TECHNICAL PAPERS

Australian Water & Wastewater Association

PLATFORM PAPERS

15TH FEDERAL CONVENTION

HOTEL CONRAD • GOLD COAST
QUEENSLAND • 18–23 APRIL 1993

"Water - Every Drop is Precious"

VOLUME 2

PATHOGENS IN WASTEWATER SLUDGE

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SUMMARY

Information concerning pathogen densities in wastewater sludge is scarce, particularly for Australian sludges. As part of a project investigating the die-off of pathogens in stored sludge pathogen densities in sludge from Perth wastewater treatment plants were determined. Faecal coliform, faecal streptococci, enterovirus and *Salmonella* concentrations in sludge were quantified. *Campylobacter, Shigella, Legionella* and helminths were not detected in sludge using qualitative tests. *Giardia* and *Entamoeba* were present in raw sludge but were not detected in digested and dewatered sludge. There was no consistent relationship between the die-off of indicator organisms and pathogens through sludge treatment. Pathogens generally survived treatment better than faecal coliforms and faecal streptococci. On the basis of these results the use of indicator bacteria to predict pathogen densities in wastewater sludge may not be appropriate.

1. INTRODUCTION

The disposal of wastewater sludge is a growing problem within Australia (Varjavandi, 1991) and throughout the world (Davis, 1987; Scheltinga, 1987; Yanko, 1988). Estimated Australian sludge production is 250 000 dry tonnes annually and increasing (AWRC, 1992). Increases are due to the connection of greater numbers of households to sewerage networks and improvements to wastewater treatment standards. The provision of better quality effluents through greater solids removal in wastewater treatment results in more sludge production.

A number of disposal options are available for wastewater sludge and these include ocean disposal, incineration, minesite rehabilitation, landfilling and use as a soil amendment (Williams *et al.*, 1990). Ocean disposal has a limited future as it has been banned in the United States and is expected to cease in Australia by the end of 1993 (AWRC, 1992). Incineration is associated with high energy and running costs and concerns about air pollution (Yanko, 1988). The use of sludge as a soil amendment is an attractive alternative disposal method as the sludge is utilised as a resource. Sludge contains organic matter and nutrients so is useful as a soil conditioner and low grade fertiliser (Awad *et al.*, 1989). There are potential problems associated with the use of wastewater sludge as a soil amendment. It may contain unacceptably high levels of trace metals and organic chemical contaminants such as pesticides. It may also contain microorganisms which are dangerous to human health.

Research on pathogens in wastewater sludge has been scarce and this was highlighted by literature reviews conducted by Lewis Jones and Winkler (1991), Pederson (1981) and Sorber and Moore (1987). Most of the available data was restricted to *Salmonella* and indicator organisms, with little data available for viruses and parasites. In a more recent literature review conducted for this project (Gibbs and Ho, 1992) similar conclusions were reached. Of particular concern was the lack of information about pathogen densities in Australian sludges and uncertainty about the relationship between indicator organisms and pathogens in sludge.

This paper describes the results from the first stage of a project examining pathogen die-off in stored wastewater sludge and sludge applied to land. Pathogen densities in sludge taken from wastewater treatment plants in Western Australia were determined.

2. METHODS

2.1 Description of Wastewater Treatment Plants

Sludge samples were collected from three wastewater treatment plants in Perth, Western Australia. Table 1 summarises the treatment stages and indicates sample collection points at the treatment plants.

