Factors affecting decay of *Salmonella* Birkenhead and coliphage MS2 during mesophilic anaerobic digestion and air drying of sewage sludge

Tania Mondal, Duncan A. Rouch, Nerida Thurbon, Stephen R. Smith and Margaret A. Deighton

ABSTRACT

Factors affecting the decay of *Salmonella* Birkenhead and coliphage, as representatives of bacterial and viral pathogens, respectively, during mesophilic anaerobic digestion (MAD) and air drying treatment of anaerobically digested sewage sludge were investigated. Controlled concentrations of *S.* Birkenhead were inoculated into non-sterile, autoclaved, γ-irradiated and nutrient-supplemented sludge and cultures were incubated at 37 °C (MAD sludge treatment temperature) or 20 °C (summer air drying sludge treatment temperature). Nutrient limitation caused by microbial competition was the principal mechanism responsible for the decay of *S.* Birkenhead by MAD and during air drying of digested sludge. The effects of protease activity in sludge on MS2 coliphage decay in digested and air dried sludge were also investigated. MS2 coliphage showed a 3.0–3.5 $\log_{10}$ reduction during incubation with sludge-protease extracts at 37 °C for 25 h. Proteases produced by indigenous microbes in sludge potentially increase coliphage inactivation and may therefore have a significant role in the decay of enteric viruses in sewage sludge. The results help to explain the loss of viability of enteric bacteria and viral pathogens with treatment process time and contribute to fundamental understanding of the various biotic inactivation mechanisms operating in sludge treatment processes at mesophilic and ambient temperatures.

Key words | coliphage, indigenous flora, nutrient limitation, protease activity, *Salmonella* Birkenhead, sewage sludge

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INTRODUCTION

One of the principal aims of sewage sludge treatment is to protect public health by reducing the numbers of pathogenic and indicator microorganisms in sludge recycled to farmland as an agricultural fertiliser. Sewage sludge treatment processes, including, for instance, mesophilic anaerobic digestion (MAD), thermophilic aerobic digestion, lagooning, air drying and composting, are capable of significantly reducing pathogen and indicator numbers in treated sludge, although the relative extent of the removal is dependent upon the specific process employed (Watanabe *et al.* 1997; Gantzer *et al.* 2001; Amahmid *et al.* 2002; George *et al.* 2002; Tanji *et al.* 2002; Malack Muhammad *et al.* 2007; Rouch *et al.* 2011). Sewage sludge treated to achieve a microbiological standard acceptable for use on land is increasingly referred to as biosolids, to distinguish the product from untreated material (Pepper *et al.* 2006).

Air drying beds and lagoons provide low cost options for treating sewage sludge, and these extensive treatment methods are commonly used for small- to medium-sized populations and where sufficient space is available. Air drying is predominantly used in warmer climatic regions of the USA and Europe, and also in the Middle East, Asia and Australia (Hall & Smith 1998; Idris *et al.* 2002; US EPA 2003; Ahn & Choi 2004; NRMMC 2004). For example,

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in Victoria, Australia, sludge treatment generally involves air drying and stockpiling of dried biosolids. In metropolitan areas of Australia, and other countries, MAD is also widely practised as a treatment process for sludge prior to the transfer to lagoons or drying beds.

Pathogen decay in sewage sludge treatment processes involving exposure to elevated temperature conditions in the thermophilic range, or higher, is readily defined by established time–temperature relationships (Strauch 1990). However, the mechanisms influencing pathogen decay in processes that operate at cooler mesophilic or ambient temperatures and environmental conditions, such as MAD and air drying processes, are poorly understood (Lang & Smith 2008). Factors potentially influencing pathogen decay and loss of viability under these conditions include temperature (Smith *et al.* 2005), retention time (Lang & Smith 2008), pH (Feng *et al.* 2003), moisture content (Ward *et al.* 1981; Yeager & Ward 1981) and the activities of indigenous flora (Yeager & Ward 1981; Sidhu *et al.* 2001). Indigenous flora may reduce the survival of enteric bacteria by predation, antagonism and competition for nutrients or, in the case of viruses, by producing enzymes capable of degrading viral capsids (Nasser *et al.* 2002).

Yeager & Ward (1981) reported that several species of faecally associated bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Proteus mirabilis*, *Salmonella* Typhimurium and *Streptococcus*, now *Enterococcus faecalis*) were able to grow or survive for long periods when inoculated into sterile, raw sewage sludge. Further studies with *S.* Typhimurium showed that growth was inhibited in unsterilised sludge (containing indigenous flora). In a similar study, Hussong *et al.* (1985) found that *S.* Typhimurium was capable of growing in composted sewage sludge irradiated to remove the indigenous flora, but the pathogen was suppressed in the presence of the indigenous microbial community in compost. Sidhu *et al.* (2001) also concluded that the indigenous flora was important in controlling the growth of *Salmonella* spp. in composted sewage sludge following an inoculation experiment with *S.* Typhimurium introduced into stockpiles of composted, dewatered anaerobically digested biosolids. These studies used an inoculation approach to calculate the bacterial decay coefficients due to the low numbers of pathogens present in sludge from industrialised countries (Rouch *et al.* 2012). For example, sludge sampled during the summer period from mesophilic anaerobic digesters and from air drying pans of various ages at the metropolitan wastewater treatment plants (WWTPs) operating in Melbourne, Australia contained maximum numbers of *Salmonella* spp. equivalent to 1.34×10^2 colony-forming unit (CFU)/g dry solids (DS) (Rouch *et al.* 2012).

Coliphages comprise a large heterogeneous group of bacterial viruses that infect coliform bacteria and have been used as indicators for the presence of enteric viruses in wastewater (Harwood *et al.* 2005; Costan-Longares *et al.* 2008). The loss of viability of coliphages in sewage sludge is affected by the presence of indigenous flora as well as by process pH, temperature and retention time (Feng *et al.* 2003; Nappier *et al.* 2006), but the specific mechanisms controlling viral decay in sewage sludge are also poorly understood. Nasser *et al.* (2002) suggested that bacterial proteases may contribute to the loss of viability of coliphages and enteric viruses by degrading their protective protein coat. During the microbiological stabilisation of sewage sludge, for instance, by anaerobic digestion, a dynamic bacterial population degrades organic matter through a series of hydrolytic reactions, to produce smaller molecules that can be taken up and metabolised by bacterial cells. This process requires an array of enzymes, including proteases, which are produced by various species of bacteria (Dueholm *et al.* 2000; Gessesse *et al.* 2003; Gerardi 2000). Nasser *et al.* (2002) examined the effect of a pure protease, pronase and extracellular enzymes produced by *Pseudomonas aeruginosa*, on the inactivation of MS2 coliphage and a range of enteric viruses. They also determined the antiviral activity of soil saturated with secondary effluent from the activated sludge process. All of the protease sources tested showed antiviral activity; however, the activity depended on the virus type.

A better understanding of the inactivation factors influencing pathogenic bacteria and viruses in sewage sludge treatment processes will improve hazard analysis of critical control point measures aimed at ensuring the microbiological quality and safety of biosolids for agricultural application. Since different mechanisms of decay are likely to operate and influence the survival of bacteria and bacterial viruses in sewage sludge treatment processes operating at or below the mesophilic temperature range, the aims of this study were twofold. The first major aim was to investigate the effects and significance of (1) indigenous flora and (2) nutrient availability, in mesophilic anaerobically digested and air dried sewage sludge on the survival of bacterial pathogens in these sludge treatment processes. The second major aim was to examine the effects of microbial proteases present in sewage sludge on coliphage inactivation.

The four specific objectives were to: (i) examine the effects of indigenous flora and nutrient availability in sludge from mesophilic anaerobic digesters and from early, middle and late phases in air drying treatment of digested sewage sludge on the fate and survival of inoculated *Salmonella* Birkenhead; (ii) measure protease activity (PA) in sewage sludge at different stages of treatment, including directly after MAD, and in digested sludge sampled from early and late stages of air drying in pans; (iii) determine the effect of temperature and pH on sludge PA; and (iv) investigate the effects of sewage sludge (as a potential protease source) on MS2 coliphage inactivation.

