DETECTION OF SALMONELLA IN COMPOSTED WASTEWATER SLUDGE

A report on a research project carried out for the Water Authority of Western Australia

by

Catherine J. Hu, Robyn A. Gibbs and Goen E. Ho
Institute for Environmental Science
Murdoch University

August 1995

ISBN No. 0-86905-429-5
Summary

It is likely that Australian sludge management guidelines will require monitoring of composted wastewater sludge for the presence of salmonellae. However, methods to perform this monitoring are not well established and it appears that methods used in the past may severely underestimate *Salmonella* numbers.

The aim of this project was to evaluate methods for detecting *Salmonella* in composted sludge. The project consisted largely of a comparison of available culture media. The enrichment media tested were Rappaport-Vassiliadis broth (RV), mannitol selenite cystine broth (MSE), tetrathionate broth (TT) and strontium chloride B broth (SCB). The plating media tested were xylose lysine deoxycholate agar (XLD), bismuth sulphite agar (BSA) and lysine mannitol glycerol agar (LMG). These media were chosen on the basis of methods recommended in Australia and the USA.

Comparison of the four enrichment broths indicated that RV was more effective than any of the other media tested in the study. There were no differences in the performance of the three plating media tested.

Several compositions of selenite broth were also compared. The type of sugar and peptone and the addition of yeast extract were tested for their effects on the growth of several *Salmonella* serotypes. Some improvements in the performance of selenite broths were achieved and these may warrant further investigation. However, at this stage no changes to the suggested selenite broth are recommended.

On the basis of the results of this and previous projects, a method for detection of salmonellae in composted wastewater sludge is suggested. The recommended protocol for detecting the presence or absence of salmonellae in 50 g samples of composted sludge is as follows:

1. **Sampling**

Five samples should be collected and 100 g of each combined and blended with 500 mL phosphate buffered saline.
2. **Pre-enrichment**

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

3. **Enrichment**

Samples should be enriched for 48h 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. **Isolation**

The recommended isolation medium is lysine-mannitol-glycerol agar (LMG). The enrichment cultures should be subcultured onto this medium after 24 and 48h incubation. LMG plates should be examined for suspected salmonellae after 24h incubation at 37°C.

5. **Purification and identification**

All suspected salmonellae must be confirmed. Presumptive salmonellae should be purified on MacConkey agar without salt and confirmed by biochemical and serological tests.
Acknowledgements

This project was funded by the Water Authority of Western Australia (WAWA) and their support is gratefully acknowledged. Particular thanks go to the project manager, Mr Ivan Unkovich from WAWA. We would also like to thank the additional members of the steering committee, Mr Hugh Rule (WAWA) and Dr Richard Lugg (Health Department of Western Australia) for their support.

The research work at Murdoch University was carried out with the assistance of Mr Daryl Elphick and Mrs Wendy Lund and we deeply appreciate the high level of technical support provided by them.

We would also like to thank the staff of the clinical enteric section of Pathcentre (formerly State Health Laboratory Services). In particular, thanks go to Mr Brian MacKenzie and Ms Denise Bolger for their patience and helpfulness.

Samples were collected from the Soiland composting facility at Canning Vale, and WAWA's Woodman Point Wastewater Treatment Plant. We appreciate the help provided by the staff at these treatment facilities.

Disclaimer

Throughout this report 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 United States Environmental Protection Agency (1989, 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 results of this study should therefore not be interpreted as an indication of the efficacy of composting to remove pathogens.
# Table of Contents

Summary................................................................................................................................. i
Acknowledgements.................................................................................................................. iii
Disclaimer................................................................................................................................. iii
Table of Contents...................................................................................................................... iv
List of Tables................................................................................................................................. vi
List of Figures................................................................................................................................. vi
List of Abbreviations................................................................................................................... vii

1: Introduction................................................................................................................................................. 1
  1.1 Background........................................................................................................................................... 1
  1.2 Aims and Scope of the Study.................................................................................................................. 2

2: Comparison of Enrichment and Plating Media......................................................................................... 3
  2.1 Introduction........................................................................................................................................ 3
  2.2 Materials and Methods.......................................................................................................................... 4
    2.2.1 Samples........................................................................................................................................... 4
    2.2.2 Pre-enrichment media.................................................................................................................... 5
    2.2.3 Enrichment media .......................................................................................................................... 5
    2.2.4 Isolation media............................................................................................................................. 5
    2.2.5 Methods......................................................................................................................................... 6
  2.3 Results....................................................................................................................................................... 6
    2.3.1 Comparison of enrichment and plating media............................................................................... 6
    2.3.2 *Salmonella* serotypes isolated from composted sludge............................................................ 8
  2.4 Discussion................................................................................................................................................. 9
    2.4.1 Enrichment media....................................................................................................................... 9
    2.4.2 Plating media............................................................................................................................. 10

3: Composition of Selenite Broth..................................................................................................................... 11
  3.1 Introduction......................................................................................................................................... 11
    3.1.1 Selenite broths............................................................................................................................. 11
    3.1.2 Mode of action of selenite broths............................................................................................... 12
    3.1.3 Selenite broth composition.......................................................................................................... 13
    3.1.4 Conclusions.................................................................................................................................. 16
  3.2 Materials and Methods.......................................................................................................................... 16
    3.2.1 Composition of selenite broths tested....................................................................................... 16
    3.2.2 Method......................................................................................................................................... 17
  3.3 Results....................................................................................................................................................... 17
3.4 Discussion ........................................................................................................ 19

4: The Importance of Replication in Salmonella Monitoring ............................. 21
   4.1 Replication During Sampling ................................................................. 21
   4.2 Replication of Pre-enrichments .............................................................. 21
   4.3 Replication of Enrichments ................................................................. 22
   4.4 Multiple Plating ..................................................................................... 22

5: General Discussion and Recommendations .................................................... 23
   5.1 The Importance of S. typhi and Paratyphi ............................................ 23
   5.2 Choice of Enrichment Media ................................................................. 23
   5.3 Choice of Isolation Medium ................................................................. 24

6: Conclusions ....................................................................................................... 25
7: Recommendations ............................................................................................. 25

References ............................................................................................................. 26
Appendix I: Statistical comparison of enrichment and plating media ............. 30
Appendix II: Statistical comparison of selenite broths .................................. 32
Appendix III: Statistical comparisons of replication of enrichments ............ 35
List of Tables

Table 2.1  Media tested in this study ................................................................. 4

Table 2.2  Number of positive replicates detected by four enrichment media and three plating media ................................................................. 7

Table 2.3  *Salmonella* serotypes isolated from composted wastewater sludge by four enrichment broths ................................................................. 9

Table 3.1  Fermentation of dulcitol and mannitol by members of the enterobacteriaceae ......................................................................................... 15

Table 3.2  Composition of selenite broths .......................................................... 17

Table 3.3  Log increase of *Salmonella* populations grown in six types of selenite broth ......................................................................................... 18

List of Figures

Fig. 2.1  Mean number of positive replicates detected by four enrichment and three plating media ................................................................. 8

