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Disruption of cells in biosolids affects *E. coli* dynamics in storage

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Abstract

Achieving microbial compliance during biosolids storage can be complicated by the unpredictable increase of *Escherichia coli*. Thermal treatment during anaerobic digestion (AD) and the effects of dewatering may be a significant factor contributing to indicator survival. Shear forces present during dewatering may promote cell damage, releasing nutrient for *E. coli* growth. The effect of cell damage on *E. coli* survival was assessed in laboratory-scale thermal and physical disruption experiments. *E. coli* growth curves for disrupted treatments were compared with control conditions and quantified using flow cytometry and membrane filtration techniques. A significant difference ($p < 0.05$) in the level of damaged cells between control and disrupted conditions was observed. For thermal and physical disruption treatments, the peak of *E. coli* concentration increased significantly by 1.8 Log and 2.4 Log (CFU (colony forming units) g^{-1} DS), respectively, compared with control treatments. Research findings contribute to the understanding of bacterial growth and death dynamics in biosolids.

Key words: biosolids, cells, dewatering, *E. coli*, storage

INTRODUCTION

Monitoring levels of pathogenic indicators in biosolids validates the effectiveness of treatment processes and ensures the safe recycling of biosolids to agricultural land (European Commission 2015). Concerns of pathogen removal in biosolids treatment during storage have been raised where post-dewatering levels of microbial indicators can exceed compliance levels (Monteleone *et al.* 2004; Higgins *et al.* 2007a; Chen *et al.* 2011).

Centrifuge dewatering is a common method used to remove the liquid fraction of sludge. Prior to dewatering, *E. coli* indicator concentrations in the digested liquid sludge tend to be (in the case of study sites examined here) approximately 4 Log (CFU (colony forming units) g^{-1} DS) (operational

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data, Severn Trent plc). However, after dewatering operations, particularly in the first 48 h of storage, indicator concentrations have been observed to increase rapidly, reaching levels of approximately 7 Log (CFU g^{-1} DS) in some cases (operational data, Severn Trent plc). This observation is not isolated and has been observed on other treatment sites (Monteleone *et al.* 2004; Higgins *et al.* 2007a; Qi *et al.* 2007; Chen *et al.* 2011). This rapid increase in *E. coli* levels may indicate the presence of pathogenic bacteria within the biosolids product and is a concern for utility operators.

The reasons for indicator increase in stored biosolids post-dewatering have not yet been clearly identified (Dentel *et al.* 2008). One possible explanation is that following suppression during thermal anaerobic digestion (AD) treatment, the release of readily bioavailable nutrients during mechanical dewatering processes may provide substrates for bacterial growth during subsequent storage periods (Higgins *et al.* 2006, 2007a; Chen *et al.* 2011; Sun *et al.* 2015). The pre-conditioning of the digested sludge with polyelectrolyte to form flocs aggregates the sludge organic matter, which could favour the growth of bacterial cells held within the floc matrix. In addition, the floc structure can provide protection from environmental stressors, prolonging cell survival (Mahendran *et al.* 2012; Lin *et al.* 2014).

Although the mechanical dewatering process is conditioned to support aggregation and the formation of the cake product (Higgins *et al.* 2007b), researchers suggest that the effects of mechanical shear during dewatering treatments can cause floc disruption and even bacterial dispersal, elevating indicator concentrations. Research by Chen *et al.* (2011) suggested that reasons for the bacterial increase were explained by the release of soluble proteins and other organics during shearing, which serves as substrate sources for microbial organisms. Higgins *et al.* (2007b) suggested that the cause of indicator increase may be cell reactivation as the shear force exerted on the sludge during centrifuge dewatering releases bioavailable nutrients which supports the culturability of bacterial cells previously held in a viable but non-culturable (VBNC) state. This nutrient release may be from cell lysis. Sun *et al.* (2015) investigated dewatering processes on cyanobacteria-containing sludge and attributed cell lysis to flocculation turbulence and pressure from mechanical operations on flocs.

The effects of methanogen cell lysis resulting from centrifuge dewatering were studied by Chen *et al.* (2005) who identified that a higher level of shearing led to greater cell lysis and inhibition of methanogenesis (Chen *et al.* 2005). The sludge AD environment contains a complex community of bacteria, many of which are obligate anaerobes (Town *et al.* 2004; Shah *et al.* 2004). During dewatering, oxygen exposure may be increased in the sludge matrix (Chen *et al.* 2011), which could additionally have lethal effects on cells preferentially adapted for the anaerobic environment. The growth of *E. coli* bacteria may, therefore, be enhanced as obligate anaerobes decay and provide a food source for the viable *E. coli* bacteria to grow.

