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Characterisation and Control of the Biosolids Storage Environment: Implications for *E. coli* Dynamics

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Abstract

E. coli survival in biosolids storage may present a risk of non-compliance with guidelines designed to ensure a quality product safe for agricultural use. The storage environment may affect *E. coli* survival but presently, storage characteristics are not well profiled. Typically biosolids storage environments are not actively controlled or monitored to support increased product quality or improved microbial compliance. This two-phased study aimed to identify the environmental factors that control bacterial concentrations through a long term, controlled monitoring study (phase 1) and a field-scale demonstration trial modifying precursors to bacterial growth (phase 2). Digested and dewatered biosolids were stored in operational-scale stockpiles to elucidate factors controlling *E. coli* dynamics. *E. coli* concentrations, stockpile dry solids, temperature, redox and ambient weather data were monitored. Results from ANCOVA analysis showed statistically significant ($p < 0.05$) *E. coli* reductions across storage periods with greater die-off in summer months. Stockpile temperature had a statistically significant effect on *E. coli* survival. A 4.5 Log reduction was measured in summer (maximum temperature 31°C). In the phase 2 modification trials, covered stockpiles were able to maintain a temperature >25°C for a 28 day period and achieved a 3.7 Log *E. coli* reduction. In winter months *E. coli* suppression was limited with concentrations >6 Log_{10} CFU g^{-1} DS maintained. The ANCOVA analysis has identified the significant role that physical environmental factors, such as stockpile temperature, has on *E. coli* dynamics and the opportunities for control.

Keywords

Biosolids, Sewage Sludge, *E. coli,* Pathogens, Log Reduction, Temperature

Introduction

Biosolids recycling to agricultural land is one of the processes in the sludge value chain (Ofwat, 2015). The requirement to monitor faecal indicator microorganisms before land application acts as a safeguard for public health. However, *E. coli* resurgence is frequently observed in biosolids storage and therefore poses a risk of non-compliance with sludge quality requirements (Sprigings and Le, 2011). Although anaerobic digestion provides stabilisation and additional benefits of reduced biological activity in sludge (Chen *et al.,* 2011), high concentrations of *E. coli* in anaerobically digested biosolids have been observed immediately after mechanical dewatering (Monteleone *et al.,* 2004; Higgins *et al.,* 2007a; Dentel *et al.,* 2008; Qi *et al.,* 2008; Chen *et al.,* 2011; Fane *et al.,* 2019a). Post-dewatering coliform bacteria changes have been known to range from -0.4 Log to +6.4 Log units (Qi *et al.,* 2007) emphasising the highly variable bacterial behaviour amongst treatment plants. This unpredictable indicator fluctuation in storage causes much concern for utility operators and can affect the confidence of users of sludge products. Higgins *et al.,* (2007a) and Fane *et al.,* (2019a) argue that environmental conditions after mechanical dewatering in the biosolids matrix can favour the growth of faecal indicator organisms. A study of biosolids produced after thermophilic and mesophilic digestion treatments and centrifuge dewatering found that *E. coli* concentrations during storage at 35°C increased within the first three days to between 10^8 and 10^9 colony forming units (CFU) /g DS (Higgins *et al.,* 2007a).

Although regulations apply for final product quality following storage, limited regulation or control of the dewatered sewage sludge storage environment exists. Controls on the storage environment focus upon preventing surface and ground water contamination (Environment Agency (UK), 2012) with no advisory comments on the best practice to support bacterial die-off. A typical sludge cake bay holds between 200 to 250 tonnes of biosolids and is constructed with a concrete base with run off channeled to surface drains. Concrete side walls segregate bays and no top cover is used. When evaluated in the context of up-stream sludge treatment processes, where environmental parameters are highly monitored and controlled, the storage treatment of the biosolids material could be enhanced to meet microbial compliance requirements. The European Union (2000) requires >2 Log_{10} *E. coli* reduction for conventional treatments. In the United Kingdom the Environment Agency (UK) (2003a) requires a minimum 2 Log_{10} *E. coli* reduction (99% pathogens destroyed) with a maximum allowable concentration threshold value of 10^5 *E. coli* CFU per grams dry solid (ADAS 2001). The United States Environmental Protection Agency (2003) has a minimum standard requiring the geometric mean of 7 samples to be $<2 \times 10^6$ most probable number (MPN) faecal coliforms per gram of total solids at the time of use or disposal.

Limited research has been completed on the characteristics of the biosolids storage environment in temperate climates and the effects of physical parameters on *E. coli* bacteria dynamics. The key factors responsible for indicator growth and survival in stored dewatered biosolids and their relative importance have not yet been clearly identified (Dentel *et al.,* 2008). The immediate elevation in indicator numbers post-dewatering and the prolonged survival during subsequent biosolids storage, suggests parameters of the storage environment may be controlling factors (Higgins *et al.,* 2007a; Fane *et al.,* 2019a). However, previous studies are often limited to laboratory scale trials and short term experiments (Iranpour *et al.,* 2005; Lang and Smith, 2008; Qi *et al.,* 2008; Sprigings and Le, 2011), due to the practicalities involved with operational scale studies and the reduced level of experimental control achieved when working at such scales. This prevents an understanding of the seasonal impact

on stockpile characteristics and of the environmental factors controlling *E. coli* dynamics. Conducting operational-scale trials is critical as measurements in smaller biosolids stockpiles are prone to fluctuation.

An understanding of *E. coli* behaviour in biosolids could be inferred from studies in analogous environments (Fane *et al.,* 2019b). Plachá *et al.,* (2001) studied the survival of *Salmonella typhimurium* during summer and winter field storage of solid fraction pig slurry. Findings showed temperature to be the most important factor affecting the survival of microorganisms in the environment (Plachá *et al.,* 2001). In the 17-26°C summer and -1 to 30°C winter/spring tests the maximum survival period of *Salmonella typhimurium* was 26 days and 85 days, respectively. Similarly, results obtained by Nicholson *et al.,* (2005) on the survival times of pathogens in livestock solid manure heaps showed *E. coli* 0157 concentrations reached undetectable levels in less than 10 days of storage between June and July where temperatures >55°C were maintained (Nicholson *et al.,* 2005).

Findings on temperature were reported by Sprigings and Le, (2011) on biosolid treatment sites. The cake pad temperature immediately after centrifuge dewatering was recorded as 30 ±2.5°C on two treatment sites, with this temperature remaining for approximately 12 hours, probably due to residual heat from the digestion process. Over the following 48 hours the temperature profile gradually reduced and fell below ambient temperature (Sprigings and Le, 2011). This temperature dataset is not representative of the biosolids storage environment as treatment requires a minimum 14 day storage period after mesophilic digestion (DEFRA, 2006) and impact of seasonality was not investigated.

Empirical statistical models were developed by Lang *et al.,* (2007) which summarised the relationship between soil temperature, moisture content, time and *E. coli* populations in biosolids-amended soil. Analysis confirmed 'soil temperature' to be the key environmental parameter responsible for the general seasonal patterns in background *E. coli* numbers observed between field trials. The work also showed that enteric bacteria survival in biosolids amended soil was shorter in moist soils and prolonged in dry conditions (Lang and Smith, 2007). Changes in moisture content can affect oxygen and nutrient pools available for organisms (Banach *et al.,* 2009; Peralta *et al.,* 2014). As conditions become more anoxic, microorganisms switch to alternative electron acceptors such as nitrate, iron or sulphur, which alters the dominant metabolic activity (Keddy, 2000; Mitsch and Gosselink, 2007). In up-stream sludge treatment processes, such as digestion, the combination of an oxygen limiting environment and other pernicious reactor conditions has a controlling effect on pathogen concentrations. In particular, oxygen availability has a substantial effect on cell respiration and consequential energy production, regulating most cell activities (Alberts *et al.,* 2010). Mechanical dewatering causes substantial change of environmental conditions in the sludge product (Higgins *et al.,* 2007b; Chen *et al.,* 2011) and can often amplify oxygen exposure (Chen *et al.,* 2011). Oxygen can disrupt methanogens (Chen *et al.,* 2007; Chen *et al.,* 2011) and therefore provide a selective advantage for *E. coli* bacteria growth (Fane *et al.,* 2019a*)*. Redox potential measurements can give an indication of the oxygen availability and a shift in microbial metabolic pathways (Peralta *et al.,* 2014), however redox datasets for biosolids storage environments are limited.

