DECAY OF *ESCHERICHIA COLI* IN BIOSOLIDS APPLIED TO AGRICULTURAL SOIL

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ABSTRACT
There is little scientific data available on the survival patterns of pathogenic microorganisms introduced into the soil through the broad acre application of biosolids. This study was conducted to investigate the decay rates of *Escherichia coli* in agricultural soil amended with biosolids during two different growing seasons in a dry temperature cropping region in Western Australia.

Biosolids-amended and unamended soil were inoculated with *E. coli* (ACM 1803), inserted into sentinel chambers and placed into the topsoil (0-10 cm) of a wheat crop. Biosolids were applied to designated biosolids plots, according to normal district practice, and *E. coli* numbers within the sentinel chambers were monitored over time. *E. coli* numbers in biosolids-amended soil reached detection limits (>10 cfu/mL) within 6 to 7 months. The decay patterns of *E. coli,* by treatment difference (biosolids-amended or unamended), linear and quadratic relationships of sampling time, and their interactions were highly significant. The T_{90} or time taken for a 90% reduction in numbers in the biosolids-amended soil was estimated to be 74, 143, 183 days (2006) and 173, 211 days (2008) as compared with 188 days (2006) and 156, 242 days (2008) in the unamended soil. This research provides scientific data on the survival times of *E. coli* in agricultural soil, with and without biosolids and can thus be helpful to public health policy.

Keywords: Biosolids, *E. coli,* enteric pathogens, agriculture.

INTRODUCTION

Biosolids is the term given to sewage sludge that has undergone further treatment and stabilisation processes such as dewatering and anaerobic digestion, to enable it to be applied for beneficial reuse. In Australasia approximately 360,000 dry tonnes of biosolids are produced annually and are predominantly used in agriculture, forestry, composting, blending, land rehabilitation and energy recovery (LeBlanc *et al.* 2008). In 2006/2007 Western Australia (WA) produced 20,100 dry tonnes, of which approximately 52% was used for broadacre agriculture (i.e. cereal grains and oilseed crops), 30% for composting and 17% for forestry (LeBlanc, Matthews, and Richard 2008).

The use of biosolids in agriculture holds benefits for both the water industry and primary producers. For the water industry, the need for waste disposal is eliminated and biosolids are therefore, considered a resource; for agricultural producers as a source of fertiliser and a soil conditioner. While there are many benefits from biosolids reuse, biosolids may contain contaminants of concern such as heavy metals and enteric pathogens which may affect water quality and the safety of agricultural (food) produce (LeBlanc *et. al*, 2008). Pathogens may pose an immediate and often severe risk to human health by causing rapid illness in infected individuals (Sidhu and Toze 2009). However, there is limited information on the survival of enteric pathogens in field crops (Horswell *et al.* 2007; Lang *et al.* 2007). Uncertainty therefore exists, as to the potential risk to humans consuming cereal grain products fertilized with biosolids.

Few studies have been conducted on the survival of pathogens in biosolids (and livestock manures) applied to soil. Eamens *et al.* (2006) studied the prevalence of *E. coli, Clostridium perfringens* and *Salmonella* spp. in soil amended with anaerobically-digested biosolids at Goulburn, New South Wales (NSW) and found that bacterial numbers were above detection limits for 10 to 17 months. Previous work by Crute *et al.* (2005) found that *E. coli* and *Enterococci* were detectable for up to 6 months in biosolids-amended soil applied to dryland agricultural land in WA. Internationally there have also only been limited studies on pathogens in soils amended with biosolids. Examples of these studies include Lang *et al.* (2007) who measured the survival of *E. coli* in agricultural soil amended with conventionally treated and enhanced-treated biosolids in a wet temperate environment in the United Kingdom (UK). They determined that *E. coli* in the conventional biosolids treatment reached detection limits by 3 months whereas enhanced-treated biosolids were not a source of *E. coli.* In New Zealand, Horswell *et al.* (2007) examined the decay rates of *E. coli* and *Salmonella* spp. in sewage sludge applied to young and old pine forests (*Pinus radiata*) and found that *E. coli* numbers returned to background levels after 3 weeks in the spring but did not significantly decrease until weeks 5 and 13 in the autumn/winter.