MATERIALS AND METHODS

Bacterial isolates and coliphage

An environmental isolate of *S.* Birkenhead (isolated using standard methods from a calf with diarrhoea) was used in the bacterial investigations. *Salmonella* spp. are important causes of gastroenteritis worldwide (CDC 2011) and are used as representative enteric pathogens for monitoring the efficacy of sludge treatment processes for pathogen removal. The F-specific MS2 coliphage was selected as a common surrogate for enteric viruses (ISO 1995) to examine the effect of proteases on the loss of viral infectivity in sludge.

Sample collection

Composite samples of sludge from MAD processes were collected from three WWTPs in the greater Melbourne area, designated as E, T and M. Most samples were obtained from Site E, but two samples of digested sludge from Site M and Site T were included for comparison between different plants. Composite samples of air dried anaerobically digested sludge of different ages were also available from Site E (Table 1). All samples were collected aseptically, transported to the laboratory on an ice pack and stored at 4 °C. The DS content was determined from the loss of weight on heating in a forced-air oven at 105 °C for 24 h (SCA 1984). The volatile solids (VS) content was determined by loss on ignition for 2 h at 500 °C using a muffle furnace. Samples used in this study contained 2–14% of DS and 58–76% VS.

Table 1 | Sewage sludge samples

WWTP site	Sample type	Sample age (days)[a]	Sample abbreviation	DS (%)	VS (%)
E	MAD	N/A	EMADa, b, c	2–3	70
	Early pan	30–81	EEPa, b	3–4	69
	Middle pan	188	EMP	3	Unavailable
	Late pan	262–420	ELPa, b	4–14	58
T	MAD	N/A	TMAD	2	76
M	MAD	N/A	MMAD	2	70

DS, dry solids; VS, volatile solids; WWTP, wastewater treatment plant; MAD, mesophilic anaerobic digestion; EP, early pan; MP, middle pan; LP, late pan; N/A, not applicable.
[a]Ages of drying pan samples were defined as the time since the start of filling of the pan with sludge from MAD.

Sample preparation and inoculation

Controlled concentrations of *S.* Birkenhead, as a representative bacterial pathogen, were used to investigate the effects of indigenous microbial flora and nutrient availability on pathogens exposed to MAD and pan drying processes. Sludge samples from Site E (see Table 1 for explanation of sample abbreviations) were used to examine the decay of *S.* Birkenhead in sludge from MAD (EMADc) and from early, middle and late air drying pans (EEPa, EMP and ELPa). Each sample type was divided into three portions of 500 g. One portion of each sample was autoclaved at 121 °C for 40 min, the second portion was sterilised by γ-irradiation at 50 kGy (performed by a specialist company: Steritech, Dandenong South, Victoria Australia), and the third portion was maintained as a control sample, in a non-sterile condition.

An overnight culture of *S.* Birkenhead was prepared in nutrient broth (NB) (Oxoid Limited, Basingstoke, UK). After adjusting to a concentration of 10^7 CFU mL^{-1}, 500 μL of the culture was added to 50 mL of each sludge type; the final concentration of *S.* Birkenhead in each sludge sample was therefore equivalent to 10^5 CFU mL^{-1}. An aliquot of the overnight culture was also diluted to 10^5 CFU mL^{-1}, as a positive control. The digested sludge samples (EMADc) were incubated at 37 °C for 24 h and air dried samples (EEPa, EMP and ELPa) were incubated at 20 °C for 24 and 48 h. The different temperature regimes were selected to simulate operational MAD and field ambient temperature conditions, respectively. There were three replicate samples of each sludge type treatment and the temperature conditions were each evaluated twice.

Nutrient supplementation of digested sludge

Anaerobically digested sludge from Site T (TMAD) (Table 1) was used in this experiment. Four experimental treatments were applied to quantify the effects of nutrient availability on the growth of *S.* Birkenhead in digested sludge; these included (1) sterilised sludge (20 mL) following the autoclaving procedure described above, (2) unsterile sludge (20 mL), (3) nutrient supplementation of unsterile sludge and (4) NB control (6 mL of NB at ×10 the standard concentration (×10 NB) and 14 mL reverse osmosis (RO) water). Nutrient supplementation was performed by mixing 6 mL of ×10 NB with 14 mL of unsterile digested sludge; thus, the nutrient supplementation increased the availability of organic carbon in the amended sludge, but was calculated to maintain an overall concentration of organic carbon equivalent to sludge samples without NB addition. The prepared experimental samples were inoculated with *S.* Birkenhead to the target final concentration (10^5 CFU mL^{-1}). Cultures were incubated at 37 °C for 24 h and *S.* Birkenhead was enumerated as described below. The experimental treatments were performed in triplicate and the experiment was repeated twice.

Enumeration of *Salmonella* Birkenhead

Unsterile, sterilised and nutrient-supplemented unsterile sludge samples were diluted 1:10 in maximum recovery diluent (MRD) (Oxoid Limited, Basingstoke, UK) in sterile Falcon tubes containing 1 g of sterile glass beads (diameter 4 mm). The tubes were placed on an orbital shaker at 200 rpm for 4 min. Following a series of 10-fold dilutions in MRD, viable counts of *S.* Birkenhead were determined by a membrane filtration technique (based on SCA 2004). Portions of the diluted samples (1.0 mL) were filtered in triplicate using nitro-cellulose grid membranes (pore size 0.45 μm, diameter 47 mm) and a triple-head filtration system. Two types of filtration system (steel funnels: Sartorius AG Goeftingen, Germany, or disposable funnels: Merck Millipore, Victoria, Australia) were used for this procedure. Phosphate buffered saline (20 mL) was applied to each membrane prior to filtration to ensure even distribution of bacteria. Prepared membranes were placed on Rappaport-Vassiliadis soya peptone broth (RVSB) agar (prepared from RVSB and Agar Bacteriological: Oxoid Limited) and incubated for 16–20 h at 41.5 °C, followed by incubation on xylose lysine desoxycholate (XLD) agar plates (Oxoid Limited) for a further 18–24 h at 37 °C. *Salmonella* spp. were detected as black coloured colonies on XLD. Numbers of colonies were counted and expressed as CFU mL^{-1}. For the control NB culture, *S.* Birkenhead was enumerated in NB cultures by a standard plate count methodology.

Assay for proteases in digested sludge supernatant

The PA experiments were completed with digested sludge samples collected from Site E and Site M (Table 1; EMADa and MMAD, respectively) and also with early and late air dried samples from Site E (Table 1; EEPa and ELPa, respectively). Sludge supernatants were prepared following the procedure described by Gessesse *et al.* (2003). Anaerobically digested sludge and air dried samples (20 mL) were transferred into sterile Falcon tubes and maintained in ice. Triton X-100 was added at a rate of 0.5% (v/v) and the mixtures were stirred at 200 rpm for 1.5 h at 4 °C using a rotary shaker. This procedure was designed to release and activate enzymes bound to the surface of cells or adsorbed into extracellular polymeric substances, since most of the enzymes in sludge are located at these sites (Gessesse *et al.* 2003; Xia *et al.* 2007; Yu *et al.* 2007). Activated samples were centrifuged at 4,700 rpm for 10 min at 4 °C and supernatants were collected for analysis.

