DECAY OF HUMAN ENTERIC PATHOGENS IN AGRICULTURAL SOIL AMENDED WITH BIOSOLIDS

Key findings from a comprehensive research project to examine potential health risks

K Schwarz, S Toze, D Pritchard, JPS Sidhu, Y Li

ABSTRACT

A comprehensive study was undertaken to examine the survival potential of enteric microorganisms in biosolids-amended soil, wheat plant phyllosphere, and stored grains. The presence of these microorganisms in the dust at harvesting time was also evaluated. *In situ* field experiments were conducted to examine the decay of *E. coli* (indicator bacteria), *Salmonella enterica*, bacteriophage MS2 and human adenovirus in biosolids-amended soils and in dust generated during harvesting of wheat. Glasshouse experiments were conducted to determine the survival potential of enteric microorganisms in the wheat phyllosphere and stored grains to determine any possible risks to humans or livestock through consumption of contaminated grains or fodder.

The results of this study suggest that the target microorganisms decayed faster in the biosolids-amended soil compared with the unamended soil in the field, that the decay times were specific to the microorganism type; and that microorganism decay was correlated to declining soil moisture levels and increasing soil temperature. The risk of transmission of disease-causing microorganisms (human pathogens) from cereal crops fertilised with biosolids was considered to be low.

Keywords: biosolids-amended soil, agriculture, adenovirus, phyllosphere, bio-aerosols, wheat grains and cereal crops.

INTRODUCTION

Biosolids are a valuable resource that can be used sustainably as a soil amendment to improve the chemical and physical properties of soil. The application of biosolids onto agricultural land introduces substantial organic matter and is a rich source of plant-available nutrients and trace elements (Joshua *et al.*, 1998; Epstein, 2003; Horan *et al.*, 2004; LeBlanc *et al.*, 2008). Several studies have shown the many benefits to agriculture such as increased crop yields, improved soil fertility, soil conditioning, improved cation exchange, an increase in soil porosity, decreased bulk density and increased soil water-holding capacity (Epstein, 1998; Nicholson *et al.*, 2005; LeBlanc *et al.*, 2008).

Approximately 360,000 dry tonnes of biosolids are produced in Australia annually and the bulk of these biosolids (60–70%) are applied to agricultural land for beneficial purposes, particularly as fertiliser to amend nutrient-depleted soils (LeBlanc *et al.*, 2008). However, biosolids are known to contain a diverse range of human pathogens such as adenovirus, norovirus, *Salmonella enterica,* Cryptosporidium and Giardia (Sidhu and Toze, 2009).

Although properly treated biosolids are a safe and effective fertiliser, any pathogens present may contaminate foods produced from the field. Awareness of [potential?] health hazards associated with biosolids in agriculture has long been acknowledged, however, for better management of health risks, quantitative data on pathogen numbers and survival potential in the environment is required (Gerba and Smith, 2005).

The environmental impacts associated with the use of biosolids as a fertiliser need to be evaluated in the interest of protecting public health from transmissible diseases, since there is currently no monitoring system in place to track pathogen decay following the application of biosolids onto land (Gerba and Smith, 2005). The major direct sources of exposure risks are municipal wastewater treatment plant workers, biosolids handlers, onsite treatment systems, biosolids application sites and animal feeding operations (Figure 1). Land application of biosolids may also generate bio-aerosols (Gerba and Smith, 2005), airborne particles containing living microorganisms such as pathogens.

Minimal data has been collected on the fate of human viruses in the soil, particularly where cereal crops are grown. In the process of identifying potential human health risks, three key areas were identified where contact with pathogens may occur; thus the research objectives were to assess the decay of enteric pathogens: i) in the biosolids-amended soil where cereal crops are grown; ii) in the plant phyllosphere (leaves and

