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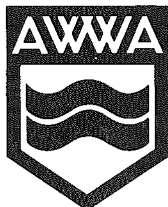

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DIE-OFF OF HUMAN PATHOGENS IN SLUDGE AMENDED SOIL

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ABSTRACT

Anaerobically digested sludge is commonly used both in Australia and overseas as an agricultural soil amendment. Even though a number of studies have examined the die-off of faecal indicator bacteria in sludge amended soil, there is less information concerning the die-off of human pathogens. This study examined the die-off of enteroviruses, *Salmonella* and *Giardia* in sandy soil amended with sludge which had been treated by anaerobic digestion and mechanical dewatering. Enteroviruses were not detected in sludge amended soil even though they were present in sludge in low numbers. *Giardia* cysts were initially present but decreased in number and were not detected after 8 weeks. *Salmonella* decreased to non-detectable concentrations within 8 weeks but were then re-detected after 36 weeks. It appeared that *Salmonella* regrew as a result of an increase in soil moisture content at the beginning of winter. Faecal coliforms also appeared to regrow after a long period of non-detection. The potential regrowth of bacterial indicator organisms and human pathogens in soil amended with wastewater sludge is of concern.

KEY WORDS

Wastewater sludge, *salmonella, giardia*, faecal coliforms, faecal streptococci, regrowth, amended soil

1 INTRODUCTION

Raw wastewater sludge contains a variety of microorganisms which are potential human pathogens. It was established previously (Gibbs *et al.*, 1993) that mesophilic anaerobic digestion, a treatment commonly used in Australia, does not result in the total removal of pathogens from wastewater sludge. Concentrations of pathogens in anaerobically digested sludge are such that this sludge is not considered suitable for unrestricted marketing.

Anaerobically digested wastewater sludge is commonly used both in Australia and overseas as an agricultural soil amendment. Australia does not at present have guidelines for sludge management but draft guidelines allow the use of sludge in agriculture which has been treated by aerobic digestion, anaerobic digestion or lime stabilisation. In the US (US EPA, 1992) and UK (DOE, 1989) the use of anaerobically digested sludge in agriculture is also allowed. Guidelines contain restrictions on use and withholding periods to minimise the microbial risk.

A number of studies have examined the die-off of *Salmonella* and faecal indicator bacteria in sludge amended soil and these were well reviewed by Sorber and Moore (1987). Less information was available concerning the die-off of viruses in sludge amended soil and no studies were found which had investigated the die-off of protozoa.

This study aimed to examine the die-off of enteroviruses, *Salmonella* and *Giardia* in sludge amended soil. These pathogens were selected on the basis of a risk assessment based on infection rates in Western Australia (Gibbs and Ho, 1993). Faecal coliforms and faecal streptococci were also examined in this study.

2 METHODS

2.1 Description of Study

Two soil amendment field trials were carried out using small scale plots. A control plot was also prepared. Each of the plots had dimensions of 5 m by 7 m and contained 80 sections of 0.5 m by 0.5 m. The soil type was Bassendean sand (described previously by Ho *et al.*, 1991). Sludge applied to the plots was from the Subiaco Wastewater Treatment Plant. This sludge had been treated by mesophilic anaerobic digestion and mechanical dewatering (Gibbs *et al.*, 1993).

The trials commenced in August 1993 and November 1993. On each occasion sludge was spread on the surface of the plots at a rate equivalent to 10 tonnes/hectare, as recommended in guidelines of the NSW Agriculture and Fisheries Department (Ross *et al.*, 1991). After application the sludge was immediately incorporated into the soil to a depth of 7 cm using a rotary hoe.

2.2 Sampling

Samples were collected from just below the soil surface to a depth of 10 cm using a sterile trowel. Samples were collected from five sections on each sampling occasion based on a randomised design. Soil temperature was measured at a depth of 10 cm on each sampling occasion.

For each of the five samples collected on each sampling occasion, 100 g amounts were combined and blended with 500 mL of phosphate buffered saline. The blended sample was analysed for the concentrations of faecal coliforms, faecal streptococci, enteroviruses, *Salmonella* and *Giardia*. A combined unblended sample was analysed for solids, volatile solids (APHA *et al*, 1989) and pH.

2.3 Microbiological Analyses

Faecal coliform and faecal streptococci concentrations were determined using membrane filtration as described previously (Gibbs *et al.*, 1993).

Enterovirus concentrations were determined using the elution and concentration method described previously (Gibbs *et al.*, 1993) with the following modifications. After elution and concentration 200 µL of the concentrate was placed onto four different cell lines. The cell lines used were HF_{32}, LLC.MK_2, Fl Amnion and Hep_2 cells. Each cell had 6 repeat tubes. Tubes were maintained in a medium containing trypsin and extra antibiotics. Tubes were left for 3 to 4 days and then passed onto tubes not containing trypsin or extra antibiotics. Tubes were incubated for 28 days and cytopathic effect recorded.

