Microbial safety of air-dried and rewetted biosolids

Duncan A. Rouch, Tania Mondal, Sneha Pai, Florian Glauche, Vennessa A. Fleming, Nerida Thurbon, Judy Blackbeard, Stephen R. Smith and Margaret Deighton

ABSTRACT

To assess microbial safety of treated sewage sludge (biosolids), we examined the inactivation of microbial indicators for potential bacterial, viral and protozoan pathogens. The levels of indicators were determined throughout the air-drying and storage phases of anaerobically digested sewage sludge. Samples were collected from two wastewater treatment plants (WWTPs) in Victoria, Australia. Established methods were applied for analysis of bacteria and coliphages, based on membrane filtration and layered plates, respectively. In the pan drying phase, the prevalence of *Escherichia coli* was reduced by >5 $\log_{10}$ compared with sludge entering the pan. Thus, after pan drying of 8-11 months at WWTP A and 15 months at WWTP B, the numbers of *E. coli* were reduced to below 10^2 cfu/g dry solids (DS). This level is acceptable for unrestricted use in agriculture in Australia (P1 treatment grade), the UK (enhanced treatment status) and the USA (Class A pathogen reduction). Coliphage numbers also decreased substantially during the air-drying phase, indicating that enteric viruses are also likely to be destroyed during this phase. *Clostridium perfringens* appeared to be an overly conservative indicator. Survival, but not regrowth, of *E. coli* or *Salmonella* was observed in rewetted biosolids (15–20% moisture content), after being seeded with these species, indicating a degree of safety of stored biosolids upon rewetting by rain.

Key words | air-dried biosolids, *Escherichia coli*, microbial indicators, microbial safety, regrowth, rewetting

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INTRODUCTION

Stabilized sewage sludge (biosolids) is produced by the biological treatment of sewage. Biosolids are highly beneficial natural resources, that can provide plant nutrients and improve soil structure when applied to land (Natural Resource Management Ministerial Council 2004). Sewage sludge is treated and dewatered to reduce the volume, aid removal of pathogens, and diminish the cost of subsequent management processes (US EPA 1982; 2003). The use of solar drying beds and lagoons provide a low cost dewatering option, and are commonly used in small to medium sized systems, or where land-use issues are not a factor. Solar drying is predominantly used in warmer climatic regions, such as in the USA, Europe, Middle-East, Asia and Australia (Hall & Smith 1997; Idris *et al.* 2002; US EPA 2003; Natural Resource Management Ministerial Council 2004; Ahn & Choi 2004; Seginer & Bux 2006; Muhammad *et al.* 2007; Salihoglu *et al.* 2007; Tamimi *et al.* 2007). In Victoria, Australia dewatering treatment generally involves solar-drying and stockpiling of dried biosolids. In order to assure the safety of land application of biosolids, it is critical that levels of potentially pathogenic microbes and potentially toxic chemicals are within safe limits (Englande & Reimers 2001; Singh & Agrawal 2008; US EPA 2003; Natural Resource Management Ministerial Council 2004).

To maintain public health, the use of biosolids is regulated in many countries through the concept of fit-for-purpose. For unrestricted use of biosolids on agricultural land, the Australian national guidelines require $<10^2$ *Escherichia coli*

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cfu/g dry solids (DS) (Treatment Grade P1, Natural Resource Management Ministerial Council 2004). This is more restrictive than the limits applied in the USA for Class A biosolids (<=103 cfu/g DS; US EPA 2003), while in the UK the Safe Sludge Matrix defines an enhanced treatment category as $\leq 10^3$ cfu/g DS and 6 log reduction of *E. coli* (Godfree & Farrell 2005). Lower treatment grades allow higher numbers of pathogens, but with reduced types of application, and may require withholding periods, to provide multi-barriers.

During storage, the nutrient value of biosolids diminishes owing to the loss of mineral nitrogen (N) by volatilization of the ammoniacal content, stabilization of the organic N fraction, reduced solubility of phosphorus (P) and degradation of the organic matter content. Over time, this compromises the quality and value of the treated material as a soil improver and fertilizer. Therefore, shortening the storage period has the benefit of increasing the agronomic value of the biosolids, but account must be taken of the implications for the microbiological quality of the product. Storage after mechanical or solar/air drying for periods of between 3 to 6 months to meet appropriate microbiological criteria for agricultural application is commonly practiced in countries with climates ranging from temperate (e.g. UK) to arid (e.g. Egypt) (Hall & Smith 1997; Lang *et al.* 2003). However guidelines in Victoria, Australia, stipulate a storage period of 3 years for production of material that can be used on land without restriction (T1 grade, equivalent to the national P1 grade) (EPA Victoria 2004).

In addition, the potential for regrowth of pathogens in certain types of treated sewage and composts has been recognised as a microbial safety issue (US EPA 2003). Additional controls, such as in Victoria, Australia, include the requirement for testing regrowth of microbial pathogens in treated biosolids (EPA Victoria 2004).

The species *E. coli* and *Salmonella* are accepted as a means of assessing the presence of bacterial pathogens in environmental samples. *E. coli* is a useful indicator of the presence and re-growth capacity of enteric bacteria. *Salmonella* is an important enteric pathogen of humans that could conceivably re-grow if undetectable levels of viable bacteria or viable but non-culturable bacteria were present in dewatered biosolids. Moreover recontamination of stockpiles by *Salmonella* could occur following exposure to faecal material from either domestic or wild animals (Zaleski *et al.* 2005a). Coliphages, such as MS2, are represented as an indicator for the removal of enteric viruses in wastewater treatment, and *Clostridium perfringens* has been proposed as an indicator for the removal of protozoan pathogens. In addition, enterococcal species as a group may be considered as a potential indicator in pathogen removal, due to their tolerance of adverse environmental conditions (Fisher & Phillips 2009).