Fig. 3.1  Mean log increase of *Salmonella* populations after 48h in six formulations of selenite broth ................................................................. 19
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPW</td>
<td>buffered peptone water</td>
</tr>
<tr>
<td>BSA</td>
<td>bismuth sulphite agar</td>
</tr>
<tr>
<td>DSE</td>
<td>dulcitol selenite enrichment</td>
</tr>
<tr>
<td>LMG</td>
<td>lysine mannitol glycerol agar</td>
</tr>
<tr>
<td>MSE</td>
<td>mannitol selenite enrichment broth</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RV</td>
<td>Rappaport-Vassiliadis broth</td>
</tr>
<tr>
<td>SCB</td>
<td>strontium chloride B broth</td>
</tr>
<tr>
<td>TT</td>
<td>tetrathionate broth</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>WAWA</td>
<td>Water Authority of Western Australia</td>
</tr>
<tr>
<td>XLD</td>
<td>xylose lysine deoxycholate agar</td>
</tr>
</tbody>
</table>
1: Introduction

1.1 Background

The Australian Water Resources Council and the Australian and New Zealand Environment and Conservation Council are currently in the process of formulating a set of sludge management guidelines for the treatment and disposal of wastewater sludge (Alan Maus, pers. comm.). It is proposed that sludge products will be classified, and their end use regulated, largely on the basis of the risks posed to human health by pathogens in sludge. To be available for unrestricted use, sludge 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.

Once legislated, these guidelines will almost certainly contain a requirement for generators of sludge products such as compost, to monitor for the presence of Salmonella. Unfortunately there is no standard method available for analysis of compost. Guidelines are likely to stipulate that Salmonella should be monitored using the method recommended for food analysis (A. Maus, pers. comm.). However, it is not necessarily appropriate to apply a method designed for one type of sample to another. This is because variations in the sample can significantly affect Salmonella detection. In particular, sample types vary in the number of Salmonella likely to be present and in the number of closely related bacteria (competitors). Competitors can interfere with Salmonella detection if present in high numbers.

The general technique for culturing Salmonella from any type of sample is comprised of the four steps outlined below.

1. Pre-enrichment - Pre-enrichment of samples in a non-selective medium allows sub-lethally damaged salmonellae to recover and multiply to a level that will ensure their survival upon transfer to a selective medium.

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.
3. Selective isolation - Selective isolation media are solid agar media which allow the growth of salmonellae while inhibiting non-salmonellae. In addition, selective isolation media usually utilise mechanisms which allow salmonellae to be visually distinguished from non-salmonellae.

4. Confirmation - Biochemical and serological tests are performed to ensure that suspected isolates are in fact *Salmonella*.

Pre-enrichment of environmental samples in buffered peptone water is generally accepted, although it is not specified in USEPA sludge management guidelines (USEPA, 1992), and confirmatory tests are well developed. However, many media are available for the selective enrichment and selective isolation stages and it appears that they have not been sufficiently evaluated for their ability to isolate *Salmonella* from compost.

1.2 Aims and Scope of the Study

In this project several aspects of *Salmonella* detection in composted wastewater sludge were examined. The efficiency of four enrichment media and three plating media were compared in terms of their ability to isolate *Salmonella* from composted sludge (section 2). In addition, several formulations of selenite enrichment broth were tested (section 3). Some aspects of the reproducibility of the recommended method are considered in section 4. A general discussion of the project is contained in section 5. Finally, recommendations for a *Salmonella* analysis method are made in section 6.
2: Comparison of Enrichment and Plating Media

2.1 Introduction

A number of media are available for the selective enrichment and isolation of Salmonella. Several studies have compared the relative efficiencies of various enrichment media, although none appear to have used composted sludge samples. There appears to be a consensus that Rappaport-Vassiliadis broth (RV) is the most suitable enrichment broth for use with environmental samples as it gives a greater percentage of positive results, supports the growth of more serotypes and increases inhibition of more competitors than tetrathionate or selenite broths (Carrington, 1980; Morinigo et al., 1993; Rhodes and Quesnel, 1986; van Schothorst and Renaud, 1983; Vassiliadis et al., 1974; Watson, 1985). However, a recent study conducted at Murdoch University (Gibbs et al., 1995) indicated that dulcitol selenite broth detected a greater number of Salmonella-positive samples from composted sludge than RV.

Several studies have also compared the relative merits of a variety of selective isolation media. Once again, few have used sludge samples and none appear to have used composted sludge. The data from these experiments has been conflicting, with Carrington (1980) and Rhodes and Quesnel (1986) recommending xylose lysine deoxycholate agar (XLD) and Edgar and Soar (1979) and Fricker (1984) recommending brilliant green agar (BGA). Recently, a new medium known as lysine-mannitol-glycerol agar (LMG) has been developed and is reported to be able to detect a wider range of salmonellae than other media (Cox, 1993).

The purpose of the experiments described in this chapter was to determine appropriate culture media for isolation of salmonellae from composted wastewater sludge. This study was largely based on a comparison of methods recommended in the USA and in Australia. The US Environmental Protection Agency (USEPA) has specified methods for detection of Salmonella in sludge products (USEPA, 1992) while Australia is in the process of formulating sludge management guidelines. At this stage proposed methods are similar to the Australian food standard (Standards Australia, 1991). The media tested in this study and the reason for their inclusion is shown in Table 2.1.
Table 2.1. Media tested in this study

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reason for inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enrichment broths</strong></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>Recommended in the Australian food standard (Standards Australia, 1991). Previously found to be effective for other forms of wastewater sludge.</td>
</tr>
<tr>
<td>MSE</td>
<td>Recommended in the Australian food standard (Standards Australia, 1991). Similar to dulcitol selenite broth recommended by the USEPA (1992).</td>
</tr>
<tr>
<td>TT</td>
<td>Recommended by the USEPA (1992).</td>
</tr>
<tr>
<td>SCB</td>
<td>Used by WA State Health Laboratories for detection of Salmonella in environmental samples.</td>
</tr>
<tr>
<td><strong>Plating media</strong></td>
<td></td>
</tr>
<tr>
<td>XLD</td>
<td>Recommended in the Australian food standard (Standards Australia, 1991) and by the USEPA (1992). Previously found to be effective for other forms of wastewater sludge.</td>
</tr>
<tr>
<td>LMG</td>
<td>Reported by Cox (1993) to be more efficient than XLD.</td>
</tr>
<tr>
<td></td>
<td>Has the ability to detect atypical salmonellae.</td>
</tr>
<tr>
<td>BSA</td>
<td>Recommended in the Australian food standard (Standards Australia, 1991).</td>
</tr>
</tbody>
</table>

**Abbreviations:**

- RV: Rappaport-Vassiliadis broth
- MSE: Mannitol selenite cystine enrichment broth
- TT: Tetrathionate broth
- SCB: Strontium chloride B broth
- XLD: Xylose lysine deoxycholate agar
- LMG: Lysine-mannitol-glycerol agar
- BSA: Bismuth sulphite agar
2.2 Materials and Methods

2.2.1 Samples

Eleven samples of composted wastewater sludge were obtained from Soiland's composting facility at Canning Vale. At this composting facility dewatered sludge from Perth wastewater treatment plants is composted by combining sludge with shredded tree waste in a ratio of 1:3 (v/v). This mixture is heaped and allowed to dry for two to three weeks after which it is mixed and placed in compost windrows for approximately six weeks. During composting the windrows are turned every three to four days. The composted sludge is then shredded and placed in a pile ready for distribution. It was from this final pile that samples were taken for this experiment. On each occasion five samples were collected from different sections of the pile using a sterile scoop.