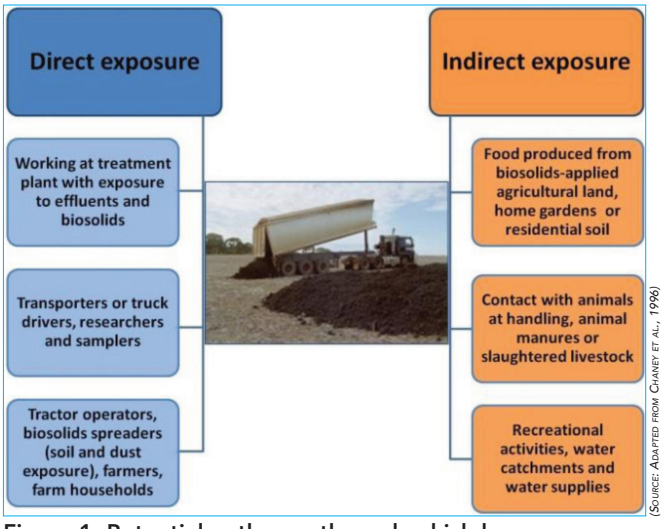

Figure 1. Potential pathways through which human pathogens in biosolids could be ingested by humans.

spikelets or grain heads) where contact with contaminated parts may occur in handling and consumption and; iii) the background bacteria levels present in the field at biosolids-applied sites where inhalation or contact with contaminated aerosols or dust may occur at harvest time.

Enteric microorganisms were laboratory-cultured and then inoculated into the soil, wheat phyllosphere, grains and matured wheat stems. The change in numbers of seeded microorganisms was then monitored over time to obtain the estimated decay time for a one-log reduction of pathogen count (T_{90} values) to occur under ambient environmental conditions.

This paper reports on the key findings from a comprehensive research project designed to examine the potential health risks from biosolids-amended soil, pathogens surviving in plant phyllosphere, stored grains and in the dust at harvesting time.

MATERIALS AND METHODS

SOIL DECAY EXPERIMENTS

Three field sites for broadacre wheat (*Triticum aestivum*) cultivation were established in Moora, Western Australia (WA) and Mt Compass, South Australia (SA). Soil at each site was amended with biosolids according to the nitrogen-limited biosolids application rate (NLBAR) as 1 x NLBAR at Moora in 2006 and 1.5 x NLBAR at Moora and Mt Compass in 2008, as determined by Crute (2004), according to district practice (DEC, 2012), to create normal external environmental conditions. Thus, application rates at the Moora sites were 6 t dry solids (DS) ha-1 in May 2006, and 19 t DS ha-1 in May 2008 (using air-dried biosolids from Bolivar Wastewater Treatment Plant (WWTP) at 66% moisture to 1.8% solids), and at Mt Compass, 28 t DS ha-1 in May 2008 (using anaerobically digested biosolids from Beenyup WWTP at 20% moisture to 7.7% solids).

Topsoil (0–10cm depth) collected from each site was mixed with biosolids from the nearest wastewater treatment plants (i.e. Beeynup, WA and Bolivar, SA) and inoculated with laboratory-cultured *E. coli*, *S. enterica*, bacteriophage (MS2) and adenovirus as described in Schwarz *et al.* (2010). Then amended soil was placed into sentinel chambers (Figure 2). The constructed sentinel chambers with membranes (0.2 µm pore size) on both sides were sufficiently large to allow exchange of gases and moisture without the loss of bacteria and viruses from the chambers. Individual chambers were filled with 25% biosolids to 75% soil collected from each site. The chambers (120) were placed into the topsoil at the beginning of the crop-growing season. Control chambers were also set up using soil only (unamended).

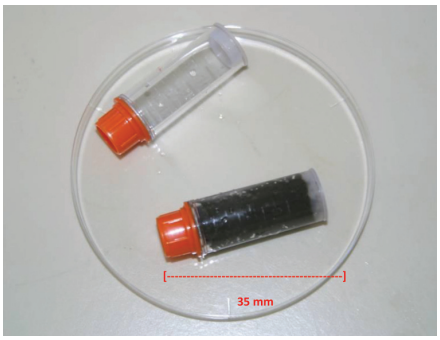

Figure 2. A commercial 3.5mL Microsep™ centrifugal device (35mm x 10mm) used as a sentinel chamber; and (bottom right) filled with the sample contents of soil, biosolids and laboratory-cultured microorganisms.

Figure 3. Wheat crop at harvest time at the Moora site in WA. The pink tags mark the location of buried sentinel chambers.