In response to other stressors such as heat, Noor (2015) identified lysis of VBNC *E. coli* cells as a response to heat stress. Findings suggested a possible survival output from the cell lysis, contributing to the removal of damaged cells able to serve as nutrients for the remaining population. Research by Higgins *et al.* (2007b) suggests that the effects of AD cause bacterial cells, including *E. coli* cells, to enter a VBNC state. The VBNC state of *E. coli* cells may be reversed during mechanical dewatering as the environment favours the growth of the indicator bacteria. Based on research by Noor (2015), it may be feasible to suggest that a proportion of the bacterial cells in a VBNC state lyse and provide cellular nutrients for the remaining reactivated bacteria. Confirmation of cellular nutrients supporting viable cell survival is highlighted in Murate *et al.* (2012) who showed that cytoplasmic materials are released into the culture medium as a result of cell lysis and may act as a nutrient supplement for remaining cell population survival. Murate *et al.* (2012) suggested that further research is required to clarify whether cell lysis increases available nutrients able to support remaining cell survival. The disruption of cells may enhance *E. coli* replication and the recovery of dormant cells as cellular nutrients are released and added to the sludge matrix. Therefore, the hypothesis tested in this study is that cell disruption provides a substrate source able to support the growth of *E. coli* bacteria in sewage sludge cake stored under favourable conditions.

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METHODOLOGY

Sludge sampling and storage

Samples of sludge cake, which had been both mesophilic digested and centrifuge dewatered, were collected from a wastewater treatment works serving a population equivalent of 440,000. The characteristics of the cake at the treatment works showed a sludge of 20% DS with a pH of 8 and a typical *E. coli* concentration of 5.5 CFU Log_{10} g^{-1} DS at time 0 (day of sample collection). Representative sub-samples were taken to form a bulk sample of approximately 26 kg and were collected at a depth of 0.5 m using a spade or 'corer' device from central locations on the sludge stockpile after the 0.5 m surface 'crust' had been discarded. From this bulk (26 kg) sample, sub-samples were taken for the subsequent experimental treatments. The sludge cake samples collected from the treatment site were less than 24 h old from the point of centrifuge output and enabled laboratory-tested storage environments to begin from 'day 1' assumption. Samples were placed in screw lid sampling pots (Nalgene, Rochester, USA) and transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2 and 8 °C to suppress biological activity. Samples were processed within 24 h of sampling.

Samples collected from the treatment site were then used in the two sets of experiments described below.

Experimental treatments

To reduce bacterial concentrations, two methods of disruption were applied to samples.

Thermal disruption

Samples of 6 × 50 g were subjected to treatment at 62 °C (±2 °C) for 20, 80 and 120 min (Gallenkamp, A. Gallenkamp, London, UK). An additional comparative treatment included 50 g × 4 samples subject to autoclaving at 121 °C for 15 min at 1.03 bar (±0.03 bar) (Priorclave, London, UK). For the experimental set-up, 100 g (2 × 50 g) of each thermally treated sludge cake sample was placed into a glass beaker (FisherBrand, Loughborough, UK). One (100 g) autoclaved sample was mixed with an additional 0.5 g of the undisrupted sludge cake to reintroduce a sludge-derived bacterial community including *E. coli*. Alongside the thermal treatments, an undisrupted sample of sewage sludge cake (100 g) was tested as a control condition. All samples were well mixed, covered with Parafilm M™ (FisherScientific, Loughborough, UK) and stored at 22 °C (±2 °C) for 10 days. Flow cytometry analysis distinguished intact and damaged cell concentrations and membrane filtration determined *E. coli* bacterial levels. All sample conditions were repeated in triplicate.

Physical disruption

Microcosms of 3 g of fresh sewage sludge cake sample were mixed with 15 mL of deionised water and sonicated for 7 min at 33 W (MicroTip, Virsonic 600, VirTis, USA). Additional microcosms were set up and not subjected to sonication treatment, remaining as control conditions. All samples were covered with Parafilm M™ (FisherScientific, Loughborough, UK) and stored at 22 °C (±2 °C) for 10 days. Flow cytometry analysis distinguished intact and damaged cell concentrations, and membrane filtration determined *E. coli* bacterial levels. Dissolved organic carbon (DOC) analysis was conducted on samples before and after physical disruption treatment. All sample conditions were replicated in triplicate, and each microcosm was used sacrificially on the day of testing.