The studies above highlight the effects of physical parameters on bacterial survival in biosolids and analogous environments. However, previous studies tend to be based on short term measurements and are often focussed upon indictor bacteria growth immediately after mechanical dewatering. Hence there is limited understanding of the characteristics of the biosolids storage environment at an operational scale and of the multiple variables controlling *E. coli* dynamics, which in turn limits our ability to design storage areas that consistently ensure compliance with biosolids quality requirements. This 2-phased study investigated the characteristics of the biosolids storage environment through (phase 1) a long term, operational-scale storage monitoring programme and

(phase 2) a storage modification demonstration trial (phase 2). The aim of phase 1 was to monitor the environmental conditions of the sludge matrix and increase understanding of the parameters controlling *E. coli* concentrations. Phase 2 built upon the outputs of phase 1 by testing biosolids storage environment modifications to control *E. coli* growth and death rates.

1. Methodology

2.1 Sludge Samples Source

Representative bulk samples of mesophilic digested and centrifuge dewatered sludge cake were collected from a wastewater treatment works serving a population equivalent (PE) of 440,000. Mesophilic sludge was the focus of the study due to this treatment being most common across UK wastewater sludge treatment sites. The samples were transported to the trial sites in 25 tonne capacity haulage trucks. The cake was taken immediately after centrifuge dewatering to ensure the product was less than 24 hours old when reaching the trial site, this allowed tested storage environments to begin from a day 1 assumption.

2.2 Phase 1: Monitoring

The monitoring programme was based on 1.5 m^3 sewage sludge stored for a period of 12 months. Monitoring was undertaken on a fresh load of sludge cake each quarter to enable seasonal comparisons. Quarter 1 ran from October to December, Quarter 2 January to mid-April, Quarter 3 mid-April to June and Quarter 4 July to September. Although three months storage is substantially greater than the post-digestion 14 day storage period recommended on treatment sites operating mesophilic digestion (DEFRA 2006), it is not uncommon for sewage sludge cake to be stored for up to six months if the product fails compliance tests. Additionally, stockpiles can be stored on agricultural land for long periods of time before spreading.

Three galvanised steel cages (1.5 m^3) were designed to contain the dewatered sewage sludge cake (J.R. Trolleys, Bedfordshire, England) and situated on an open concrete pad at Cranfield University (Bedfordshire, England). Cages were lined with an interlocked, knitted high-density polyethylene (HDPE) mesh to allow drainage and gas exchange whilst holding the sludge cake in the cages (Secure Covers, Shropshire, UK).

Temperature and redox potential data were collected at hourly intervals from the three cage stockpiles during the monitoring study, using a CR800 measurement and control system powered by a 12 V alkaline battery (Campbell Scientific, Loughborough, UK). Temperature (CS655-DS, Campbell Scientific, Loughborough, UK) and redox (Paleo Terra, Amsterdam, Netherlands) sensors were fitted into 1 m length probes, with 3 sensors per probe and a total of 3 probes for temperature and 3 for redox vertically inserted per cage as illustrated in Figure 1. This arrangement allowed for a grid-type characterisation of the stockpile (nine data points), recording temperature and redox for low (Y1), middle (Y2) and top (Y3) positions across cage depth and front (X1), middle (X2) and back (X3) across cage width (side section view, Figure 1). This grid was designed to capture sufficient spatial variations across each of the cages whilst maintaining a realistic biosolids stockpile density. The grid was based on the theory that there would be stockpile variance across the X and Y (horizontal and vertical) axis. It was acceptable to estimate that independent samples could be taken from the nine data points on the grid as parameter measurements would independently vary. The grid formation created three replicates in a single cage for depth assessment (Y axis) (Figure 1). Separate reference electrodes (Paleo Terra, Amsterdam, Netherlands) were installed into each stockpile and individually connected to the data logger, maintaining a separate redox data recording system for each of the three stockpiles. The final redox potential value (E_h) was calculated by adding the potential from the

reference electrode (E_{ref} = -144 mV) to the measured potential (E_m) (Vorenhout van der Geest and Hunting 2011). Probes were connected to the data logger system through two AM16/32B 16/32 channel relay multiplexers (Campbell Scientific, Loughborough UK), which were housed in mounted enclosures to protect the equipment during the 12 month study (TM-ENC-MOUNT, Campbell Scientific, Loughborough, UK). All temperature and redox probes were placed into the cages during filling and data was downloaded at the end of each storage period.

To obtain monthly samples for microbial enumeration from the stockpiles, acrylic plastic containers (Amari Plastics, Bedford, UK) were designed and installed across the cage during filling. These consisted of semi-circular tubes with 15 mm diameter holes to allow drainage (tube detail, Figure 1). The tubes were fitted with baffles every 150 mm to (i) ensure sludge was retention when removed for sampling and (ii) to guarantee the independence of the samples. A total of 12 tubes were installed per cage (Figure 1). The tubes were used sacrificially, with one tube extracted from the top, middle and bottom section of the stockpile for each sampling time point, again allowing for a nine point grid characterisation of the stockpile. This sampling approach ensured a low level of disturbance within the tested stockpiles.

The four quarters studied across the 12 month period gave a temporal factor to the study allowing seasonal variations to be determined.

