A RECOMMENDED METHOD FOR DETECTING SALMONELLAE IN COMPOSTED BIOSOLIDS

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ABSTRACT

It has been found in Australia and in the United States that composting does not always result in the complete removal of salmonellae from biosolids. It is therefore likely that monitoring of composted biosolids for salmonellae will be required in Australia to ensure the safety of biosolids products. At present rapid methods of detection such as PCR and ELISA are not sufficiently developed to monitor environmental samples. The relative efficiency of various culture methods for detecting salmonellae in composted biosolids was therefore investigated. On the basis of the results a presence/absence method is recommended for the detection of salmonellae in biosolids products. The recommended technique involves pre-enrichment of samples, followed by enrichment in Rappaport-Vassiliadis and mannitol selenite enrichment broths, and isolation on lysine mannitol glycerol agar.

KEY WORDS

Biosolids, compost, guidelines, salmonellae methods

1 INTRODUCTION

The Agriculture and Resource Management Council of Australia and New Zealand (ARMCANZ) and the Australian and New Zealand Environment and Conservation Council are formulating a set of biosolids management guidelines for the treatment and disposal of wastewater sludge (A. Maus, pers. comm.). It is proposed that biosolids products will be classified, and their end use regulated, partially on the basis of the risks posed to human health by pathogens in biosolids. To be available for unrestricted use, biosolids products must contain no detectable salmonellae in a 50g sample, and should have been composted for a specified length of time at a particular temperature.

Due to the potential risks it is likely that guidelines will contain a requirement for generators of biosolids products such as compost to monitor for the presence of salmonellae. This approach has already been taken by the US EPA (1992) which has produced requirements for a standard salmonellae monitoring process. However, our experiments (Hu et al., 1995) suggest that the salmonellae detection method recommended by the US EPA (1992) may not result in optimal detection. Guidelines in Australia are likely to stipulate that salmonellae should be monitored using the method recommended for food analysis (A. Maus, pers. comm.). The use of a food method to detect salmonellae in biosolids may also not be appropriate. This is because variations in the sample type can significantly affect salmonellae detection. In particular, sample types vary in the number of salmonellae likely to be present and in the number of competitors. Competitors can interfere with salmonellae detection if present in high numbers. Biosolids samples are expected to contain higher numbers of competitors than food samples.

We have carried out several studies to optimise detection of salmonellae in wastewater biosolids and composted biosolids (Hu, et al, 1995). On the basis of these studies, suggestions for a standard method for detecting salmonellae in composted biosolids are made in this paper.

2 RECOMMENDED CULTURE METHOD FOR SALMONELLAE IN COMPOSTED BIOSOLIDS

The traditional salmonellae culture method involves four stages. Although this is a time consuming and expensive process, more rapid methods such as PCR and ELISA are not yet at a stage where they can be used with biosolids products. The four stages of the culture method are described below. Recommendations for best techniques for use with biosolids are given on the basis of our results and results from previous studies.

2.1 Step 1 - pre-enrichment

Pre-enrichment of samples in a non-selective medium is presumed to allow sub-lethally damaged salmonellae to recover and multiply to a level which will ensure their survival upon transfer to a selective medium. Pre-enrichment of environmental samples is widely accepted, although there appears to have been only one previous study which compared pre-enrichment with direct enrichment using biosolids samples. In a study by Edgar and Soar (1979) it was found that pre-enrichment improved isolation of salmonellae when used in conjunction with certain enrichment broths but had no effect when used with others. We carried out a small study to compare direct enrichment with pre-enrichment in buffered peptone water (BPW) (Gibbs et al., 1995), the results of which are summarised in Fig. 1. In this study five presence/absence tests of 10 g were carried out for five samples. Direct enrichment of raw sludge in Rappaport-Vassiliadis broth (RV) was statistically equally effective as pre-enrichment of the sample followed by enrichment in RV. However, because of the limited nature of this study and the possibility that salmonellae could be more likely to be sub-lethally injured in composted biosolids than in raw sludge, we feel that it is prudent to continue pre-enriching samples of sludge and biosolids products. Pre-enrichment was used in all subsequent studies from which conclusions in this paper are drawn.