There is a gap in the scientific data as to the presence and survival of enteric bacteria in agricultural soil following the application of anaerobically digested dewatered biosolids cake (Gerba and Smith 2005; Lang *et al.* 2007; Sidhu and Toze 2009), particularly in Australia. This paucity of quantitative data on the pathogenic bacteria and virus numbers in biosolids has been a major obstacle in determining the impact that pathogens have to human health. Pathogenic microorganisms may present a serious health risk to consumers of agricultural products (Nasser *et al.* 2003) even though the risk may seem minor where biosolids have been used on field crops, processing crops or crops not used directly for human consumption (Epstein 1998).

In the present study we examined the survival patterns of *E. coli* in biosolids applied to agricultural soil in Western Australia. The survival patterns and decay times of *E. coli* were examined as a function of time, the presence of biosolids, climate and soil conditions.

METHODS AND MATERIALS:

Site description

Two field experiments were carried out in the central west dry land cropping zone at Moora, Western Australia (30º 50'27.73"S, 116º 06'15.24"E) during the winter-summer season (May to Dec.) over two years, 2006 and 2008. Moora is 175 km north-east of Perth, with Mediterranean climate, characterised by a mean annual rainfall of 450-500 mm per annum, with mild winters and warm to hot summers. The topography at the site was undulating with medium slope and soil type was gravely-loam.

Experimental design

Survival experiments comprised two treatments: biosolids-amended soil and unamended soil (nil-biosolids). A total of six plots (2m x 5m with 2m buffer zone) were established in triplicate using a randomized-block design to minimise random sampling error in the field and hence determine decay rates more accurately. Anaerobically digested dewatered biosolids cake from Beenyup Wastewater Treatment Plant (Perth, WA) was applied to three of the plots at 19 tonnes per ha^{-1} dry solids (or 100 kg per 10 m^2), the other three left unamended as the control. The application rate was higher than the required nitrogen limited biosolids application rate (NLBAR) so that any treatment effect could be observed. Both treatments were incorporated with a disc-seeder and then wheat (*Triticum aestivum cv.* Calingiri) was sown at 60 kg/ha, 18 cm width and 2.5 cm depth.

The survival experiments were undertaken using sentinel chambers (Jenkins *et al*. 1999) placed into each of the plots. The sentinel chambers were constructed using 3.5 mL Microsep™ centrifugal devices with a membrane pore size of 300,000 molecular weight cut-off (MWCO) (PALL Life Sciences, New York USA) along with 0.2 µm Eppendorf Lid-Bac membrane lids to close the top of the columns. The pore size of the Microsep membrane and the Eppendorf lids were sufficiently large to allow exchange of gases and moisture without the loss of bacteria from the sentinel chambers.

The sentinel chambers were constructed using soil from the experimental site which was seeded with washed *E. coli* culture. The *E. coli* culture was prepared by growing the *E. coli* strain ACM 1803 in 100 mL nutrient broth (Oxoid) in a shaking platform incubator overnight at 37ºC. Prior to inoculation, overnight cultures were washed in phosphate buffer as described in Gordon and Toze (2003). The final suspension was determined to have a final cell count of approximately 1 x 10^9 colony forming units (cfu) mL^{-1}. To fill the chambers, collected unamended soil was sieved (<2mm) and then split into two equal portions. One portion was amended with biosolids to a final ratio of 1:4 (2006 experiment) and 1: 3 (2008 experiment). The other portion was left unamended with no addition of biosolids. Each of the portions was then seeded with the washed *E. coli* suspension to achieve a final number in the soil of approximately 1 x 10^7 cfu g^{-1} soil. The amended and unamended soils were then used to fill the sentinel chambers. Over 240 chambers were prepared so that destructive sampling could occur throughout the experiment. Forty chambers (20 x 20) were also prepared for testing moisture content in both treatments. Once constructed the chambers were positioned vertically in the soil, in each of the plots, to a depth from the surface to 10 cm below ground; the biosolids-amended chambers in the biosolids-applied plots and the nil-biosolids chambers in the control plots where no biosolids had been added. Each of the plots had 120 sentinel chambers at the start of the experiment.