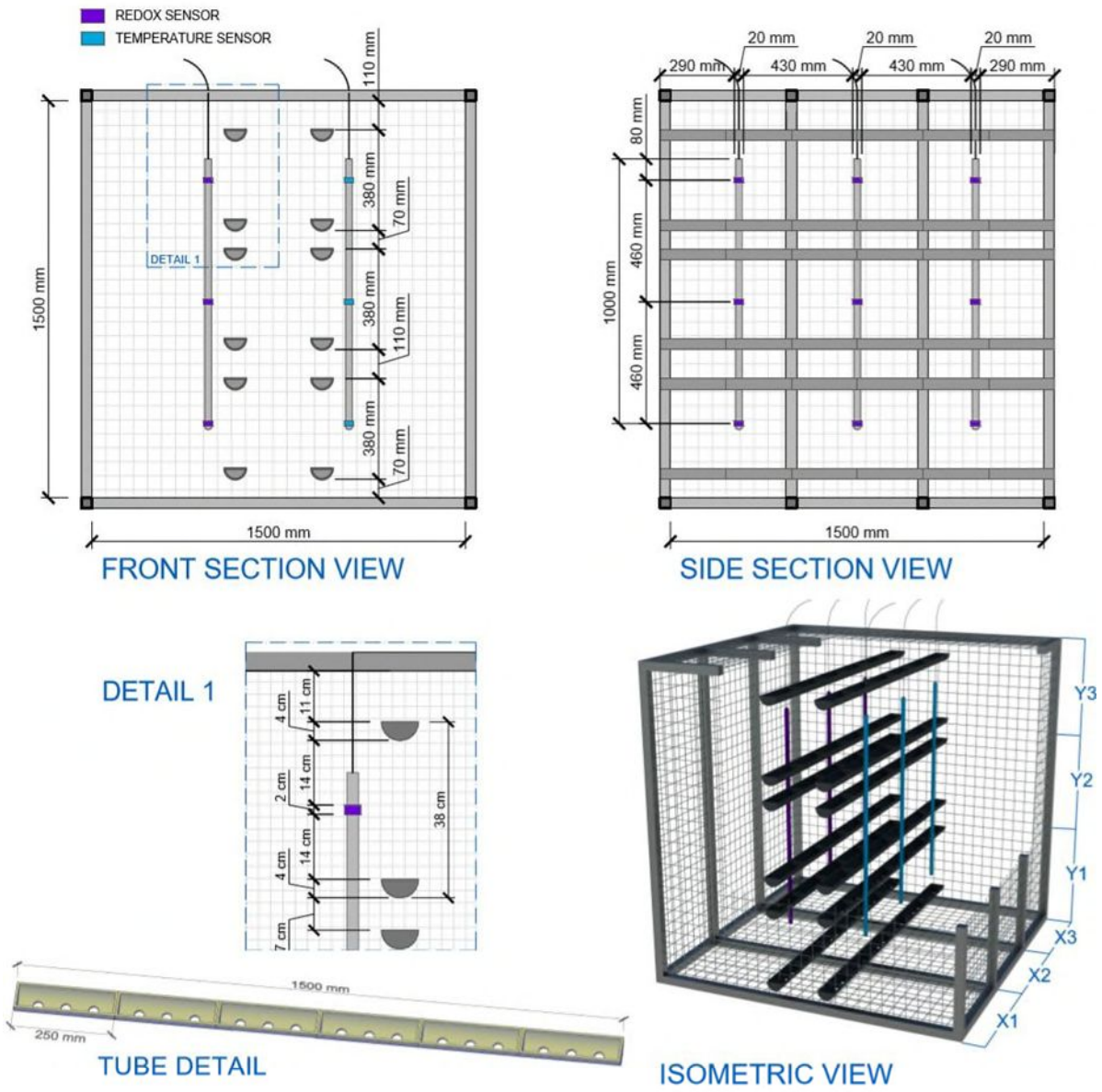


Figure 1: Schematic of temperature and redox monitoring probes and sample tubes arrangement within the 3 x 1.5 m^3 cages. Dimensions are approximate values. Key: sample tubes for microbial enumeration, redox sensor/probe and temperature sensor/probe.

Ambient weather fluctuations were monitored for the duration of each storage period using a wireless weather centre (Maplin Electronics, Rotherham, England). Specific focus was given to precipitation levels and ambient temperature, which were recorded at 30 minute intervals.

2.3 Phase 2: Modification

The storage modification trial was repeated in winter and summer months. The winter trial ran for 69 days between January and March and the summer trial ran for 28 days from June to July. Sewage sludge cake was placed in triplicate 20 tonne stockpiles with two storage conditions tested in each season. These included an open control (typical of storage practices on operational treatment sites) and a covered treatment using a membrane designed to insulate the stockpile and keep rain off for improved stockpile integrity (Airbeam Roller Stockpile Cover, Tim Evans Environment, Surrey, UK). The demonstration trial test site was a flat agricultural field in the North West Midlands, England, chosen

because of its open surroundings, field accessibility for heavy plant equipment and free draining soil which would reduce the impact of water clogging on the tested biosolids stockpiles.

Weekly recording of environmental parameters during the demonstration trials was conducted using hand held meter readers. Monitoring probes recorded temperature (TPA1-N 1 AWE Ltd, Stafford, UK), moisture content (SM 150 soil moisture sensor, Delta-T, Cambridge, England) and redox potential measurements (Paleo Terra, Amsterdam, Netherlands). When taking measurements, the probes were inserted individually into the 20 tonne stockpiles on a horizontal plane and to a depth of 0.5 m following a standard W-shape sampling regime (Carter and Gregorich 2008) before recording the output, this gave 5 replicates per stockpile. To ensure consistency, a timer was used and readings recorded after 1 minute when the probe output had settled. The final redox potential value (E_h) was calculated by adding the potential from the reference electrode (E_{ref} = -144 mV) to the measured potential (E_m) (Vorenhout van der Geest and Hunting 2011).

Weather fluctuations were monitored for the duration of each demonstration trial at 30 minute intervals using a wireless weather centre (Maplin Electronics, Rotherham, England). Specific focus was given to precipitation depth and ambient temperature. Stockpile measurements were temporally co-located with the 30 minute weather centre data using time stamps. The weather centre was located in a field adjacent to the demonstration trial and set up according to the manufacturer's instructions.

2.4 Microbial Analysis

Over each quarter during the 12 month storage monitoring programme measurements of *E. coli* concentrations and dry solids (DS) were taken at four week intervals with the first sample collection completed on day 1 of storage. Samples of approximately 20 g were taken from the sacrificial acrylic sampling tubes (Figure 1) and placed into autoclaved, screw cap, glass universal tubes (FisherBrand, Loughborough, UK). The samples were transported to the laboratory in insulated cool boxes and stored in the dark at temperatures between 2 to 8°C to suppress biological activity. Samples were processed on the day of sampling following the standard protocol for membrane filtration and DS determination (Environment Agency (UK), 2003).

For the storage modification demonstration trial, weekly samples were collected for *E. coli* enumeration and DS determination from each 20 tonne stockpile. Five replicates of approximately 30 g were taken from each of the three stockpiles at a depth of 0.5 m on a horizontal plane using a clean spiral auger (Delta-T, Cambridge, England) following a standard W-shape sampling regime (Carter and Gregorich 2008). This gave 15 replicates per condition for each week of the summer or winter trial duration. Samples were placed in individual 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 to 8°C to suppress biological activity. Samples were processed within 24 hours of sampling.

Membrane filtration (Environment Agency (UK) 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, 3 g of material was mixed with 15 mL of maximum recovery diluent (MRD) (OXOID, Basingstoke, UK) in a universal tube and homogenized by vortexing for 1 minute (Scientific Industries Vortex-Genie 2 50 Hz, New York, USA.) prior to settling for 20 seconds. The supernatant was then removed diluted in a tenfold series 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 (± 1) hours and 37°C (± 0.5°C) for 18 (± 2) hours as described by the Environment Agency (Environment Agency (UK), 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 normalized against percentage dry solids (DS %). A 3 g sludge sample was used for DS determination and analysis was performed as per Environment Agency (UK) (2003) guidance.

The characteristics of the raw data collected were analysed via graphical outputs (error bar plots). In the phase 1 study the *E. coli* values from the replicates were used to calculate the average *E. coli* concentration for Y1, Y2 and Y3 results and associated standard errors. In phase 2, the *E. coli* values from the replicates were used to calculate the average *E. coli* result for each tested storage condition (control and covered) and their associated standard errors. Each replicate was treated as an individual, independent measurement. Section 3 reviews the original raw data values from the phase 1 and 2 studies.