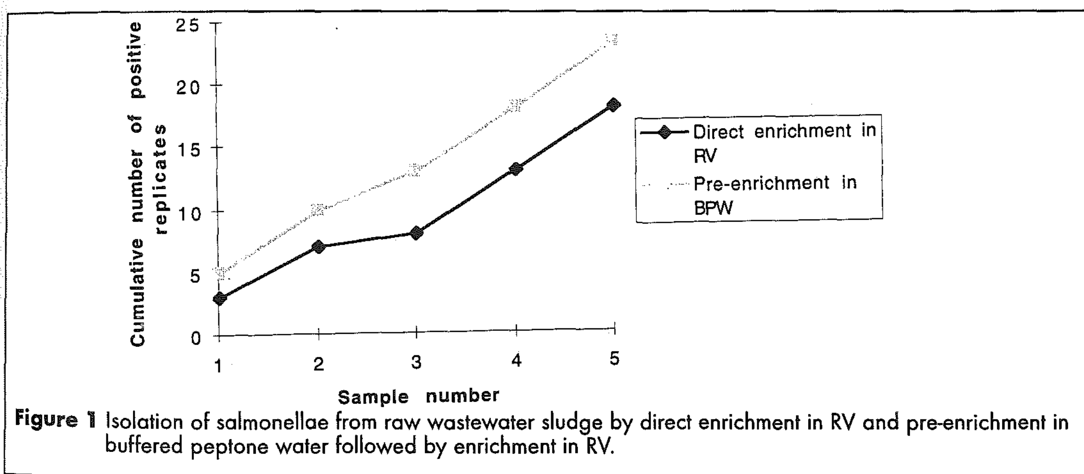

Figure 1 Isolation of salmonellae from raw wastewater sludge by direct enrichment in RV and pre-enrichment in buffered peptone water followed by enrichment in RV.

2.2 Step 2 - selective enrichment

Selective enrichment media are liquid media containing substances which inhibit the growth of non-salmonellae while allowing or encouraging the growth of salmonellae. A wide variety of enrichment media are available and numerous previous studies have compared the relative efficiency of these broths. However, few studies have used sludge samples and none appear to have used composted biosolids samples.

We compared media recommended in Australia and in the USA (US EPA, 1992). Specifically, the media tested were RV and mannitol selenite enrichment broth (MSE), recommended in Australia for analysis of food samples (Standards Australia, 1991); tetrathionate broth (TT), recommended by the USEPA for analysis of water and wastewater (US EPA, 1992) and strontium chloride B broth (Iveson & MacKay-Scollay, 1972) which is used by Pathcentre (formerly the West Australian State Health Laboratories) for detection of salmonellae in environmental samples. The results of this experiment are summarised in Table 1.

Table 1 Number of salmonellae - positive replicates detected by four enrichment broths and three plating media

Plating Media	Enrichment Media			
	RV	MSE	TT	SCB
XLD	115	38	0	5
LMG	128	36	3	1
BSA	72	17	4	0

Abbreviations: RV - Rappaport-Vassiliadis broth; MSE - mannitol selenite broth; TT - tetrathionate broth; SCB - strontium chloride B broth; XLD - xylose lysine deoxycholate agar; LMG - lysine mannitol glycerol agar; BSA - bismuth sulphite agar.