Salmonella concentrations were determined using a five tube most probable number method. Samples were pre-enriched in buffered peptone water, enriched in Rappaports Vassiliadis (RV) medium, and isolated on xylose lysine deoxycholate (XLD) plates. Pre-enrichment was for 24 hours at 37°C, enrichment was for 24 and 48 hours at 43°C, and XLD isolation plates were incubated at 37°C for 24 hours. Four dilutions of sludge with five replicates were prepared for the pre-enrichments. Pre-enrichment tubes contained 10 g of sludge in 100 mL of buffered peptone water, and 1 g, 0.1 g or 0.01 g of sludge in 9 mL of buffered peptone water. Aliquots of 0.1 mL of each pre-enrichment were transferred to 9 mL of RV for enrichment. After isolation on XLD plates and purification on MacConkey plates, presumptive *Salmonella* isolates were confirmed by serology and biochemical tests.

To determine *Giardia* concentrations, duplicate samples of approximately 1 g of blended sludge were analysed. These samples were vortexed for 30 seconds with 30 mL of 0.5% Tween 80/0.5% dodecyl sulphate and one drop of antifoam B in 40 mL centrifuge tubes. The vortexed solutions were both poured into one 100 mL beaker and refrigerated for 2 hours. After refrigeration the supernatant was decanted into a 100 mL centrifuge tube and centrifuged at 800 g for 5 minutes. The supernatant from the centrifugation was discarded and the pellet

Table 1 Results From Control Plot

Date	FC/g	FS/g	Entero-viruses (IU/g)	*Salm.* (MPN/g)	*Giardia* (cysts/g)	pH	Air Temp. (C)	Soil Temp. (C)	VSS (% of dry weight)	% Moist.
24.8.93										
	23	12	0	0	0	7.1	18.2	16.5	1.4	2.7
20.9.93										
	0	0	NT	0	94	5.9	20.5	16	1.1	26
1.8.94										
	29	0	0	0	0	6.1	17.5	12.8	2.3	0.03

NT= Not tested

Table 2 Results From Trial

Date (Week)	FC/g	FS/G	Entero-viruses (IU/g)	*Salm.* (MPN/g)	*Giardia* (cysts/g)	pH	Air Temp (C)	Soil Temp. (C)	VSS (% of dry weight)	% Moist.
9.8.93 (Before Amendment)										
	0	63	0	0	0	8.5	19.4	14.8	NT	NT
24.8.93 (Week 0)										
	440	780	0	0	66300	8.7	20.7	16.4	NT	NT
30.8.93 (Week 1)										
	73	340	0	0	160370	7.6	13	15.6	NT	NT
6.9.93 (Week 2)										
	$1.0x10^3$	$1.0x10^3$	0	0	6478	8.4	NT	NT	NT	NT
20.9.93 (Week 4)										
	230	160	NT	0	0	7.8	20.5	16.1	1.8	24
18.10.93 (Week 8)										
	173	0	NT	NT	30,0	7.35	NT	NT	NT	NT

NT=Not tested

transferred to a 10 mL centrifuge tube with deionised (DI) water. The volume was made up to 10 mL with DI water, the contents vortexed for 30 seconds and centrifuged for 5 minutes at 800 g. The volume was made up to exactly 5 mL with DI. The concentrate was analysed for the presence of *Giardia* cysts using a monoclonal antibody stain as described by Rose *et al.* (1991).

3 RESULTS

Results from the control plot are shown in Table 1. Results from trial 1 are shown in Table 2. Trial 1 commenced on 24 August 1993 and the trial plot was monitored for a total of 8 weeks. This trial was discontinued after 8 weeks because no enteroviruses or *Salmonella* were detected in the sludge amended soil. As described previously (Gibbs *et al.*, 1993), dewatered sludge usually contained both enteroviruses and *Salmonella*. After 8 weeks it was decided to start another trial with fresh dewatered sludge.

The second trial commenced on 15 November 1993 and the sludge was monitored for 37 weeks, finishing on 1 August, 1994. Results for faecal coliforms, faecal streptococci, *Salmonella* and *Giardia* are shown in Figure 1.

Figure 1 shows that faecal coliforms and faecal streptococci were present in sludge amended soil at initial concentrations of 63 000/g and 28 000/g respectively. Faecal streptococci then reduced in number through the weeks of storage and were present at low or non detectable concentrations from weeks 2 to 37. Faecal coliforms also reduced in number and were at low or non detectable concentrations from weeks 4 to 21. However, at week 29 faecal coliform concentrations were 110 000/g, which was higher than at the beginning of the study.