We have investigated the inactivation of microbial indicators for enteric pathogenic bacteria, in biosolids during the air-drying phase and storage of anaerobically digested biosolids at two wastewater treatment plants in Victoria, Australia. We have also quantified the survival and extent of the potential regrowth of bacteria in biosolids under simulated environmental conditions.

Preliminary data of this study was presented at the Biosolids Specialty Conference IV, 11–12 June 2008, Adelaide.

MATERIALS AND METHODS

Sites

Biosolids samples were taken from two wastewater treatment plants (WWTPs) in the Melbourne metropolitan area, Victoria, Australia, coded here as WWTP A and WWTP B. These two plants have similar process sequences, which culminate in anaerobic digestion and air-drying in open pans, followed by stockpiling uncovered for a period of at least three years. WWTP A serves a population of about 1.5 million people in Melbourne's South-Eastern and Eastern suburbs (42% of Melbourne city). About 92% of the sewage that flows into this plant is from homes and businesses. The remaining 8% is from industry. After grit removal, the sewage undergoes screening and sedimentation to remove solids (primary treatment), followed by an activated sludge process, clarification and thickening. Sludge entering the anaerobic digester contains a mixture of thickened and primary sludge. At WWTP B 90% of the sewage inflow consists of domestic waste and 10% industrial waste, from a smaller regional area (population about 72,000). WWTP B uses a similar treatment system compared to WWTP A, but at a substantially smaller scale. Due to the focus on open pan air-drying, brief details of the air-drying processes at the two

plants are outlined. The drying pans sampled at WWTP A included pans with either cement or clay bases, while those at WWTP B had only clay bases. There were also differences in the treatment of biosolids between the two pans. At WWTP A the sampled pans were serially filled, with settling and decanting over 5 to 10 weeks, until the dry solids (DS) value reached about 4%, before being air-dried over 6 months (November to April), a 'summer pan', to 10 months, a 'full year pan'. In contrast, at WWTP B, serial filling, sampling and decanting of the sampled pan took place over 18 weeks. At the end of filling a water cap was left in place for 6 months to facilitate secondary digestion, then the water was decanted and the pan was air-dried for 7 months. The pans were uncovered during the entire filling, settling, decanting and drying period. In addition, pans were regularly stirred vertically, about once per week, during the drying phase at WWTP A, but not at WWTP B. At a weather station located in the region of these two WWTPs the mean minimum and maximum daily temperatures in winter were 8.3 and 13.3 °C, and in summer these were 15.0 and 23.9 °C, respectively (Bureau of Meteorology 2010).

Biosolids sampling and analysis

At WWTP A, samples were collected during the air-drying treatment in three pans, two 'full year pans', one in each of two periods, SDP 33 (2007–08) and SDP 23 (2009–10), and a 'summer pan' SDP 41, during 2009–10. At WWTP B samples were taken only during 2007–08, of a 'full year pan', Pan 3, on four separate occasions, from October to April, during the Australian summer months. The ages of pans and stockpiles were recorded as the time since the start of filling of the pan or, in the case of stockpiles, the time of filling the associated pan. Generally, on a day of sampling, triplicate samples were taken from the pump output of anaerobic digesters and three composite samples were taken from air-drying pans. Stockpiles were sampled three times at three different depths to provide composite samples at, 0–0.2 m (surface), 0.4–0.6 m, and 0.9–1.1 m.

Microbial enumeration

Biosolids samples were stored at 4 °C and analysed within 72 h for the presence of *E. coli*, enterococci, *C. perfringens*, and coliphages. To count viable bacteria, biosolids were diluted 1:10 in Maximum Recovery Diluent (MRD, Oxoid) in sterile glass bottles that also contained 10 g sterile glass beads, then placed on an orbital shaker at 200 rpm for 4 min. Following a series of 10-fold dilutions in MRD, the viable counts were determined by the membrane filtration technique. Enumeration by membrane filtration of *E. coli* and confirmation were by methods modified from those detailed by the UK Environment Agency (Environment Agency UK 2002a; 2003). *Escherichia coli* was detected as green coloured colonies on membrane lactose glucuronide agar (MLGA, Oxoid), incubated at 30 °C for 2–3 h and 44 °C for 18–24 h. The enumeration by membrane filtration and confirmation of enterococci and *C. perfringens* were performed based on standard methods (Environment Agency UK 2002b, c).

For membrane filtration, 1.0 mL samples of diluted biosolids were filtered in triplicate using 0.45 μm 47 mm nitro-cellulose grid membranes (Millipore), supplied by an Ez-Pak membrane dispenser (Millipore), and applied to two triple-head filtration systems, (a) Sartorius, with steel funnels, and (b) Millipore, with disposable funnels (Microfil V). After adding samples, 20 mL of phosphate buffered saline (PBS, Sigma-Aldrich) was applied to each membrane to ensure efficient distribution of bacteria. After filtration the membranes were placed on the media appropriate to each bacterial species and incubated as described above. After incubation, colony counts were determined and the concentration of organisms calculated as cfu/g DS, for field samples, or cfu/mL, for rewetted biosolids. The DS content was assessed according to Rothamsted (2004).

Coliphages (including F-specific phages) were enumerated by standard methods using the *E. coli* HS(pFamp)R host (ISO 1995). The host strain was grown in Tryptone Yeast-extract Glucose Broth (TYGB) media at 37 °C with shaking to an OD 600 nm value of 0.4, then stored on ice until use. 3.5 mL aliquots of Semisolid Tryptone Yeast-extract Glucose Agar (ssTYGA) media were distributed in test tubes and kept at 50 °C. In general, to each tube was added 20 μl of nalidixic acid solution (25 mg/mL), 48 μl of ampicillin solution (15 mg/mL), 1 mL of sample and 0.25 mL of host. Each mixture was lightly mixed before pouring over a 90 mm plate of Tryptone Yeast-extract Glucose Agar (TYGA) media. Plates were incubated at 37 °C for 16 h before

plaques were enumerated. TYGB, pH 7.2, contained, per L, 10 g tryptone (Oxoid, LP0042), 1 g yeast extract (Oxoid, LP0021), 8 g NaCl. TYGA and ssTYGA additionally contained 14 g and 7 g of agar (Oxoid, LP0011) per L. After autoclaving, ssTYGA was amended with 10.0 mg glucose, 3 mg $CaCl_2.2H_20$ and 3 mg $MgSO_4.7H_20$ per L, supplied in 100 × concentrations in stock solutions.