2.2.2 Pre-enrichment media

Samples were pre-enriched in Buffered Peptone Water (BPW) (Oxoid).

2.2.3 Enrichment media

Four enrichment media were compared. Rappaport-Vassiliadis broth (RV) (Oxoid) and tetrathionate broth (TT) (Gibco BRL) were prepared according to the manufacturer's instructions.

Strontium chloride broth (SCB) was prepared according to Iveson (1971, cited by Iveson and MacKay-Scollay, 1972) and contained tryptone (Difco) 5.0 g/L; NaCl (Univar) 8.0 g/L; KH₂PO₄ 1.0 g/L and strontium chloride (Sigma) 60.0 mL/L of a 35.7% solution in distilled water. SCB was sterilised by steaming for 30 min and stored at 4°C.

Mannitol selenite cystine enrichment broth (MSE) was prepared according to Standards Association of Australia (1982). It contained mannitol (Sigma) 4.0 g/L; tryptone (Difco) 5.0 g/L; NaHSeO₃ (Sigma) 4.0 g/L and K₂HPO₄ 10.0 g/L. MSE was sterilised by steaming for 10 min, after which 10.0 mL/L of a 1 g/L in a 1M NaOH solution of cystine was added. The broth was stored for no longer than one day at 4°C.
2.2.4 Isolation media

Three isolation media were tested. Xylose lysine deoxycholate agar (XLD) and bismuth sulphite agar (BSA) (both Gibco BRL) were prepared according to the manufacturer's instructions. LMG was prepared according to Cox (1993). LMG contained proteose peptone (Difco) 3.0 g/L; yeast extract (BBL) 5.0 g/L; lysine (Sigma) 5.0 g/L; mannitol (Sigma) 5.0 g/L; glycerol 5.0 g/L; NaCl (BDH) 5.0 g/L; sodium deoxycholate (Sigma) 1.0 g/L; sodium thiosulphate (Ajax) 4.0 g/L; ferric ammonium citrate (Ajax) 1.0 g/L; phenol red (Sigma) 0.1 g/L and agar (Gibco) 15.0 g/L.

2.2.5 Methods

Each sample was prepared by combining five 100 g lots of composted sludge with 500 mL of phosphate buffered saline (NaCl 8.0 g/L, K2HPO4 1.2 g/L and KH2PO4 0.34 g/L) and blending with a hand-held electric blender. A 60 g aliquot of the combined sample was added to 540 mL BPW and shaken. This mixture was subdivided into five lots of 100 mL which were incubated at 37°C overnight.

Following pre-enrichment, samples were enriched in 9 mL of the four enrichment broths. Five replicates of each enrichment were inoculated from each pre-enrichment culture, giving 25 replicates of each enrichment in total. Inoculation ratios and incubation temperatures were selected from relevant literature. RV was inoculated with 0.1 mL of pre-enrichment culture (Standards Australia, 1991), while all other enrichments were inoculated with 1 mL (APHA et al., 1989, Iveson and MacKay-Scollay, 1972 and Standards Australia, 1991). RV and SCB were incubated at 43°C (Standards Australia, 1991 and Iveson and MacKay-Scollay, 1972) and TT and MSE were incubated at 37°C (APHA et al., 1989 and Standards Australia, 1991). Enrichments were incubated for 48h.

Each enrichment culture was subcultured onto the three isolation media after 24 and 48h incubation. All plates were incubated at 37°C for 24h, except for BSA which was incubated for 48h.

Suspected salmonellae were purified on MacConkey agar without salt (Difco) and identified using GLISSUDA tubes (from the WA State Health Laboratories) and agglutination with Salmonella O groups A-S and Salmonella H phase 1 and 2 antisera (Murex Diagnostics). If the results of these tests conflicted isolates were identified using API 20E biochemical tests (bioMérieux).
All confirmed salmonellae were serotyped by the WA State Health Laboratories.

2.3 Results

Of the eleven samples of composted sludge tested, ten contained *Salmonella*. Qualitative observations suggest that the density of *Salmonella* was variable.

2.3.1 Comparison of enrichment and plating media

Nine out of ten positive samples were detected by RV and MSE and two were detected using strontium chloride B and tetrathionate.

The distribution of positive replicates detected by each media combination is shown in Table 2.2 and this data is shown graphically in Fig. 2.1.

**Table 2.2.** Number of positive replicates detected by four enrichment and three plating media

<table>
<thead>
<tr>
<th>Sample</th>
<th>RV XLD</th>
<th>LMG</th>
<th>BSA</th>
<th>MSE XLD</th>
<th>LMG</th>
<th>BSA</th>
<th>TT XLD</th>
<th>LMG</th>
<th>BSA</th>
<th>SCB XLD</th>
<th>LMG</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>21</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>21</td>
<td>20</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>23</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>24</td>
<td>10</td>
<td>12</td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Abbreviations: see Table 2.1

Analysis of variance and multiple comparisons (Appendix 1) indicated that RV detected significantly more *Salmonella* - positive replicates than any of the other
enrichment media tested (α = 0.05). There were no significant differences between the plating media. The superiority of RV over other enrichment media is clearly illustrated in Fig. 2.1.

Using RV and MSE with XLD, rather than the RV/XLD or RV/LMG combination alone would have detected 100% of the positive samples. The latter combinations detected 90% of the positive samples. However, analysis of variance indicated that there was no statistical advantage in using more than one enrichment or plate (see Appendix I).

Fig. 2.1 Mean number of positive replicates detected by four enrichment and three plating media. Bars denote one standard deviation. Refer to Table 2.1 for abbreviations.

2.3.2 *Salmonella* serotypes isolated from composted sludge

All salmonellae isolated from samples 1 to 8, and three isolates from sample 10, were serotyped by the WA State Health Laboratories. The serotypes detected and the enrichments that they occurred in are shown in Table 2.3. At least eight serotypes were detected and in two samples (3 and 5) only one serotype (*S. idikan*) was isolated. Full identifications for isolates belonging to group I were not available at the time of writing this report. All of the isolates for which full
identifications were available belonged to subgenus I, except for \( S. \) \textit{mobeni} (subgenus II).

\textbf{Table 2.3.} \textit{Salmonella} serotypes isolated from composted wastewater sludge by four enrichment broths

<table>
<thead>
<tr>
<th>\textit{Salmonella} serotype</th>
<th>RV</th>
<th>MSE</th>
<th>TT</th>
<th>SCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>( S. ) \textit{infantis}</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{havana}</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{idikan}</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{mbandaka}</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{senftenberg}</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{ardwick}</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>( S. ) \textit{mobeni}</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Salmonella} O group I</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>( S. ) \textit{cubana}</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The serotypes which were not isolated from RV were inoculated into this medium and it was verified that they were able to grow in RV.