Replicate chambers (three) were collected fortnightly from each site (Figure 3) to week four, then at monthly intervals up to seven months (the duration of the wheat-growing season) or until target microorganisms fell below the detection limit. Soil moisture levels were also monitored inside and outside the chambers using oven-drying and automated tensiometers. Daily air temperature and other climatic variables were recorded using Tinytag Plus 2 monitors.

PHYLLOSPHERE AND GRAIN EXPERIMENT

Wheat plants were grown in pots in the glasshouse using soil from Moora, WA. The spikelets (grain heads) and leaves of wheat plants were inoculated with *E. coli*, *S. enterica* and bacteriophage MS2 at flowering stage as described in Schwarz *et al.*, 2013. Plant leaves and spikelets were sampled hourly for up to nine days. Samples were processed within 24 hours of collection in the laboratory using phosphate buffer, processed in a stomacher (laboratory paddle homogeniser) and plated onto agar as described in Sidhu *et al.* (2008) to quantify microorganisms.

For the grains experiment, two grain varieties – biscuit wheat (ASW) and pasta wheat (NN) – were inoculated with *E. coli*, *S. enterica* and bacteriophage MS2 using an atomiser. Grain samples were stored in tins with the lids on to represent grain silos. Samples were collected daily for up to 35 days. Grain samples were suspended in phosphate buffer, processed in the stomacher and plated onto agar, as described in Sidhu *et al.* (2008).

COLLECTION OF HARVESTER DUST SAMPLES

The dust experiments were carried out over two years (2008 and 2009) at Moora, WA, at four cropping sites during harvest time. Biosolids had been previously applied to the first site in May 2006 (three years earlier) and the second site in May 2009 (seven months earlier). No biosolids were applied to the nil-biosolids sites. Ambient air samples were collected and tested for the background levels of bacteria present at the site (*E. coli*, enterococci and heterotrophic bacteria) using SKC BioSamplers® with Vac-U-Go pumps. No experimental spiking was carried out for this study. Samples were taken downwind from the operating axial-flow harvester. At each site, soil, spikelet, chaff and grain samples were also tested for the background bacteria (as mentioned above). Samples were analysed within 24 hours of collection on selective agar plates, as previously described.

WHEAT AND GRAIN SAMPLING FROM THE THRESHER

A separate experiment with a thresher was carried out in an undercover area (Northam, WA) to determine the effect of threshing on microorganism numbers. Pathogens could transfer from contaminated biosolids-amended soil onto wheat plants during threshing and avert a health risk at harvest either from contact with bio-aerosols or from consumption of contaminated grain products. Matured wheat was spray-inoculated with *E. coli*, *S. enterica* and bacteriophage MS2 using an atomiser for a more controlled experimental environment. The wheat plants were fed into the thresher and plant parts

Table 1. Time (T_{90}) for a one-log10 reduction to occur for enteric microorganisms in the soil, from the phyllosphere and from stored grains.

Source	Estimated T90 times (days)			
	E. coli	*S. enterica*	MS2	Adeno-virus
Biosolids-amended soil	5-56	4-37	22-36	>180
Unamended soil	8-83	21-57	29-108	>180
Wheat leaves	3	2	1.5	#
Wheat spikelets	2	1	1	#
Grains - noodle	9	10	60	#
Grain (ASW)	10	12	71	#

(spikelets, chaff, grains and dust) sampled to quantify microorganism numbers.Samples were suspended in phosphate buffer, processed in the stomacher and plated onto agar plates to quantify colony-forming units (cfu) or plaque-forming units (pfu), as described for the soil and grains experiments.

DATA ANALYSIS

Pathogen counts were normalised into log10 cfu or pfu g-1 from the raw data. Origin® 6.1 was used to perform standard deviations, trend lines and logarithmic transformations. ANOVA was used to identify sources of variation (i.e. site, treatment) affecting final pathogen counts (log10 Count) and all analyses were performed using SAS 9.1. Based on the decay rates for either 'biosolids' or 'nil' treatment in the soil experiments, the decay time for a one-log reduction of pathogen count (*T90* values) was estimated using quadratic equations (Schwarz *et al.*, 2010).