In addition, the $\log_{10}$ reduction of *E. coli*, coliphages and enterococci was compared to DS contents of the biosolids during the pan drying phase. For data calculations the full pan treatment times were taken, starting from the time of beginning of filling.

Bacterial strains used for regrowth experiments

Escherichia coli M34 was previously isolated from a drying-pan at an Australian WWTP. *Salmonella enterica* subsp. *enterica* serovar Birkenhead was isolated from a calf with diarrhoea. Bacteria were routinely grown in Nutrient Broth No. 2 (NB, Oxoid, CM0067) and plated on Nutrient Agar (NA, Oxoid, CM0003).

Regrowth experiments

The four basic steps followed in the regrowth analysis were biosolids preparation, bacterial growth, sample incubation, and sample analysis. The six biosolids samples used were drying pan sample A-P-13, and stockpile samples A-S(0.1)-25, A-S(0.5)-25, A-S(1.0)-25, B-S(0.1)-25 and B-S(0.5)-25 (Table 1) except that in the case of being challenged with *E. coli*, biosolids A-S(0.1)-25 and A-S(0.5)-25 were not included due to lack of sufficient material. The six biosolids samples were chosen as representing possible products for land application. To prepare these samples, aliquots of approximately 500 g were dried at 37 °C for 4 days, and aseptically broken into fragments of 10 mm diameter or less, using a mortar and pestle. Aliquots of 5 g, of each sample were used to determine the moisture content (Rothamsted 2004). Two conditions were set up per sample; 5–10% moisture (dry), and 15–20% moisture (moist). The moist level was chosen to provide similar physical structure to the dry samples, without any free water. For each sample, 6 × 15 g aliquots were measured into 70 mL sterile plastic screw-top containers. Three of these remained as the dry aliquots, while for moist biosolids three of the aliquots were amended with 3 mL sterile reverse osmosis (RO) water and thoroughly mixed.

Table 1 | Properties of Biosolids Used for Regrowth Test

Code[a]	Type	Age[b] (months)	Depth	DS%	MC%	VS%
A-P-13	Drying pan	13	NA	96.9	3.1	ND
A-S(0.1)-25	Stockpile	25	0.0-0.2 m	90.4	9.6	ND
A-S(0.5)-25	Stockpile	25	0.4-0.6 m	89.8	10.2	ND
A-S(1.0)-25	Stockpile	25	0.9-1.1 m	83.4	16.6	59.1
B-S(0.1)-25	Stockpile	25	0.0-0.2 m	96.6	3.4	26.3
B-S(0.5)-25	Stockpile	25	0.4-0.6 m	96.1	3.9	29.5

[a]Sample code: plant [A or B] -type [P, pan or S, stockpile (depth, m)] -age [months]. MC, moisture content; VS, volatile solids; NA, not applicable; ND, not determined.
[b]time since the start of pan filling.

Overnight cultures of each strain in NB, were diluted in fresh NB to 5×10^8 cfu/mL, by measuring the OD600 nm values, which had been previously calibrated to plate counts on NA. Aliquots of 30 μL of each of the bacterial cultures were added directly to the surface of 15 g biosolids, in the plastic containers, giving a final concentration of 1×10^6 cfu/mL. For samples without added bacteria, 30 μL sterile RO water was added to each 15 g sample. The mixtures were shaken vigorously on an orbital shaker, at 400 rpm for 4 minutes. Thus, the following treatments were applied to the dry and moist biosolids: (1) without added bacteria, (2) seeded with *E. coli* at 10^6 cfu/g biosolids, (3) seeded with *Salmonella enterica* subsp. *enterica* serovar Birkenhead at 10^6 cfu/g biosolids. Mixtures were incubated at 20 °C for 4 weeks and 5 g samples were collected at 0, 2 and 4 weeks for enumeration. Triplicate microbiological analysis of samples was performed. The levels of *E. coli* were determined as described above. Enumeration of *Salmonella* was based on the membrane filtration method of the UK Environment Agency (Environment Agency UK 2002d). *Salmonella* was resuscitated on Rappaport-Vassiliadis Soya Peptone Broth Agar (RVSA) (Oxoid, plus agar at 1.5%), with incubation at 41.5 °C for 2–3 h, and enumerated on xylose lysine desoxycholate agar (XLD, Oxoid), incubated at 37 °C for 18–24 h, on which *Salmonella* were primarily detected as red colonies with black centres.

RESULTS

Indicator die-off in air-drying pans

The numbers of *E. coli* were reduced logarithmically with time in uncovered air-drying pan at each WWTP (Figure 1). At WWTP A, the P2 grade limit ($<10^3$ *E. coli* cfu/g DS) was reached by about 230 days after start of filling the 'full year pans' in both seasons, 2007–08 (SDP 33) and 2009–10 (SDP 23), while the P1 grade limit ($<10^2$ *E. coli* cfu/g DS) was reached by 300 days in both cases. In contrast for the 'summer pan', SDP 41, the P2 and P1 grades were reached in 170 and 230 days, respectively. Interestingly, composite samples from sampling at days 70 and 105 of the 'summer pan' showed substantially more decreased levels of *E. coli* than were expected by the trend line (Figure 1). At WWTP B a slower removal trend was observed for the 'full year pan', Pan3, compared to all pans at WWTP A. In this case the P2 grade limit was reached by 320 days after filling, while the P1 grade limit was reached by 420 days.