2.5 Statistical Analysis

A repeated measures analysis of co-variance (ANCOVA) was performed to identify statistically significant differences ($p < 0.05$) between the conditions tested. In individual tests '*E. coli* concentration' was set as the dependent variable and 'stockpile temperature', 'stockpile DS%' and 'stockpile redox' were set as the independent variables. Experimental factors including 'quarter', season ('winter' or 'summer') and 'week', 'month' or storage condition ('control' or 'covered') were classified as categorical. Factors set as co-variates for the ANCOVA analysis included 'ambient temperature' and 'rainfall' data. The assumption of normality for each of the independent variables was visually assessed via histograms and normal probability plots using STATISTICA (Version 12, Tulsa, USA). Normality was accepted if standard deviation and skewness was close to zero and the kurtosis gave a standard bell curve. In all instances, variables departing from normality were transformed using a Box-Cox transformation (Marques de Sá, 2007). The validity of the ANCOVA assumptions was tested through residual analysis which confirmed that the residuals were independent, followed a normal distribution with mean zero and constant standard deviation (homoscedasticity) (Gujarati 2011). A post-hoc analysis (Fisher LSD) ($p<0.05$) was completed to assess the distinct differences between groups based on *E. coli* concentrations (Marques de Sá, 2007). Individual parameter datasets were assessed as part of the statistical analysis using Least Square Means to determine significant differences (0.95 confidence interval) between categories such as treatments or storage quarter.

2. Results

3.1. Phase 1: Monitoring

E. coli concentrations (Figure 2) in study quarters 1 and 4 showed a gradual and statistically significant ($p < 0.05$) reduction between the start and the end of each storage period. No statistically significant difference was identified between the starting *E. coli* concentrations in each of the 4 storage quarters, with an average of 6.8 Log_{10} CFU g^{-1}DS. In quarters 1 and 4 a significant reduction of 0.8 Log and 4.5 Log was shown, respectively, between months 1 and 2 of storage. For quarters 1, 2 and 3 no statistically significant change in *E. coli* concentration was identified between month 2 and the end of the storage quarter. Quarter 4 showed an enhanced level of die-off with *E. coli* concentrations significantly reducing from 6.5 Log_{10} CFU g^{-1}DS to 2 Log_{10} CFU g^{-1}DS over the 3 month storage period. The greater standard deviation observed in quarter 4 was due to the enhanced *E. coli* die-off with some data points measuring 0 Log_{10} CFU g^{-1} DS. Statistical analysis confirmed no effect of depth on the *E. coli* concentrations measured across the Y sampling points in the tested stockpiles (Figure 2).

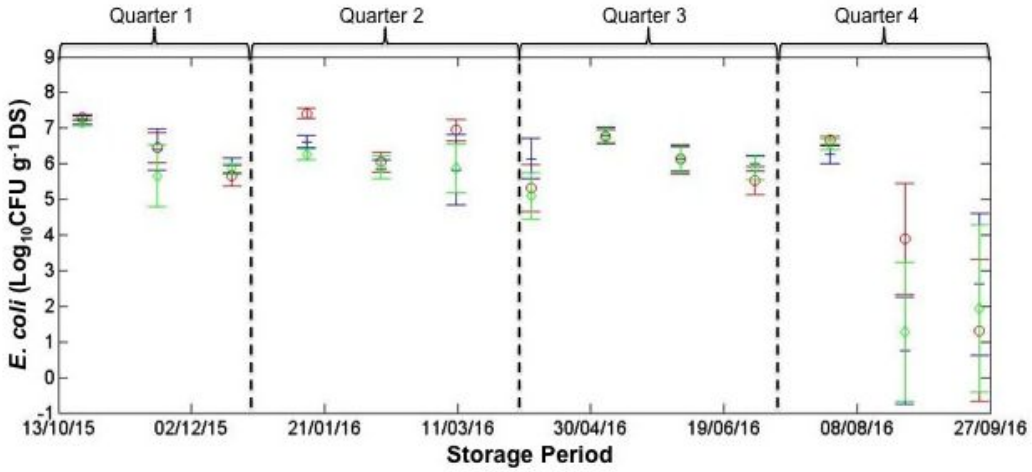


Figure 2*: E. coli* concentration data recorded during the long term biosolids storage monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured data points show the average concentration of 9 replicates. Key: ○ samples taken from Y1, + samples taken from Y2, ◊ samples taken from Y3 (~0.1 m below surface). Error bars represent the standard deviation of 9 replicates.

A statistically significant reduction of 3.6°C in average was displayed between quarters 1 and 2 followed by a significant stockpile temperature increase between quarters 2 and 3 of 6.6°C (average) (Figure 3a). A significant increase of 7.5°C was measured between quarters 3 and 4 which coincided with the enhanced level of *E. coli* die off (observed in Figure 2). No statistically significant change in stockpile temperature was identified with stockpile depth for each individual quarter (Figure 3a). Across the months of storage in each quarter a significant decrease of 5.8°C was shown in quarter 1 and no significant change in temperature was shown from the start to end of quarter 2. In quarter 3 a significant 6.2°C increase in stockpile temperature was displayed between months 1 and 4 and in quarter 4 no difference was shown between the months of storage but stockpile temperatures persisted above 18°C. For ambient temperature (Figure 3a), variation was high between daily measurements. A notable increase in measurement variability occurred on 12/04/2016 and continued for the remaining 6 months of testing as a result of the weather station being moved to a higher elevation due to access restrictions. The average quarterly ambient temperatures observed were 10°C, 5.5°C, 12.3°C and 17.9°C for quarters 1, 2, 3 and 4 respectively. Observations from Figure 3a show that the average stockpile temperature always remained above the average ambient values by a minimum of 0.4°C. ANCOVA analysis showed *E. coli* values to be affected by stockpile temperature ($p= 0.017$).

E. coli concentrations were not significantly affected by DS content across the 12 month study period, despite the significant change in DS content observed between each of the consecutive quarters studied (Figure 3b). Additionally a significant DS difference was identified across the Y axis (Y1, Y2, Y3) of the stored biosolids material. Within study quarters, a significant change between month 1 and month 3 was shown in quarter 1 with the average DS decreasing by 1.3%. Quarter 2 showed an increase in DS between months 1 and 3 of storage, whilst results from quarter 3 displayed a 2% decrease that coincided with more frequent rainfall events. In quarter 4, which had the highest stockpile temperature recording (31°C), a significant increase of 1.9% DS was observed between month 1 and month 2, without further difference thereafter. The total rainfall measured for each consecutive quarter was 235 mm, 224 mm, 343 mm and 176 mm, respectively, and analysis confirmed a significant difference between each of the 4 quarters measured (Figure 3b). The peak rainfall events in quarter 3 coincided with the low DS of 18.6% in month 3.

Redox potential demonstrated a high degree of temporal variability (Figure 3c) across the study period ranging from -165 to -587 mV. Only redox data from probes in the top level of the stored biosolids (Y3) are displayed for quarter 1 as equipment failure restricted full data collection. No significant difference was identified between redox potentials across the Y axis of the stored stockpiles in quarter 1, 2 and 4 (grid points; Y1, Y2, Y3). Only in quarter 3 was a statistical difference found across stockpile depth, with a reduced average redox potential of -362.8 mV recorded in the Y2 (middle) point of the stockpile compared with -332.7 mV in the Y1 (lower) point. No difference was shown between quarters 1, 2 and 4 for redox measurements. However, a significant difference between quarters 1 and 3 was identified with the average redox level changing from -496.4 mV in quarter 1 to -551.5 mV in quarter 3. In quarters 1 and 4 a distinct reduction in the first 48 hours of storage was displayed. The average redox potential in the first 48 hours for quarter 1 reduced from -266 to -502.4 mV, while the quarter 1 average was -496.4 mV. Similarly, in quarter 4 the redox potential after 1 hour of storage was -362.9 mV while a value of -563.5 mV was recorded following 48 hours. The average redox potential for quarter 4 was -630.9 mV. ANCOVA analysis confirmed no significant relationship between redox potential and *E. coli* concentration measured over the study quarters.