To determine any relationship between changes in soil temperatures or soil moisture (within individual microorganisms), correlations were calculated using the CORRE function (Excel) and any significance determined using Student *t-tests* (*P*-value at 0.05). A one-tailed Student *t-test* was used to determine any significant differences between the soil moisture levels in the chambers and the soil outside the chambers.

An ANOVA (linear) was applied and *T90* values were estimated for phyllosphere, grains (Schwarz *et al.*, 2013) and thresher experiments.

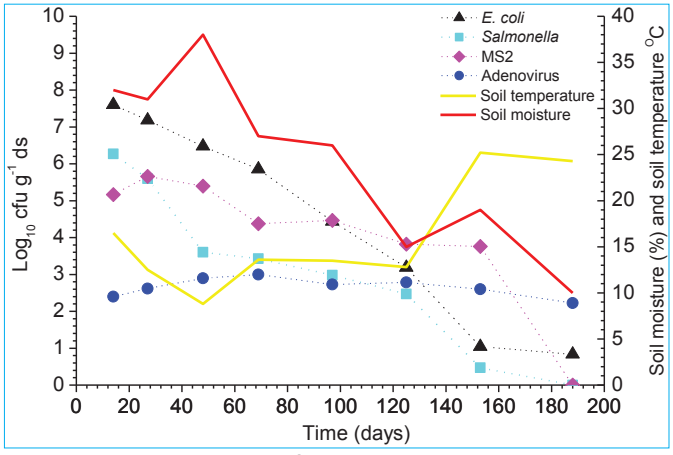

Figure 4. Decay patterns of spiked microorganisms in biosolids-amended soil (chambers) at the Moora site in 2008, with soil moisture patterns (in chambers) and soil temperature.

RESULTS

Key findings from the soil experiments were:

- Most of the target microorganisms (apart from adenovirus) decayed faster in the biosolids-amended soil than in the unamended soil (Table 1) and could not be detected in the soil after six to seven months (Figure 4);
- Decreasing soil moisture and increasing soil temperature significantly influenced most microorganisms at Moora ($P<0.05$) and Mt Compass ($P<0.01$) in 2008, particularly in the unamended soils (Figure 4). Increasing soil temperature also influenced *E. coli* decay at Moora in 2008 in both soils.

Key findings from the phyllosphere and grains experiments were:

- Microorganism type influenced decay times, i.e. bacteria spiked onto the wheat plant had shorter decay times than the bacteriophage MS2;
- The location of the microorganisms on the plant made a difference to decay times. The microorganisms on the spikelets decayed faster than the microorganisms on the leaves, although this was not statistically significant (Figure 5);

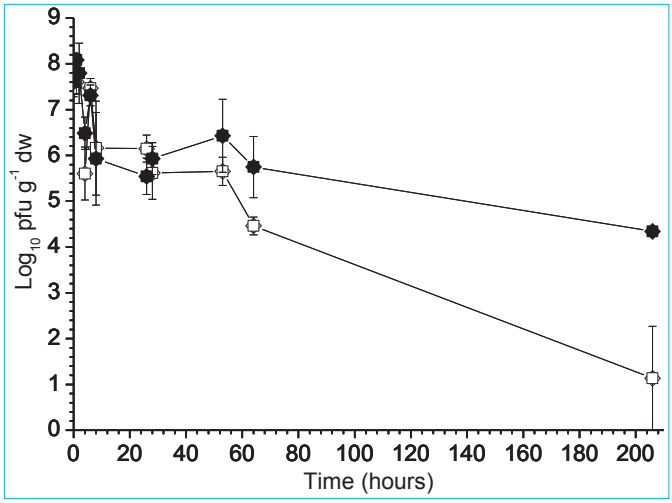

Figure 5. Decay pattern of virus (MS2) on spikelets (◊) and leaves (♦) of wheat.

- The grain variety made a significant difference to the decay times of the target microorganisms. The microorganisms on the biscuit variety of grains (ASW) persisted significantly longer than those on the pasta variety (NN) (Figure 6);
- The bacteria spiked onto stored grains had shorter decay times (Table 1) than the bacteriophage MS2, which persisted longer than the bacteria.