Table 1. Description of Wastewater Treatment Plants

Treatment Stages	Subiaco	Beenyup	Woodman Point
Input to plant	56.25 ML/day	59.2 ML/day	82.5 ML/day
Pre-chlorination for odour control	25 mg/L average	15 mg/L average	dosed in summer as needed at approx. 6 mg/L
Primary treatment	Yes	Yes	Yes
Raw sludge sample collected	Yes	Yes	Yes
Secondary treatment (activated sludge)	Yes	Yes	No
Dissolved air flotation of sludge from secondary treatment	Yes	Yes	No
Raw sludge and secondary DAF sludge mixed together	Yes	Yes	No
Primary anaerobic digestion - heated	13 days, 35°C	27 days, 35°C	16 days, 35°C
Secondary anaerobic digestion - no heating	30 days, open digesters, approximately ambient temperature	17 days, closed digesters, approx. 30°C	6 days, closed digester, approx. 30°C
Digested sludge sample collected	Yes	Yes	Yes
Dewatering			
Filter belt presses production	80 m^3/day	70 m^3/day	No
$KMnO_4$ addition for H_2S control	4.3 g/m^3 of sludge	55 g/m^3 of sludge	No
Centrifuge production	No	No	30 to 40 m^3/day
Sand drying beds and lagoons feed	No	No	150 m^3/day
Dewatered sludge sample collected	Yes	Yes	No

2.2 Microbiology

Faecal coliforms were analysed at Murdoch University using the membrane filter procedure and M-FC medium described in Standard Methods (APHA *et al.*, 1985). Faecal streptococci were analysed using the membrane filter procedure and m-Enterococcus agar (APHA *et al.*, 1985).

Salmonella densities were determined at Murdoch University using a modification of the method recommended for sludge samples by Carrington (1980). A five tube most probable number method (MPN) was used with the following 10 fold successive enrichments of sludge in Rappaports Vassiliadis (RV) enrichment medium.

Enrichment 1:
5 g of sludge in 45 mL of RV medium

Enrichment 2:
0.5 g of sludge in 9 mL of RV medium

Enrichment 3:
0.05 g of sludge in 9 mL of RV medium

Enrichment 4:
0.005 g of sludge in 9 mL of RV medium

Enrichment 5:
0.0005 g of sludge in 9 mL of RV medium

Enrichments 1, 2 and 3 were prepared from a master dilution of 30 g of sludge in 270 mL of RV medium. Enrichment 1 contained 50 ml of the master dilution, enrichment 2 contained 5 mL and enrichment 3 contained 0.5 mL. Enrichments 4 and 5 were prepared from two successive dilutions of the master dilution in buffered peptone water (BPW). The inoculated enrichment media were incubated at 39°C. Each of the five replicates for each enrichment was streaked to xyline lysine deoxycholate (XLD) plates after 24 and 48 hours incubation. Presumptive *Salmonella* isolates from XLD plates were confirmed by agglutination and biochemical tests.

The media and methods described above were compared to other methods including tetrathionate enrichment medium, strontium chloride B enrichment medium, brilliant green agar plates, bismuth sulphite agar (BSA) plates, incubation of media and plates at 37°C and pre-enrichment of sample dilutions for 24 hours at 37°C in BPW. The method described above was found to give more positive *Salmonella* isolations than alternative methods tested.

Enterovirus analysis was carried out by the virology section of the State Health Laboratory Services, Western Australia. Enteroviruses were initially analysed by a method used for extracting viruses from faeces. A sample of sludge was thoroughly mixed with virus transport medium. The preparation was then frozen overnight and allowed to thaw. The thawed specimen was centrifuged at 3500 g for 20 minutes. The supernatant was collected and inoculated into MK, HF32 and FL Amnion cell lines in the presence of mycostatin.

