8.99	8.51	8.00	7.98	7.98	7.51	7.47	7.46	7.46	7.45	7.42	7.02	7.01
35.3-NaOH	10.35			8.25				7.56							6.92

Table 2
Sulfite-reducing clostridia and *E. coli* data for WWTP digestion, raw wastewater and human feces. Mean ± standard deviation. Data in $\log_{10}$ (CFU/g TS)[a] or $\log_{10}$ (CFU/mL)[b]. Analyses were performed twice.

	Human feces		WWTP[1]			WWTP[2]		
	A[a]	B[a]	Raw wastewater[b]	Sludge before AD[a]	Sludge after AD[a]	Raw wastewater[b]	Sludge before AD[a]	Sludge after AD[a]
TSRC	4.14±0.02	4.31±0.21	3.35±0.10	7.23±0.03	7.19±0.14	3.34±0.02	6.75±0.32	7.08±0.11
SSRC	3.73±0.13	4.48±0.17	3.44±0.06	6.78±0.07	6.75±0.05	3.43±0.04	6.77±0.05	7.04±0.19
E. coli	6.22±0.13	6.49±0.18	4.26±0.04	6.50±0.04	5.40±0.01	4.73±0.05	7.35±0.06	4.79±0.04
SSRC/*E. coli*	0.60	0.69	0.81	1.04	1.25	0.73	0.92	1.47

Figure 1:

Figure 2:

(a)