Key findings from the harvesting experiment were:

- Total heterotrophic bacterial levels were highest on the chaff, especially at the unamended site. The same bacteria in the dust and air were higher at the biosolids-amended site than at the unamended site;
- No *E. coli* was detected in any of the samples in 2008; however, low levels of enterococci were detected in dust samples in 2009 where biosolids had been applied seven months earlier. These levels were slightly higher at the biosolids-amended site (2.71 x 10^3 cfu per m3), but not statistically significant;
- Total heterotrophic bacterial numbers were higher in the dust samples during harvesting than in the clean air where no

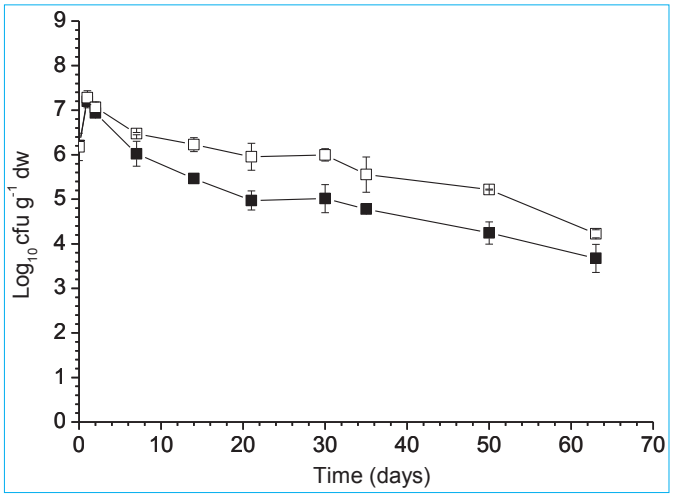

Figure 6. Decay pattern of *S. enterica* on pasta variety (■) and biscuit variety (□) of wheat.

harvester was in operation; and

- In the field, heterotrophic bacterial numbers were highest on the chaff samples.

Key findings from the thresher experiment were:

- The spiked microorganisms were higher on chaff than the grains after threshing;
- Low levels of *E. coli* and bacteriophage MS2 passed through the thresher into the dust samples. *S. enterica* was not able to be detected in the dust after threshing; and
- Bacteriophage MS2 numbers were more stable than bacteria throughout the process of threshing. As a result, numbers were higher on the grains and chaff following threshing.

DISCUSSION

Natural attenuation of enteric microorganisms in biosolids-amended soil can provide a barrier to the potential transmission of human diseases in a multi-barrier risk management approach. This, in turn, can influence the permissibility for biosolids to be applied to agricultural land.

The reduction of pathogen numbers during sludge treatment is essential in a multi-barrier approach of risk management, along with the crop types to which biosolids are applied (i.e. high-risk crops consumed raw, or low-risk crops consumed following processing). Currently, the level of pathogenic contamination in biosolids is graded according to indicator bacteria numbers.

The extent of microbial contamination and resulting grading may currently restrict the possible end uses for the product. For example, in Western Australia the minimum pathogen grade for direct land-applied biosolids for use in agriculture (P3 for non-root crops) is 2,000,000 thermo-tolerant coliforms (DEC, 2012). While these levels are the guideline values, they do not represent the whole suite of pathogens that may be present in any batch of biosolids. Moreover, no data is available on the individual survival times or the types of the pathogens present in sludge (or biosolids).

This information is important for land application of biosolids since some viruses, helminths and protozoa have been reported to persist in the soil for longer than bacteria (Sorber and Moore, 1987; Sidhu and Toze, 2009). Efficient processes to remove high levels of pathogenic contaminants at treatment plants, along with recognised rates of inactivation once introduced into the soil, can decrease the risk of transmission of disease-causing microorganisms and optimise the opportunities for biosolids reuse.

In the present study, a general trend was observed where the decay of the target microorganisms was higher in the biosolids-amended soil, thus indicating that biosolids application to agricultural land may have a positive influence on the natural decay of pathogens in the soil. The decay times (*T90*) of enteric bacteria inoculated into biosolids-amended soil in the field were four to 12 d for *S. enterica* (at Moora, WA) and five to seven d for *E. coli* at Moora in 2006 and Mt Compass, SA in 2008 (Table 1). Similar *T90* decay times of four d for *E. coli* and 12 d for enterococci were reported in our previous study by Crute (2004) in biosolids-amended soil at Toodyay, WA.