Samples were collected at Time 0 and then every second week until week 4. Sampling frequency was then reduced to monthly intervals up to a maximum of 7 months or until *E. coli* fell below the detection limit. At each sample event, 3 chambers from 3 plots in each treatment were randomly selected. All samples were transported on ice to the CSIRO Microbiology Laboratory, Floreat, WA and were processed within 24 hours of collection.

Daily air temperature and relative humidity were recorded every 20 mins using a Tinytag Plus 2 (Gemini Data Loggers (UK) Ltd). Soil temperature and soil moisture were recorded at hourly intervals using a Watermark Monitor (Irrometer Company Riverside, CA USA). Rainfall was recorded every 20 mins with a tipping bucket rain gauge (Davis Instruments Corp, Hayward CA USA) and Tinytag data logger (Gemini Data Loggers (UK) Ltd). Soil

moisture was manually determined by oven-drying (105ºC for >24hrs) soil samples from the field and from moisture chambers (sentinel chambers set up to test moisture levels with no *E. coli*) taken at each sample event.

Enumeration of E. coli from the sentinel chambers
Sample contents (approximately 2 to 5 g) of each chamber were transferred into pre-weighed sterile tubes and net weights were obtained. Phosphate buffer (pH 7.2) was added (30 mL), and the samples vortexed for 2 mins, left to settle, then vortexed again for 1 min. A 1 mL portion of the resulting supernatant above the soil was then collected and serially diluted ten-fold in phosphate buffer. Triplicate 100 µL volumes of appropriate dilutions based on the anticipated number of viable *E. coli* cells present were spread-plated onto Chromocult coliform agar (Merck) using sterile glass spreaders. Agar plates were incubated at 37ºC overnight and the number of dark-violet *E. coli* colonies present on each media plate was recorded. The cfu per gram were then calculated on a dry soil weight basis from the original weight of the soil contents, inside the sentinel chamber.

Statistical analysis
E. coli counts were normalised from the raw data by transformation into $\log_{10}$ cfu g^{-1}. The counts from Time 0 were removed from all trials prior to any statistical analyses as they may not be consistent with results taken at later times.

The linear mixed effect model analysis of variance was initially conducted using SAS Program (version 9.1) to identify significant variation sources affecting final *E. coli* counts ($\log_{10}{}^{Count}$) in individual experiments as well as across experiments. These variation sources included the fixed effects (experiment, treatment, linear and quadratic terms of a covariate - sampling date, and their interactions) and random effects (block and chambers nested within block). Since random effects were not significant in individual experiments or across experiment analyses, the final ANOVA model included the significant fixed effects only. The least-square effects of all the fixed factor comparisons were then produced. The regression coefficients of sampling date (linear, quadratic and cubic terms) within each treatment were used as the indication of pathogen inactivation rate or decay time. Based on the decay times and intercept of the final model, the predicted equation for individual pathogen survival patterns was established for either 'biosolids' or 'unamended treatment. T_{90} *values (*the time taken for a 90% reduction in pathogen numbers) were estimated by solving the cubic equations.

Multiple regression analysis was then performed using the R program (Version 10.0) on the residual values derived from the final model mentioned above to determine if there was any influence of climate parameter changes on pathogen numbers within individual experiments. These parameters included the measurement on rainfall, air temperature, humidity, soil moisture and soil temperature.

RESULTS:

Decay of E. coli in sentinel chambers
The changes in *E. coli* numbers in the soil chambers during the field inactivation experiments are shown in Figures 1 and 2. Across both experiments, *E. coli* numbers declined significantly with time. The decay rates did not strictly follow simple linear relationship as non-linear relationships (quadratic and cubic terms of sampling time) were also identified to be significant factors impacting on pathogen numbers. The *E. coli* numbers in the chambers at the beginning of each experiment were 2 x 10^7 cfu g^{-1} in the biosolids-amended soil and 2 x 10^8 cfu g^{-1} in the unamended soil in 2006 (Fig. 1); and 4 x 10^7 cfu g^{-1} in the biosolids-amended soil and 2 x 10^6 cfu g^{-1} in the unamended soil in 2008 (Fig. 2). *E. coli* numbers in the biosolids-amended soil (outside the chambers) in 2008 at the same time were 3 x 10^6 cfu g^{-1}. There was no significant plot or chamber effect identified within individual experiments.