At WWTP A destruction of coliphage was similar to that of *E. coli* (Figure 2), with the reduction of $>10^3$ pfu/g DS by 350 days in the two full year pans, and by 230 days in the summer pan. At WWTP B, removal of $<10^2$ pfu/g DS occurred over 500 days. The similarity in removal of *E. coli* and coliphage in the full year pans between the two seasons at WWTP A indicated that air-drying was a robust process for removal of enteric bacteria and viruses, especially as the treatment season 2007–08 was exceptionally dry. There was a reduction of 20% in rainfall during the drying period (September to March), compared to the 2009–10 treatment season (data not shown). Faster removal was seen on average in the summer pan, as would be expected given that its complete drying occurred only during the drying season. Slower removal of *E. coli* and coliphage was observed at WWTP B, possibly due to the differences in the treatment of biosolids in pans between the two plants.

Numbers of enterococci in pans at WWTP A in the 2009–10 season decreased in a similar fashion to *E. coli* (Figure 3). In contrast to the presence of *E. coli*, enterococci and coliphages, *C. perfringens* was detected at similar levels in all samples, at 10^6 to 10^7 cfu/g DS. The numbers of *C. perfringens* increased by about 0.5 $\log_{10}$ in the anaerobic digester at WWTP B, and this species showed only a 0.5 $\log_{10}$ reduction in the drying pan after 8 months.

No *E. coli* or *E. coli* bacteriophages were detected (limit 20 cfu/g DS or pfu/g DS) in stockpiles aged 6 months to 3 years. During February 2008, a small number of *E. coli*

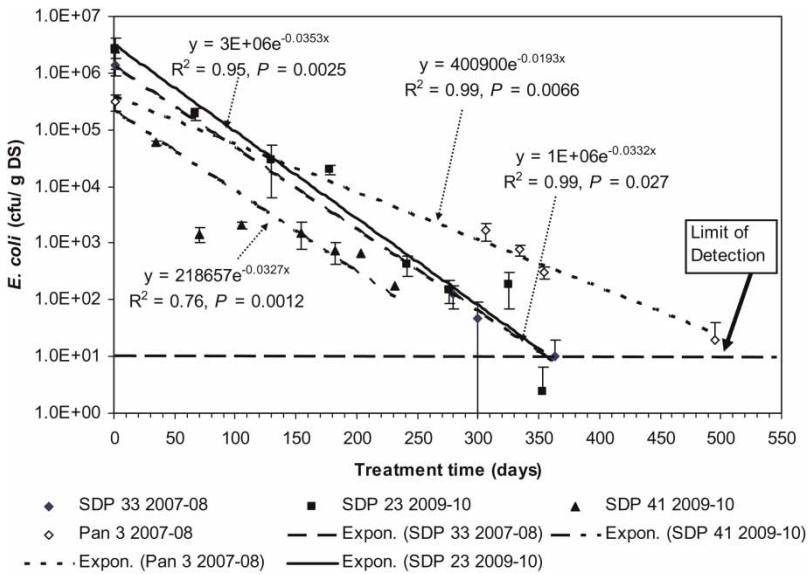

Figure 1 | Effect of air-drying in pans on removal of *E. coli* in digested sludge at WWTP A over two seasons 2007–08 (pan 33) and 2009-10 (pans 23 & 41) and at WWTP B in season 2007–08 (pan 3). R^2, coefficient of determination; *P*, *P*-value. Error bars show 1 standard deviation (sd).

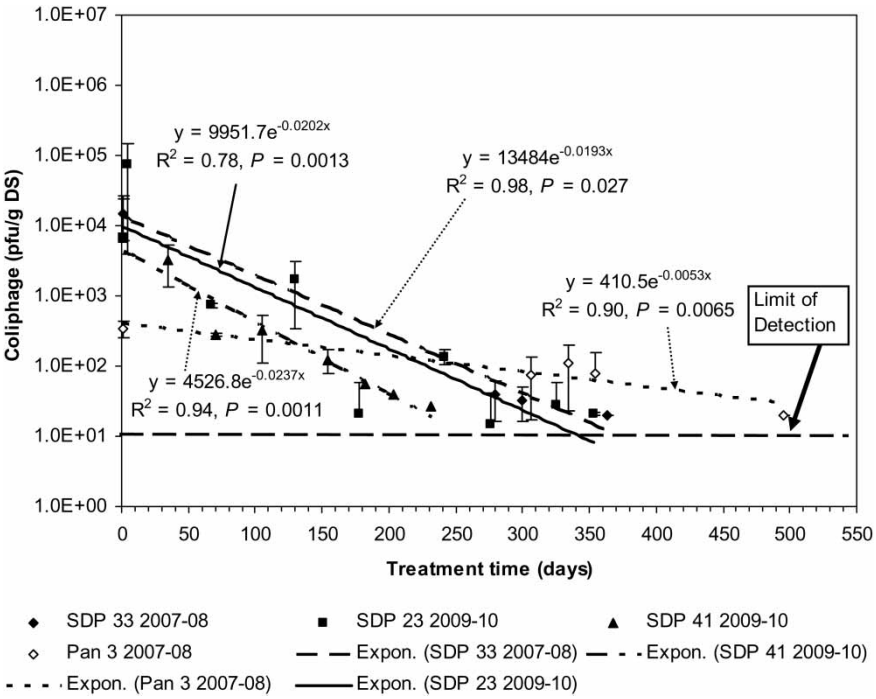

Figure 2 | Effect of air-drying in pans on removal of coliphage in digested sludge at WWTP A over two seasons 2007–08 (pan 33) and 2009–10 (pans 23 & 41) and at WWTP B in season 2007–08 (pan 3). R^2, coefficient of determination; *P*, *P*-value. Error bars show 1 sd.