One serotype, \( S. \) \textit{idikan}, which was the only serotype detected in samples 3 and 5, appeared to have a variable colony morphology when grown on XLD. Although it had a typical \textit{Salmonella} appearance on XLD (pink colony with a black centre) when initially isolated, it grew as a yellow colony when it was re-inoculated onto the same medium. It also showed an atypical reaction in GLISSUDA tubes (one component of our identification system). It is possible that this serotype has been overlooked in our previous experiments.

\section*{2.4 Discussion}

\subsection*{2.4.1 Enrichment media}

RV was consistently the most effective enrichment broth for detecting \textit{Salmonella} in composted sludge. In comparison with the other enrichments
tested it detected a greater number of positive samples and a greater number of positive replicates. The latter suggests that RV improved reproducibility compared to the other enrichments.

The superiority of RV over MSE as demonstrated in this study appeared to conflict with the results of a similar earlier study (Gibbs et al., 1995) which indicated that dulcitol selenite enrichment (DSE) was more effective than RV. This may have been due to differences between DSE and MSE but may also have reflected the poor reproducibility of the detection method. When numbers of salmonellae in the compost samples appeared to be low, as suggested by a low number of positive RV replicates (eg samples 2 or 5), the distribution of positive replicates was erratic. In the previous study there were only five replicates of each enrichment broth (one replicate from each of five pre-enrichments) and it is therefore possible that the apparent difference between RV and DSE was due to the erratic distribution of low numbers of salmonellae. The possibility that there were significant differences in the performance of DSE and MSE was also investigated during this project and is described in section 3.

2.4.2 Plating media

Although there were no statistical differences between the effectiveness of the isolation media tested in this experiment, there are other factors which influence their suitability for use in routine monitoring. For example, salmonellae were difficult to distinguish from non-salmonellae on BSA in comparison with XLD and LMG. This probably accounts for the fact that the number of positive replicates detected by BSA was lower than with the other isolation media. BSA has a further disadvantage in that it must be incubated for 48 h as opposed to 24 h for XLD and LMG. BSA has traditionally been included in monitoring protocols because of its ability to detect S. typhi, which does not grow on many other media. This is discussed further in section 5.
3: Composition of Selenite Broth

3.1 Introduction

As mentioned in section 2.4.1, results of two separate comparisons of enrichment media have reached conflicting conclusions about the relative efficacy of selenite broths. In a previous experiment (Gibbs et al., 1995) more positive samples were detected by dulcitol selenite broth than by RV, while in the experiment described in section 2, RV outperformed MSE. One explanation for this is that the performance of selenite broth is affected by its composition. Certain serotypes may also be inhibited by some formulations of selenite broth. These hypotheses were tested in the experiment described in this chapter.

In the following sections (3.1.1 to 3.1.4) literature concerning the composition of selenite broths is reviewed. Unfortunately, very little information has been published recently.

3.1.1 Selenite broths

Selenite broths are commonly recommended for enrichment of Salmonella. However, many changes have been made to the original selenite broth formula proposed by Leifson in 1936 and this has resulted in a variety of media based on selenite. For example, the broth recommended in the Australian food standard for isolation of salmonellae from food, and the broth that is recommended in proposed Australian sludge management guidelines, is mannitol selenite cystine broth. This contains mannitol, tryptone, and L-cystine. The selenite broth recommended by the USEPA for sludge products, on the other hand, is dulcitol selenite enrichment (DSE), containing dulcitol, peptone and yeast extract.

In general, selenite broths contain

1) nutrient substances - eg meat or yeast extract, meat or soya peptone, lactose, mannitol or other carbohydrates.

2) selenite selective inhibitor - eg. sodium hydrogen selenite (NaHSeO₃)

In addition they may or may not contain the amino acid L-cystine.
3.1.2 Mode of action of selenite broths

The selective properties of selenite against certain bacteria have been known for over one hundred years. Gram positive organisms such as *Staphylococcus aureus* and *Bacillus* spp. are particularly susceptible to selenite while *Salmonella* and other enterobacteria are relatively resistant (Arroyo and Arroyo, 1995; Chen et al., 1994).

Selenite-resistant bacteria are able to reduce selenite to elemental selenium while sensitive bacteria can not (Smith, 1959a). Reduction of selenite can be observed as a reddening of the medium, with selenium granules visible in the medium and inside the bacteria (Smith, 1959a, Weiss et al., 1965).

Reduction of selenite is strongly associated with production of H$_2$S (Lapage and Bascomb, 1968). Smith (1959a) observed that reduction of selenite began at approximately the same time as the onset of H$_2$S production. H$_2$S is known to react with selenious acid in the following manner:

$$\text{H}_2\text{SeO}_3 + 2\text{H}_2\text{S} = 2\text{S} + \text{Se} + 3\text{H}_2\text{O}$$

and Smith (1959a) suggested that a similar reaction occurred with sodium hydrogen selenite (NaH$_2$SeO$_3$).

However, the selective action of selenite broth is not dependent on reduction of sodium selenite. Smith (1959a) showed that the inhibitory action of selenite occurred early in the growth period, before selenite reduction took place. This is consistent with the findings of Weiss et al. (1965) who demonstrated that a high susceptibility to selenite corresponded with a high rate of uptake of selenium in the early stages of incubation. Selenite is believed to inhibit susceptible bacteria through competition with preferred sulphur sources. This leads to incorporation of selenium, instead of sulphur, into amino acids and other cellular components. These amino acids cannot be used for growth. Evidence for this theory was provided by Smith (1959a) who demonstrated that addition of different sulphur sources to selenite broth annulled the toxic effects of selenite, presumably due to the provision of a preferred sulphur source. Using radioautography, Weiss et al. (1965) were able to demonstrate the presence of seleno analogues of sulphur-containing amino acids, particularly seleno-cystine, in organisms grown in selenite media.
Selenite broth is therefore not a simple nutrient medium in which free selenite acts as an inhibitor (Smith, 1959a). The role of H₂S in selenite resistance and the formation of seleno-amino acids in selenite-sensitive bacteria suggests that the action of selenite is closely linked to bacterial nutrition. In turn, this suggests that the effectiveness of selenite broths may be affected by the nature of components other than selenite.

3.1.3 Selenite broth composition

**Nutrient substances**

The only undefined component of selenite broth is the nutrient substances such as peptone and tryptone. Smith (1959a) demonstrated the importance of peptone in selenite broth. He treated selenite broth with charcoal, producing a biuret-negative (protein-negative) medium. In this medium, levels of selenite which were otherwise withstood were highly toxic to the *Salmonella* and non-*Salmonella* spp tested. The presence of peptone thus provided some protective effect and allowed the use of higher concentrations of selenite which were then selective against some non-salmonellae.