In 2006 (Fig.1), *E. coli* numbers at the start of the experiment were higher in the unamended soil and decayed significantly faster (P<0.01) than the *E. coli* in the biosolids-amended soil. This was illustrated by the T_{90} value or time taken for a 90% reduction in *E. coli* numbers (Table 1). In the biosolids-amended soil, despite significantly faster linear decay rate (P<0.001) than the unamended soil and due to much higher positive quadratic inactivation rate in the biosolids-amended soil, it was observed that T_{90} times fluctuated and these times were observed to change on three separate time points (74 days, then at 143 days and then at 183 days). In the unamended soil, despite higher *E. coli* numbers at the start, a 1 log loss based on the T_{90} time was expected to be reached at 188 days which was very similar to the final value of the biosolids-amended soil. *E. coli* fell below the detection limit by day 205 (or week 29) in both treatments.

In 2008 (Fig.2.), *E. coli* numbers at the start of the experiment were higher in the biosolids-amended soil. Despite this *E. coli* in the biosolids-amended soil decayed significantly faster, given significantly less positive linear relationship and more negative quadratic regression (P<0.001), than *E. coli* in the unamended soil. The T_{90} time in the biosolids-amended soil was 173 days and then 211 days as compared with 156 days and then 242 days in the unamended soil (Table 1). *E. coli* was below detection limits in the unamended soil by day 188 (or week 27). *E. coli* in the biosolids-amended soil was still detectable (at 5 x 10^1 cfu g^{-1}) by day 188.

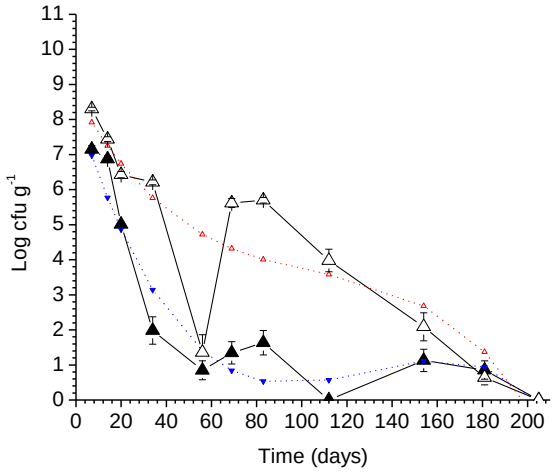

Figure 1: E. coli decay in 2006 in biosolids (▲) and unamended (∆) soil in chambers with predicted values for biosolids (--▲--) and unamended (--∆--) soil.

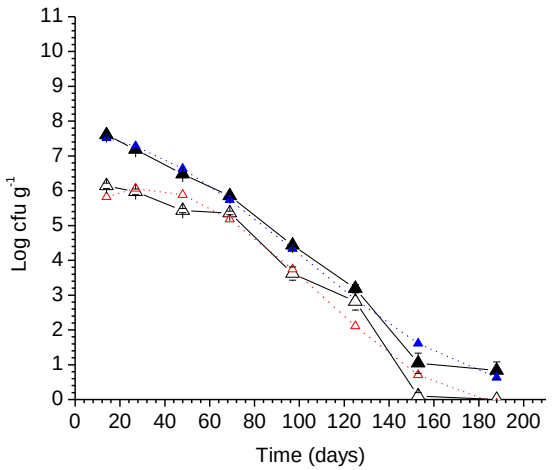

Figure 2: E. coli decay in 2008 in biosolids (▲) and unamended (∆) soil chambers with predicted values for biosolids (--▲--) and unamended (--∆--) soil.

Climatic results

The climatic conditions during the inactivation experiments are shown in Table 2. Mean average daily air temperatures ranged from 14-16ºC across both experiments. Minimum daily temperatures were 9-10ºC with maximum daily temperatures of 21-22ºC. Mean soil temperatures were 17-18ºC. Average relative humidity was 63-73% and average soil moisture content in 2008 was 12%. Cumulative rainfall was 262 mm in 2006 and 275 mm in 2008.