In comparison, one-log10 reduction times of 15 d for *E. coli* and 10 d for *Salmonella* have been reported in soils irrigated with farm effluent in a study from Victoria (Chandler and Craven, 1980), and eight to 15 d for *S. enterica* in a column study with sewage sludge amended soil from New Zealand (Horswell *et al.*, 2010). The maximum time before the microorganisms fell below the detection limit in this study was well within the time taken for the crops to grow and be harvested (i.e. <3 to 4 months).

Based on results from the present study, the main microorganisms of concern are the enteric viruses, since they showed slower decay patterns than bacteria in the biosolids-amended soils (*T90* of 22–180 days for bacteriophage MS2 and >180 days for adenovirus). In Australia, the growing season of cereal crops is approximately six months from seeding to harvest, and grain crops are generally not consumed raw. Therefore, the risk of transmission of disease-causing microorganisms from biosolids-amended soil can be considered to be low, based on these reported decay patterns.

In addition, the climatic conditions in southern Australia (in particular, declining soil moisture and increasing soil temperature) caused an onset of more rapid inactivation processes for the microorganisms in the present study (Figure 4). This, however, may not be the case in tropical crop-growing regions such as Queensland and, as such, these areas require further research to determine if there are similar or different decay patterns in different climatic regions.

Based on the results of this study, the risk that enteric pathogens may persist on wheat plants and grains until consumed is considered to be low. Target microorganisms inoculated into the soil were at three- to four-log10 by springtime (Figure 4), and environmental strains of *E. coli* were approximately one-log10. Given that the decay times (*T90*) for the same microorganisms from the leaves and spikelets of a wheat plant were equivalent to one to three d (Table 1), these microorganisms would be below the detection limit within a matter of weeks.

In addition, the changes in climate in typical spring to summer months are not favourable for the survival of enteric pathogens. Should any microorganisms survive in the field to harvest and be present on the grains, the time from harvest to consumption is usually more than four months due to transport, warehousing, shipping and storage. In this study maximum decay time *T90* of 71 d was observed for bacteriophage MS2, which suggests that grain storage time of four months is a significant barrier in reducing the number of any surviving pathogens. Moreover, most foods produced from wheat involve some form

of processing such as grinding, milling, rolling, steaming and baking, [therefore?] the risk of human enteric pathogens originating from land-applied biosolids and transmitting to humans at consumption (of cereal grains) is considered to be very low.

In the dust study at harvesting time, highest numbers of microbial contaminants were found on the chaff in the field and, thus, the authors recommend that this region of the plant should be tested first for any potential contaminants that could become airborne in wheat dust. Overall, since the process of threshing was found to reduce enteric microorganism numbers, the risks of unsafe levels of bio-aerosols in the dust at harvest was considered to be low.

From the results of this research work, it was reasoned that pathogens from biosolids are of greatest risk to humans immediately following dispatch from the wastewater treatment plant. As microbial contamination levels are highest during this time, transport providers, handlers, spreaders, farmers and farm workers are at greatest risk of exposure to pathogens (Figure 1). After the biosolids have been incorporated with the soil, the pathways to ingestion are low where withholding periods are maintained. Therefore, the main pathway to transmission may be more prevalent from poor hygienic practices such as food consumption following handling, or the transfer of biosolids into vehicles or homes.

CONCLUSION

This study provides data that can be used in management systems designed to reduce the transmission of human pathogens that may originate from biosolids reuse in agriculture. The key outcomes of this research were that enteric microorganisms are highest in numbers following application of biosolids, and that the enteric microorganisms decayed faster in soils amended with biosolids compared with unamended soil.

In addition, it was found that decay times were specific to microorganism type. Microorganism decay was correlated to the changes in soil moisture and soil temperature in the field. Results of this study also suggest that there is very limited potential for enteric pathogens survival on wheat phyllosphere and grains. Overall, the risk of transmission of disease-causing microorganisms (pathogens) from land-applied biosolids in a wheat crop was considered to be low where withholding periods are maintained.

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