The method to determine PA in sludge extract was modified from Gessesse *et al.* (2003). Solutions containing

2.5% (w/v) of azocasein substrate (Sigma–Aldrich, Victoria, Australia) and a buffer with 0.5% (w/v) of sodium bicarbonate at pH 8 were prepared; 2.5 mL of substrate was added to 1.5 mL of the buffer solution and the mixture was maintained at 37 °C for 30 min for temperature equilibration. Prepared, enzyme reactive sludge supernatant (1.0 mL) was added to the buffered substrate, mixed by vortexing for ~2 sec and incubated at 37 °C and samples were removed after 0.5, 2, 20, 48 and 96 h of incubation. The incubation temperature was selected to maximise PA present in the samples. After incubation, a 1 mL aliquot was transferred into another tube, 4 mL of 5% (v/v) trichloroacetic acid was added to terminate the reaction and the mixture was stored at 4 °C. The acidified solution was centrifuged at 4,700 rpm for 1 min at 4 °C and 1 mL of the supernatant was mixed with 500 mM sodium hydroxide solution (3 mL) to neutralise the reaction mixture. A 350 μL aliquot of the neutralised mixture was transferred to a microtitre plate and the absorbance of the orange coloured product was measured against a blank using a spectrophotometer (Thermo Scientific Multiskan, Ascent, Thermofisher Scientific, Victoria, Australia) set at a wavelength of 407 nm. The reaction blank test used to calculate the background optical density (OD) of the reagent matrix consisted of equal quantities of azocasein substrate and buffer without the addition of sludge enzyme extract. The experiments also included a positive control treatment containing 30 μg mL^{-1} of proteinase K (PK) in RO water. One unit of PA was defined as the amount of enzyme increasing the solution absorbance by 0.01. The experimental conditions were replicated twice using triplicate tests for treatments for each incubation time.

Effect of pH and temperature on PA of sludge supernatants

Sludge supernatants, prepared as described previously, were obtained from digested sludge (Table 1; EMADb) and from early and late air dried samples (Table 1; EEPb and ELPb, respectively), collected from Site E, and these were used to determine the effects of sludge pH and temperature conditions on PA. Stock solutions at different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) were prepared by adding the appropriate amount of HCl or NaOH to 0.5% (w/v) sodium bicarbonate buffer (500 mg in 100 mL RO water). Aliquots of buffer (1.5 mL) were mixed with 2.5 mL of 2.5% (w/v) azocasein substrate and sludge supernatant (1 mL). Mixtures of buffered substrate and sludge supernatant were incubated for 24 h at 37 or 20 °C; the incubation period corresponded with the maximum PA measured in the PK positive control treatment and the temperature regimes were selected to correspond with mesophilic sludge digestion conditions and summer air drying temperatures, representative of the southern Victoria region of Australia, respectively. Protease activity was measured by the spectrophotometric procedure described earlier. Two independent experiments were performed in triplicate.

Effect of filtered supernatants on the decay of MS2 coliphage

The coliphage MS2, a recognized surrogate for enteric viruses, was used to investigate the role of proteases in the decay of viruses during sewage sludge treatment. Supernatants prepared as described previously from digested sludge samples from Site E and Site M (Table 1; EMADb and MMAD) and early and late air drying pans (Table 1; EEPb, ELPb) were filter sterilised at 0.45 μM to remove bacterial cells. MS2 coliphage stock was retrieved from storage (at −20 °C) and diluted to a concentration of 10^7 plaque-forming units (PFU) mL^{-1}. Each reaction mixture contained 900 μL of sludge extract, 90 μL of buffer ($NaHCO_3$, pH 8.0) and 10 μL of MS2 coliphage, to provide a final concentration of 10^5 PFU mL^{-1}. MS2 coliphage in the sludge extract was quantified directly, as described below, and after incubation at 37 °C (for maximum enzyme activity) for periods of 4, 8, 22 and 26 h. Proteinase K (30 μg mL^{-1}) was used as the positive control and tryptone yeast extract glucose broth (TYGB) (Oxoid Limited), without an enzyme source, as the negative control. The positive control was prepared for optimal enzyme activity by adding 100 mM Tris HCl and 100 mM $CaCl_2$ to PK and MS2 coliphage in a total volume of 1 mL; the reaction tube contained 5 mM calcium at pH 8.0, 10^5 PFU mL^{-1} of MS2 coliphage and 30 μg mL^{-1} PK. There were three replicates of each sludge sample at each time period. Positive and negative controls were repeated twice.

Enumeration of MS2 coliphage

MS2 coliphage was enumerated using *E. coli* NZRM 4027 as the host strain and following the procedure described by Rouch *et al.* (2011). The host strain was grown in TYGB (Oxoid Limited) at 37 °C with shaking at 150 rpm to an OD_{600} nm value of 0.4, and was stored on ice until use. Semi-solid tryptone yeast extract glucose agar (ssTYGA) was prepared by adding 2.5 mL of 20 mM $MgSO_4$, 2.5 mL of glucose-calcium chloride solution (glucose 10%, calcium chloride 30%), 1.25 mL of nalidixic acid solution (25 mg mL^{-1}) and 1 mL of ampicillin solution (30 mg mL^{-1}) to 250 mL of TYGB and 0.5% Agar Bacteriological (Oxoid Limited). Aliquots of ssTYGA (3.5 mL) were transferred to test tubes and maintained at 50 °C until needed. In the meantime, dilutions of sludge extracts were prepared by adding 100 μL of sludge extract to 900 μL of MRD, followed by further 10-fold dilutions up to 10^{-6}. Next, 1 mL of diluted sludge and 0.25 mL of the host culture were added to each tube of warmed ssTYGA. After lightly mixing, the agar containing MS2 coliphage was poured onto 90 mm TYGA plates, which were incubated at 37 °C for 16 h. Plaques were enumerated and the numbers of PFU mL^{-1} were calculated.

Statistical analysis

The three sets of experimental results, i.e. (i) effect of removal of indigenous flora on the growth of *S.* Birkenhead in sludge, (ii) effect of added nutrients on the growth of *S.* Birkenhead in sludge, and (iii) effect of pH and temperature on PA of sludge supernatants, were initially examined by single factor analysis of variance (ANOVA), with statistical significance indicated by $P \leq 0.05$. Means comparisons were completed using a *t*-test procedure, assuming unequal variances, also indicating statistical significance at $P \leq 0.05$. Mean values for two sets of data were assessed to be statistically different if t Stat $> P(T < =t)$ two-tail, or t Stat $< -P$ $(T < =t)$ two tail, and $P \leq 0.05$. $\alpha = 0.05$, was used to assess all replicated data sets in pairs. Protease activity (U mL^{-1}) was calculated using the formula: optical density (OD_{407} sample – OD_{407} blank)/0.01. Means and standard deviations of PA (U mL^{-1}) and PFU mL^{-1} of coliphage were calculated. Linear regression analysis was used to quantify both the protease activities and the exponential decay rates of MS2 coliphage with time, by assessing the rates of activity or decay, respectively, and their 95% confidence limits: for protease activities net U mL^{-1} values were regressed against time (*h*), and for MS2 decay natural log (Ln) transformed enumeration data were regressed against time (*h*). The data processing and statistical tests were performed using the Microsoft Excel spreadsheet computer programme (versions 2007 and 2010).

The decay of MS2 followed first order kinetics and was modelled by the equation

$$N = N_0 e^{-kt} \quad (1)$$

where N = number of MS2 phages at time t (PFU/g DS), N_0 = initial number of MS2 phages (PFU/g DS), k = decay coefficient (Ln(PFU/g DS)/day) and t = treatment time (h).

While Ln data were required for decay equations, $\log_{10}$-data were utilised for graphs, to calculate log reduction values, and as commonly used in water and wastewater reports.