RV was the enrichment medium of choice because it detected a significantly ($p < 0.0001$) greater number of positive enrichments than the other enrichment media tested. Although there was no statistically significant advantage in using more than one enrichment broth the use of a second broth is also recommended because of the possibility that RV, or indeed any enrichment broth, will not be able to detect all salmonellae serotypes. (Over 2000 serotypes have been identified). While it is not necessary to identify serotypes in routine monitoring of composted biosolid products, there is the possibility that only one serotype could be present and not be detectable if only one enrichment broth is used. Of particular concern are S. *typhi* and *paratyphi*. These serotypes cause a potentially more serious illness (typhoid fever) than other *Salmonella* serotypes, are biochemically atypical and are not able to grow in RV (Merck,1988). For this reason MSE is also recommended. This broth does support the growth of S. *typhi* and *paratyphi* and, as will be discussed in section 2.3, promotes the production of H_2S in these serotypes which is useful in their identification on lysine mannitol glycerol agar (LMG) (Cox, 1993).

2.3 Step 3 - selective isolation

Selective isolation media are agar media which allow the growth of salmonellae while inhibiting the growth of non-salmonellae. In addition, selective isolation media usually utilise mechanisms which allow salmonellae to be visually distinguished from non-salmonellae. Again, variety of selective isolation media are available. Several studies have also compared the relative merits of a variety of media but again, few have used sludge samples and none appear to have used composted biosolids. The data from these experiments has been conflicting, with Carrington (1980) and Rhodes and Quesnel (1986) recommending xylose lysine deoxycholate agar and Edgar and Soar (1979) and Fricker (1984) recommending brilliant green agar (BGA). In our experiments (Hu et al., 1995) we compared XLD, recommended in Australia for analysis of food samples (Standards Australia, 1991) and in the USA for analysis of water and wastewater (US EPA, 1992); lysine mannitol glycerol agar (LMG), which was reported by Cox (1993) to be superior to XLD; and bismuth sulphite agar (BSA), also recommended for food samples (Standards Australia, 1991). We had previously tested brilliant green agar (BGA) which is also recommended by the US EPA (1992), and found that this medium was inferior to XLD (Gibbs et al., 1995). The results of our experiments are summarised in Table 1. There was no statistical difference between the plating media tested but LMG was chosen because it has the ability to detect *S. typhi* and other atypical salmonellae (Cox, 1993). Atypical salmonellae are not detectable on XLD, and BSA has several disadvantages, these being

1) *S. typhi* is best isolated on fresh BSA, which is inhibitory to other salmonellae serotypes
2) plates must be incubated for 48 h for typical colonies to develop
3) salmonellae are often difficult to distinguish from competitors and reactions vary between strains (Cox, 1993)

Although *S. typhi* colonies have an atypical appearance on LMG in that they are yellow rather than pink, they will have the typical black centre if previously grown in a selenite broth (Cox, 1993).

2.4 Step 4 - purification and confirmation

Purification and confirmation of presumptive salmonellae isolates is essential, even for experienced technicians. Isolates should undergo biochemical tests and be screened for agglutination with polyvalent *Salmonella* antisera to confirm their identification as salmonellae because several competitors have similar morphological characteristics to salmonellae.

2.5 Summary of the recommended procedure

The recommended procedure for isolation of salmonellae from composted biosolids therefore is:

1 Five samples should be collected, and 100 g of each combined and blended with 500 mL of phosphate buffered saline.

2 120 g of the blended samples should be added to 480 mL of buffered peptone water, mixed and subdivided into five lots of 100 mL (each therefore containing 10 g of compost). Pre-enrichments should be incubated overnight at 37°C.

3 Samples should be enriched for 48 h in RV and MSE. RV (9 mL) should be inoculated with 0.1 mL pre-enrichment culture while 9 mL MSE should be inoculated with 1 mL pre-enrichment culture. RV should be incubated at 42±1°C and MSE at 37°C.

4 The recommended isolation medium is lysine mannitol glycerol agar. The enrichment cultures should be subcultured onto this medium after 24 and 48 h incubation. LMG plates should be examined for suspected salmonellae after 24 h incubation at 37°C.
5 All suspected salmonellae must be confirmed. Presumptive salmonellae should be purified on MacConkey agar without salt and confirmed by biochemical and serological tests.