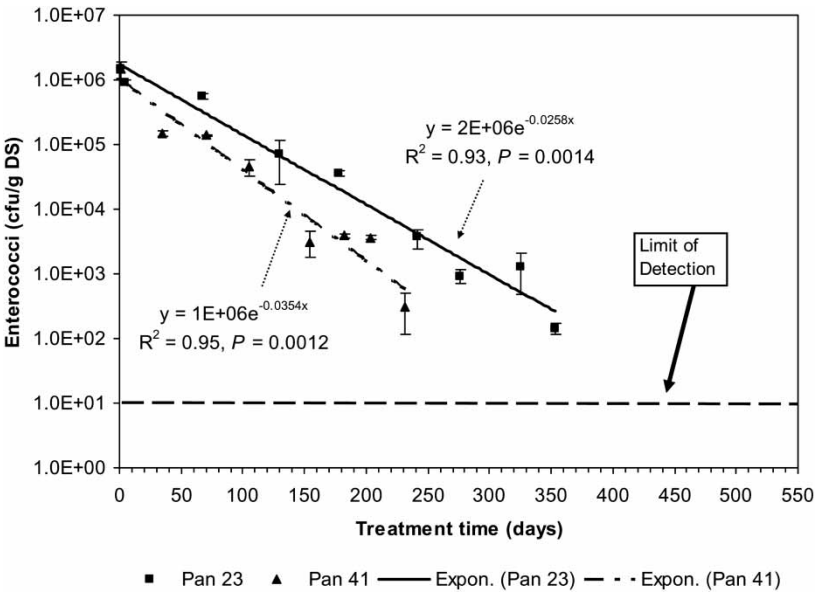

Figure 3 | Effect of air-drying on removal of enterococci in digested sludge at WWTP A, 2009–10 (pans 23 & 41). R^2, coefficient of determination; *P*, *P*-value. Error bars show 1 sd.

were detected in the surface level of a stockpile set up in February 2007 at WWTP B, which previously had not shown any detectable levels of this indicator. As no *E. coli* were detected at a lower level (0.5 m), the results suggested that contamination of the stockpile had occurred recently, presumably due to faecal contamination by birds or land animals.

The results indicate $>4\ \log_{10}$/g DS removal of *E. coli* during the air drying phase due to destruction related to

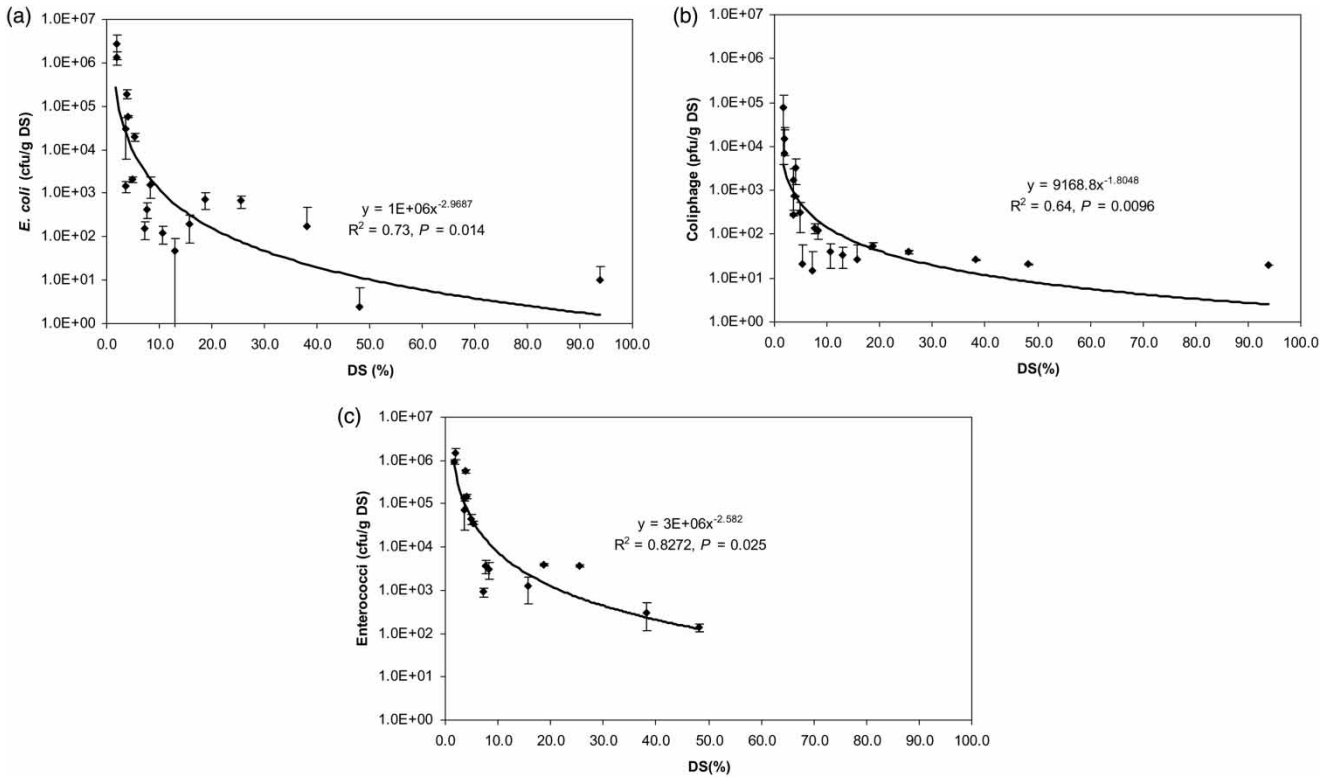

Figure 4 | Removal of indicators vs DS: (a) *E. coli*, (b) coliphage and (c) enterococci. R^2, coefficient of determination; *P*, *P*-value. Error bars show 1 sd.

retention time and drying of the sludge. Moreover, in the stockpiles, the prevalence of *E. coli* was reduced by 6 $\log_{10}$/g DS compared to primary sludge entering the plants. Coliphage numbers also decreased substantially during the air-drying phase, indicating that enteric viruses are also likely to be destroyed during this phase.