(b)
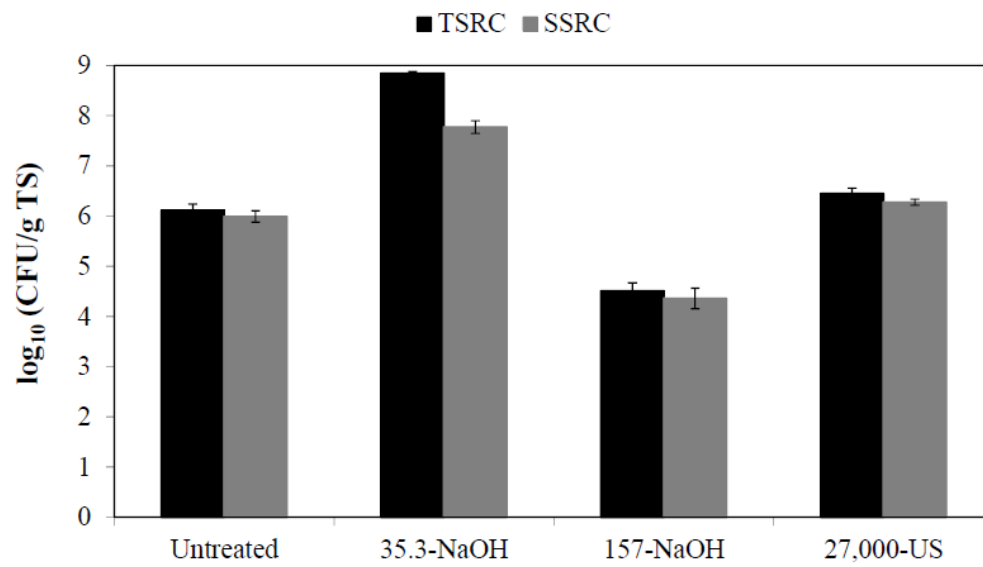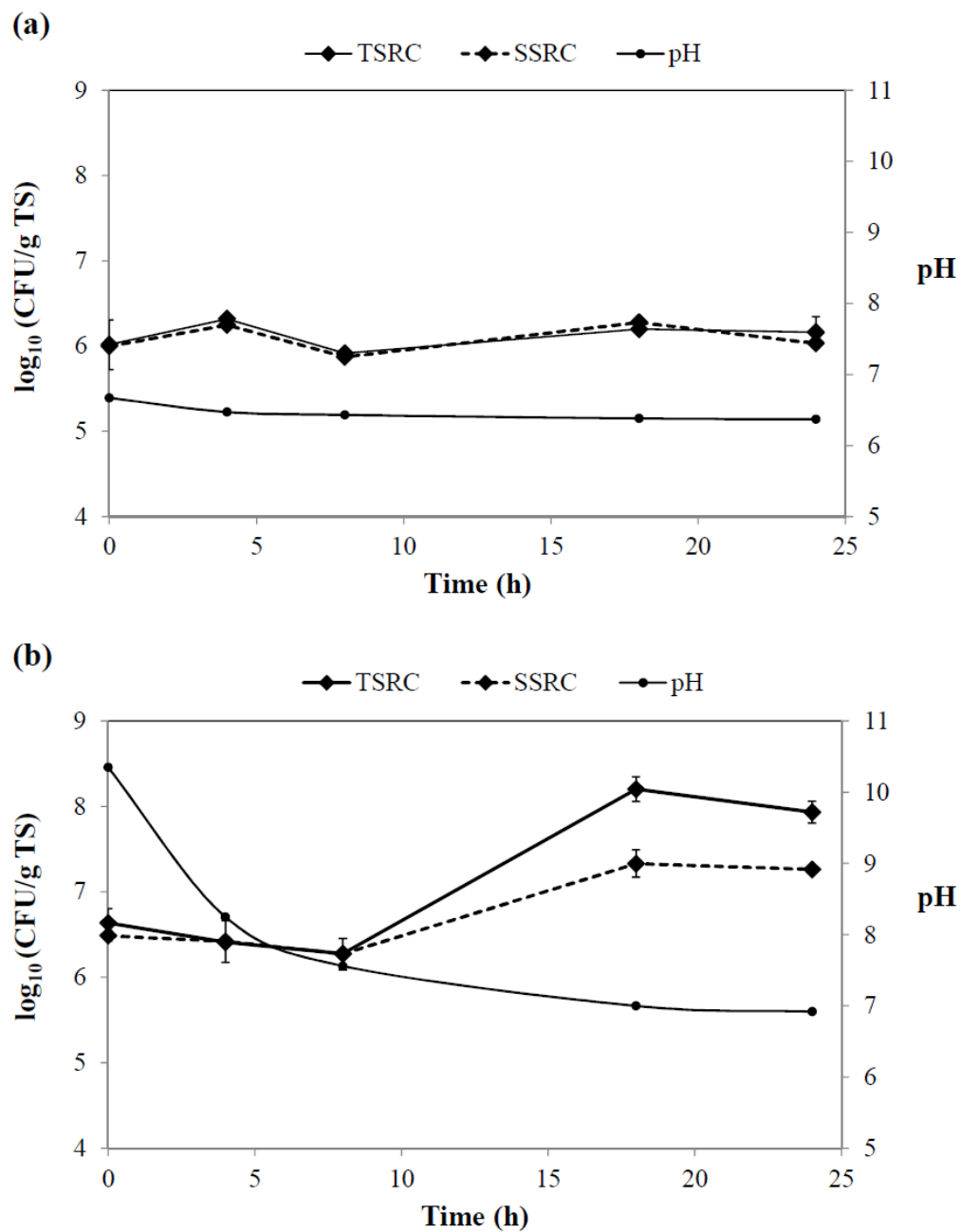
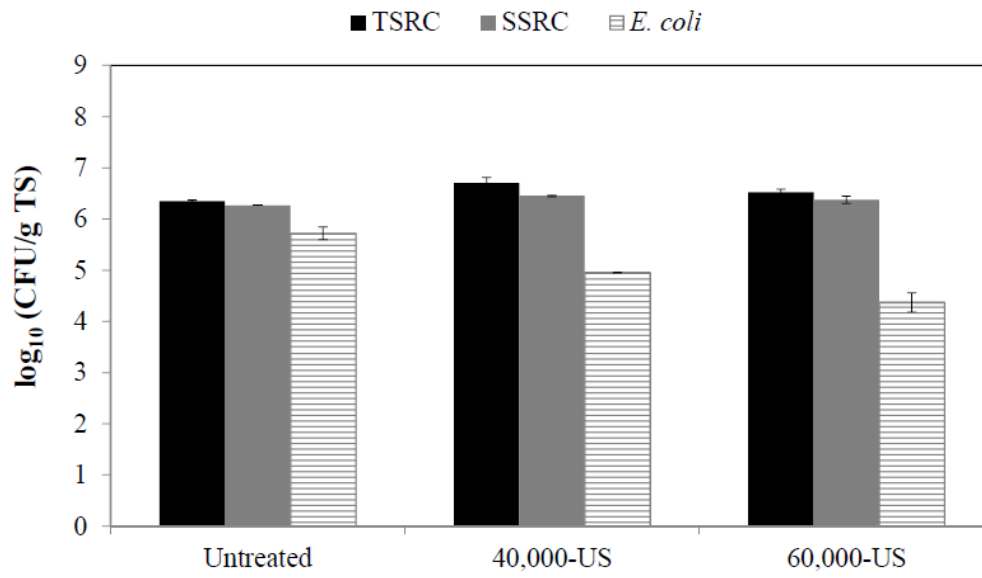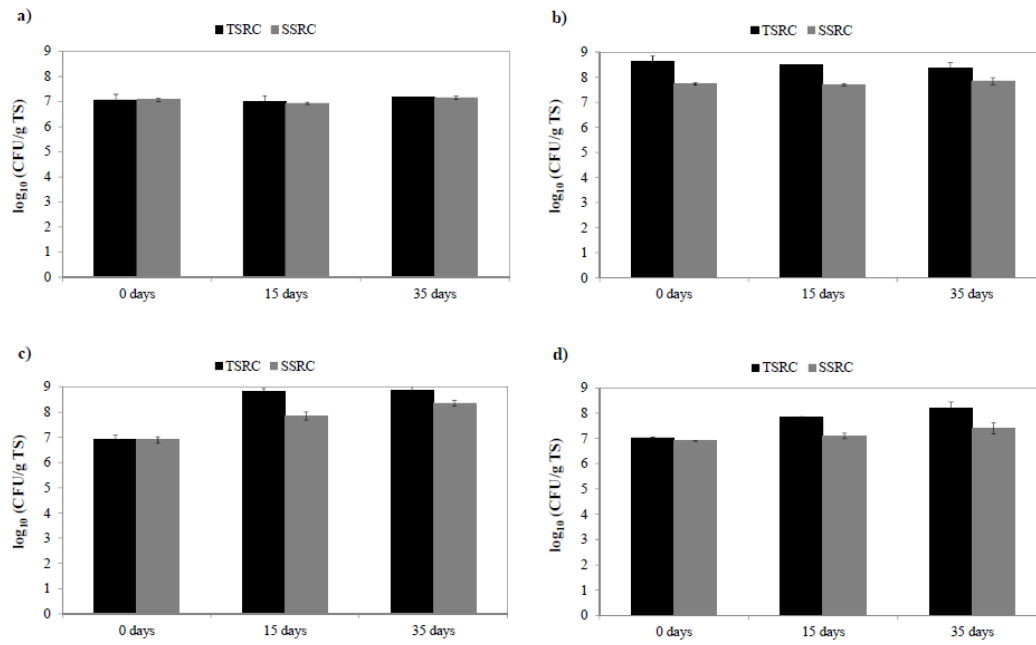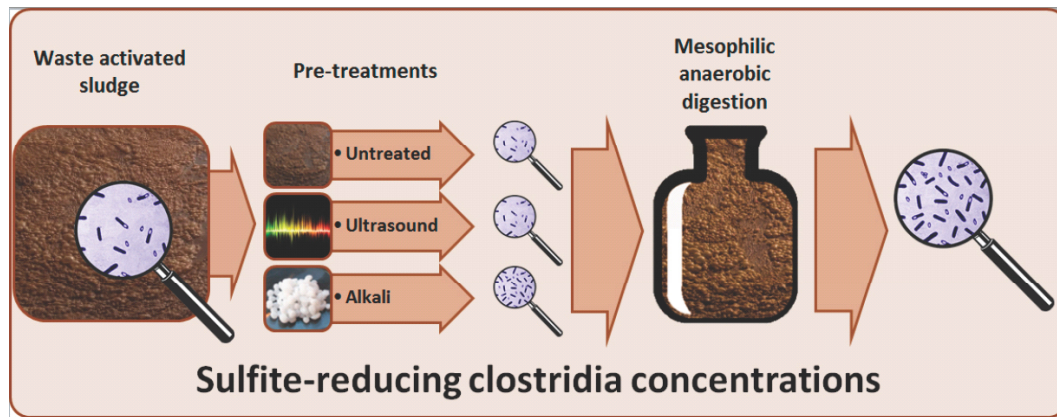**Figure 3:**

Figure 4:

Highlights

- Clostridia and *E. coli* were monitored during sewage sludge anaerobic digestion

- Results question the usefulness of clostridia as sewage sludge treatment

  indicators

- Alkali pre-treatment (35.3 g NaOH/kg TS; 24h) increased clostridia levels

- Clostridia also increased after digesting NaOH or ultrasound pre-treated sludge

- *E. coli* levels did not increase under any of the tested conditions

Waste activated sludge · Pre-treatments · Untreated · Ultrasound · Alkali · Mesophilic anaerobic digestion

Sulfite-reducing clostridia concentrations