The presence of peptone is known to increase the production of H₂S (Miller *et al.*, 1994). As discussed above, there is a correlation between the ability to produce H₂S and resistance to selenite. Smith (1959a) suggested that peptone provided a source of cystine (and therefore sulphur) which is necessary for H₂S production. Raj (1966) found that the selectivity of selenite broth was improved when free cystine was replaced by proteose peptone. Presumably proteose peptone provided an adequate source of sulphur to those organisms that were able to derive it from peptone. The growth of *Proteus* was reduced when peptone was used instead of free cystine. Smith (1959a) also noted that the growth of *E. coli* was suppressed when peptone, and not free cystine, was present. It seems plausible that some organisms are unable to extract cystine from peptone.

The protective effect of peptone cannot be completely attributed to increased production of H₂S. In an experiment by Smith (1959a) peptone provided a protective effect to *E. coli* even though this organism did not produce H₂S in the test medium. Therefore, peptone reduces the toxic effects of selenite in some other way, possibly by binding to the selenite and effectively reducing its concentration.
Raj (1966) also incorporated yeast extract into his dulcitol selenite enrichment medium. Very little growth of salmonellae or competitors was obtained using dulcitol without yeast extract. However, when yeast extract was added there was very good growth of salmonellae and a few competitors. Raj (1966) hypothesised that yeast extract provided a source of co-enzymes, particularly B complex vitamins, needed for utilisation of dulcitol. North and Bartram (1953) also found that yeast extract improved *Salmonella* isolation.

Nutrient substances therefore play an important role in the action of selenite broth. Although the composition of nutrient substances is undefined, certain characteristics of these substances are known. For example, proteose peptone has approximately three times as much cystine as tryptone (Raj, 1966). The efficiency of selenite broth could therefore be affected by changes in the nutrient substance due to changing the selenite - binding properties or altering the cystine content.

**Cystine**
North and Bartram (1953) found that *Salmonella* isolation from selenite broth was improved by the addition of cystine. The action of cystine is probably due to the provision of a sulphur source that *Salmonella* have a high preference for but that is not utilised by some other competitors (Smith, 1959a).

**Carbohydrates**
Reduction of selenite causes the pH of the medium to rise and this is accompanied by a decrease in the toxicity of selenite. Fermentation of carbohydrates helps to maintain the pH of the medium (Leifson, 1936, cited by Fricker, 1987). The original selenite broth proposed by Leifson in 1936 contained lactose (Merck, 1988) but Leifson also demonstrated that other sugars could be used and that satisfactory results could be obtained without a carbohydrate (Smith 1959b). Leifson's original formula was modified by Hobbs and Allison (1945, cited by Smith 1959b), who substituted the lactose with mannitol, and by Raj (1966), who replaced lactose with dulcitol.

Smith (1959b) grew four bacterial species, *S. typhimurium*, *Proteus vulgaris*, *Citrobacter freundii* and *Escherichia coli*, in selenite broths containing one of three carbohydrates (lactose, sucrose and mannitol). Of these sugars, *S. typhimurium* could only ferment mannitol. *P. vulgaris* could only ferment sucrose, while *C. freundii* and *E. coli* were able to utilise both lactose and mannitol. The presence of a fermentable carbohydrate did not alter the pattern of growth but tended to increase the amount of growth of organisms able to utilise
the carbohydrate. However, when *C. freundii* and *S. typhimurium* were grown together in selenite broth containing mannitol *C. freundii* was able to outgrow *S. typhimurium*. Hence, the presence of a fermentable carbohydrate may not always enhance *Salmonella* recovery.

The choice of carbohydrate therefore hinges not only on finding a sugar that salmonellae can ferment but also lies in the nature of the competitors that may be present. The ideal carbohydrate source in a selenite enrichment broth would be one that was utilised only by salmonellae. This would allow the salmonellae to reach higher concentrations than competitors and would therefore increase the chance of isolation on selective plating media. Given the relatedness of the enterobacteria this is difficult to achieve in practice. Table 3.1, adapted from Cowan (1974), shows the fermentation habits of *Salmonella* and several common competitors.

**Table 3.1. Fermentation of dulcitol and mannitol by members of the enterobacteriaceae (adapted from Cowan, 1974)**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Dulcitol</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhi</em></td>
<td>(d)</td>
<td>+</td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>S. gallinarum</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. choleraesuis</em></td>
<td>(d)</td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella</em> subgenus I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td>d</td>
<td>+</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>d</td>
<td>+</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>d</td>
<td>d</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>d</td>
<td>+</td>
</tr>
</tbody>
</table>

- d different reactions by different strains
- 0-15% strains negative
+ 85-100% strains positive
( ) positive reactions delayed
From Table 3.1 it can be seen that although all *Salmonella* sp. can utilise mannitol, so do the majority of their competitors. Although dulcitol is only variably fermented by competitors not all *Salmonella* serotypes are able to use it either. In practice use of dulcitol in selenite broth may not be detrimental to *Salmonella* isolation because

i) the presence of fermentable carbohydrate is not essential for *Salmonella* growth in selenite broth,

ii) the proportion of salmonellae that do not ferment dulcitol is small and

iii) the growth of competitors may be reduced.

### 3.1.4 Conclusions

The performance of selenite broth is clearly related to bacterial nutrition and is therefore dependent on the nature of its components. It is likely that the composition of selenite broth can be optimised.

### 3.2 Materials and Methods

#### 3.2.1 Composition of selenite broths tested

Six formulations of selenite broth were compared. Their compositions are shown in Table 3.2.
Table 3.2. Composition of selenite broths

<table>
<thead>
<tr>
<th>Component</th>
<th>Broth 1 (MSE)</th>
<th>Broth 2 (DSE-USEPA std)</th>
<th>Broth 3</th>
<th>Broth 4</th>
<th>Broth 5</th>
<th>Broth 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>mannitol (g/L)</td>
<td>4.0</td>
<td>-</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>dulcitol (g/L)</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>tryptone (g/L)</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>proteose (g/L)</td>
<td>-</td>
<td>4.0</td>
<td>4.0</td>
<td>-</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>peptone (g/L)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yeast extract (g)</td>
<td>-</td>
<td>1.5</td>
<td>-</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NaHSeO₃ (g)</td>
<td>4.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>K₂HPO₄ (g)</td>
<td>10.0</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>KH₂PO₄ (g)</td>
<td>-</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>L-cystine (g)</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

3.2.2 Method

Growth of salmonellae that had been previously isolated from compost and raw sludge was assessed in the six formulations of selenite broth described in section 3.2.1. In addition, *S. dublin* was tested. This was kindly supplied by Sue Holliday of the School of Biological and Environmental Science at Murdoch University.

Salmonellas were grown overnight in BPW and their concentration was determined by a viable count on XLD. Each selenite broth (9 mL) was inoculated with approximately $10^2$ CFU by adding 1 mL of the appropriate dilution of pre-enrichment culture. (Dilutions were made in phosphate buffered saline.) The selenite broths were incubated at 37°C for 48 h and viable counts were performed on XLD after 24 and 48 h.

3.3 Results

The growth of the *Salmonella* serotypes in several formulations of selenite broth is shown in Table 3.3. and growth after 48h incubation is shown graphically in Fig. 3.1. The log increase in salmonellae is the log (final number / initial
number). From Fig. 3.1 it appears that growth of salmonellae in some broths was more variable than in others. There were, in fact, significant differences in the variance (see Appendix II), and as such, a non parametric test for analysis of variance was used. This test indicated that there were significant differences between the formulations of selenite broth. Non parametric multiple comparisons indicated that broth 4 was significantly different to broths 1 and 3.