RESULTS

Effect of indigenous flora and nutrients on the growth of *Salmonella* Birkenhead

Microbiological examination confirmed the absence of *Salmonella* spp. in the digested and air dried digested sludge samples collected for the research. The numbers of *S.* Birkenhead in inoculated samples of unsterile, digested sludge from Site E and Site T (EMADc and TMAD, respectively), and in air dried sludge of increasing age (three drying times were tested) from Site E (EEPa, EMP and ELPa, respectively), were generally consistent with the initial inoculum concentration of *S.* Birkenhead supplied, equivalent to 10^5 CFU mL^{-1}, indicating there was no detectable growth observed in sludge samples containing an indigenous microbial community (Figures 1–3). By contrast, bacterial numbers increased significantly ($P < 0.005$), by 2–3 $\log_{10}$ compared with non-sterile conditions, in autoclaved (Figures 1–3) and γ-irradiated (Figures 1 and 2) digested sludge (EMADc), and in air dried materials, except for autoclaved early pan samples (AEEPa), where there was no

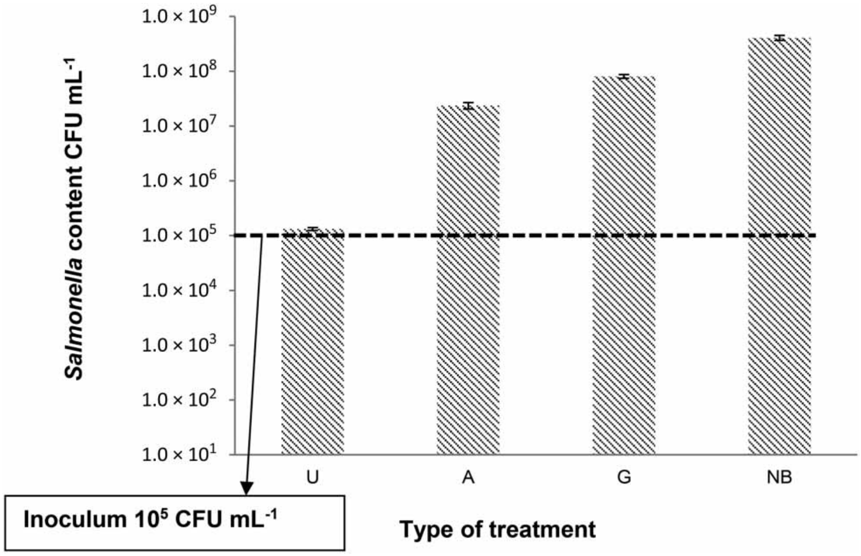

Figure 1 | Growth of *S.* Birkenhead in unsterile (U) and sterilised (autoclaved, A; γ-irradiated, G) mesophilic anaerobically digested sludge from WWTP E (EMADc) incubated at 37 °C for 24 h compared to a NB control. Mean values are shown for triplicate tests and two replicates per treatment; error bars show 1 SD. The dashed line indicates the initial concentration of *S.* Birkenhead inoculum.

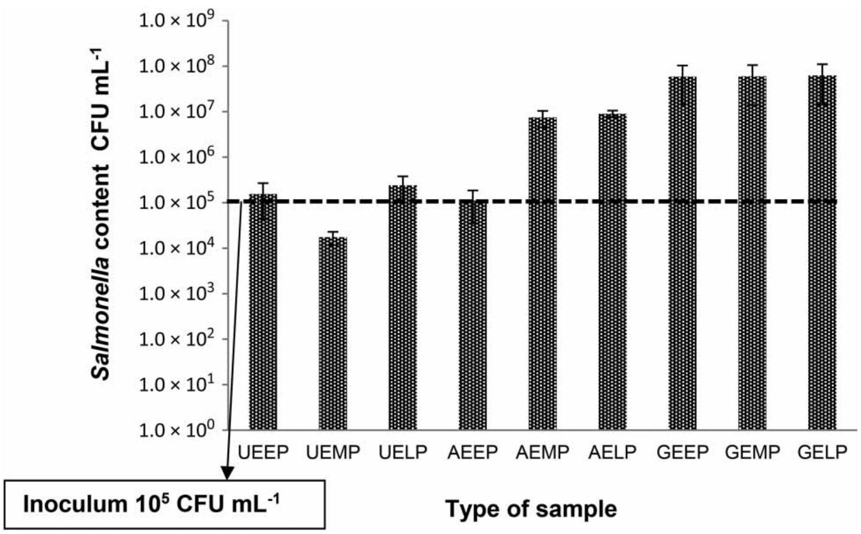

Figure 2 | Fate of *S.* Birkenhead in unsterile (U) and sterilised (autoclaved, A; γ-irradiated, G) air dried pan sludge samples (early, EEPa; middle, EMP; late, ELPa) collected from WWTP E incubated at 20 °C for 48 h. Mean values are shown for triplicate tests and two replicates per treatment; error bars show 1 SD. The dashed line indicates the initial concentration of *S.* Birkenhead inoculum.

evidence of a population increase. Bacterial growth in γ-irradiated sludge was consistently greater than in autoclaved sludge ($P < 0.005$) (Figures 1 and 2). Bacterial growth also increased in NB control treatments, compared to sterile and unsterile sludge, but in these cases the population was raised by 4 $\log_{10}$ to approximately 10^9 CFU mL^{-1} in the absence of nutrient limitation or other growth inhibition (Figures 1 and 3). In contrast to unsterile digested sludge (EMADc and TMAD) where no growth was observed, the addition of NB significantly increased the numbers of *S.* Birkenhead in autoclave sterilised sludge (TMAD; Figure 3) by approximately 2 $\log_{10}$ ($P < 0.005$), and this response was comparable to the increase measured in the autoclaved, sterile condition (Figure 3).

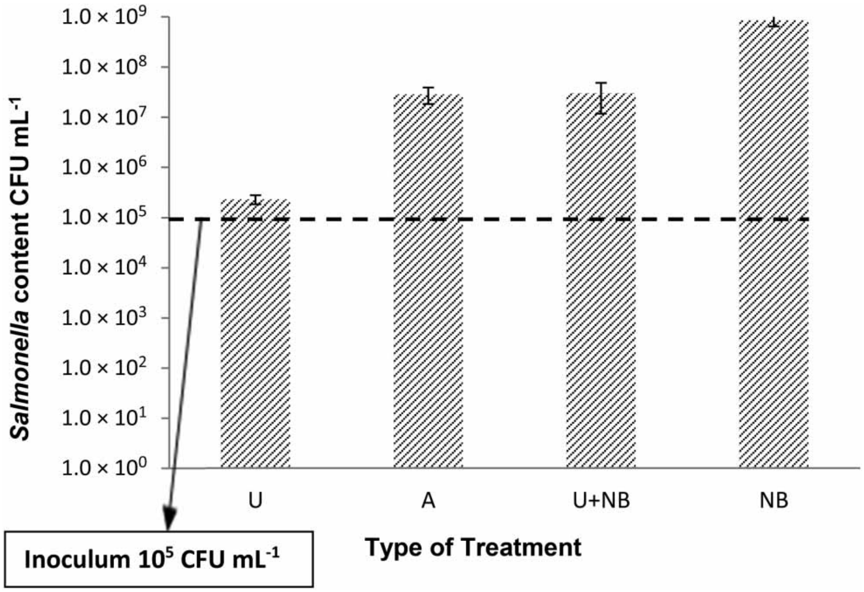

Figure 3 | Effect of NB supplementation and sterilisation treatment (unsterile, U; autoclaved, A) of mesophilic anaerobically digested sludge from WWTP T (TMAD) on growth of *S.* Birkenhead after incubation for 24 h at 37 °C, compared to a NB control. Mean values are shown for triplicate tests and two replicates per treatment; error bars show 1 SD. The dashed line indicates the initial concentration of *S.* Birkenhead inoculum.

Protease activity in digested and air dried sludge

Anaerobically digested sludge extracts and air dried sludge extracts from Site E demonstrated the highest rate of PA overall (EMADa > EEPa > ELPa) (Figure 4). Protease activity was highest in fresh anaerobically digested sludge and decreased by approximately 36% with air drying. The lowest activity in sludge from Site E was measured in the late air dried sample, ELPa. Low activity was also measured in the digested sludge sample collected from Site M (MMAD) (Figure 4).