The survival values of *E. coli*, enterococci and coliphages showed an inverse relationship to the DS contents of the biosolids (Figure 4). For *E. coli* removal similar inverse relationships to DS content were shown at both plants, so the data were combined (Figure 4(a)). Removal of coliphage and enterococci showed similar inverse relationships to DS content, see Figures 4(b) and 4(c), respectively.

Regrowth

Survival of *E. coli* in wetted biosolids

No *E. coli* or *Salmonella* were detected at any time over the incubation period (4 weeks) in biosolid-soil mixtures without added bacteria, whether dry or wetted. Under moist conditions, the levels of *E. coli* generally remained relatively stable over 4 weeks, without any significant growth, with changes of <0.5 $\log_{10}$ compared to the initial inoculum concentration of 1×10^6 cfu/mL (Figure 5(a)), however, one stockpile sample, B-S(0.5)-25, showed a rapid decline in bacterial numbers (5 $\log_{10}$ over 4 weeks).

Survival of *E. coli* in dried biosolids

In dried biosolids challenged with *E. coli*, the levels of this species declined dramatically, by 2.5 to 4.5 $\log_{10}$, in all four mixtures before the initial sampling time (2–4 h post mixing) (Figure 5(b)). By 2 weeks, the levels of *E. coli* in three mixtures, A-P-13, A-S(1.0)-25 and B-S(0.5)-25, were reduced below the detection threshold and remained so at 4 weeks. One mixture, B-S(0.1)-25, showed a similar decrease at 2 weeks, followed by an increase at 4 weeks. This may have been due to incomplete mixing of the amended biosolids before sampling, nevertheless, the level

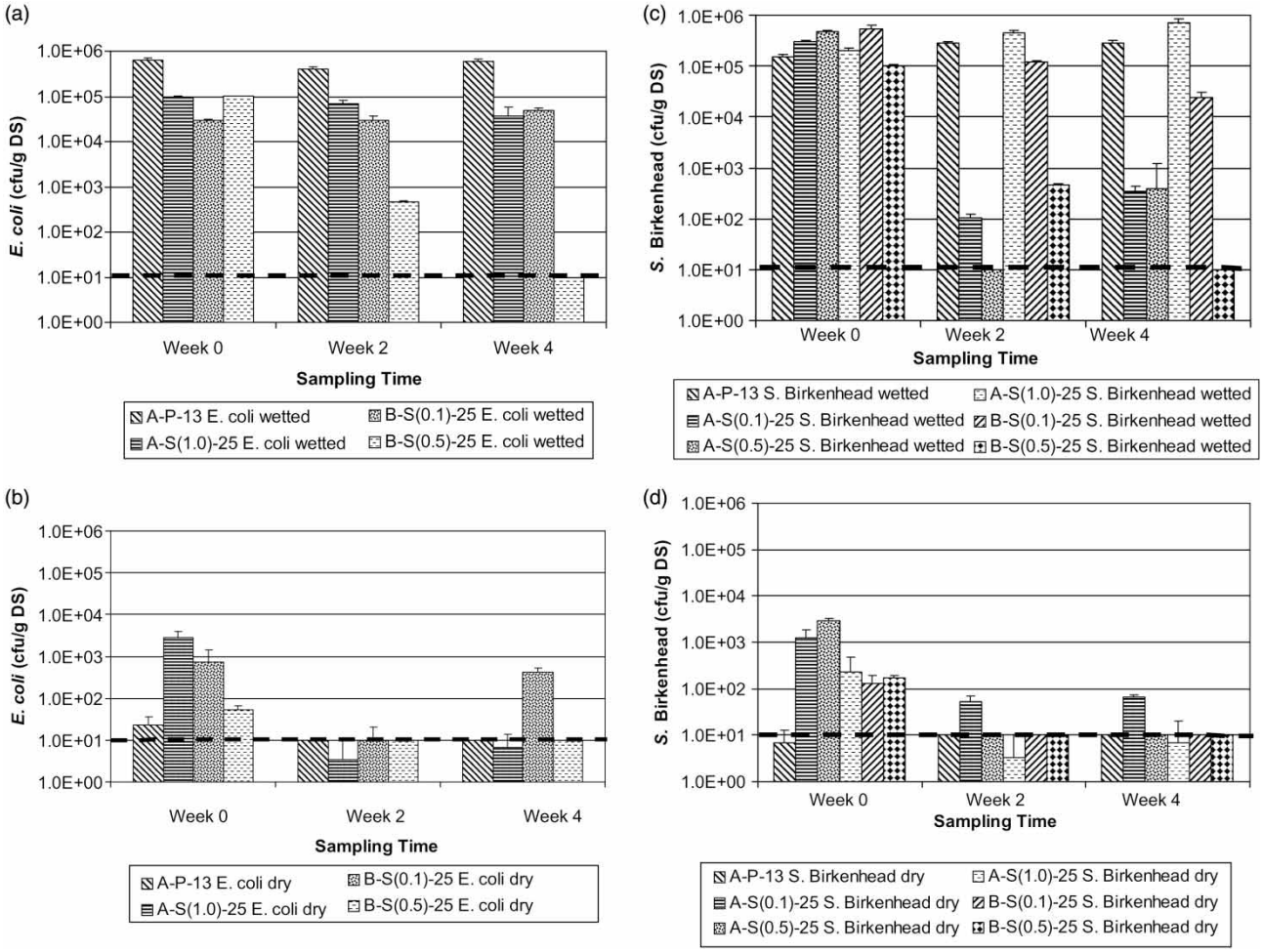

Figure 5 | Survival of *E. coli* and *Salmonella* in wetted and dried biosolids: (a) Survival of *E. coli* in wetted biosolids, (b) survival of *E. coli* in dried biosolids, (c) survival of *Salmonella enterica* subsp. *enterica* serovar Birkenhead in wetted biosolids and (d) survival of *Salmonella enterica* subsp. *enterica* serovar Birkenhead in dried biosolids. The horizontal broken line indicates the minimum detection level. Bars on the columns show one statistical deviation value for each sample.

at 4 weeks remained about 3.5 $\log_{10}$ below the initial amended concentration.