Table 3.3. Log increase of Salmonella populations grown in six types of selenite broth.

<table>
<thead>
<tr>
<th>Serotype*</th>
<th>Broth</th>
<th>1 2 3</th>
<th>4 5 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h 48h</td>
<td>24h 48h</td>
<td>24h 48h</td>
</tr>
<tr>
<td>S. infantis a</td>
<td>4.8 5.8</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>- 6.9</td>
<td>3.3</td>
<td>7.5</td>
</tr>
<tr>
<td>S. idikan a</td>
<td>7.3 6.8</td>
<td>6.7 6.6</td>
<td>7.5 7.5</td>
</tr>
<tr>
<td></td>
<td>7.1 6.8</td>
<td>6.8 6.6</td>
<td>7.5 7.5</td>
</tr>
<tr>
<td></td>
<td>7.1 6.2</td>
<td>6.4 6.4</td>
<td>7.3 7.3</td>
</tr>
<tr>
<td>S. mobeni a</td>
<td>3.6 6.8</td>
<td>3.9</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>- 6.9</td>
<td>- 6.9</td>
<td>- 7.7</td>
</tr>
<tr>
<td>S. havana a</td>
<td>7.0 6.8</td>
<td>6.9 6.6</td>
<td>7.6 7.6</td>
</tr>
<tr>
<td>S. senftenberg a,b</td>
<td>7.4 6.8</td>
<td>6.8 6.6</td>
<td>7.5 7.1</td>
</tr>
<tr>
<td>S. ardwick a</td>
<td>7.3 7.4</td>
<td>6.9 6.7</td>
<td>7.6 7.5</td>
</tr>
<tr>
<td>S. dublin c</td>
<td>7.4 6.7</td>
<td>6.8 6.6</td>
<td>7.5 7.5</td>
</tr>
<tr>
<td>S. anatum b</td>
<td>7.2 6.6</td>
<td>6.8 6.6</td>
<td>7.5 7.5</td>
</tr>
<tr>
<td>S. tennessee b</td>
<td>7.3 6.7</td>
<td>6.8 6.6</td>
<td>7.7 7.4</td>
</tr>
<tr>
<td>S. agona b</td>
<td>7.3 6.4</td>
<td>6.8 6.6</td>
<td>7.8 7.5</td>
</tr>
<tr>
<td>S. mbandaka a</td>
<td>7.6 7.0</td>
<td>7.0 6.9</td>
<td>7.9 7.4</td>
</tr>
</tbody>
</table>

- = no result
ng = no growth

*Source: a composted sludge, b raw sludge, c laboratory strain
3.4 Discussion

There was a high degree of variability in the growth of salmonellae in some formulations of selenite broth. Growth was least variable in broth 4 and most variable in broth 3 (Fig 3.1). Variability appeared to be reduced by addition of 1% yeast extract since growth was less variable in broths containing this concentration than in broths with little or no yeast extract. The exception to this generalisation was broth 1 (MSE) which contained no yeast extract but which displayed relatively low variability. The addition of L-cystine and the use of one phosphate instead of two may have played a role in reducing variability in broth 1 but the effects of these factors were not in investigated in this experiment. Tryptone also appeared to reduce variability, since growth was less variable in broth 4 than in broth 5.

Some of the Salmonella strains tested in this experiment displayed poor growth in broths 2 and 3. S. infantis showed slow growth in these broths. Low or
undetectable numbers were present after 24 h although numbers were higher (but undetermined) after 48 h. One strain of *S. mobeni* was also unable to grow in broths 2 and 3. Ideally, the growth of *S. typhi* and *S. paratyphi* would have been tested in each of the selenite broth formulations, since it is for this serotype that selenite broths are usually recommended. (This is discussed further in section 5.) Unfortunately, isolates of these serotypes could not be obtained during this project.

Although no statistical difference could be detected, the MSE recommended in the Australian food standard (Standards Australia, 1991) appeared to perform better than the DSE recommended by the USEPA (USEPA, 1992). The mean log increase in *Salmonella* populations was higher in MSE than in DSE and growth was less variable. DSE also failed to support the growth of some serotypes.

This experiment did not yield sufficient evidence to warrant changing composition of the selenite broth recommended by Standards Australia (1991). However, the addition of yeast extract to MSE may improve its performance and this could be the subject of further investigation.
4: The Importance of Replication in Salmonella Monitoring

4.1 Replication During Sampling

To determine the number of samples needed to ensure reliable detection of Salmonella, the distribution of Salmonella in compost must be known (Haas, 1993). At present this information is not available. It seems safest to assume that salmonellae are not evenly distributed and it logically follows that several samples should be combined before being analysed. The decision to combine five samples in this project was judged to be 'reasonable' but was not scientifically established. The Salmonella monitoring process would benefit from research into distribution of Salmonella in compost.

4.2 Replication of Pre-Enrichments

An experiment that compared the efficacy of one pre-enrichment culture containing a large amount of sludge to that of several pre-enrichments containing smaller amounts of sludge has been presented previously (Gibbs et al., 1995). This experiment suggested that using five pre-enrichments each containing 10 g of sludge detected marginally more positive samples than one pre-enrichment containing 50 g of sludge. However, this result may have been due to the fact that only one enrichment was set up from the 50 g pre-enrichment whereas five enrichments were effectively taken from the five 10 g pre-enrichments. The experiment was repeated during this project, this time taking five enrichments from the 50 g pre-enrichment, but a small number of positive samples prevented statistical comparison of the two methods (data not shown).

Previous experience in our laboratory has also shown that salmonellae were sometimes not recovered from pre-enrichments containing larger amounts of sludge despite being recovered from pre-enrichments containing less sludge. It is possible that there is some threshold level of toxic contaminant(s) which is reached when larger amounts of sludge are pre-enriched.

Given the results of the previous experiment (Gibbs et al., 1995) and the anecdotal evidence mentioned above, it appears that the recommendation contained in the current draft sludge management guidelines (AWRC et al., 1993) to pre-enrich five 10 g samples should remain at this stage.
4.3 Replication Of Enrichments

In the experiment described in section 2 five replicate enrichments were set up from each pre-enrichment. This enabled a statistical assessment of the advantages of taking multiple enrichments from one pre-enrichment. Each pre-enrichment was considered as a separate sample (thus giving a sample size of 50) and only the data from the RV/LMG combination was used as this was the optimum combination tested (see section 5). The analyses used are detailed in Appendix III.

Two analyses were performed. The first analysis considered the number of replicate enrichments which would have been positive if one, two, three, four or five enrichments had been taken from each one. This showed that there was no significant advantage ($p=0.8920$) in taking more than one enrichment. The second analysis compared the number of positive samples obtained when taking one, two, three, four or five enrichments from one pre-enrichment. Again, there was no significant advantage in taking more than one enrichment. It therefore appears to be adequate to set up one enrichment from each pre-enrichment.