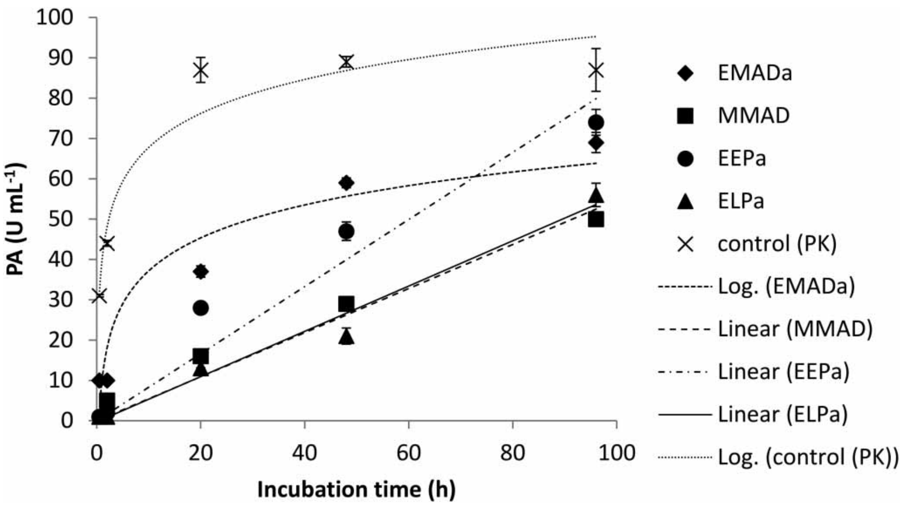

Figure 4 | Protease activity in mesophilic anaerobically digested (EMADa and MMAD) and air dried pan (early, EEPa; late, ELPa) sludge samples collected from WWTP E and M. Mean values are shown for triplicate tests and two replicates per treatment; error bars show 1 SD. Log relationships are shown for Control (PK) ($r^2 = 0.93$) and EMADa ($r^2 = 0.91$), otherwise linear relationships are shown, for: EEPa ($r^2 = 0.95$), ELPa ($r^2 = 0.98$), MMAD ($r^2 = 0.95$). Rates for PA (U mL^{-1} h^{-1} ± 95% confidence limit) were determined by linear regression from linear sections of data: Control (PK), 24.1 ± 6.2; EMADa, 1.33 ± 0.14; EEPa, 0.83 ± 0.05; ELPa, 0.56 ± 0.03; MMAD, 0.55 ± 0.03. *P* values for all fitted curves were <0.005.

Effect of pH and temperature on PA of sludge supernatants

As would be expected, the results showed that PA in sludge extracts increased in warmer conditions (37 °C) compared with the cooler incubation temperature (20 °C) (Figure 5). In general, PA was also raised with increasing pH values up to pH 8, but declined in more alkaline conditions. The magnitude of the response to increasing pH value was also greater in the warmer temperature environment. In this experiment, using the series of sludge types collected from Site E, the greatest PA at all pH values tested was detected in the early air dried sample, EEPb, and the activities detected in fresh digested sludge (EMADb) and a late air dried sample (ELPb) were smaller.

Effect of sludge extracts on the viability of MS2 coliphage

Numbers of MS2 coliphage were reduced by approximately 3 $\log_{10}$ in filtered extracts of digested sludge (EMADb) and late air dried sludge (ELPb) after incubation at 37 °C for 25 h (Figure 6). By contrast, sludge samples from an early air drying pan (EEPb) showed approximately a 4 $\log_{10}$ reduction of MS2 under the same conditions. Thus, the largest decay rate (−0.35) was measured in the early air dried sample (EEPb) and the smallest overall rate of decay (−0.26) of MS2 coliphage was obtained for recently digested sludge (EMADb) (Figure 7). However, no statistically significant difference was detected in the decay coefficients determined for any of the different sludge samples tested.

DISCUSSION

Effect of indigenous flora and nutrients on the growth of *S.* Birkenhead in biosolids

The mechanisms influencing the decay of bacterial and viral pathogens in treatment processes, such as MAD and air drying, are poorly understood. In this study, we attempted to elucidate the significance and role of some identified mechanisms potentially responsible for reducing the viability of enteric bacterial and viral pathogens in these types of sludge treatments that support active microbial ecologies.

There are several possible explanations why the growth of *S*. Birkenhead was suppressed in unsterile digested sludge (incubated at 37 °C), or in air dried anaerobically digested sludge (incubated at 20 °C), compared to the same sludge types following sterilisation by autoclaving or γ-irradiation, where the population numbers increased by 2–3 $\log_{10}$ CFU mL^{-1} (Figures 1–3). One possibility is that the activities of the indigenous microbial flora in unsterile sludge suppressed the growth of the bacterial pathogen, to maintain numbers at a uniform level. We hypothesise that the situation is likely to be a dynamic one, in which growth and decay of the introduced microbial population occurred simultaneously and was controlled through microbial competition for essential nutrients. In addition, predatory activities of protozoa and other higher organisms, or inactivation by toxic and antimicrobial substances produced by indigenous bacteria may have contributed to the population balance of the introduced pathogen. This is consistent with the conclusions reached by Sidhu *et al.* (2001) for composted biosolids, where a complex interaction was also demonstrated between the growth of introduced *S.* Typhimurium and the suppression of the bacterial population due to nutrient limitation and the production of inhibitory compounds by indigenous microorganisms.

As nutrient supplementation (NB) had a highly significant impact increasing numbers of *S.* Birkenhead in unsterile digested sludge (Figure 3), the results presented here strongly suggest nutrient limitation and competition were major factors limiting growth in unsterile digested sludge. Indeed, the growth ability of *S.* Birkenhead in sterile digested sludge (Figures 1 and 3) could therefore be partly explained by the release of nutrients from dead biomass fractions following sterilisation treatment, as well as the removal of the competing microbial flora. Smith *et al*. (2005) also concluded that both substrate limitation and microbial competition were potentially major factors responsible for pathogen decline during MAD of biowastes. Other studies also consider the activities of the indigenous microbial population as being a major cause of decay of *Salmonella* spp. in composted biosolids (Hussong *et al*. 1985; Sidhu *et al.* 2001), biowaste composts (Lemunier *et al*. 2005) and raw sludge (Yeager & Ward 1981), although in these