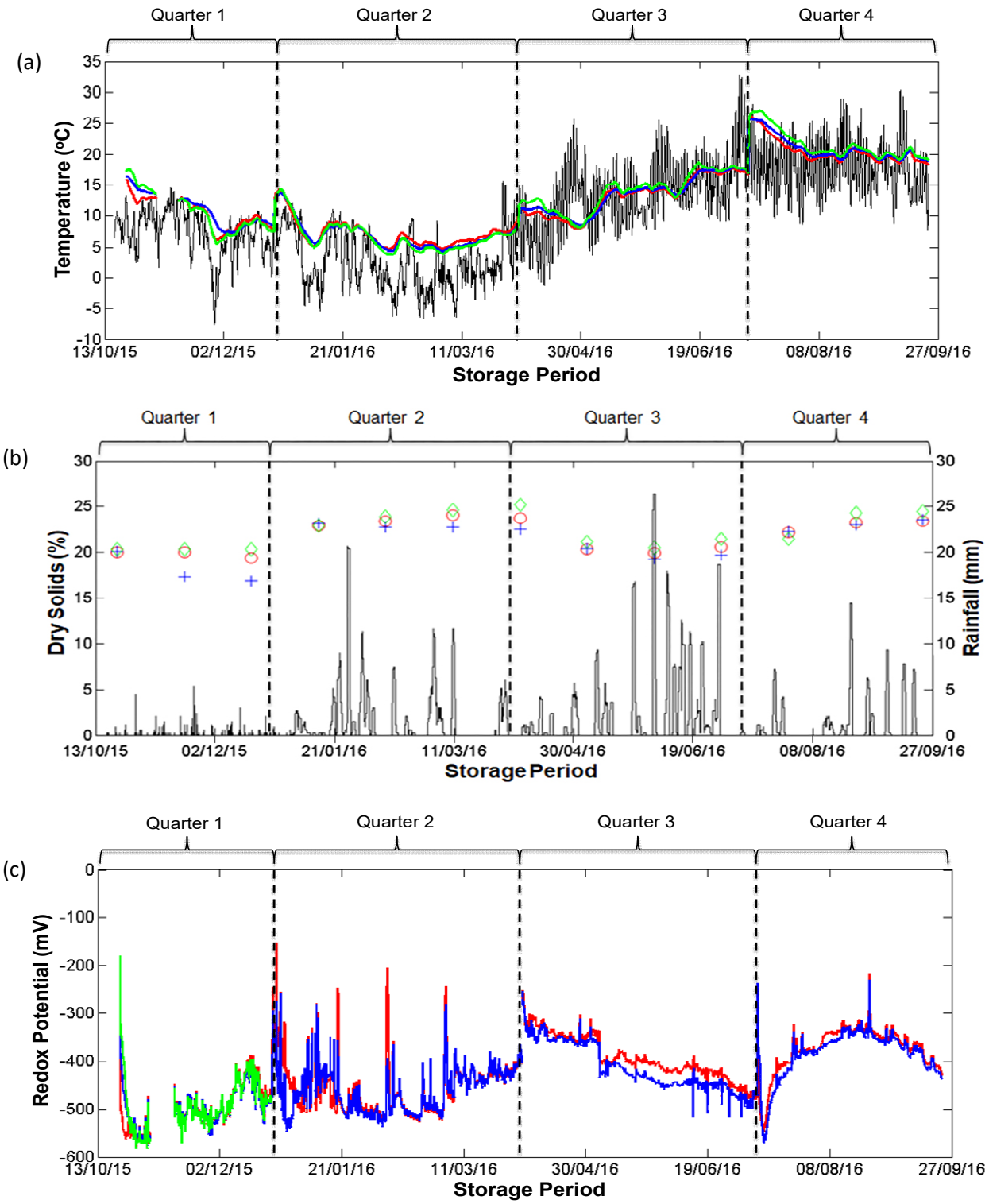


Figure 3: Temperature (a), dry solids and rainfall data (b) and redox potential (c) recorded during the long term biosolids storage monitoring programme. Dashed lines indicate end of study quarter where biosolids were removed and replenished. Coloured lines show the average parameter value of 9 replicates. Cage temperature and redox potential data was recorded at hourly intervals. Key: — data recorded from Y1 (low depth), — data recorded Y2 (middle depth), — data recorded from Y3 (top depth, ~0.25 m below surface). — Ambient temperature recorded on-site every 30 minutes. — Rainfall measurements recorded on-site over 24 hours are displayed. Redox probes measuring Y3 data were corrupted after first quarter and therefore Y3 data for quarters 2, 3 and 4 are not shown.

3.2. Phase 2: Modification

The *E. coli* density gradually reduced during storage over the course of both the summer and winter trials (Figure 4). The greatest decrease in *E. coli* density was observed in the summer trial with the modified storage condition (insulating membrane cover), which showed a reduction of 3.7 Log over the 28 day period (time 0 = 4.9 Log_{10} CFU g^{-1}DS). A statistically significant reduction of approximately 2.1 Log from time 0 to day 28 was observed across the summer open control. In the winter trial at day 69 the open control and covered treatment conditions contained *E. coli* concentrations of 6.1 Log_{10} CFU g^{-1}DS, a reduction of 1.8 Log over the trial period (Figure 4).