These stages are illustrated in Fig. 2.

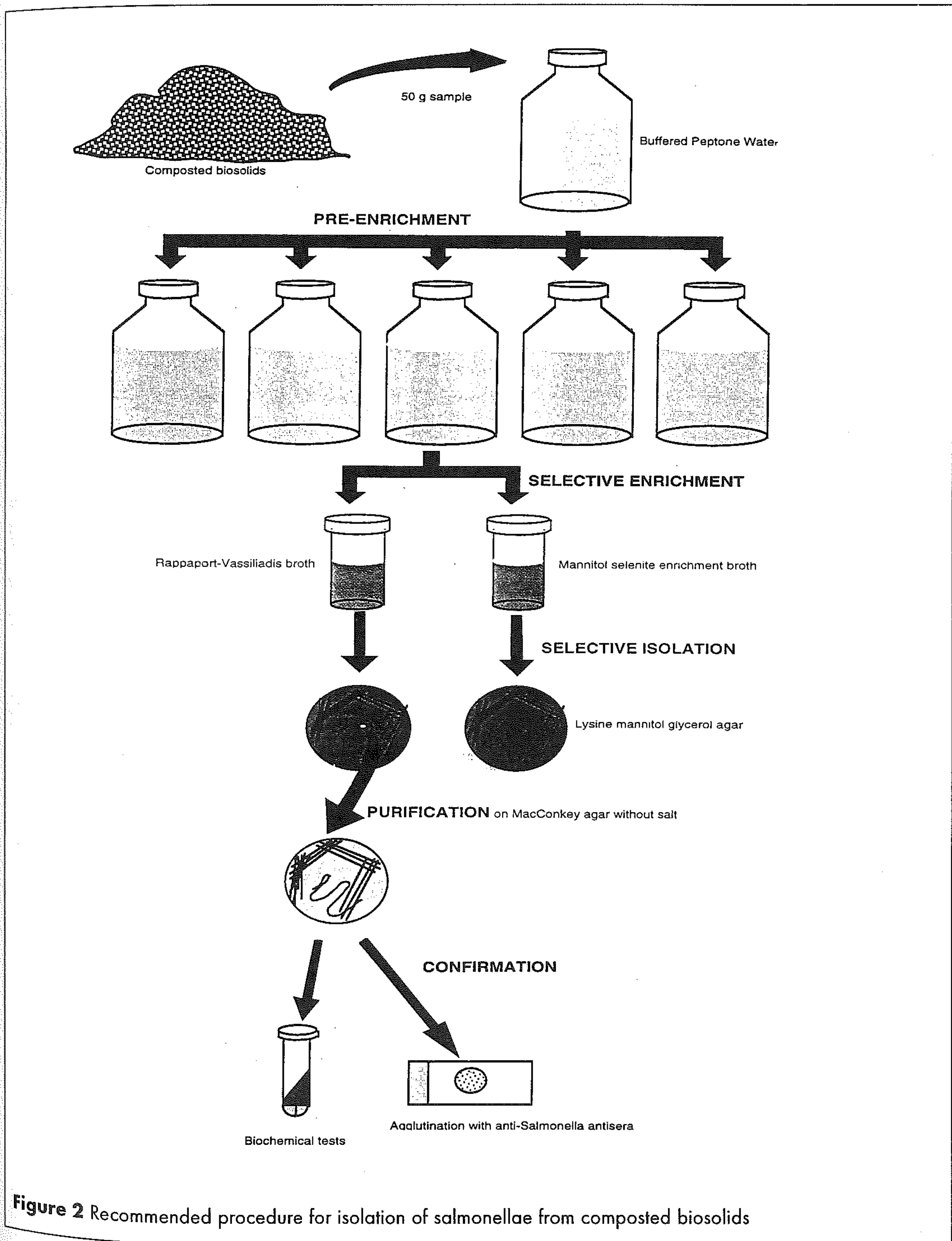

Figure 2 Recommended procedure for isolation of salmonellae from composted biosolids

3 COMPARISON OF PROPOSED METHOD AND THE US EPA METHOD

The major differences between the USEPA method and the method recommended here are the enrichment and plating media used. The US EPA recommends tetrathionate and dulcitol selenite enrichment broths and XLD and BGA. We have found that RV enrichment results in the isolation of higher numbers of salmonellae than tetrathionate and selenite enrichment broths so RV and MSE enrichment media and LMG agar are recommended.