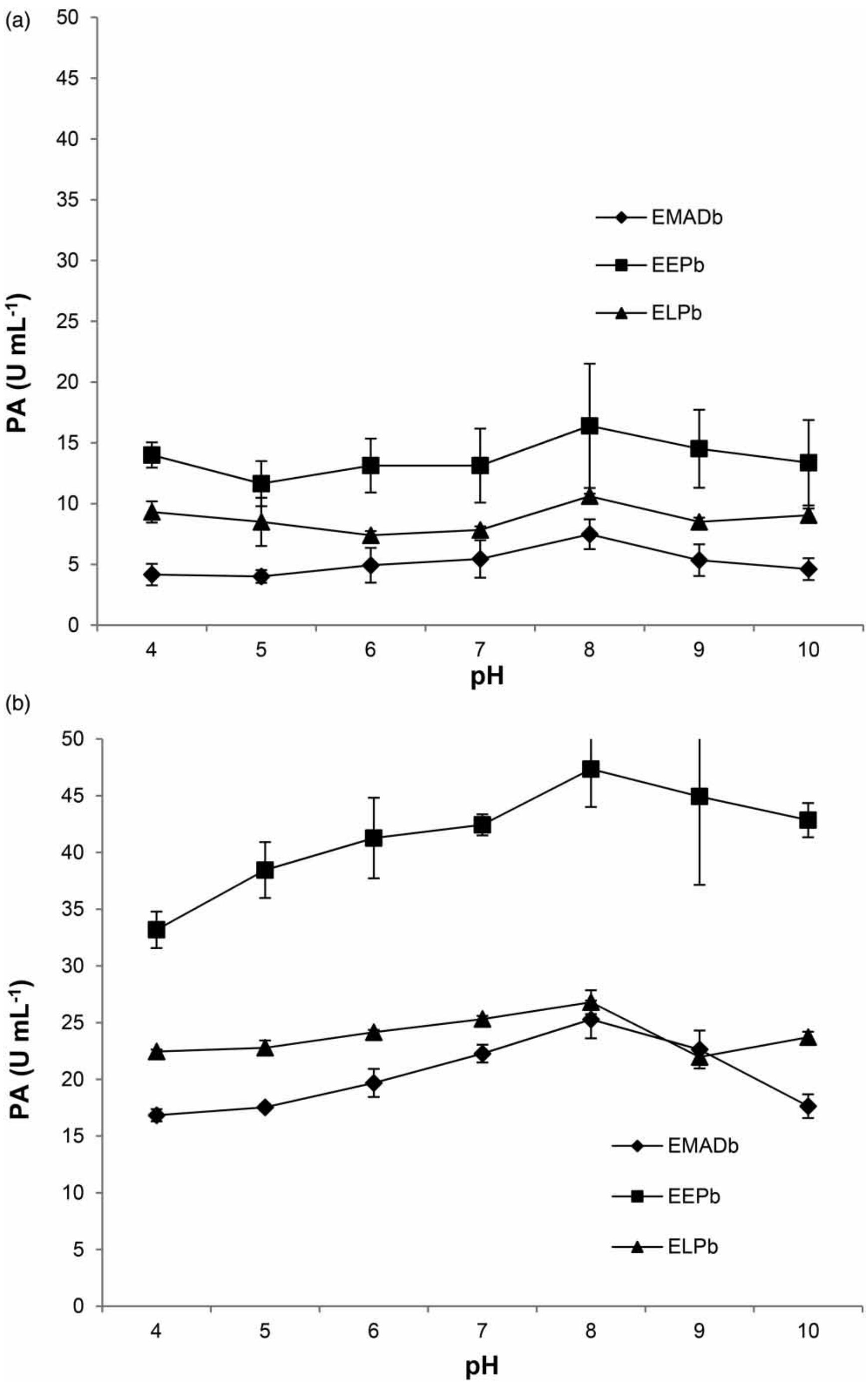

Figure 5 | Effect of pH on PA of extracts from different sludge types (mesophilic anaerobically digested, EMADb; early air dried pan, EEPb; late air dried pan, ELPb) from WWTP E incubated for 24 h at (a) 20 °C and (b) 37 °C. Mean values are shown for triplicate tests and two replicates per treatment; error bars show 1 SD.

examples the effects of nutrient supply and competition were not examined directly.

Further evidence of the significance of nutrients for the growth of *S.* Birkenhead was provided by the microbial response in γ-irradiated air dried sewage sludge which was consistently greater, compared to the autoclaved treatments (Figure 2). Both autoclaving and irradiation destroy indigenous flora; however, they influence the physicochemical

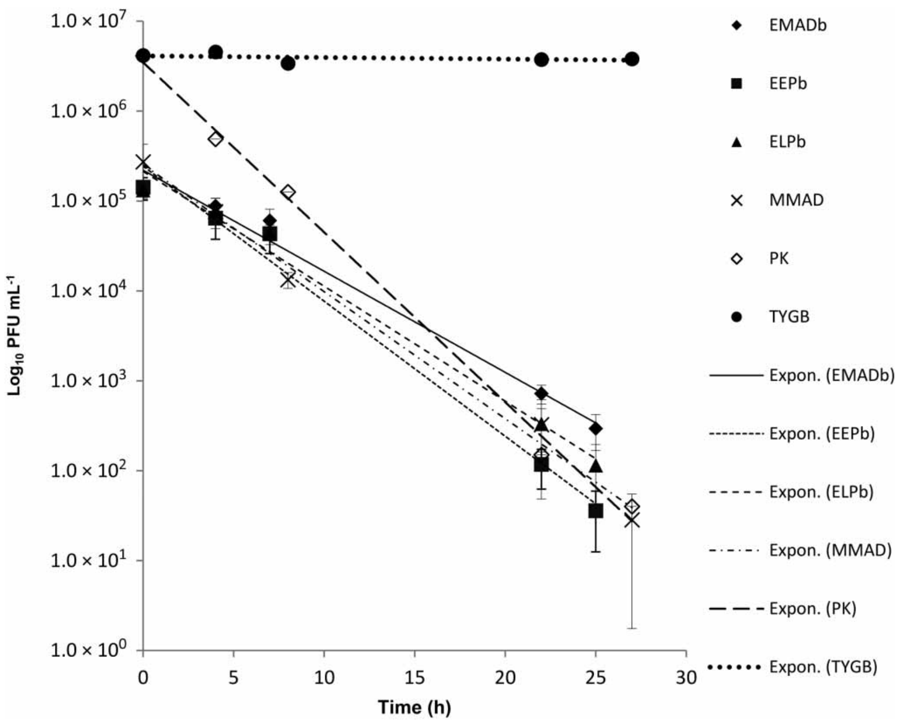

Figure 6 | Effect of extracts from different sludge types (mesophilic anaerobically digested, EMADb and MMAD; early air dried pan, EEPb; late air dried pan, ELPb) as protease sources for the removal of MS2 coliphage compared to positive (PK) and negative (TYGB) controls. Error bars show 1 SD. Exponential trend lines were fitted to the data: EMADb ($r^2 = 0.97$), EEPb ($r^2 = 0.97$), ELPb ($r^2 = 0.94$), MMAD ($r^2 = 0.97$), PK ($r^2 = 0.99$), TYGB ($r^2 = 0.12$). *P* values for data in all incubations were <0.005, except for the negative control, $P = 0.11$.

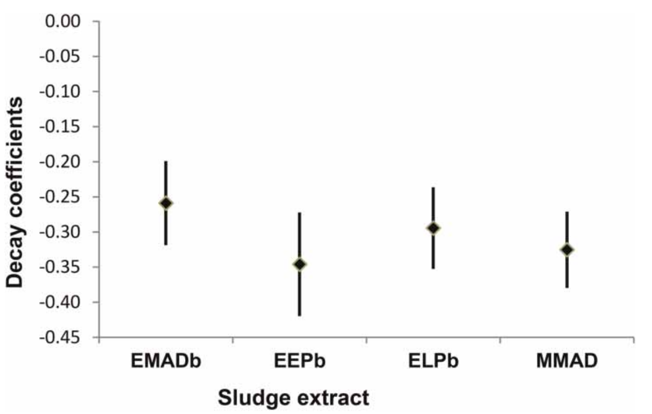

Figure 7 | Decay coefficients of MS2 coliphage in extracts of mesophilic anaerobically digested sludge from WWTP E (EMADb) and WWTP M (MMAD) and early and late air dried pan sludge from WWTP E (EEPb and ELPb, respectively). Symbols denote mean decay coefficients and vertical bars indicate 95% confidence limits.

environment in the sludge in different ways. For example, autoclaving (130 °C with a holding time of 5 min) of waste activated sludge solubilised about half of the solids, reduced the lipid and carbohydrate content by 20–30%, increased the protein content slightly (by ~5%), increased the volatile fatty acid content substantially and increased the pH of the sludge from 6.4 to 7.0 (Tanaka & Kamiyama 2002). Heat also causes protein unfolding (Haque *et al.* 2013), decreasing the availability of active bacterial enzymes. By contrast, γ-irradiation (25 kGy) disintegrated and solubilised sludge flocs, caused a slight decrease in proteases, catalase and superoxide dismutase, and a significant increase in the levels of protein and carbohydrates in the soluble fraction, indicating the transfer of large amounts of insoluble organic material into the soluble fraction. Nitrate concentrations were also greatly reduced, while there was a small increase in ammonia content (Chu *et al.* 2011). Therefore, γ-irradiation treatment of sludge may improve the overall bioavailability of proteins and carbohydrates for microbial growth, which is consistent with the increased numbers of bacteria observed in the irradiated sludge samples compared to autoclaved sludge (Figure 2).