A modified elution and concentration method was developed from methods recommended by Albert and Schwartzbrod (1991), Goyal *et al.* (1984) and Lewis and Metcalf (1988). The modified method was as follows. Raw and digested sludge samples were centrifuged at 1500 g for 15 minutes (dewatered samples were not centrifuged). 40 g of sludge pellet was mixed with a solution of 3% beef extract in 0.1 M borate buffer pH 9. The mixture was magnetically stirred for 15 minutes, sonicated at 100 W for 1 min and centrifuged at 10 000 g for 45 minutes. The supernatant was collected and neutralised. Polyethylene glycol 6000 was added to give a final concentration of 15% (w/v). The suspension was stirred for 1.5 to 2 hours at 4°C and centrifuged at 10 000 g for 20 minutes. The supernatant was discarded and the pellet suspended in 0.15 M Na_2HPO_4 (pH 9.0), sonicated for 30 seconds, shaken for 20 min and centrifuged at 10 000 g for 30 minutes. The supernatant was adjusted to pH 7.4 and detoxified with chloroform. For the chloroform detoxification a 100 g sample of diphenylthiocarbazone (dithizone) was dissolved in 1000 mL of chloroform and 10 mL of this working dilution added to the suspended floc. After blending at high speed for 1 minute the mixture was centrifuged at 10 000 g for 30 minutes. The upper aqueous layer was gently removed and placed in a sterile test tube containing 0.05 mL of 0.15 $CaCl_2$. The aqueous supernatant was gently aerated with filtered air fed through a sterile pasteur pipette for 10 minutes to remove residual chloroform. The concentrated end products were inoculated into HF_{32}, FL Amnion, RD and BGM cell lines in the presence of mycostatin, vanomycin, streptomycin, gentamycin and fungizone. The tubes were observed for toxicity or cytopathic effect and passaged as appropriate.

Tests for the presence or absence of parasites, *Campylobacter, Shigella, Salmonella* and *Legionella* were carried out by the Western Australian State Health Laboratory Services using methods for faecal specimens as follows.

The presence of parasites was determined using formal saline ethyl acetate concentration. Approximately 1 mL of sample was mixed with 7 mL of 10% formal saline in centrifuge tubes. To this was added 2 mL of ethyl acetate, the tube was

shaken and then centrifuged at 1500 g for three to five minutes. The top layers were poured off and the bottom pellet removed. The pellet was stained with Lugol's iodine and examined using microscopy.

For *Campylobacter* determination the sludge was streaked onto Campylobacter blood-free selective agar and plates incubated at 43°C for 48 hours in a 10% CO_2 atmosphere with a balance of nitrogen.

For *Shigella* determination sludge was streaked onto XLD and deoxycholate agar (DCA) plates. Plates were incubated at 37°C for 24 hours.

For *Salmonella* determination sludge was streaked onto XLD and deoxycholate plates which were incubated at 37°C for 24 hours. In addition approximately 1 mL of sludge was inoculated into 10 mL of strontium chloride B broth and incubated for 24 hours at 43°C. The enrichment medium was then streaked onto BSA and DCA plates which were incubated at 37°C for 24 hours. Presumptive colonies were tested using agglutinations and biochemical tests.

The presence of *Legionella* was determined by first making 1 in 100 and 1 in 1000 dilutions of sludge in sterile distilled water. Buffered charcoal yeast extract agar (BCYE) and modified Wadowsky Yee plates (MWY) were spread with 0.1 mL of the 1 in 100 dilution and 0.1 mL of the 1 in 1000 dilution . A 2 mL volume of the 1 in 100 dilution was added to a wide necked McCartney bottle and placed in a 50°C water bath for 30 minutes. BCYE and MWY plates were inoculated with 0.1 mL of the heat treated sample. Plates were incubated at 36°C in a sealed canister for up to 10 days. Plates were examined on the 3rd, 5th, 7th, 10th and 14th days.

3. RESULTS

Legionella, Campylobacter, Shigella and helminths were not detected in sludge using the described methods. In six initial samples tested using the faecal specimen method no enteroviruses were detected.

Entamoeba spp. were present in 7 of 12 raw sludge samples and *Giardia intestinalis* present in 8 of 12 raw sludge samples examined. No protozoa were detected in digested and dewatered sludge samples.

Results from the faecal coliform, faecal streptococci, *Salmonella* (quantitative and faecal specimen methods) and enterovirus analyses are shown in Tables 2, 3 and 4.

Percentage reductions from raw to digested sludge (Woodman Point) and raw to dewatered sludge (Subiaco and Beenyup) for the indicator organisms and pathogens shown in Tables 2, 3 and 4 are summarised in Table 5.