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Analytical methods

Microbial enumeration

Membrane filtration (Environment Agency 2003) was used for bacterial enumeration using Membrane Lactose Glucuronide Agar (MLGA) (OXOID, Basingstoke, UK) plates to distinguish *E. coli* from coliforms. From each sample condition, 3 g of material was removed and mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube. To ensure effective homogenisation, samples were subjected to vortexing for 1 min (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA) prior to settling for a further 20 s. The supernatant was then removed for serial dilution. Samples were serially diluted with MRD to ensure CFUs were <80/plate. Samples were passed through 0.45 µm filters (Millipore S-PAK® 47 mm, Watford, UK) using a 3-way vacuum manifold (CombiSart®, Sartorius UK Ltd, Surry, UK). Filters were placed on MLGA plates and incubated at 30 °C (±1 °C) for 4 h (±1) h and 37 °C (±0.5 °C) for 18 (±2) h as described by the Environment Agency (2003). Arising green colonies were counted as presumptive *E. coli*, yellow colonies were counted as coliforms, and pink colonies were recorded as non-coliforms. All *E. coli* enumeration results were normalised against the percentage of dry solids (DS%). Three gram of the sludge sample was used for DS determination and analysis was performed as per the Environment Agency (2003) guidance.

Flow cytometry measurement

For flow cytometric analysis, 1 g of the sample was vortexed for 30 s with 9 g of MRD to form a liquid-sludge mix. Samples were sonicated twice for 2 min (Grant Ultrasonic Bath XUBA1, Grant Instruments Ltd, Cambridgeshire, UK) to enhance the disaggregation of sludge flocs within the sample and disperse bacteria from the sludge particle attachment. Once sonicated, samples were passed through a 20 µm filter (Cat. No.: 1004-070, Whatman™, GE Healthcare, Buckinghamshire, UK) using a vacuum pump (No35.1.2AN.18, KNF Neuberger Ltd, Witney, UK) to remove large sludge particles from the sample, preventing interference with flow cytometry measurement (FCM) (Foladori *et al.* 2010).

Serial dilution was completed on the prepared sample suspension using filtered, cell-free (0.1 µm; Millex, Merck Millipore Ltd, Tullagreen, Ireland) bottled mineral water (EVIAN, EVIAN-les-Baines, France), so that the concentration measured with the flow cytometer was always less than 10^6 counts mL^{-1}. Filtered mineral water is often used as a diluent for flow cytometric analysis as it provides osmotic stability (Hammes *et al.* 2012; Gillespie *et al.* 2014).

FCM was completed as described by Gillespie *et al.* (2014) and Foladori *et al.* (2007, 2010). SYBR Green I (10,000 × stock, Cat. No.: S-7567, Life Technologies Ltd, Paisley, UK) was diluted with dimethyl sulfoxide (DMSO, Fisher Scientific, Fair Lawn, NJ, USA) to obtain a working stock solution of 100×. For the measurement of intact and damaged cell concentrations, a dye mix containing five parts of 100 × SYBR Green I and one part of propidium iodide (PI; 1.0 mg/mL) (Cat. No.: P3566, Life Technologies Ltd, Paisley, UK) was prepared. About 2.76 µL of this dye mix was added to 230 µL of the sample. Following the addition of the dye, samples were placed in an incubator shaker (Grant-Bio PHMP, Grant Instruments Ltd, Cambridgeshire, UK) set at 620 rpm at 37 °C for 13 min with the lid closed to ensure homogeneous temperature conditions and to avoid light exposure (Gillespie *et al.* 2014). Following incubation, sample volumes of 50 µL were analysed using a BD Accurri C6 flow cytometer equipped with a 488 nm solid state laser (Becton Dickinson UK Ltd, Oxford, UK).

Green fluorescence was collected in the 'FL-1' channel at 533 nm, and red fluorescence was collected in the 'FL-3' channel at 670 nm with the trigger set on FL-1. Only signals above an FL-1 threshold of 2,000 were recorded. No colour compensation was used. A fixed gate, previously defined

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by Gatza *et al.* (2013), was used as a template with the corresponding instrument settings to determine the intact cell concentration (ICC). Data were processed using the Accurri C6 software (Becton Dickinson UK Ltd, Oxford, UK). Damaged cell concentrations were defined by a second fixed gate using the guidance from Foladori *et al.* (2007, 2010). Figure 1 shows the gate positions used.