Sterilisation treatment generally increased the growth of inoculated *S.* Birkenhead in both digested and air dried sludge (Figures 1–3), except in the case of AEEP where no bacterial growth occurred (Figure 2). Possible reasons for the apparently less favourable environment for growth in autoclaved early pan sludge (30–80 days of drying, VS = 69%) compared to autoclaved older pan material (188–420 days of drying, VS up to 58%) could be related to the release of inhibitory substances from microbial cells damaged during autoclaving or to an increase in the availability of one or more key nutrients during sludge drying and ageing. Furthermore, *Salmonella* spp. are facultative anaerobic bacteria and aerobic microbial growth may improve due to increased aeration and organic matter degradation as the sludge slowly dries and crumbles (Ulfig *et al.* 2006).

In addition to the biological factors suppressing the growth of bacterial pathogens in sludge, the experimental results also provided evidence of physicochemical limitations to growth. Thus, numbers of *S.* Birkenhead in unsterile digested sludge did not decline during the relatively short experimental incubation employed (24 h) and were stimulated by nutrient supplementation. However, the growth of *S.* Birkenhead in the unsterile sludge samples receiving nutrient supplementation was significantly smaller compared to the large population increase measured in NB alone (Figure 3). As would be expected, NB is designed to provide ideal growth conditions for a variety of bacteria. Thus, the data presented here suggest that competition and other microbiological factors, coupled with physicochemical constraints, such as the high electrolyte concentration of sludge, can be detrimental to the growth of *Salmonella* spp. (Foster & Hall 1991; Mattick *et al.* 2001; Lang & Smith 2008).

Effect of proteases on the fate of coliphage in sewage sludge

The fresh digested sludge from WWTP E demonstrated significant PA, which decreased significantly with air drying time (Figure 4). This behaviour is typical of environmental media showing high levels of heterotrophic and hydrolytic microbiological activities associated with the degradation of nitrogenous organic materials (Lenhard 1965). The observed reduction in PA during air drying may be linked to the declining availability of substrates as the degradation and stabilisation of organic matter increases during treatment, since it was greatest in sludge sampled directly from MAD and from early air drying pans (VS ~ 70%) and lowest in late air dried sludge (VS 58%). The PA of sludge also varied between different WWTPs (Figure 4) suggesting the microbial communities present in the sludge samples comprise different microbial populations. Indeed, the PA of sludge collected from various stages of wastewater and sludge treatment (activated sludge, anaerobically digested, aerobic granules and lagoon sludge) has been attributed to many different species of bacteria (Lenhard 1965; Gaddad *et al.* 1987; Chen *et al.* 2004; Xia *et al.* 2007; Adav *et al.* 2009). These properties could be influenced by the input wastewater characteristics and particularly by differences in the management and operation of similar sludge treatment processes between sites, as well as physicochemical factors, including temperature and pH (Figure 5). Physicochemical dynamics inducing changes in microbiological community structure would also be expected to influence potential PA during air drying of sewage sludge.

The very significant reduction (3–4 $\log_{10}$) of MS2 coliphage in sludge extracts (Figures 6 and 7) strongly suggests this behaviour is linked to bacterial proteases, although other antiviral mechanisms cannot be discounted. The observed loss of viable MS2 coliphages demonstrates the potential intrinsic inactivation of enteric viruses in sludge, which may be particularly relevant to treatment processes involving microbiological stabilisation mechanisms. Enteroviruses are susceptible to destruction by bacterial proteases, but the response varies greatly between different viruses and proteases (Cliver & Herrmann 1972; Nasser *et al.* 2002). Consequently, the susceptibility of enteric viruses to protease inactivation may vary compared to MS2 coliphage. However, it is possible that dynamic bacterial populations present during sewage sludge treatment may produce different proteases, potentially active against specific viral coat proteins. A further question is whether a reduction in virus numbers, similar to that observed here with sludge extract, could be achieved in whole sludge, since viruses generally attach to solid material to different degrees, depending on the virus (Nasser *et al.* 2002), where they may be protected from bacterial proteases. Laboratory simulations of air drying treatment of anaerobically digested sewage sludge demonstrated 4–6 $\log_{10}$ reductions in spiked MS2, at 20 °C, over periods of ~100 days (Rouch *et al.* 2012). However, in the present study, the rate of decay measured for sludge extracts under controlled laboratory conditions at

37 °C was about 300 times higher compared to air drying treatment simulation at ambient temperatures. This difference is consistent with MS2 being protected from decay in whole sludge by attachment to solid material.

CONCLUSIONS

The results presented in this paper suggest that the decay of *Salmonella* spp. during MAD and air drying treatment of sewage sludge depends mainly on nutrient limitation and competition with indigenous flora. Furthermore, the decay of coliphage, and by extension, enteric viruses, in sludge, is likely to be due, at least in part, to the proteases produced by mixed and dynamic populations of indigenous microbes in sludge. These findings contribute further understanding of the mechanisms of decay of bacteria and viruses in sludge and of the fundamental factors potentially responsible for their decay. They emphasise the microbiological and chemical environment in sludge is intrinsically aggressive to survival of bacterial and viral pathogens. Further research is needed to replicate these studies under representative sewage sludge treatment environments; nevertheless, the data presented here will contribute to and inform forecasting of pathogen decay in operational treatment conditions.

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REFERENCES

Adav, S. S., Lee, D. J. & Lai, J. Y. 2009 Proteolytic activity in stored aerobic granular sludge and structural integrity. *Bioresour. Technol.* **10**, 68–73.

Ahn, Y. H. & Choi, H. C. 2004 Municipal sludge management and disposal in South Korea: status and a new sustainable approach. *Water Sci. Technol.* **50** (9), 245–253.

Amahmid, O., Asmama, S. & Bouhoum, K. 2002 Urban wastewater treatment in stabilization ponds: occurrence and removal of pathogens. *Urban Water* **4**, 255–262.

CDC 2011 *Trends in Foodborne Illness, 1996–2010*. Center for Disease Control and Prevention, Atlanta, GA, USA.

Chen, X.-G., Strabnikova, O., Tay, J.-H., Wang, J.-Y. & Tay, S. T.-L. 2004 Thermoactive extracellular proteases of *Geobacillus caldoproteolyticus*, sp. nov., from sewage sludge. *Extremophiles* **8**, 489–498.

Chu, L., Wang, J. & Wang, B. 2011 Effect of gamma irradiation on activities and physicochemical characteristics of sewage sludge. *Biochem. Eng. J.* **54**, 34–39.

Cliver, D. O. & Herrmann, J. E. 1972 Proteolytic and microbial inactivation of Enteroviruses. *Water Res.* **6**, 797–805.

Costán-Longares, A., Montemayor, M., Payán, A., Méndez, J., Jofre, J., Mujeriego, R. & Lucena, F. 2008 Microbial indicators and pathogens: removal, relationships and predictive capabilities in water reclamation facilities. *Water Res.* **42**, 4439–4448.

Dueholm, T. E., Andreasen, K. H. & Nielsen, P. H. 2000 Transformation of lipids in activated sludge. *Water Sci. Technol.* **43** (1), 165–172.

Feng, Y. Y., Ong, S. L., Hu, J. Y., Tan, X. L. & Ng, W. J. 2003 Effects of pH and temperature on the survival of coliphage MS2 and Qβ. *J. Ind. Microbiol. Biotechnol.* **30**, 549–552.

Foster, J. W. & Hall, H. K. 1991 Inducible pH homeostasis and the acid tolerance response of *Salmonella* Typhimurium. *J. Bacteriol.* **173**, 5129–5135.

Gaddad, S. M. & Rodgi, S. S. 1987 The effect of temperature on the growth and biochemical activities of *Escherichia coli* in sewage. *Environ. Pollut.* **43**, 313–321.