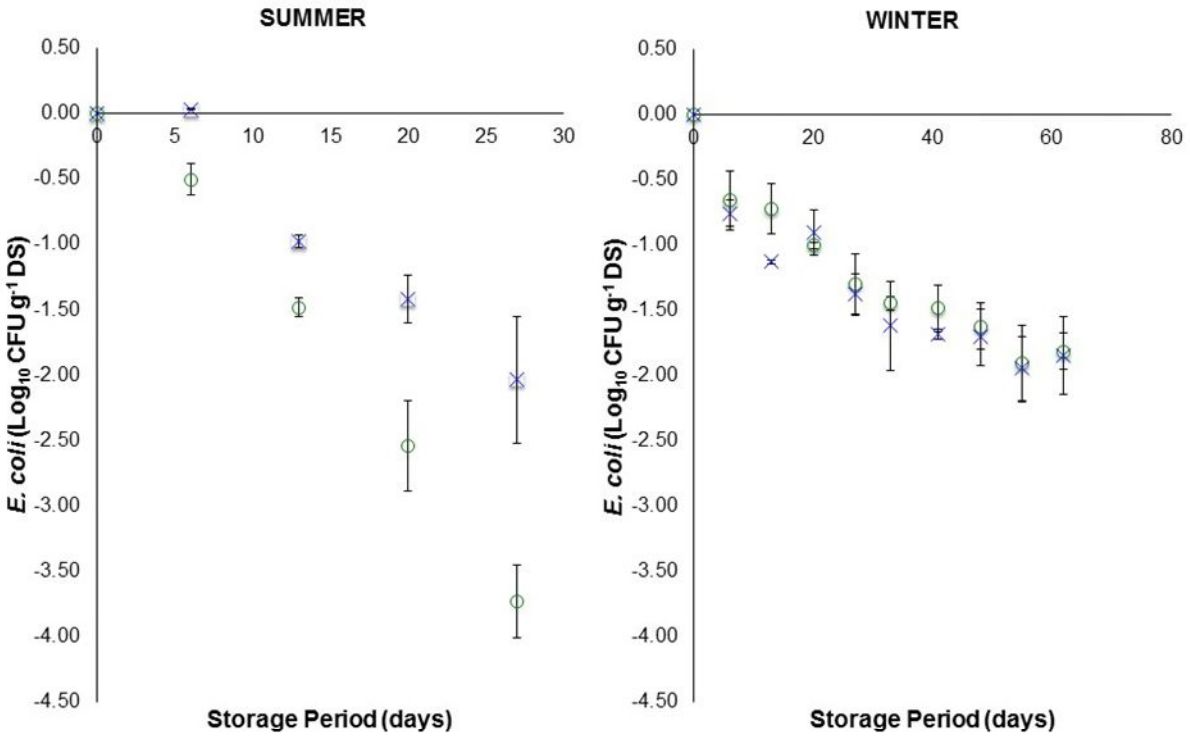


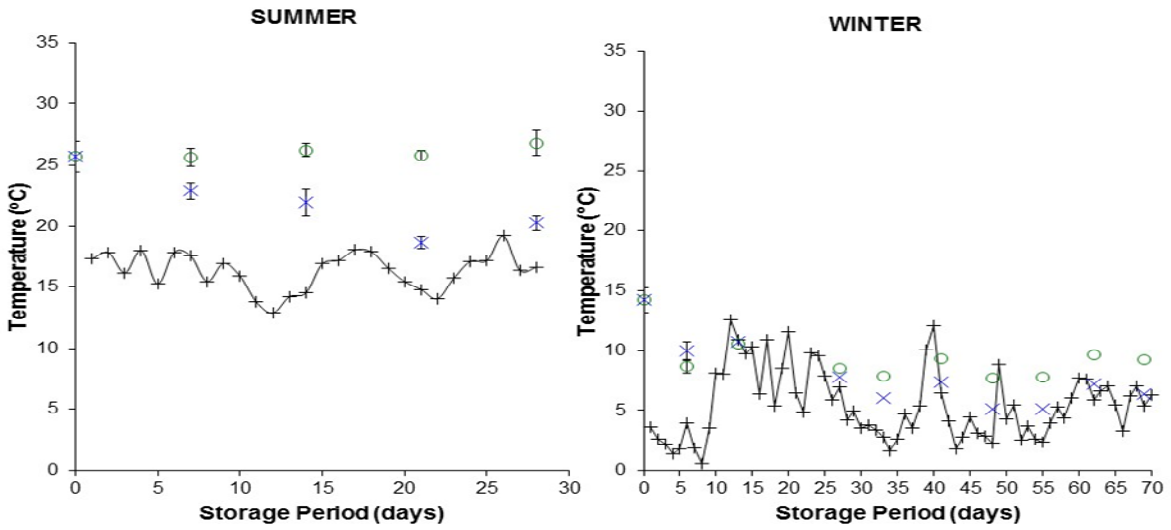
Figure 4: *E. coli* concentrations recorded during summer (28 day) and winter (69 day) demonstration trials under open control and covered stockpile test conditions. Results are normalised against starting concentration at time zero. Key: open control, covered stockpile. Error bars represent the standard deviation of 15 replicates.

ANCOVA analysis identified *E. coli* concentrations in the stockpiles to be significantly affected by stockpile temperature (p= 0.008). Stockpile temperatures were significantly different between the winter and summer trials with a reduction of approximately 12.2°C between the seasons (Figure 5a). This coincided with an average 13.7°C reduction in ambient temperature at time 0. The open control condition maintained a high temperature during the summer trial with a 20.3°C stockpile temperature recorded on day 28. In the summer months the stockpile temperatures consistently remained above the ambient field site temperature. The insulated covered condition in the summer months successfully modified the stockpile storage temperature and was significantly different from the open control treatment by day 7 of storage. The modified storage environment maintained a temperature above 25°C throughout the 28 day test period. Fluctuations in ambient temperature over the 28 day summer test period were limited, with the range experienced between the lowest and highest temperatures recorded of 6.3°C. In the winter months, ambient temperature and stockpile temperatures were more similar with the greatest difference (10.6°C) at time 0. In the winter trial the ambient temperature range was 12.1°C. The covered treatment was able to maintain the highest stockpile temperature and was significantly different from the open control treatment, showing successful modification to stockpile temperature.

Stockpile moisture content changed between the summer and winter trials and was significantly lower by 0.03 $m^3 \cdot m^{-3}$ at time 0 in the winter trial (Figure 5b). The open control summer condition remained at approximately 0.46 $m^3 \cdot m^{-3}$ with no significant change for the duration of the tested storage period. Significantly higher stockpile moisture content in the covered condition averaging at 0.48 $m^3 \cdot m^{-3}$ was maintained over the 28 day summer trial. Moisture content significantly changed under the covered condition between time 0 and day 28 with a higher moisture content on day 14 (0.5 $m^3 \cdot m^{-3}$) in comparison to the open control. Throughout the summer test the total rainfall was 60.6 mm with a peak precipitation event in week 2 where 20.7 mm fell over one 24 hour period (Figure 5b). Rainfall over the winter test amounted to 223.2 mm and the peak precipitation event was in week 3 with 40.8 mm recorded over one 24 hour period. Stockpile moisture content showed no significant change over the winter trial between the conditions tested (covered and open) with the average value recorded at 0.43 $m^3 \cdot m^3$. ANCOVA tests confirmed no significant effect of stockpile moisture content on *E. coli* concentrations.

Similarly, redox potential was found to have no significant effect on the level of *E. coli* die-off and overall no treatment effect was identified between the modified and controlled storage conditions across the summer and winter tests. Redox data recorded over the summer period displayed no significant change, with the average redox level recorded as -402 mV. During the winter trial the average redox level was -325 mV. A significant difference in the redox potential between the summer and winter trials was observed, with an increase to -309.6 mV displayed (Figure 5b).

(a)