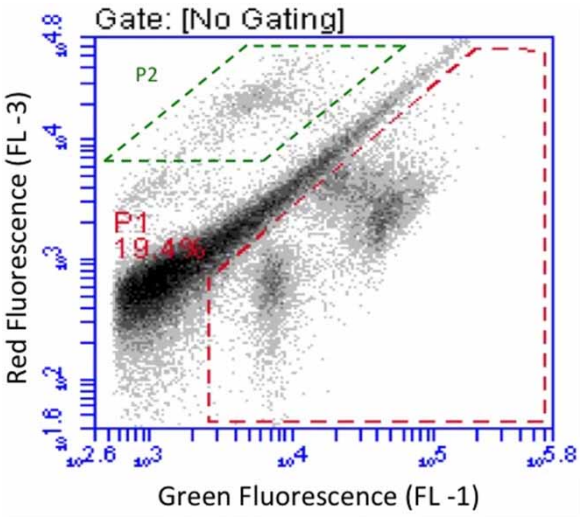

Figure 1 | Representative FCM cytogram showing distribution of microbial clusters in control sample. Signals from intact cells are within the P1 (red) gated region (Gatza *et al.* 2013) and signals from damaged bacteria (propidium iodide stained cells) are shown in P2 (green) gated region (Folardori *et al.* 2007, 2010). Other signals are caused by organic and inorganic background (Harry *et al.* 2016).

All measurements were performed in triplicate and FCM analysis at time 0 was always taken pre- and post-disruption treatment.

All bacterial numbers, both from membrane filtration and FCM, were referred as the percentage dry solids (DS%) content for normalisation based on Environment Agency (2003) methods.

Dissolved organic carbon analysis

Dissolved organic carbon (DOC) determination was conducted using a Series 2000 autosampler (Burkard, Uxbridge, UK) following methods supplied by Burkard Scientific. For sample preparation, 1 g of the sludge cake was vortexed for 30 s with 9 g of deionised water to form a liquid-sludge mix. Samples were sonicated twice for 2 min (Grant Ultrasonic Bath XUBA1, Grant Instruments Ltd, Cambridgeshire, UK) to enhance the disaggregation of sludge flocs within the sample. Once sonicated, samples were passed through a 20 μm filter (Cat. No.: 1004-070, Whatman™, GE Healthcare, Buckinghamshire, UK) using a vacuum pump (No35.1.2AN.18, KNF Neuberger Ltd, Witney, UK) to remove sludge particles. The persulfate ultraviolet oxidation method was used to measure DOC concentrations in the prepared sample (APHA 1995).

Statistical analysis

Using STATISTICA (Version 12, Tulsa, USA), a normality assessment was conducted on the dataset using the histogram and normal probability plot programme function. Variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá 2007). A repeated-measures ANOVA was performed to identify statistically significant difference ($p < 0.05$) between and within the conditions tested. In individual tests, the dependent variable was set as either 'damaged cell concentration', '*E. coli*' or 'DOC'. Treatment conditions such as 'undisrupted' and 'disrupted' were set as the categorical factors. The validity of the ANOVA assumptions, including normality of residuals

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(Gujarati 2011), were tested through residual analysis. A post-hoc analysis (Fisher's LSD) was carried out to assess the distinct differences between experimental conditions (Marques de Sá 2007).

RESULTS

Thermal disruption

A significant difference was found between levels of damaged bacteria in undisrupted (control) and disrupted treatments (Figure 2). For thermal treatments at 62 °C for 80 min, results show a significant increase of 24.7% in the proportion of damaged cells. A gradual increase in damaged cells is observed up to 80 min of exposure. Autoclave treatments showed a significant increase of 36% in damaged cell concentration when compared with undisrupted samples. In comparison with samples exposed to 62 °C for 120 min, levels of damaged cells were 11.3% higher in autoclaved treatments.

Thermal disruption treatments significantly reduced levels of *E. coli* bacteria. The greatest reduction was observed after 80 min of exposure where *E. coli* concentrations fell to 0.8 Log and were significantly different from other treatments at day 1 (Figure 3(b)). In contrast, after 120 min of exposure, *E. coli* concentrations only showed a reduction to 2.8 Log (Figure 3(c)). For samples exposed to a greater degree of thermal disruption, a longer recovery time was necessary for *E. coli* populations to return back to pre-disrupted levels or above. Undisrupted samples retained a relatively stable concentration of *E. coli*, averaging 5.9 Log throughout the 10-day test period.

Thermal disruption of samples consistently resulted in an increase in peak *E. coli* concentration when compared with undisrupted treatments. A disruption time of 20 min peaked at 7.7 Log, 80 min at 7.2 Log, and 120 min showed a peak *E. coli* concentration of 7.7 Log. Peak *E. coli* concentrations for each respective treatment were significantly different ($p < 0.05$) from undisrupted samples. By day 10, observations of the 20- and 80-min disruption treatments showed a plateau in *E. coli* concentrations at 7.3 and 7 Log, respectively. This result was significantly higher from the undisrupted sample concentration. For 120 min of exposure, no significant difference was shown with undisrupted samples at day 10.