Gantzer, C., Gaspard, P., Galvez, L., Huyard, A., Dumouthier, N. & Schwartzbrod, J. 2001 Monitoring of bacterial and parasitological contamination during various treatment of sludge. *Water Res.* **35**, 3763–3770.

George, I., Crop, P. & Servais, P. 2002 Fecal coliform removal in wastewater treatment plants studied by plate counts and enzymatic methods. *Water Res.* **36**, 2607–2617.

Gerardi, M. H. 2000 Wastewater Bacteria (M. H. Gerardi, ed.), Part V. Fermentation and methane production. John Wiley & Sons, Hoboken, NJ, 153–161.

Gessesse, A., Dueholm, T., Petersen, S. B. & Nielsen, P. H. 2003 Lipase and protease extraction from activated sludge. *Water Res.* **37**, 3652–3657.

Grant, E. J., Rouch, D. A., Deighton, M. A. & Smith, S. R. 2012 Pathogen risks in land-applied biosolids. Evaluating risks of biosolids produced by conventional treatment. *AWA Water J.* **391**, 72–78.

Hall, J. E. & Smith, S. R. 1998 Cairo sludge disposal study. *Water Environ. Manage. J.* **11**, 373–376.

Haque, M. A., Aldred, P., Chen, J., Barrow, C. J. & Adhikari, B. 2013 Comparative study of denaturation of whey protein isolate (WPI) in convective air drying and isothermal heat treatment processes. *Food Chem.* **141**, 702–711.

Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J. & Farrah, S. R. 2005 Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Appl. Environ. Microbiol.* **71**, 3163–3170.

Hussong, D., Burger, W. D. & Enkiri, N. K. 1985 Occurrence, growth and suppression of *Salmonella* in composted sewage sludge. *Appl. Environ. Microbiol.* **50**, 887–893.

Idris, A., Yen, O. B., Hamid, M. H. A. & Baki, A. M. 2002 Drying kinetics and stabilization of sewage sludge in lagoon in hot climate. *Water Sci. Technol.* **46** (9), 279–286.

ISO 1995 *Water Quality. Detection and Enumeration of Bacteriophages – Part 1: Enumeration of F-Specific RNA Bacteriophages. ISO 10705–1. Report of the International Organisation for Standardisation ISO*, Geneva, Switzerland.

Lang, N. L. & Smith, S. R. 2008 Time and temperature inactivation kinetics of enteric bacteria relevant to sewage sludge treatment processes for agricultural use. *Water Res.* **42**, 2229–2241.

Lemunier, M., Francou, C., Rousseaux, S., Houot, S., Dantigny, P., Piveteau, P. & Guzzo, J. 2005 Long-term survival of pathogenic and sanitation indicator bacteria in experimental biowaste composts. *Appl. Environ. Microbiol.* **7**, 5779–5786.

Lenhard, G. 1965 *Determination of Protease Activity in Bottom Deposits of Sewage Stabilization Ponds. Report of the National Institute of Water Research, South African Council for Scientific and Industrial Research*, Pretoria, pp. 67–79.

Malack Muhammad, H., Bukhari Alaadin, A. & Abuzaid Nabil, S. 2007 Fate of pathogens in sludge sand drying beds at Qateef, Khobar and Dammam: a case study. *Int. J. Environ. Res.* **1**, 19–27.

Mattick, K. L., Jørgensen, F., Wang, P., Pound, J., Vandeven, M. H., Ward, L. R., Legan, J. D., Lappin-Scott, H. M. & Humphrey, T. J. 2001 Effect of challenge temperature and solute type on heat tolerance of *Salmonella* serovars at low water activity. *Appl. Environ. Microbiol.* **67**, 4128–4136.

Nappier, S. P., Aitken, M. D. & Sobsey, M. D. 2006 Male-specific coliphages as indicators of thermal inactivation of pathogens in biosolids. *Appl. Environ. Microbiol.* **72**, 2471–2475.

Nasser, A. M., Glozman, R. & Nitzan, Y. 2002 Contribution of microbial activity to virus reduction in saturated soil. *Water Res.* **36**, 2589–2595.

NRMMC 2004 *Guidelines for Sewerage Systems. Biosolids Management*. Natural Resource Management Ministerial Council, Commonwealth of Australia, Canberra, Australia.

Pepper, I. L., Brooks, J. P. & Gerber, C. P. 2006 Pathogens in biosolids. *Adv. Agronomy* **90**, 1–41.

Rouch, D. A., Fleming, V., Mondal, T., Glauche, F., Smith, S. R., Blackbeard, J. & Deighton, M. A. 2011 Microbial safety of air-dried and rewetted biosolids. *J. Water Health* **9**, 403–414.

Rouch, D. A., Smith, S. R., Thurbon, N., Fleming, V. & Deighton, M. A. 2012 *Verifying Microbial Safety in Pan-Dried and Stockpiled Biosolids Treatment*. Smart Water Fund Project 611–001, Melbourne, Australia.

SCA 1984 *Methods for the Examination of Waters and Associated Materials: The Conditionability, Filterability, Settleability and Solids Content of Sludge. A Compendium of Methods and Tests. Report of the Standing Committee of Analysts, Methods for the Examination of Waters and Associated Materials Number 83*. DEFRA, UK.

SCA 2004 *The Microbiology of Sewage Sludge – Part 4: Methods for the Detection, Isolation and Enumeration of Salmonellae*. Environment Agency, UK.

Sidhu, J., Gibbs, R. A., Ho, G. E. & Unkovich, I. 2001 The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Res.* **35**, 913–920.

Smith, S. R., Lang, N. L., Cheung, K. H. M. & Spanoudaki, K. 2005 Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manage.* **25**, 417–425.

Strauch, D. 1990 Microbiological treatment of municipal sewage sludge and refuse as means of disinfection prior to recycling in agriculture. *Environmental Biotechnology: Studies in Environmental Science* (A. Blazej & V. Privarova, eds). Elsevier, Amsterdam, vol. 42, 121–136.

Tanaka, S. & Kamiyama, K. 2002 Thermochemical pretreatment in the anaerobic digestion of waste-activated sludge. *Water Sci. Technol.* **46** (10), 173–179.

Tanji, Y., Mizoguchi, K., Yoichi, M., Morita, M., Hori, K. & Unno, H. 2002 Fate of coliphage in a wastewater treatment process. *J. Biosci. Bioeng.* **94**, 172–174.

Ulfig, K., Płaza, G., Terakowski, M. & Janda-Ulfig, K. 2006 Sewage sludge open-air drying affects on keratinolytic, keratinophilic and actidione-resistant fungi. *Rocz. Panstw. Zakl. Hig.* **57**, 371–379.

US EPA 2003 *Environmental Regulations and Technology – Part 503: Control of Pathogens and Vector Attraction in Sewage Sludge*. United States Environmental Protection Agency, Cincinnati, OH, USA.

Ward, R. L., Yeager, J. G. & Ashley, C. S. 1981 Response of bacteria in wastewater sludge to moisture loss by evaporation and effect of moisture content on bacterial inactivation by ionizing radiation. *Appl. Environ. Microbiol.* **41**, 1123–1127.

Watanabe, H., Kitamura, T., Ochi, S. & Ozaki, M. 1997 Inactivation of pathogenic bacteria under mesophilic and thermophilic conditions. *Water Sci. Technol.* **36** (6–7), 25–32.

Xia, Y., Kong, Y. & Nielsen, P. H. 2007 In situ detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbiol. Ecol.* **60**, 156–165.

Yeager, J. G. & Ward, R. L. 1981 Effects of moisture content on long-term survival and regrowth of bacteria in wastewater sludge. *Appl. Environ. Microbiol.* **41**, 1117–1122.

Yu, G. H., He, P. J., Shao, L. M. & Lee, D. J. 2007 Enzyme activities in activated sludge flocs. *Appl. Microbiol. Biotechnol.* **77**, 605–612.

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