Table 2. Woodman Point Results

Date and Sampling Point	Faecal Coliforms (FC/g)	Faecal Streptococci (FS/g)	*Salmonella* (Quantitative Method) (MPN/g)	*Salmonella* (Faecal Specimen Method)	Enteroviruses (Infectious Units/g)
<u>26.2.92</u>					
Raw	2.4×10^8	1.2×10^5	NT*	*S. cerro*	NT
Digested	5.1×10^3	2.0×10^3	NT	-ve	NT
<u>10.4.92</u>					
Raw	8.0×10^7	3.1×10^5	NT	*S. typhimurium*	107
Digested	1.1×10^3	245	NT	-ve	1
<u>18.5.92</u>					
Raw	3.6×10^7	4.4×10^5	34	*S. cerro* *S. chester*	15
Digested	2.2×10^3	89	0.4	-ve	170
<u>10.8.92</u>					
Raw	2.2×10^7	7.0×10^5	0.46	*S. give*	65
Digested	6.7×10^3	810	0.08	-ve	15

*NT: Not tested

shaken and then centrifuged at 1500 g for three to five minutes. The top layers were poured off and the bottom pellet removed. The pellet was stained with Lugol's iodine and examined using microscopy.

For *Campylobacter* determination the sludge was streaked onto Campylobacter blood-free selective agar and plates incubated at 43°C for 48 hours in a 10% CO_2 atmosphere with a balance of nitrogen.

For *Shigella* determination sludge was streaked onto XLD and deoxycholate agar (DCA) plates. Plates were incubated at 37°C for 24 hours.

For *Salmonella* determination sludge was streaked onto XLD and deoxycholate plates which were incubated at 37°C for 24 hours. In addition approximately 1 mL of sludge was inoculated into 10 mL of strontium chloride B broth and incubated for 24 hours at 43°C. The enrichment medium was then streaked onto BSA and DCA plates which were incubated at 37°C for 24 hours. Presumptive colonies were tested using agglutinations and biochemical tests.

The presence of *Legionella* was determined by first making 1 in 100 and 1 in 1000 dilutions of sludge in sterile distilled water. Buffered charcoal yeast extract agar (BCYE) and modified Wadowsky Yee plates (MWY) were spread with 0.1 mL of the 1 in 100 dilution and 0.1 mL of the 1 in 1000 dilution . A 2 mL volume of the 1 in 100 dilution was added to a wide necked McCartney bottle and placed in a 50°C water bath for 30 minutes. BCYE and MWY plates were inoculated with 0.1 mL of the heat treated sample. Plates were incubated at 36°C in a sealed canister for up to 10 days. Plates were examined on the 3rd, 5th, 7th, 10th and 14th days.

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Percentage reductions from raw to digested sludge (Woodman Point) and raw to dewatered sludge (Subiaco and Beenyup) for the indicator organisms and pathogens shown in Tables 2, 3 and 4 are summarised in Table 5. Quantitative pathogen results from the wastewater treatment plants are summarised in Table 6.

Table 3. Subiaco Results

Date and Sampling Point	Faecal Coliforms (FC/g)	Faecal Streptococci (FS/g)	*Salmonella* (Quantitative Method) (MPN/g)	*Salmonella* (Faecal Specimen Method)	Enteroviruses (Infectious Units/g)
14.1.92					
Raw	$3.0x10^6$	$8.4x10^4$	NT	*S. livingstone** *S. anatum*	NT
Digested	$7.0x10^3$	440	NT	*S. adelaide* *S. chester*	NT
Dewatered	$2.6x10^4$	$3.1x10^4$	NT	*S. livingstone* *S. montevidea* *S. arion*	NT
6.4.92					
Raw	$8.3x10^5$	$5.2x10^4$	13	-ve	483
Digested	137	320	NT	-ve	NT
Dewatered	537	537	NT	-ve	toxicity†
25.5.92					
Raw	$3.9x10^6$	$2.0x10^4$	16	*S. infantis* *S. muenchen*	350
Digested	$1.1x10^2$	$3.9x10^2$	-ve	-ve	NT
Dewatered	$1.4x10^2$	$1.5x10^3$	-ve	-ve	$1.1x10^3$
17.8.92					
Raw	$5.6x10^5$	$2.1x10^4$	8	-ve	19.7
Digested	620	$2.8x10^3$	0.16	-ve	NT
Dewatered	650	$3.0x10^3$	0.4	-ve	6