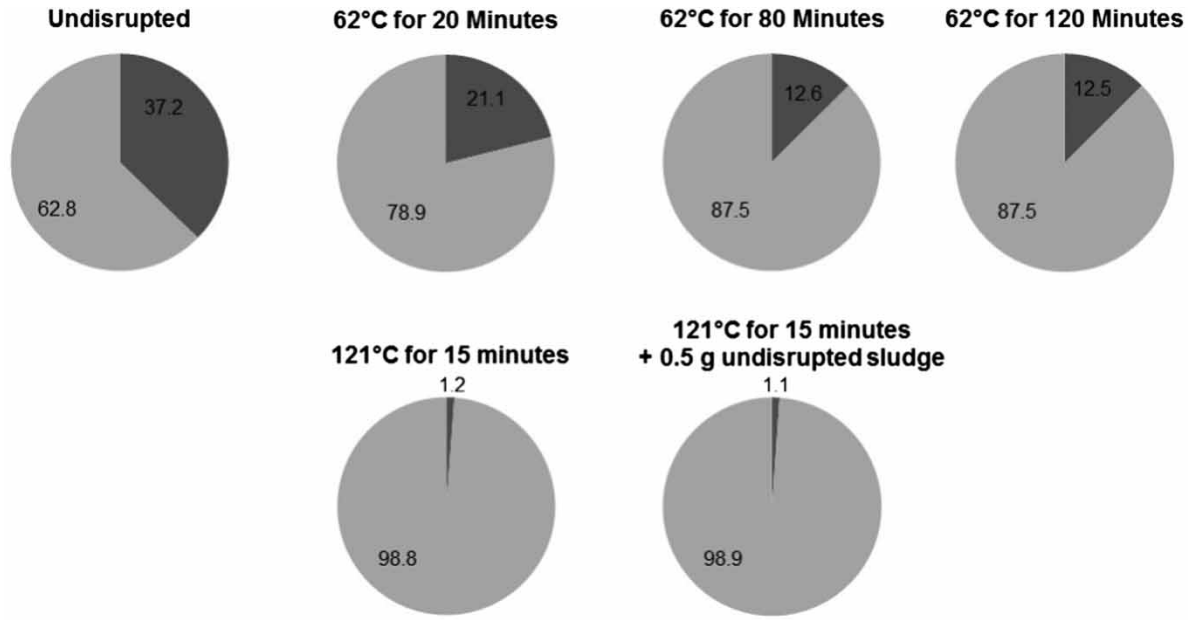

Figure 2 | Proportions of intact and damaged cells in samples after thermal disruption treatments. Samples thermally disrupted at 62 °C for 20, 80 and 120 min or autoclaved at 121 °C for 15 min at 1.03 bar (±0.03 bar). A second autoclaved sample included 0.5 g of undisrupted sludge in addition to reintroduced sludge-derived *E. coli*. Chart segment numbers indicate the % of intact or damage cells. Key: ■ % intact, ■ % damaged.

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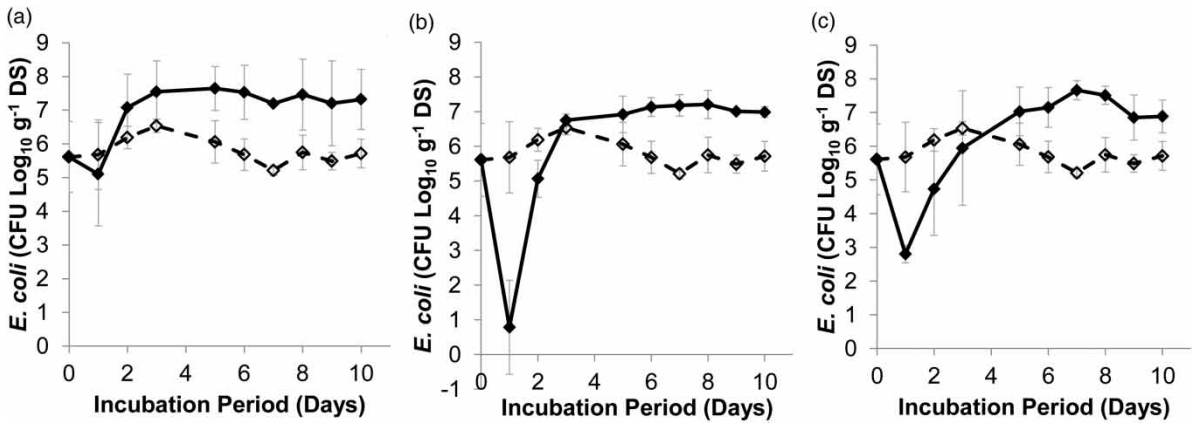

Figure 3 | *E. coli* concentration during controlled storage at 22 °C for 10 days after thermal disruption treatment at 62 °C for (a) 20 min (—◆—), (b) 80 min (—◆—) and (c) 120 min (—◆—). All plots include *E. coli* concentrations recorded from undisrupted control condition (—◇—). Time 0 represents *E. coli* concentration prior to disruption treatments. Error bars represent the standard deviation of three repeats ($n = 3$).