Survival of *Salmonella* Birkenhead in wetted biosolids

In wetted conditions, two biosolids, A-P-13 and A-S(1.0)-25, showed relatively stable levels of *Salmonella* over the 4-week sampling period, with slight increases of less than ~0.5 $\log_{10}$, being within 3 standard deviations of the mean (Figure 5(c)). In contrast, one stockpile sample, A-S(0.1)-25, exhibited a mild reduction in the bacterial count (2 $\log_{10}$ reduction over 4 weeks), while three samples showed a reduction of ~2–4 $\log_{10}$ by 2 weeks, with two showing a slight increase by 4 weeks.

Survival of *Salmonella* Birkenhead in dried biosolids

All six dry biosolids samples showed substantial initial decreases in *Salmonella* levels, of 3 to 5 $\log_{10}$ (Figure 5(d)). Subsequently, five of the six samples exhibited non-detectable levels of *Salmonella*, with only stockpile sample A-S(0.1)-25 showing a quantifiable amount, though still 4 $\log_{10}$ below the initial amended level.

Comparison of survival of *E. coli* and *S.* Birkenhead

The survival of *E. coli* and *Salmonella* in wetted biosolids generally showed similar trends, although *E. coli* appeared to survive better than *S.* Birkenhead under the experimental

conditions used. Although for three treatments, the bacterial numbers of both species generally remained constant or declined slightly over the incubation period, three others behaved differently, with a more rapid decline in numbers of one or both species, followed by an apparent small level of re-growth in the case of two samples (Figures 5(a) and 5(c)).

The survival of *E. coli* and *Salmonella* in dried biosolids also followed similar patterns. Initially the levels of bacteria were in the order of 1×10^3 to 1×10^4 cfu/mL. These levels gradually declined and most samples had undetectable levels of bacteria by 4 weeks, except for *E. coli* in sample B-S(0.1)-25 and *Salmonella* in sample A-S(0.1)-25.

DISCUSSION

Pathogen die-off

At the two plants examined in this study, air drying for 15 months or less in open pans, at ambient temperatures ranging from 8.3 to 23.9 °C, resulted in a reduction of the numbers of *E. coli* to below 1×10^1 cfu/g. These levels are compliant with the P1 treatment grade in Australia and are below Class A biosolids requirements in the USA for unrestricted use, as well as the enhanced treatment status for agricultural use of biosolids in the UK. In addition, the results indicate a $>4 \log_{10}$/g DS reduction of *E. coli* during the air drying phase which was related to retention time and drying of the sludge. This would exceed the conventional treatment requirements for agricultural use of biosolids prescribed in the UK Safe Sludge Matrix. Although we did not investigate the mechanism of indicator die-off, multiple factors are likely to be involved. These include moisture availability, nutrient depletion, predation and competition with indigenous flora (Rudolfs *et al.* 1950; Sidhu *et al.* 2001; Zaleski *et al.* 2005a). Levels of *E. coli* observed in the 'summer pan' at WWTP A (SDP-41) decreased more than expected during November and December, which might possibly be due to increased predation or competition in the relatively fresh sludge of this pan during increased environmental temperatures at this time, at the beginning of summer.

Desmier *et al.* (2004) have reported substantially higher reduction of *E. coli* content in an agitated air drying method. In this technique tertiary treated sludge was centrifuged before being windrowed, and was then turned every one or two days. The higher rate of *E. coli* reduction in this case is likely to be due to the increased aerobic conditions provided by this process, compared to pan-drying. Nevertheless, higher aerobic conditions would be expected towards the end of pan drying, and might contribute to the reduction of *E. coli* content, and perhaps other bacterial pathogens at that phase.

Clostridium perfringens has been suggested as an indicator in water treatment for removal of parasites such as *Cryptosporidium* and *Giardia*. In this study, however, little change in content of *C. perfringens* was observed during the drying pan treatment. This suggests that *C. perfringens* might survive substantially during pan drying treatment. Alternatively, as *C. perfringens* is a common organism found in the environment, it is possible that while some *C. perfringens* might have died during treatment, these might have been replaced from environmental sources. Since *Cryptosporidium* oocysts are highly susceptible to desiccation when suspended in water on glass slides and allowed to dry (Robertson *et al.* 1992), these are likely to be substantially more sensitive to pan drying treatment than *C. perfringens*. Moreover $\log_{10}$ removal of clostridial spores was much lower than that of *Cryptosporidium* in an activated sludge process (Wen *et al.* 2009) and studies of reclaimed water showed no correlation between removal rates of *Cryptosporidium* and *Clostridium* species (Harwood *et al.* 2005; Briancesco & Bonadonna 2005). The loss of infectivity of *Cryptosporidium* occurs due to the depletion of energy resources (King *et al.* 2005). Additionally, in mesophilic anaerobic digestion, reduction of *Cryptosporidium* is reported to be 2–3 $\log_{10}$ (Stadterman *et al.* 1995), substantially more than observed for *C. perfringens* in this study. Taken together, these reports support our data and suggest that *C. perfringens* is a poor indicator of the inactivation of enteric organisms in biosolids.

Hazard Analysis and Critical Control Points (HACCP) management, which is a systematic preventive approach for microbial safety, has been accorded as proactive means to ensure microbial safety of biosolids products (Godfree & Farrell 2005). The microbial safety data at each stage of the treatment process, such as described for the pan-drying

process in this study, is necessary to support management for the effectiveness of each process, as a basis for HACCP management. Of particular value is $\log_{10}$ reduction data of pathogens and indicators, which is generally independent of the initial levels of these microbes, provided that sufficient numbers are initially present to measure the overall removal efficiency. Therefore, data from similar processes from different plants can be compared to allow benchmarking and improvement of biosolids quality.