Salmonella were initially present at an average concentration of 0.09/g. *Salmonella* concentrations also reduced and from weeks 8 to 29 no *Salmonella* were detected. However, at week 36 *Salmonella* were detected at a concentration of 0.72/g, which was higher than at the beginning of the study. Nine *Salmonella* isolates from the positive samples at week 36 were serotyped. All of the isolates were *Salmonella chester.*

Giardia cysts were present at the beginning of the study and reduced in concentration through the early weeks following amendment. From week 12 to the end of the study no *Giardia* cysts were detected.

During the period of the study daily measurements were taken of the rainfall and maximum and minimum ambient temperatures. These results are summarised in Figure 2. This figure shows average weekly maximum and minimum temperatures and total weekly rainfall for the week preceding the sampling day. At the beginning of the study, maximum temperatures gradually increased and remained high through to approximately week 20. This corresponded to the summer period November to April. From week 20 to 29 temperatures decreased and this corresponded to the autumn period April to June. From week 29 to the end of the study temperatures were low and fairly constant. This corresponded to the winter period from June to August. Through the summer and early spring there was negligible rainfall. Winter rains commenced on week 27 which was at the end of May. Rain was then recorded each week for the rest of the study.

Figure 3 shows results from measurements of sludge-amended soil temperature and moisture content on each sampling occasion. Soil temperatures initially decreased from 30°C to slightly above 25°C. After week 21 soil temperatures decreased to between 10°C and 15°C where they remained for the rest of the trial. Soil moisture content decreased in the earlier part of the trial and was then low or negligible to week 21. At week 29 sludge moisture content increased to 22% and then decreased again towards the end of the trial.

3.1 Relationship Between Indicator Bacteria and Pathogens

There was no significant correlation between faecal coliform and pathogen concentrations. Significant correlations were detected between faecal streptococci and *Salmonella* concentrations, (Kendall's Tau correlation co-efficient p = 0.0201) and faecal streptococci and *Giardia* concentrations (p= 0.0201). However, salmonellae were detected in the absence of faecal streptococci on one occasion (week 36) and faecal streptococci were still detected on a number of occasions when *Giardia* were no longer detected.

Figure 1 Microbiological Results From Trial 2

Faecal Coliforms

120000
100000
80000
60000
40000
20000
0
FC/g
0 1 2 4 8 12 21 29 36 37
Sampling Week

Faecal Streptococci

30000
25000
20000
15000
10000
5000
0
FS/g
0 1 2 4 8 12 21 29 36 37
Sampling Week

Salmonella

0.8
0.6
0.4
0.2
0
Salmonella/g
0 1 2 4 8 12 21 29 36 37
Sampling Week

Giardia

700
600
500
400
300
200
100
0
Giardia/g
0 1 2 4 8 12 21 29 36 37
Sampling Week

Figure 2 Average Weekly Maximum and Minimum Ambient Temperature and Total Weekly Rainfall Results From Trial 2

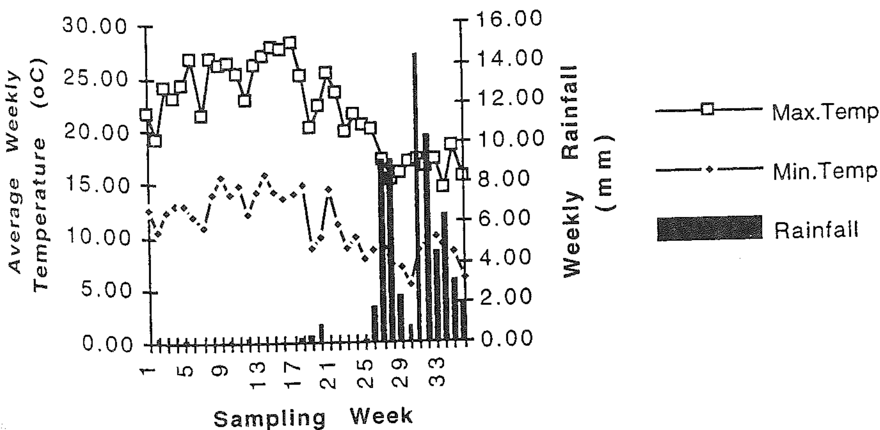
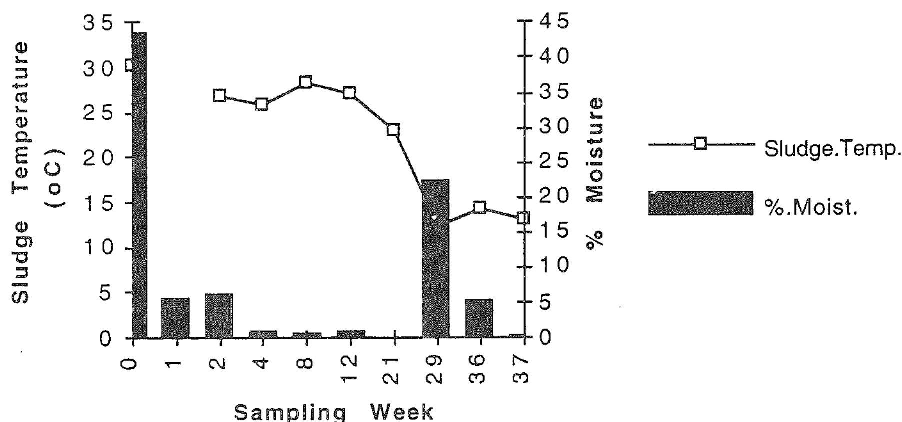