E. coli concentrations fell significantly to undetectable levels after autoclave disruption (Figure 4(a)). For samples inoculated with 0.5 g of the undisrupted sample after autoclaving, *E. coli* concentrations were raised to 2.1 Log at day 1. A significant difference ($p < 0.05$) in peak *E. coli* concentrations was observed between the two autoclaved treatments. A longer recovery time of 6 days was necessary for autoclaved *E. coli* populations to return to pre-disrupted levels. Autoclaved treatments showed a steady increase in *E. coli* concentrations over the test period reaching 6 Log on day 10. For samples inoculated with 0.5 g of the undisrupted sludge, *E. coli* recovery time was faster and peaked after 3 days at 7.3 Log. A comparison with undisrupted samples showed only the autoclaved treatment with the additional 0.5 g undisrupted sample to have a significantly higher peak *E. coli* concentration (Figure 4(b)).

Physical disruption

Physical disruption significantly increased the proportion of damaged cells in the sludge sample by 51.6% (Figure 5). A significant increase of 44,484 mg/kg DS in levels of DOC after physical disruption treatment was observed.

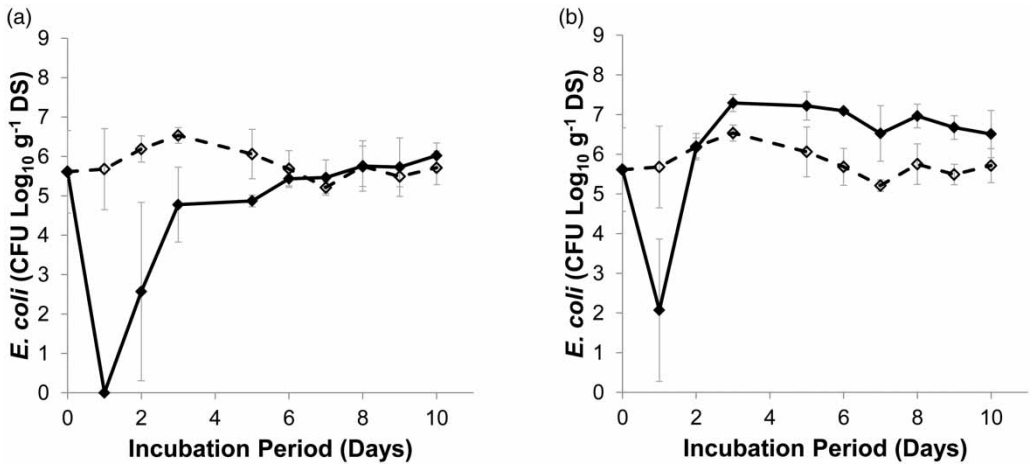

Figure 4 | *E. coli* concentration during controlled storage at 22 °C for 10 days after samples thermally disrupted through autoclaving for 15 min at 121 °C (pressure = 1.03 bar ±0.03). Key: (a) —◆— autoclaved sample, (b) —◆— autoclaved sample inoculated with 0.5 g of the undisrupted sludge sample to reintroduce sludge-derived *E. coli*. Both conditions plotted against undisrupted control conditions (—◇—). Time 0 represents the *E. coli* concentration prior to disruption treatments. Error bars represent the standard deviation of three repeats ($n = 3$).

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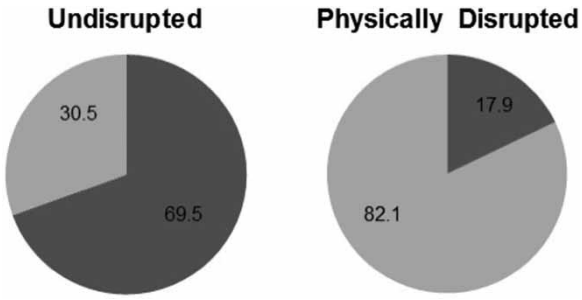

Figure 5 | Proportions of intact and damaged cells in samples after physical disruption treatment and in the undisrupted control condition. Chart segment numbers indicate the % of intact or damaged cells. Key: ■ % intact, ■ % damaged.