The method recommended in this paper is a qualititative "presence / absence" test whereas the US EPA has a quantitative approach. A qualitative method is recommended here for a number of reasons.

1) The acceptable limit of salmonellae contamination in proposed Australian guidelines is 1 salmonella in 50 g of composted biosolids. This limit readily lends itself to a presence/absence test. If salmonellae are present then the standard has been exceeded. A presence/absence test uses less culture media than the semi-quantitative "most-probable-number" procedure and therefore saves time and money.

2) It has been shown that it is statistically more reliable to evaluate the quality of a process or product by using a frequency-of-occurrence parameter rather than the mean of a number of samples (That is, a product may be said to be in compliance with a standard if the frequency of occurrence of a particular contaminant is less than a standard value.) The former has the advantages of

a) being independent of a frequency distribution The frequency distribution is unknown for salmonellae in composted biosolids
b) not being affected by data truncation which occurs when means must be taken of data which have been expressed as less than a lower limit or greater than an upper limit due to method limitations
c) it is easier to obtain a reasonable degree of precision, and therefore confidence, for frequency-of-occurrence than to achieve the same degree of precision for a mean (Pipes & Christian, 1984).

The US EPA recommended method for determining the level of salmonellae contamination in biosolids does not involve pre-enrichment. In view of the fact that there is little data on the effect of pre-enrichment of biosolids samples it seems best to include pre-enrichment in a standard monitoring procedure, as it is unlikely that pre-enrichment would have a detrimental effect on salmonellae isolation.

4 FURTHER CONSIDERATIONS NEEDED FOR A STANDARD MONITORING PROCEDURE

Included in monitoring guidelines should be requirements for a monitoring programme, including specifications for the number of samples to be taken over a certain period of time. In practice it is impossible to expect or ensure with a high degree of confidence that all 50 g samples of compost will be free of salmonellae. This is partly due to the fact that no sampling process or microbiological enumeration process is error-free. Regulatory authorities must therefore make decisions about what frequency of occurrence of contamination is acceptable. This has been the approach taken in US Primary Drinking Water Regulations (US EPA 1989) and the Australian Drinking Water Guidelines (NH&MRC & ARMCANZ, 1994). Hamilton (1994) has shown that the acceptable frequency of occurrence of positive samples can be determined statistically once the total number of samples has been set. This statistical calculation also takes into account false fail and false pass rates. (The false fail rate is the rate at which a product which does meet criteria for acceptance is falsely rejected and the false pass rate is that at which a product which does not comply with a criterion is falsely accepted.)

While it is possible to determine an acceptable frequency occurrence statistically, choices of the level of contamination which will be tolerated should be based on epidemiological evidence and risk assessment data, which is not available for salmonellae in composted biosolids. This is an avenue into which further research will be channelled.

5 CONCLUSIONS

A culture method for detecting salmonellae in biosolids samples is recommended. The culture method consists of pre-enrichment in buffered peptone water, enrichment in RV and MSE media, isolation on LMG agar plates, and confirmation by biochemical test and serology.

The sensitivity of the method described here is unknown. One of the major limitations is that any culture method cannot be expected to detect 100% of the salmonellae in a sample. It is well known that bacterial populations contain a proportion of bacteria which are viable but non-culturable. The proportion that such

bacteria make up of the population of salmonellae in compost is not known but it has been estimated that up to 99% of bacteria in the environment may be in a viable but non-culturable state (Josephson et al., 1993). It is unlikely that significant improvements in the sensitivity of culture methods for salmonellae in composted biosolids products will be achieved in the near future and at this stage alternative methods such as PCR and ELISA have also proved insensitive when used with environmental samples.