Regrowth

Regrowth may occur due to the growth of dormant or small residual populations of bacteria in biosolids, which may become active and increase upon rewetting. In this study no detectable levels of either *E. coli* or *Salmonella* were observed in either dry or wetted biosolids that had not been challenged with bacteria. This suggests that no viable *E. coli* or *Salmonella* were present in the samples. It is unlikely that dry conditions could support the transition of these organisms to viable but not culturable status, since most of the dry biosolids that had been challenged with bacteria showed dramatic declines in bacterial numbers within a few hours of inoculation. This decline is likely due to the low water activity of the biosolids, thus leading to water stress of the bacterial cells. These results suggest that, under the conditions used for this study, a moisture content of at least 15–20% is sufficient for survival of *E. coli* and *Salmonella*, but not sufficient for growth. Upon rewetting of the biosolids which had been challenged with bacteria, levels of *E. coli* and *Salmonella* generally remained stable. Slight increases (<0.5 $\log_{10}$) were seen in a few cases, but the numbers of bacteria did not reach sufficient levels to be considered as regrowth. It is possible that the slight apparent increases were due to uneven distribution of the bacteria following the original mixing with biosolids. Consistent with our results Yeager & Ward (1981) reported that regrowth of bacteria could occur in sterilized raw sludge if the moisture level was $\geq$25%, but no growth occurred with 20% moisture.

Biosolids may also be contaminated by pathogens from external sources like faeces of wild birds and animals on stockpiles (Zaleski *et al.* 2005b). The survival or growth of pathogenic bacteria when contaminating biosolids will depend on the moisture level. If the moisture level is in the range 15–20% contaminating bacteria may survive for an extended period, for at least 4 weeks, based on our data. Nevertheless, survival alone, without growth, could be sufficient for stored biosolids to fail a microbial safety grading test. Higher moisture levels, $\geq$25%, may support growth of contaminating bacteria (Yeager & Ward 1981).

Other investigators have shown, however, that growth of pathogens in biosolids or composted biosolids is generally not favoured, in large part due to competition from indigenous flora (Fujioka *et al.* 1988). Thus, biosolids products with reduced numbers of indigenous flora, may have higher risks of pathogen growth, compared to fresh biosolids, if contaminated during storage before application (Yeager & Ward 1981; Sidhu *et al.* 2001). Suppression by indigenous flora may have been a factor in the present study, as the presence of other species was observed on selection plates for the two indicators (not shown).

In contrast to the present study Gibbs *et al.* (1997) demonstrated the regrowth of faecal coliforms and *Salmonella* in stored biosolids when wetted by rain. The initial levels of faecal coliforms and *Salmonella* were approximately 10^4–10^6 cfu/g and 10–10^2 cfu/g respectively. The levels declined to undetectable levels during the summer, however, after wetting by rain during winter, growth of both faecal coliforms and *Salmonella* recovered gradually and followed a linear pattern. The authors suggested that rewetting may have caused regrowth due to increasing the respiration rate, rather than increasing substrate availability (Gibbs *et al.* 1997). In more recent field studies, initial regrowth of *E. coli* and *Salmonella* was transient and numbers generally decreased upon further air-drying and storage (this study; Zaleski *et al.* 2005a). The substantial regrowth observed by Gibbs *et al.* (1997) may have been due to specific factors such as the use of poorly stabilised biosolids (mechanically dewatered sludge), or post-treatment contamination by animals (Zaleski *et al.* 2005b).

Reactivation and subsequent growth of faecal coliforms can occur after anaerobically digested sludge has been mechanically dewatered by centrifuges and then stored (Higgins *et al.* 2007). This may be due, at least in part, to the loss of methanogens, which may inhibit, or compete with, faecal coliforms (Qi *et al.* 2008). In the present study biosolids had also been treated by mesophilic anaerobic

digestion, though they were subsequently substantially stabilized and dewatered by solar drying over a year, rather than being quickly mechanically dewatered. Since no reactivation or growth was observed with *E. coli*, long term stabilization appears to prevent these microbial safety issues.

In addition, if reactivation of *E. coli* and *Salmonella* was to occur in the biosolids during this study, that would be expected to occur within a week of rewetting, and allow clearly detectable levels within 2 weeks. As no regrowth was observed after one month it is unlikely that re-actable *E. coli* and *Salmonella* cells were present.

CONCLUSION

The results suggest alternative processing options are possible to produce biosolids suitable for use on land, compared to storing in stockpiles for 3 years. In the reported cases at two plants, microbial safety at Australian treatment P1 grade, USA and above Class A and UK enhanced treatment status was reached before the end of the air-drying phase. This would save over three years storage for producing P1 grade biosolids in the state of Victoria, since current guidelines require three years of storage in stockpiles. The Australian guidelines also require provision of data for removal of other pathogens, including viruses and parasites. It is additionally suggested that air-dried biosolids with treatment P2 and P3 grades be suitable for direct use on agricultural land, following a standard regime of land-use restrictions, without the need for an extended storage period, as recommended by the Safe Sludge Matrix in the UK. Furthermore, across Australia the metropolitan and regional wastewater plants have a range of different process sequences, for example some do not have anaerobic digesters, which may lead to some variation in removal of pathogens. Therefore, it is considered important to assess the removal of pathogens by each type of plant. Log_{10} removal data of pathogens and indicators in specific treatment processes will support HACCP management of treatment plants to improve microbial quality of biosolids products. To reduce the chance for survival or regrowth of bacterial pathogens in stored biosolids, storage conditions should provide moisture levels below 15%, and prevent the possibility of free water pools forming over the biosolids.

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