A significant increase of 3 Log was observed in the levels of *E. coli* bacteria between time 0 and day 3 of storage for physically disrupted samples (Figure 6). Undisrupted samples remained at a consistent level of 6.6 Log over the 10-day test period. *E. coli* concentrations in physical disruption treatments increased steadily, peaking at 9 Log on day 4 of storage. The peak *E. coli* concentration in disrupted samples was significantly different from undisrupted treatments ($p < 0.05$). Between day 4 and day 10, *E. coli* concentrations in disrupted samples reduced steadily by 1 Log, despite remaining above control treatment conditions.

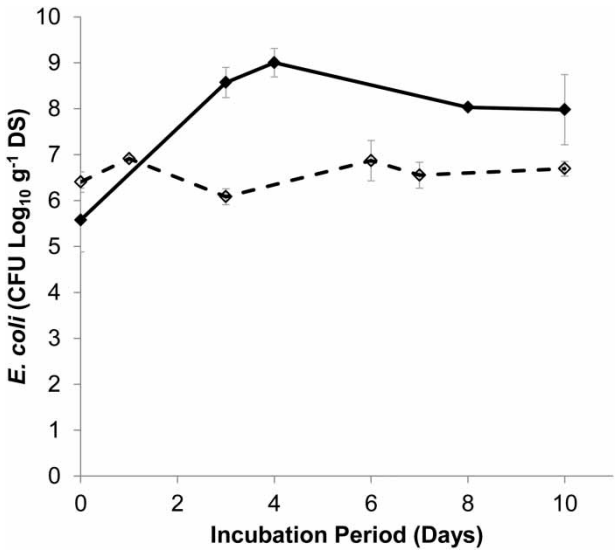

Figure 6 | Effect of physical disruption treatment on *E. coli* bacteria concentration plotted against control (undisrupted) sample. Key: —◇— undisrupted control, —◆— physically disrupted sample. Error bars represent the standard deviation of three replicates ($n = 3$).

DISCUSSION

Microbial and nutrient response to effects of disruption treatments and growth in subsequent storage

Indicator bacteria increase in stored biosolids has been attributed to the effects of mechanical dewatering. Researchers highlight possible effects of shear impacting upon the integrity of the sludge matrix and causing the release of nutrients, which may support bacterial growth (Higgins *et al.* 2007b; Chen *et al.* 2011; Sun *et al.* 2015). Flocculation turbulence and pressure from mechanical operations on flocs can cause cell damage (Sun *et al.* 2015). Further to this, researchers have identified cell lysis as a response to mechanical shear and also heat stress factors (Chen *et al.* 2005; Noor 2015). This research work assessed the impact of

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thermal and physical disruption on *E. coli* behaviour in stored biosolids after initial on-site centrifuge dewatering treatments. Laboratory treatments were conducted as a proof of principle to study the effect of cell disruption on *E. coli* dynamics and therefore were not intended to replicate on-site biosolids production processes. In order to clarify whether cell disruption increased available nutrients able to support remaining cell survival (Murate *et al.* 2012), measurements of DOC were undertaken and *E. coli* enumeration completed in the subsequent days of storage to determine peak concentrations.

Results showed a significant effect of disruption treatment, both thermal and physical, on levels of damaged cells within test samples. Longer exposure to temperature treatments (62 °C) and the combination of high temperature and pressure (autoclaving) caused intact cell numbers to fall by 36%. These ICCs were compared with *E. coli* enumeration data and reflected similar trends with a reduction in levels of culturable *E. coli* bacteria after thermal disruption treatments. A methodological limitation identified from the thermal disruption treatments was the lower level of *E. coli* bacteria inactivation identified after 120 min exposure to 62 °C where *E. coli* concentration was only reduced to 2.8 Log. This result was not comparable to the elevated level of cell damage identified in FCM data for 120 min and may have been due to sludge flocs protecting *E. coli* bacteria and reducing effects from thermal stress (Chang *et al.* 2001; Qi *et al.* 2007). The presence of highly aggregated structures or flocs in the tested sludge cake where bacteria may be embedded (Orruño *et al.* 2013) could protect cells from the 62 °C disruption treatment and enable them to remain in a viable state and grow when returned to a lower, more favourable storage temperature (such as the 22 °C incubation temperature). Therefore, pre-treatment to disrupt flocs may have been a beneficial stage of sample preparation.