Figure 3 Sludge-amended Soil Temperature and Percentage Moisture Content Results From Trial 2



4 DISCUSSION

Incorporation of sludge into sandy soil at a rate equivalent to 10 tonnes/hectare resulted in contamination of the soil with high concentrations of faecal coliforms and faecal streptococci. The pathogens *Giardia* and *Salmonella* were also introduced into the soil. In the two trials described here enteroviruses were not detected in sludge amended soil.

After 8 weeks storage faecal indicator bacteria and pathogens had reduced to non detectable concentrations. However, after 29 and 36 weeks, respectively, faecal coliforms and *Salmonella* were detected in concentrations higher than reported at the beginning of the study. It appears that these bacteria regrew from low undetectable levels of bacteria surviving in the soil or that a large amount of faecal contamination occurred from animal droppings.

It is difficult to determine from these results the cause for the observed increase in faecal coliforms and *Salmonella* concentrations later in the study. During the study it appeared that faecal contamination from animals was occurring. Animal droppings and footprints were observed on the sites. On two occasions low levels of faecal bacteria were detected in the control plot which suggested that faecal contamination from animals was occurring.

However, more evidence seems to support the view that regrowth of faecal coliforms and *Salmonella* occurred. One reason for this is that if animal droppings had been the source of the high numbers of faecal coliforms and *Salmonella* detected later in the study it was expected that faecal streptococci would also have been detected in high concentrations. In the faeces of warm blooded animals faecal streptococci would be expected in higher concentrations than faecal coliforms (APHA *et al.*, 1989). Another support for the view that regrowth occurred is that on those occasions when contamination from animal faeces occurred in the control plot the concentrations were much lower than when regrowth appeared to occur in the trial plot.

One previous study reviewed by Sorber and Moore (1987) reported results that suggested that regrowth of faecal coliforms occurred in sludge amended soil. Burge *et al.* (1981) and Skavanis and Yanko (1994) reported *Salmonella* regrowth in composted sludge but no other studies were found which reported the regrowth of *Salmonella* in sludge amended soil.

The possible faecal coliform and *Salmonella* regrowth observed at weeks 29 and 36 of the study appeared to be linked to rainfall. Through the summer months there was negligible rainfall but winter rains commenced prior to sampling at week 29. This rainfall was accompanied by an increase in the moisture content of the soil (from 1% to 22%) which may have promoted bacterial growth by providing conditions favourable for growth. Burge *et al.* (1987) reported that growth of *Salmonella* in composted sludge required a moisture content of greater than 20%.

The potential regrowth of *Salmonella* in sludge amended soil as demonstrated in this study was surprising. In this study regrowth occurred after an extended hot, dry summer when *Salmonella* were not detected. It was expected that conditions in this study would have maximised *Salmonella* die-off. It therefore seems that under these conditions the potential hazard associated with sludge application to soil may be higher than anticipated.

The results of this study do not endorse the use of faecal indicator bacteria as predictors of the presence of pathogens in sludge amended soil. There was no correlation between faecal coliform concentrations and pathogen concentrations. There was a significant correlation between faecal streptococci and pathogens. However, faecal streptococci did not appear to be adequate indicators for the presence of *Salmonella*. On one occasion *Salmonella* were detected when faecal streptococci were absent.

5 CONCLUSIONS

The main hazard associated with the application of sludge to agricultural land appeared to be the regrowth of bacterial pathogens such as *Salmonella*. Potential hazards associated with enteroviruses and *Giardia* were low. Faecal coliforms and faecal streptococci were not adequate predictors of the presence of pathogens.

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