Table 1: One log_{10} (T_{90}) reduction times of E. coli in soil chambers.

Experiment	*E. coli* T_{90} Times (days)	
	Biosolids-amended soil	Soil only
2006	74, 143, 183	**188**
2008	**173, 211**	156, 242

Table 2: Summary of seasonal parameters

Climatic variable	2006	2008
	Arithmetic Mean (SD)	Arithmetic Mean (SD)
Mean daily air temp. (ºC)	16 (± 6.4)	14 (± 3.6)
Min daily temp.(ºC)	10 (± 5.0)	9 (± 3.4)
Max daily temp. (ºC)	22 (± 5.8)	21 (± 5.0)
Mean rel. humidity (%)	63 (± 12.2)	73 (± 14.6)
Rainfall (mm)	262	275
Soil temp. (ºC)	18 (± 6.5)	17 (± 5.4)
Soil moisture (%)	-	12 (± 12.0)

Influence of climate on E. coli inactivation

In 2006, the significant relationships between the climatic factors and the *E. coli* numbers were rainfall with sample dates and soil moisture changes with sample dates ($P<0.05$). Based on the multiple regression analysis using the residual values derived from the ANOVA model which accounted for 71.4% of total variance, rainfall with sample date and soil moisture with sample date both accounted for 23% of the residual variance.

In 2008, after the ANOVA model explained 93.4% of total variance, humidity, soil temperature, rainfall with sample date and air temperature with sample date were also found to significantly affect the *E. coli* numbers ($P<0.01$), accounting for 32%, 20%, 18% and 15% of the residual variance respectively.

DISCUSSION:

Since enteric pathogens may be present in agricultural soil when biosolids are used as an alternative fertiliser (LeBlanc *et al.* 2008), the *E. coli* decay times were of interest as a measure of microbial survival in biosolids-amended soils in the present study. By introducing *E. coli* into the soil of a wheat crop, we identified the time required for a 90% reduction of organisms was in the range of 74 days to 242 days. The survival of *E. coli* cells in both experiments was highly significantly affected by the linear and non-linear inactivation rates that occurred in the presence or absence of biosolids. In addition, the pathogen numbers in the first experiment were also found to be influenced predominantly by rainfall and soil moisture changes

that occurred during the sampling period. In the second experiment, the *E. coli* numbers were mostly influenced by humidity, soil temperature and rainfall changes during the sampling period.

In the present study, *E. coli* in unamended soil had T_{90} times of 188 days and 156, 242 days (2006 and 2008, respectively) using linear and non-linear (quadratic and cubic terms) inactivation rates in the analysis models. Chandler and Craven (1980) examined the relationship between *E. coli* inactivation rates, soil moisture and soil type in an unamended grey cracking loam soil and reported inactivation times (T_{90}) of 14 to 15 days via a linear regression analysis. This was faster than the present study possibly due to the fact that Chandler and Craven used a pot experiment rather than a field experiment with different soil types and moisture content. In a study of *E. coli* in biosolids-amended soil in a pine-forest plantation, Horswell *et al.* (2007) found that T_{90} values were reached within 21 days in a mature pine stand and 77 days in a young pine stand. In the present study, *E. coli* in biosolids-amended soil had T_{90} times of 74, 143, 183 days and 173, 211 days (2006 and 2008, respectively) which was again, much slower in comparison. Again, the possible reasons for the difference could be due to different climatic conditions, different biosolids application rate and a different soil type. It should also be noted when comparing the T_{90} values obtained in the present study with other studies that quadratic and cubic regression were used for better fit in the current study compared with a linear regression analysis undertaken in the comparative studies where a single decay figure was obtained over the entire period of these decay studies.