4.4 Multiple Plating

No assessment was made of the effect of taking more than one subculture from enrichment cultures during this project. Fricker (1984) investigated the effect of multiple plating and found that detection of *Salmonella* was improved but not significantly enough to warrant the added expense of using more than one plate.
5: General Discussion

5.1 The Importance of Detection of S. Typhi and Paratyphi

Although they are only three serotypes out of over 2000, S. typhi and S. paratyphi A and B generally carry a disproportionate weight in considerations about Salmonella detection methods. This is because they cause a potentially more serious illness (enteric fever) than other salmonellae and because they are biochemically atypical and thus may not be detected using the methods used for other salmonellae.

The incidence of enteric fever in Australia appears to have remained fairly constant for the past three years. In 1992 and 1993 S. typhi and paratyphi A and B made up approximately 3.3% of all reported Salmonella infections (Powling, 1993, Powling et al., 1994). In 1991 S. typhi (not paratyphi) composed 1.6% of Salmonella infections (Anura and Hall, 1992). The case rate (annual rate of infection per 100,000 population) of typhoid infections in Australia (which excludes some paratyphi infections) was 0.5 in 1991 and 0.3 in 1992 and 1993. During these years the case rate ranged from 0.0 in some states to 1.0 in others (Anura and Hall, 1992, Powling, 1993 and Powling et al., 1994).

Although the infection rate for typhoid and paratyphoid is relatively low the possibility that these organisms will be found in sludge remains. A monitoring method should have the potential to detect these organisms.

5.2 Choice of Enrichment Media

RV enrichment broth was significantly more efficient than the other enrichment media tested in this study. However, it is not sufficient to recommend this enrichment alone because of the possibility that it will not detect some Salmonella serotypes. This is particularly true of S. typhi and paratyphi, which do not grow in RV (Merck, 1988). For this reason, MSE is also recommended. This medium does support the growth of S. typhi and, as will be discussed in section 5.3, is useful in that it promotes H2S production in this serotype on LMG agar (Cox, 1993).
5.3 Choice of Isolation Medium

The isolation media tested in this study were equally efficient in terms of the number of positive replicates detected. BSA, however, has several disadvantages, these being

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

Once again, the choice of medium is dictated by the need to detect *S. typhi*. *S. typhi* does not produce typical *Salmonella*-like colonies on XLD so BSA has traditionally been incorporated into monitoring protocols such as the Australian food standard (Standards Australia, 1991) to detect this serotype. The recent development of LMG, however, now provides an alternative approach. LMG is able to detect *S. typhi*. Although colonies are atypical in that they are yellow rather than pink, they will produce H2S (and therefore have black centres) after 24h if previously grown in selenite broth (Cox, 1993). It therefore appears feasible to replace the XLD and BSA combination with LMG. The use of one medium instead of two represents significant savings in cost and time. Unfortunately LMG is yet not commercially available in a dehydrated form, but this is being developed by Amyl Media and should be available in the near future (J. Cox, pers. comm.).
6: Conclusions

Methods of Salmonella isolation recommended in Australia and in the USA were found to have some shortcomings when used with composted sludge samples. These methods can be improved by adopting the following protocol for detecting the presence or absence of salmonellae in 50 g samples of composted sludge.

1. Sampling

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

2. Pre-enrichment

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

3. Enrichment

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

4. Isolation

The recommended isolation medium is lysine-mannitol-glycerol agar. The enrichment cultures should be subcultured onto this medium after 24 and 48h incubation. LMG plates should be examined for suspected salmonellae after 24h incubation at 37°C.

5. Purification and identification

All suspected salmonellae must be confirmed. Presumptive salmonellae should be purified on MacConkey agar without salt and confirmed by biochemical and serological tests.
7. Recommendations

The authors consider that the above method should be included in Australian sludge management guidelines as the recommended method for detecting salmonellae in composted sludge.
References


Haas, C. N. (1992) Microbial sampling: is it better to sample many times or use large samples? *Water Science and Technology* 27 (3-4) : 19 - 25.


Standards Association of Australia (1982) Methods for the Microbiological Examination of Food Part 5 - Preparation of media, diluents and reagents AS 1766.5, Standards Association of Australia.


Appendix I

Statistical comparisons of enrichment and plating media

The following statistics relate to the data shown in section 2.3. Table A1.1 shows an analysis of variance for the effects of the type of enrichment and isolation media on detection of *Salmonella* from composted sludge. The p-value of less than 0.05 indicated that there was a significant difference in the efficiency of enrichment broths. There were no significant differences between the plating media tested and no interaction between the enrichments and plates.

**Table A1.1.** Analysis of variance for effects of enrichment and isolation media on detection of *Salmonella*

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment</td>
<td>3</td>
<td>1924.424</td>
<td>641.475</td>
<td>27.947</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Plate</td>
<td>2</td>
<td>76.682</td>
<td>38.341</td>
<td>1.670</td>
<td>0.1925</td>
</tr>
<tr>
<td>Enrichment</td>
<td>6</td>
<td>106.167</td>
<td>17.694</td>
<td>0.771</td>
<td>0.5943</td>
</tr>
<tr>
<td>* Plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>120</td>
<td>2754.364</td>
<td>22.953</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant with 95% confidence interval

Table A1.2 shows multiple comparisons of the enrichment broths tested. There was a significant difference between RV and all of the other broths tested.

**Table A1.2.** Scheffe F tests for differences between enrichment broths

<table>
<thead>
<tr>
<th>Enrichments</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE vs RV</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>MSE vs SCB</td>
<td>0.1954</td>
</tr>
<tr>
<td>MSE vs TT</td>
<td>0.2139</td>
</tr>
<tr>
<td>RV vs SCB</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>RV vs TT</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>SCB vs TT</td>
<td>&gt;0.9999</td>
</tr>
</tbody>
</table>

* Significant with 95% confidence interval
The advantages of using more than one enrichment or plating media were assessed by comparing the number of positive replicates that would have been obtained if

i) MSE and RV were used instead of RV only

ii) LMG and BSA, XLD and BSA or XLD and LMG were used instead of XLD or LMG only

No significant advantages were gained by using RV and MSE or by using more than one plating medium. The p-values obtained from Scheffe test multiple comparisons are shown in Table A1.3.

Table A1.3. Scheffe F tests for improved isolation using more than one enrichment or plating medium.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV vs RV+MSE</td>
<td>0.9902</td>
</tr>
<tr>
<td>LMG vs LMG+BSA</td>
<td>0.2238</td>
</tr>
<tr>
<td>LMG vs LMG+XLD</td>
<td>0.1550</td>
</tr>
<tr>
<td>XLD vs XLD+BSA</td>
<td>0.2430</td>
</tr>
<tr>
<td>XLD vs XLD+LMG</td>
<td>0.1080</td>
</tr>
</tbody>
</table>
Appendix II

Statistical comparison of selenite broths

The following statistics relate to the data presented in section 3.3. Calculation 2.1 is Bartlett's test for homogeneity of variance. This indicated that the variance in each broth were not equal and as such a non parametric test for analysis of variance (the Kruskal - Wallis test) was employed (Table A2.1).