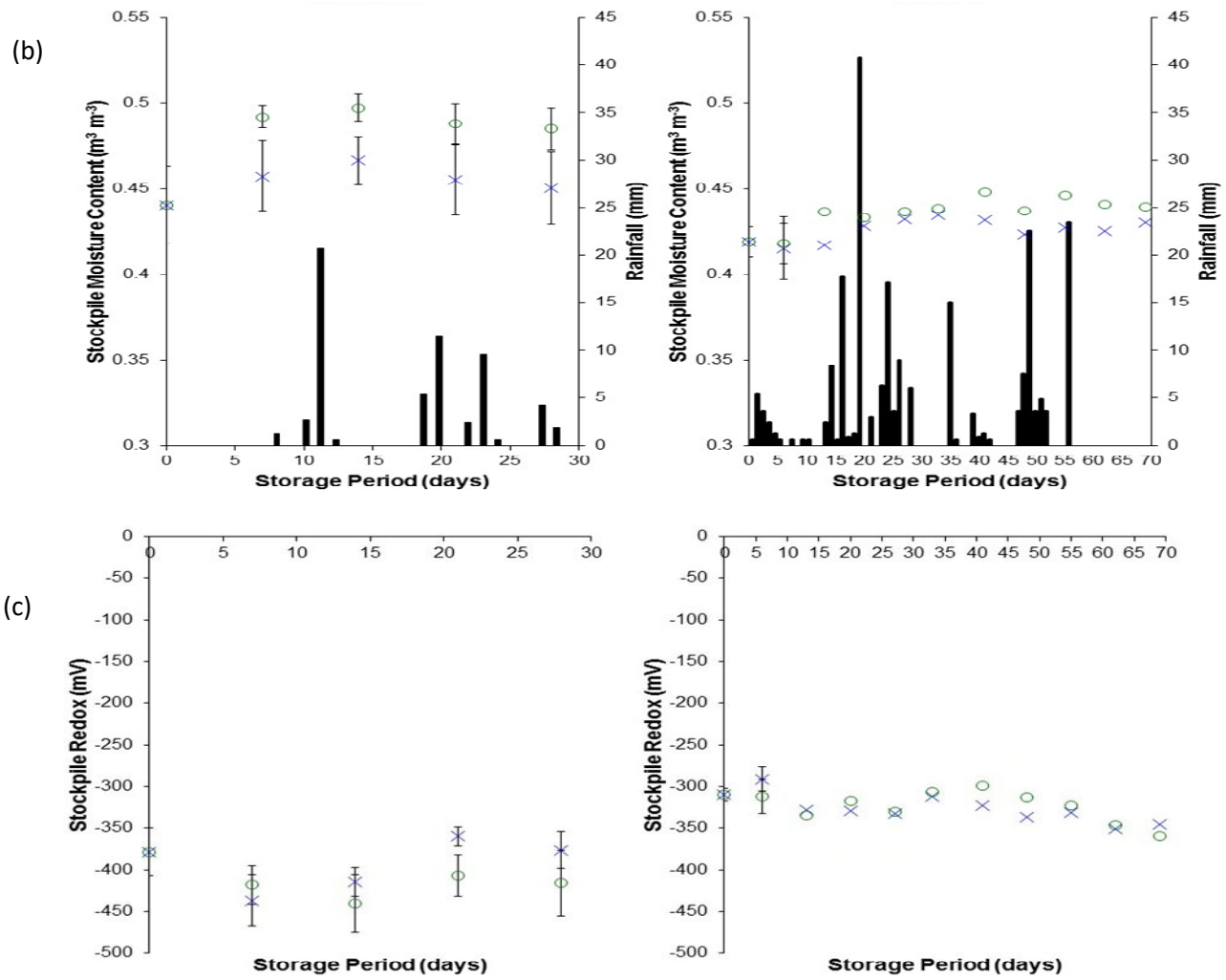


Figure 5: Stockpile temperature (a), moisture content and rainfall data (b) and redox potential (c) during summer (28 day) and winter (69 day) demonstration trials under open control and covered stockpile test conditions. Key: open control stockpile temperature, covered stockpile temperature and ambient temperature data. Ambient temperature was recorded at 30 minute intervals during the trials, with results here showing the average temperature over 24 hours. Rainfall data is displayed in bars ( ) and results show the total rainfall recorded over 24 hours. Error bars represent the standard deviation of 15 replicates.

3. Discussion

4.1 *E. coli* dynamics during storage

E. coli concentrations in the fresh cake did not vary significantly between the 4 study quarters of the 12 month storage monitoring programme (Figure 2), suggesting stability of on-site treatment processes throughout the test period. Across all 4 study quarters, *E. coli* showed a gradual reduction in concentration. Interestingly, the greatest change in *E. coli* concentrations was shown in the first 4 weeks of storage and statistical analysis confirmed significant reductions between months 1 and 2 in study quarters 1, 2 and 4, which was also further observed in the storage modification trial (Figure 4). These findings are in agreement with previous literature, where Lang *et al.,* (2007) found that time had a highly significant effect on *E. coli* numbers in agricultural soil. In addition Higgins *et al.,* (2007a) identified that continued storage of mesophilic cake decreased *E. coli* and faecal coliform concentrations substantially, with bacteria reaching a non-detectable level after 20 days of storage at 35°C. In contrast to results from Higgins *et al.,* (2007a) and Lang *et al.,* (2007), the long term study showed *E. coli* levels after 4 weeks of storage to plateau with no significant change in concentration

after the first month (Figure 2). Significant die-off in the first 4 weeks of storage was also observed by Holley *et al.,* (2006) who studied the length of *Salmonella* survival in manure-amended soils under different seasonal temperature sequences. The reduction in *Salmonella* concentrations in samples stored under the summer-winter regime was greater during the first month of storage (90% concentration reduction). In addition, prolonged *Salmonella* survival continued for >180 days after the first storage month. Similarly in this study, *E. coli* bacteria persisted at concentrations >5.2 Log_{10} CFU g^{-1}DS in quarters 1, 2 and 3 (Figure 2) and during the 69 day winter storage modification trial (Figure 4). Rogers *et al.,* (2011) suggests that the slower decay of organisms following a rapid reduction in culturable bacteria results from organisms entering a viable but non culturable (VBNC) state, leading to a rapid decay until stressed cells alter their physiology. The rapid decline is then followed by a slower decay of more resilient organisms that remain culturable or as cells transition in and out of a VBNC state (Rogers *et al.,* 2011). Findings by Li *et al.,* (2014) suggest that a VBNC *E. coli* bacterium may be resuscitated in conditions including high nutrient concentration and a temperature upshift. Methods to enumerate the *E. coli* bacteria include plating on a nutrient rich agar and incubation for 18 hours at 37°C, which could create suitable conditions for resuscitation. Therefore, the prolonged survival shown over the study may be the transition of VBNC cells into a culturable state as a result of enumeration methods. Pinto *et al.,* (2011) observed more resuscitation of VBNC *E. coli* at 37°C than at 25°C indicating the importance of a temperature upshift as a stimulating factor to reverse the VBNC state. An alternative argument for the maintenance of culturability over the long storage period (Figure 2) is the lower temperatures *E. coli* bacterial cells were exposed to during the study. Pinto *et al.,* (2011) suggests that at lower temperatures the metabolism of cells is reduced allowing them to remain culturable for longer.

Research on *E. coli* dynamics in sewage sludge suggests that the *E. coli* indicator bacteria may enter a VBNC state during digestion that is reversed after centrifuge dewatering under favourable biosolids storage conditions (Higgins *et al.,* 2006; Higgins *et al.,* 2007b; Fane *et al.,* 2019a). Evidence for this was shown by a comparison of standard culturing methods and quantitative polymerase chain reaction (qPCR) which showed a three order of magnitude increase in *E. coli* per g DS from qPCR measurements after thermophilic digestion (Higgins *et al.,* 2007b*)*. This demonstrates that cells in a VBNC state were present in the post-digestion samples but not accounted for with culturing methods. Researchers attribute the observed increase in culturable bacteria often seen after centrifuge dewatering (to levels of 10^8 *E. coli* per g DS) to the reactivation and regrowth of VBNC bacteria (Higgins *et al.,* 2006; Higgins *et al.,* 2007b). Although no significant level of growth was observed in the present monitoring study, no comparisons were made with on-site treatment works *E. coli* concentrations before transportation to the storage site. It would have been beneficial to increase the frequency of *E. coli* measurements in the first 1-3 days of storage to understand whether increases in *E. coli* density, as commonly experienced on treatment sites were occurring (Monteleone *et al.,* 2004; Higgins *et al.,* 2007a; Qi *et al.,* 2008; Chen *et al.,* 2011). For operational sites enhanced *E. coli* monitoring of biosolids immediately after dewatering, and in the three consecutive days following, will enable peak concentrations to be determined. This may serve in providing a method for forecasting the storage time required before compliance is achieved, improving stockpile management strategies.