In the present study *E. coli* cells were detectable for approximately 27 to 29 weeks in the sentinel chambers placed in the topsoil of a wheat crop in WA. Eamens *et al.* (2006) found that from summer applications of biosolids *E. coli* numbers were detectable up to weeks 40, 44 and 51; in spring applications *E. coli* was detectable up to 64 and 68 weeks. These longer survival times may have been due to the different sampling approach, that is, the sampling of biosolids 'clumps' rather than the use of chambers containing incorporated soil, biosolids and seeded *E. coli* cells. In previous work by Crute (2004) *E. coli* numbers were detectable up to 24 weeks in agricultural soil of a wheat crop at Toodyay, WA where sampling was directed towards the biosolids particles or clumps rather than the use of chambers (Crute *et al.* 2005).

Eamen *et al.* (2006) and Crute *et al.* (2004) also tested various biosolids application rates to determine if there were any differences in *E. coli* survival but found no significant effect. In contrast, Horswell *et al.* (2007) found that die-off was significantly correlated with the percent solids of sludge. In the present work, laboratory-cultured *E. coli* was inoculated into sentinel chambers to obtain higher *E. coli* numbers than would normally be expected following biosolids application; this was done so that decay times could be determined over a longer timescale in biosolids. Despite these differences, the detection times of *E. coli* in the study by Crute *et al.* (2005) were similar to the present study.

Several factors have been suggested to affect survival of enteric pathogens in the soil including time (Lang *et al.* 2007), moisture (Lang *et al.* 2007; Chandler and Craven 1980), temperature (Holley *et al.* 2006), soil type (Ross *et al.* 1991), soil nutrient supply (Estrada *et al.* 2004), organic matter (Straub *et al.* 1993), and soil pH (Stevik *et al.* 2003). Eamens *et al.* (2006) found no significant difference between survival in biosolids with or without soil incorporation, however the raw data indicated a trend towards slightly greater survival of bacteria in the incorporated biosolids and suggested that the biosolids may provide a possible 'protective effect' for bacteria under the soil. Although the present study indicates no evident trend of biosolids prolonging the survival of *E. coli,* it was observed (in unpublished data) that the addition of biosolids did significantly prolong the survival of *E. coli, Salmonella* spp., bacteriophage MS2 and adenovirus in agricultural soil in sentinel chambers. These (unpublished) results showed that the bacteria and viruses inoculated into unamended soil decayed faster than the same pathogens inoculated into biosolids-amended soil.

In our experiments, the ratio of biosolids to soil in the chambers was approximately twenty-five times greater than would actually be applied to the same soil based on agronomic guidelines. Normally the NLBAR (DEP *et al.* 2002) would equate to 1% biosolids but the rate was increased for experimental purposes, as it has been shown that this rate of application (1%) was not high enough to have any treatment effect (Crute 2004). This suggests that the addition of biosolids at application rates higher than the current agronomic guidelines (DEP *et al.* 2002) could prolong bacterial survival in the soil. However under the correct application rates, as agreed by the land application guidelines, it would be expected that there would be no prolonged survival of bacterial pathogens (compared to survival in unamended soils). It is acknowledged that while *E. coli* is commonly used as a useful indicator for predicting the decay of faecal bacteria, it is not an indicator to predict the decay of protozoa, Helminths or viruses. Further research would need to be conducted to determine the survival patterns (and subsequent health risks) of the non-bacterial enteric pathogens.

Along with rainfall with sample date (time), humidity and the addition of biosolids, soil temperature was significant in the decline of *E. coli* numbers in the present study. The experiments in this study were

conducted from May to December over the winter growing season of wheat. In both experiments, temperatures increased from August to December while rainfall events became less frequent, soil moisture levels decreased and humidity levels decreased. Cools *et al.* (2001) reported that increasing temperature caused a decrease in survival of *E. coli* at levels from 15 to 25ºC. Horswell *et al.* (2007) also observed that *E. coli* numbers reduced to background levels following a week with increased temperatures and low rainfall. In a similar study by Lang *et al.* (2007) temperature, particularly soil temperature was identified as the most influential environmental parameter.

CONCLUSION:

This study indicates that *E. coli* numbers are highest immediately following application. The persistence of enteric bacteria in agricultural soil is dependent on several factors such as application rates, time since application and climatic conditions; mainly moisture levels and temperature. The study provides data that can be used to assist strategies to reduce the transmission of infectious pathogens derived from biosolids.

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