Physical disruption was likely to be more similar to actions experienced in the sludge matrix during mechanical dewatering. Although effects of physical disruption treatments on *E. coli* were less prominent, FCM data showed damaged cells to increase by 51.6% in tested samples, indicating that other, perhaps more sensitive, sludge-derived bacteria were affected by the treatment. For *E. coli* concentrations, the reductions displayed after disruption treatments emphasised the inhibition of culturable cells. Though *E. coli* colonies were reduced on the culture media (in some cases to undetectable levels), this cannot be attributed to cell death and most likely reflect a state of VBNC in cells exposed to disruption treatments. Oliver (2010) suggested that incubation outside of the normal temperature range of growth can induce the VBNC state and is a common response to stress in bacterial cells. This becomes apparent from the *E. coli* growth curves generated in the following days of storage at 22 °C where, in all disrupted treatments, concentrations recovered to pre-disrupted levels. Oliver (2010) studied the effects of temperature on culturability in three strains of *Vibrio vulnificus* incubated at 11 °C to induce a VBNC state (*V. vulnificus* enters the VBNC state when exposed to temperature <13 °C). After a temperature upshift to 22 °C for 24 h, all three strains tested recovered to levels above 10^4 CFU mL^{-1} (Oliver 2010). The 22 °C incubation temperature may have provided an adequate environment for cell reactivation and growth where viable cells could utilise the nutrients released from cells damaged during disruption treatment. Likely evidence of the VBNC mechanism may be identified in autoclave treatments where CFUs reached undetectable levels after treatment but recovered to 2.6 Log within 24 h of incubated storage. A longer storage period may have allowed the peak *E. coli* concentration to be reached in autoclaved treatments as cell recovery time was slower. However, the significant change in concentration between time 0 and 24 h suggests that cells were able to recover and grow rapidly after such an extreme thermal treatment. Higgins *et al.* (2007a) highlighted that after dewatering, conditions develop that encourage rapid growth of coliforms with concentrations peaking in 1–3 days of storage. That study attributed reactivation of VBNC cells to shear in mechanical dewatering releasing growth factors which reactivate bacteria to a culturable state (Higgins *et al.* 2007b). In the present experiments, growth factors such as cellular nutrients have been quantified through the surrogate measure of DOC concentrations. Data showed a significant rise in levels of DOC present in the sludge matrix after disruption treatments (DOC increased by 4,448 mg/kg DS after physical disruption of samples), which coincided with elevated concentrations of *E. coli* bacteria. This finding is similar to a study by Franz *et al.* (2008) who found microbial activity

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per unit of biomass to be positively correlated with concentrations of DOC in manure-amended soils. Natural sources of carbon in sewage sludge will be present in organic compounds (Sigma Aldrich 2016); however, this may not be in a bioavailable form. Carbon is an important component of cellular material (Todar 2012) and, therefore, a critical element required for microbial growth. Tang *et al.* (2012) observed growth stimulation of *E. coli* bacteria in assays with DOC-rich samples. In biologically treated sewage effluent, Shon & Vigneswarn (2007) highlighted the constituents of DOC, which included amino acid, polysaccharide and protein. It is likely that the constituents of DOC identified by Shon & Vigneswarn (2007) are also present (perhaps to a more limited extent) in sewage sludge and, therefore, the increase in DOC observed after laboratory disruption treatments is a relevant measure of increasing nutrient levels in the biosolids tested. As sewage sludge cake is known to be nutrient limiting after stabilising up-stream AD processes (Smith *et al.* 2005), it is likely that any increase in nutrient availability will create a favourable effect on the growth of microorganisms.

In these experiments, physical and thermal stress was imposed on the biosolids material with the aim of increasing cell disruption (lysis), which was quantified using FCM of damaged cells. In the experiments conducted, cell lysis was achieved from the treatments imposed and is verified by the increase in DOC levels in disrupted samples. Shon & Vigneswarn (2007) show DOC to include cell fragment, extracellular enzyme, DNA, RNA and other components of the cellular material. The cellular material from the disrupted cells is able to be used by the remaining cell population to support growth. In the present study, the growth of *E. coli* bacteria during incubated storage of the disrupted samples consistently showed a significantly higher peak *E. coli* concentration when compared with undisrupted treatments, indicating an increase in available nutrients for growth.

These findings suggest that cell disruption does provide a substrate source able to support the reactivation and growth of *E. coli* bacteria in the sewage sludge cake stored at 22 °C for 10 days and therefore the hypothesis tested can be accepted.

CONCLUSIONS

1. Thermal and physical disruption treatments were successful methods to increase levels of cell damage within a sample.
2. Increase in damaged cells coincided with higher DOC concentrations indicating cell lysis and an increase in available nutrients for the remaining viable cells.
3. Peak *E. coli* concentration was significantly higher for disrupted samples in comparison to undisrupted treatments, suggesting the utilisation of cellular nutrients for growth when samples are placed in favourable growth conditions (22 °C).
4. The likely release of cellular nutrients from cells damaged during mechanical dewatering may be a contributory factor elevating *E. coli* concentrations in stored biosolids.

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DATA ACCESS STATEMENT

All data are provided in full in the Results section of this paper.

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