* Samples from 14.1.92 were tested using a modified *Salmonella* method
† Virus concentration was not determined because sample was toxic to cell culture

Table 4. Beenyup Results

Date and Sampling Point	Faecal Coliforms (FC/g)	Faecal Streptococci (FS/g)	*Salmonella* (Quantitative Method) (MPN/g)	*Salmonella* (Faecal Specimen Method)	Enteroviruses (Infectious Units/g)
30.3.92					
Raw	$3.2x10^6$	$4.8x10^4$	130	*S. infantis*	NT
Digested	63	18	NT	-ve	NT
Dewatered	$1.3x10^5$	$4.9x10^3$	NT	-ve	NT
13.4.92					
Raw	$9.5x10^6$	$1.3x10^5$	NT	-ve	428
Digested	$8.9x10^3$	980	NT	-ve	0.3
Dewatered	$6.8x10^4$	$5.1x10^4$	NT	-ve	2.1
2.6.92					
Raw	$3.3x10^6$	$4.8x10^4$	46	*S. tennessee*	40
Digested	10	19	4	-ve	toxicity
Dewatered	$4.5x10^4$	$9.8x10^3$	1.4	-ve	2.7
24.8.92					
Raw	$3.0x10^6$	$8.7x10^4$	8	-ve	5
Digested	$1.6x10^4$	$4.9x10^3$	0.52	-ve	NT
Dewatered	$7.8x10^4$	$2.4x10^4$	1.4	-ve	10.5

Table 5. Percentage Reduction of Indicator and Pathogen Concentrations Through Sludge Treatment

Percentage Reduction	Faecal Coliforms	Faecal Streptococci	*Salmonella*	Enteroviruses
No. of samples	12	12	6	8
Average	99.5	87.5	92.6	58.2
Minimum	97.40	60.78	82.50	0
Maximum	99.9999	99.98	96.96	99.51

Table 6. Summary of *Salmonella* and *Enterovirus* Densities in Perth Sludges

Sample Type	*Salmonella*			Enteroviruses		
	No. of Samples	Density (MPN/g)		No. of Samples	Density (Infectious Units/g)	
		Mean	Range		Mean	Range
Raw	8	32	0.46 - 130	9	168	5 - 483
Digested	6	1.0	0.08 - 40	5	46	0.3 - 170
Dewatered	4	1.1	0.4 - 1.4	6	224	$2.1 - 1.1 \times 10^3$

4. DISCUSSION

Quantitative methods for enterovirus and *Salmonella* detection in sludge developed for this study gave more positive isolations than faecal specimen methods. These results suggest that methods developed for other material, in particular faecal stools, may not be appropriate for examining sludge for pathogens. *Legionella, Campylobacter, Shigella* and helminths were not detected in Perth sludge samples using methods developed for faecal specimens. Method development for these pathogens might result in positive isolates as *Campylobacter* and helminths have been detected in sludge in other countries (Jones *et al.*, 1990; Reimers *et al.*, 1986). *Entamoeba* and *Giardia* have been found in digested sludges in other countries (Fox and Fitzgerald, 1977; Sykora *et al.*, 1991) so may also have been recovered from such sludges using different methods.

Salmonella and enterovirus densities found in this study are higher than those found in other countries. In Perth digested sludges the average densities of *Salmonella* and enteroviruses were 1.0 and 46 per g of wet sludge respectively. In studies in other counties reported average densities have ranged from 0.016 to 0.62 per g wet weight for *Salmonella* and 0.03 to 1.9 per g wet weight for enteroviruses (Gibbs and Ho, 1992). *Salmonella* and enterovirus concentrations may be higher in Australian sludges but it is also possible that differences in methods were the cause of higher recoveries in Perth sludges.