Before sensible guidelines or regulations for a salmonellae monitoring procedure can be put in place further information about risks, epidemiology and salmonellae distribution in compost is necessary in order to make decisions about acceptable levels of contamination.

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7 DISCLAIMER

Throughout this paper samples are described as having been composted. However the composting process did not conform to an approved "Process to Further Remove Pathogens", as described by the US EPA (1992). Process monitoring was not routinely carried out but on occasions when temperatures in the composting windrows were measured they were lower than required. The detection of salmonellae in our studies should therefore not be interpreted as an indication of the efficacy of the composting process to remove pathogens.

REFERENCES

Carrington, E G (1980) The Isolation and Identification of Salmonella Species in Sewage Sludge: A Comparison of Methods and Recommendations for a Standard Technique, Water Research Centre, UK Technical Report 129.

Cox, J M (1993) 'Lysine-mannitol-glycerol agar, a medium for the isolation of Salmonella spp, including S. typhi and atypical strains', Appl. Environ. Microbiol. 59,8, 2602-2606.

Edgar, D and Soar, M (1979) 'Evaluation of culture media for the isolation of salmonellas from sewage sludge', J. Appl. Bact. 47, 237-241.

Fricker, C R (1984) 'A comparison of methods for the isolation of salmonellae from sewage sludge', Zbl. Bakt. Hyg., Abt. Orig. B 179, 170-178.

Gibbs, R A, Hu, C J, Ho, G E, Unkovich, I and Phillips, P (1995) Die-Off of Human Pathogens in Stored Wastewater Sludge and Sludge Applied to Land, Research Report No. 92, Urban Water Research Association of Australia, Melbourne.

Hamilton, M A (1994) 'A statistician's view of the U.S. primary drinking water regulation on coliform contamination', Environ. Sci. Technol. 28 ,11, 1808-1811.

Hu, C J, Gibbs, R A and Ho, G E (1995) Detection of Salmonella in composted wastewater sludge, Murdoch University, Perth

Iveson, J B and MacKay-Scollay, E M (1972) 'An evaluation of strontium chloride, Rappaport and strontium selenite enrichment for the isolation of salmonellas from man, animals, meat products and abattoir effluents', J. Hygiene, Camb. 70, 367-385.

Josephson, K L, Gerba, C P and Pepper, I L (1993) 'Polymerase chain reaction detection of nonviable bacterial pathogens', Appl. Environ. Microbiol. 59, 10, 3513-3515.

Merck, E (1988) Culture Media Handbook, Darmstadt.

National Health and Medical Research Council and Agricultural and Resource Management Council of Australia and New Zealand (1994) Australian Drinking Water Guidelines (Draft, June 1994)

Pipes, W O and Christian, R R (1984) 'Estimating mean coliform densities of water distribution systems', Journal American Water Works Association 76, 11, 60-64.

Rhodes, P and Quesnel, L B (1986) 'Comparison of Muller-Kauffman tetrathionate broth with Rappaport-Vassiliadis (RV) medium for the isolation of salmonellas from sewage sludge', J. Appl. Bact. 60,161-167.

Standards Australia (1991) Food Microbiology Method 2.5: Examination for specific organisms - salmonllae AS 1766.2.5-1991, Standards Association of Australia.

US EPA (1989) 40 CFR Parts 141 and 142. Drinking Water;National Primary Drinking Water Regulations; Filtration, Disinfection, Turbidity, Giardia lamblia, Viruses, Legionella, and Heterotrophic Bacteria. Federal Register, 54, 124, 27486-27541.

US EPA (1992) Part 503 - Standards for the use or disposal of sewage sludge. US Sewage Sludge Regulations, United States Environmental Protection Agency, Cincinnati.