4.2 Effects from temperature on storage environments

Outputs from ANCOVA testing confirmed that stockpile temperature had a significant effect on *E. coli* concentrations (p= 0.0017). This effect was observed in the storage modification demonstration trial under the insulated, covered treatment where a higher temperature was maintained above 25°C throughout the 28 day storage trial (Figure 5a). In this condition *E. coli* reduced by 3.7 Log highlighting the decrease in survival times at elevated temperatures. Similarly a 4.5 Log reduction in *E. coli*

concentration was observed in quarter 4 of the 12 month storage monitoring programme (Figure 2) when stockpile temperatures were at their highest. In a study by Semenov *et al.*, (2007) a greater decline in *E. coli 0157:H7* was observed for storage temperatures changing from 7 to 33°C. For temperatures at 23°C (±4°C) final densities dropped to 5.67 (±0.27) Log CFU g DS^{-1} after 2 weeks of storage. In contrast, treatments at 7°C only decreased to 7.44 (±0.1) Log CFU g DS^{-1} (Semenov *et al.,* 2007), which is comparable with the storage modification winter results of the present study. During the winter trial *E. coli* concentrations always remained above 6 Log_{10} CFU g^{-1}DS, emphasising the reduced antagonistic activity and competition from indigenous microorganisms at lower temperatures (Cools *et al.,* 2001). Under warmer conditions microbial metabolic activity and increased competition for nutrients is expected (Cools *et al.,* 2001; Jiang *et al.,* 2002; Holley *et al.,* 2006). Nutrient exhaustion can increase cell death rate, particularly in environments such as sewage sludge cake where substrates can be limiting (Holley *et al.,* 2006; Fane *et al.,* 2019a). The microbial activity per unit of biomass showed a positive correlation with concentrations of dissolved organic carbon (DOC) in a study measuring biotic and abiotic characteristics in manure-amended soils (Franz *et al.,* 2008). It may therefore be possible that nutrients, such as DOC, released as a result of dewatering operations (Chen *et al.,* 2011; Sun *et al.,* 2015; Fane *et al.,* 2019a) are degraded and become a limiting factor to microbial growth over time. Findings suggest the rate of nutrient degradation is increased under higher temperatures, such as those recorded in the summer months of storage and under the covered treatment. The results show that increasing the stockpile temperature to a level above 25°C, which achieved the greatest level of *E. coli* die-off in the storage modification trial, may be advantageous for sites challenged by compliance limits. Storage at higher temperatures will increase cell metabolism and die-off reducing culturable *E. coli* concentrations. Controlling biosolids storage conditions and departing from the traditional open pads may be advantageous to reliably meet compliance standards.

It had been originally expected that *E. coli* bacteria survival may vary with depth in the biosolids as stockpile temperatures in the centre of the material may be greater. However, *E. coli* levels showed no significant change with depth (Figure 2) and this coincided with no significant difference in stockpile temperature between the sampling depths (Figure 3a). Stockpile temperatures recorded during the storage trials were compared with readings from an on-site treatment works biosolids storage pad (200 to 250 tonnes) taken as a reference between April to July (Severn Trent PLc.), in order to understand if the differences in stockpile sizes could lead to different temperature profiles. Only a 2.8°C difference was measured between operational sludge treatment site stockpiles and the biosolids stored in the monitoring study, suggesting that the tested storage environment was representative of operational site conditions. Findings from the present study suggest that the representative temperature of stored biosolids is approximately 11°C if generalising over a 12 month period, with an average temperature of 8°C in winter and 15°C in summer (Figure 3a). This conflicts with findings from Sprigings and Le (2011) who reported 30°C as the representative temperature of stored biosolids. However, this value was identified after only 48 hours of biosolids temperature monitoring, during which time the material may retain residual heat from up-stream digestion processes. Evaluation of how to retain the residual heat from the up-stream digestion process may be beneficial to understand if biosolids storage temperatures are to be elevated to 25°C.

Stockpile temperature changed substantially between each storage quarter and was shown to be significantly affected by the monthly average ambient temperature ($p= 0.028$) (Figure 3a). The biosolids stockpile temperature always remained above the ambient temperature suggesting a possible insulating effect of the biosolids material, which preserved higher temperatures even when conditions in winter months were below freezing.

4.3 Influence of moisture content in stored biosolids

Stockpile DS and moisture content did not significantly affect *E. coli* concentrations across the 12 month biosolids storage monitoring study and the storage modification trials. This result is in contrast to other studies (Plachá *et al.*, 2001; Holley *et al.,* 2006; Lang *et al.,* 2007; Roberts *et al.,* 2016) and is likely due to the relatively stable DS content of the biosolids material in the present study, which consistently remained within a range of 17-24 DS%. Monthly rainfall was shown to significantly affect biosolids DS levels with peak rainfall events coinciding with reduced DS concentration. Findings suggest that storage practices may benefit from weather protection if cake quality, such as stockpile integrity, is an important feature to preserve. Ability to maintain the sludge matrix integrity for stockpiling on treatment sites and agricultural land is necessary to prevent pollution incidents and stockpile DS content is a measured parameter of biosolids quality (Environment Agency (UK) 2012).

DS levels and rainfall were expected to influence the redox levels recorded over the study period, however no such effect was observed. This conflicts with previous studies which suggest that an increase in moisture content will lower redox potential and oxygen concentrations (Rubol *et al.,* 2012; Peralta *et al.,* 2014). Rubol *et al.,* (2012) argues that water content is directly linked to oxygen concentration and redox potential, which regulate microbial metabolism and chemical transformations in the environment. Findings from a long term experiment on a 1.5 m depth soil column showed increased soil moisture lowered the redox potential and oxygen concentrations of the soil (Rubol *et al.,* 2012). The redox potential levels measured in the current biosolids storage study were typically below -300 mV and above -600 mV, which is within the redox range dominated by heterotrophic anaerobic bacteria, sulphate reducing bacteria and methanogenic bacteria (Zajic, 1969). The measurements of redox confirm a homogeneous anoxic state of the biosolids stockpile, with typically no significant change in depth recorded (Figure 3c). Similar findings were reported by Hall *et al.,* (2013) who identified a limited effect of moisture on redox potential when humid tropical forest soils where exposed to extended periods of elevated moisture. No influence of temporal patterns in redox levels were shown and researchers suggest that the clay rich soils under study had a high affinity for moisture and a well-developed aggregate structure which enabled anaerobic conditions to persist.

One significant change observed in the redox levels was the steady decline over the first 48 hours of stockpiling where levels reduced by approximately -218 mV in each quarter of the 12 month monitoring study (Figure 3c). This phenomenon was also highlighted by Chen *et al.,* (2011) and Fane *et al.,* (2019a) who suggested that the shearing effects from mechanical dewatering may introduce oxygen to the sludge matrix. Over time this increase is reduced as oxygen is depleted during storage for microbial respiration (Chen *et al.,* 2011). An additional explanation for the reduction in redox potential is the possibility of stockpile settling over the first 48 hours of storage, when oxygen diffusion through the stockpile will be diminished as sludge pores become smaller and fissures across the stockpiled matrix close during the settling process.

4. Conclusion

This study profiled the biosolids storage environment and confirmed that temperature is a significant factor affecting *E. coli* concentrations. The study demonstrated that temperature can be controlled through storage modifications to achieve microbial compliance. Higher stockpile temperatures during summer months led to greater cell die-off in comparison to cooler winter stockpile temperatures. Modified biosolids storage environments using covered treatments, accelerated the suppression of *E. coli* bacteria in summer months through the maintenance of elevated stockpile temperatures >25°C with a reduction of 3.7 Log over the 28 day storage period compared to 2.1 Log observed in the open

control. Ambient temperature is a parameter that has significant influence on stockpile temperature and ultimately indicator bacterial survival. Biosolids stockpiles showed an insulating effect able to maintain a minimum core temperature of 4°C when ambient temperatures fell below freezing (-4°C). *E. coli* concentrations reduced with increased storage time in all tested conditions, with the greatest reduction exhibited in the first 4 weeks of biosolids storage. Therefore, efforts to reduce bacteria levels should target this period of increased die-off to enhance cell death mechanisms such as nutrient exhaustion, in order to achieve compliance limits. Within the ranges measured in the 12 month monitoring study and the storage modification demonstration trial; DS, moisture content and redox potential conditions did not have a significant effect on *E. coli* dynamics. This research work has contributed to the identification of factors affecting the dynamics of *E. coli* growth and death in stored biosolids with a key conclusion on temperature as a critical environmental parameter to control in storage environments. Trials demonstrated that the modification of the storage environment will ensure microbial compliance requirements are confidently achieved, safeguarding value recovery through biosolids to land application route.

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