An assessment of the risks associated with pathogens in sludge based on densities reported in other countries (Gibbs and Ho, 1992) suggested that digested sludge is not safe for unrestricted marketing to the public. This study confirms this and suggests that the risks may be greater than previously estimated. One of the Perth dewatered sludge

samples was found to contain approximately 1000 enterovirus infectious particles per gram. Infectious dose information provided by Shuval *et al.* (1986) suggests that the ingestion of 10 enterovirus infectious particles will result in an infection in approximately 1 in 4 people. With an enterovirus concentration of 1000/g in dewatered sludge, people handling dewatered sludge and ingesting 0.01 g would have a 1 in 4 chance of contracting an enterovirus infection.

One of the objectives of this study was to examine the relationship between indicator organisms and pathogens in sludge.

Bacterial indicator organisms are generally used to indicate the presence of faecal pollution and therefore the potential presence of pathogens in drinking water and natural water bodies. Wastewater sludge is slightly different in that there is no doubt that both faecal indicator bacteria and pathogens are initially present. The most important factor is their relative survival through sludge treatment. The purpose of faecal indicators in sludge should therefore be to say that if faecal indicators are below a certain concentration then we could have reasonable confidence that pathogens are absent. Previous studies have not provided data on which such a standard could be based (Lewis-Jones and Winkler, l991; Pederson, 1981).

The results of this study call into question the use of the two bacterial indicator organisms tested (faecal coliforms and faecal streptococci). It is possible that the use of a MPN rather than membrane filtration method may have given different results.

There was no consistent relationship between the reduction of bacterial indicators and pathogens through treatment. In general pathogens survived treatment better than indicator organisms. The percentage reduction in faecal coliform concentrations was in all cases higher than the reduction of *Salmonella* and enteroviruses. Faecal streptococci appeared to survive treatment better than faecal coliforms but there was no consistent relationship between faecal streptococci and pathogen densities. The relationship between viruses and bacterial indicators seemed particularly uncertain. In two cases virus concentrations were greater than faecal indicator bacteria concentrations. In three cases there was no reduction in virus concentrations through treatment but indicator organisms concentrations were reduced by at least 72%. The variability in the virus results suggests that there may be a high variation in virus concentrations through sludge products.

Proposed USA and Australian guidelines for sludge management have included the use of faecal indicator bacteria to predict pathogen densities. The USA guidelines require that faecal coliforms and faecal streptococci should be below 100/ g volatile suspended solids. The Australian guidelines suggest that E. coli should be below 100 MPN/g of final product. In one of the digested samples in this study faecal coliforms, faecal streptococci and Salmonella were 10, 19 and 4 /g respectively. In this case both faecal coliforms and faecal streptococci were below 100/g but *Salmonella* were present. Enteroviruses may also have been present but toxicity prevented any cytopathic effect being observed. The results of this study provided no evidence of a quantitative relationship between indicator organisms and pathogens. The use of faecal indicator bacteria to predict pathogen densities should not be recommended without such evidence.

5. CONCLUSIONS

Salmonella and enteroviruses were present in Perth sludges at concentrations higher than those reported from other countries. Therefore it is recommended that sludges which have only been treated by digestion and dewatering should not be available for use by the public without further treatment or restrictions. Faecal indicator bacteria were not good indicators of *Salmonella* and enterovirus survival through sludge treatment.

6. ACKNOWLEDGMENTS

This project was funded by the Water Authority of Western Australia and Urban Water Research Association of Australia. We are grateful for the advice and analyses provided by Dr Peter Phillips, Ms Val Wymer, Mr Ray Mogyorosy and Mr Frank Haverkort from the State Health Laboratory Services, WA. Ms Diedre Hughes and Ms Clara Bolle assisted with analyses.

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