Calculation 2.1

\[ H_0 : \text{All variances are equal} \]
\[ H_1 : \text{All variances are not equal} \]
Reject \( H_0 \) if \( B > X^2_{0.05;5} \)

\[ B = 2.30259 \left[ \log \left( \frac{s^2_p}{\sum v_i} \right) - \sum v_i \log s^2_i \right] \]
\[ C = 1 + \frac{1}{3(k-1)} \left[ \sum v_i - 1 - \sum v_i \right] \]
\[ B_c = \frac{B}{C} \]

where

- \( s^2_p = \frac{\sum SS_i}{\sum v_i} \)
- \( SS = \text{sum of squares} \)
- \( n = \text{sample size} \)
- \( k = \text{number of samples} \)
- \( v_i = n_i - 1 \)
- \( s^2_i = \frac{\sum s^2_i}{v_i \log s^2_i} \)

\[ s^2_p = \frac{\sum SS_i}{\sum v_i} = \frac{4961.2}{100} = 49.612 \]
\[ \log s^2_p = 1.695 \]

\[ n_1 = 18 \quad v_1 = 17 \quad s^2_1 = 0.1 \quad v_1 \log s^2_1 = 17 \log 0.1 = -17.0 \]
\[ n_2 = 16 \quad v_2 = 15 \quad s^2_2 = 3.3 \quad v_2 \log s^2_2 = 15 \log 3.3 = 7.8 \]
\[ n_3 = 19 \quad v_3 = 18 \quad s^2_3 = 4.5 \quad v_3 \log s^2_3 = 18 \log 4.5 = 11.8 \]
\[ n_4 = 17 \quad v_4 = 16 \quad s^2_4 = 0.018 \quad v_4 \log s^2_4 = 16 \log 0.018 = -27.9 \]
\[ n_5 = 17 \quad v_5 = 16 \quad s^2_5 = 1.1 \quad v_5 \log s^2_5 = 16 \log 1.1 = 0.7 \]
\[ n_6 = 19 \quad v_6 = 18 \quad s^2_6 = 0.4 \quad v_6 \log s^2_6 = 18 \log 0.4 = -7.2 \]
\[ \Sigma v_i = 100 \quad \Sigma \log s^2_i = -31.8 \]
\[ \Sigma 1/v_i = 0.3616 \]

\[ B = 2.30259 \times 1.695 \times 100 - (-31.8) \]
\[ = 463.5 \]

\[ C = 1 + \frac{1}{3}(5) \times [0.3616 - 1/100] \]
\[ = 1.0 \]

\[ B_c = 463.5 \]

\[ x^2_{0.05;5} = 11.07 \]

\[ B_c > x^2_{0.05;5} \]

Therefore, reject \( H_0 \)

---

**Table A2.1. Kruskal-Wallis analysis of variance for effect of selenite broth type on growth of Salmonella**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>5</td>
</tr>
<tr>
<td>No. groups</td>
<td>6</td>
</tr>
<tr>
<td>No. ties</td>
<td>18</td>
</tr>
<tr>
<td>( H )</td>
<td>59.215</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>( H ) corrected for ties</td>
<td>59.607</td>
</tr>
<tr>
<td>Tied p-value</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

* Significant difference with 95% confidence interval
<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference</th>
<th>Critical difference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 vs 2</td>
<td>0.281</td>
<td>1.956</td>
</tr>
<tr>
<td>3 vs 1</td>
<td>0.846</td>
<td>1.521</td>
</tr>
<tr>
<td>3 vs 6</td>
<td>1.216</td>
<td>1.550</td>
</tr>
<tr>
<td>3 vs 5</td>
<td>1.321</td>
<td>1.643</td>
</tr>
<tr>
<td>3 vs 4</td>
<td>1.615</td>
<td>1.509*</td>
</tr>
<tr>
<td>2 vs 1</td>
<td>0.565</td>
<td>1.440</td>
</tr>
<tr>
<td>2 vs 6</td>
<td>0.935</td>
<td>1.465</td>
</tr>
<tr>
<td>2 vs 5</td>
<td>1.040</td>
<td>1.562</td>
</tr>
<tr>
<td>2 vs 4</td>
<td>1.335</td>
<td>1.431</td>
</tr>
<tr>
<td>1 vs 6</td>
<td>0.370</td>
<td>0.500</td>
</tr>
<tr>
<td>1 vs 5</td>
<td>0.475</td>
<td>0.828</td>
</tr>
<tr>
<td>1 vs 4</td>
<td>0.769</td>
<td>0.271*</td>
</tr>
<tr>
<td>6 vs 5</td>
<td>0.106</td>
<td>0.883</td>
</tr>
<tr>
<td>6 vs 4</td>
<td>0.400</td>
<td>0.459</td>
</tr>
<tr>
<td>5 vs 4</td>
<td>0.294</td>
<td>0.807</td>
</tr>
</tbody>
</table>

* Significant difference with a 95% confidence interval
Appendix III

Statistical comparison of replication of enrichments

The following statistics are discussed in Chapter 4. Table A3.1 shows an analysis of variance testing the effect of taking multiple enrichments from one pre-enrichment on the number of positive replicates detected. Increasing the number of enrichments did not significantly increase the number of positive replicates detected.

Table A3.1. Analysis of variance for effect of replication of enrichments on the number of positive replicates

<table>
<thead>
<tr>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. enrichments</td>
<td>4</td>
<td>4.8</td>
<td>1.2</td>
<td>0.276</td>
</tr>
<tr>
<td>Residual</td>
<td>45</td>
<td>195.7</td>
<td>4.3</td>
<td></td>
</tr>
</tbody>
</table>

This test was able to detect a difference in the means as small as 1.5 positive enrichments with a power of ~86% (i.e., the probability of accurately rejecting the null hypothesis was 0.86). When the minimum detectable difference between population means was increased to 2 positive enrichments, the power of the test increased to 97%.

Table A3.2 and Calculation 3.1 show an alternative assessment of the effect of increasing the number of enrichments taken from one pre-enrichment. In this case, the number of positive samples detected using one, two, three, four or five enrichments were compared. Each pre-enrichment culture was considered to be a separate sample, giving a sample size of 50. Once again, the number of positive samples detected was not increased by taking more than one enrichment from one pre-enrichment.
Table A3.2. Comparison of proportion of positive samples detected by replicating enrichments

<table>
<thead>
<tr>
<th>No. enrichments taken from one pre-enrichment</th>
<th>No. of positive samples detected using RV/LMG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
</tr>
</tbody>
</table>

Calculation 3.1

H₀: The number of positive samples detected using one, two, three, four or five enrichments was the same.

H₁: The number of positive samples detected using one, two, three, four or five enrichments was not the same.

Reject H₀ if $X^2 > X^2_{0.05;4}$

\[
X^2 = \Sigma \left[ \frac{(x_i - n_ip)^2}{n_iq} \right]
\]

\[
p = \frac{\Sigma x_i}{\Sigma n_i}
\]

\[
q = 1 - p
\]

where

- $x =$ number of positive samples
- $n =$ sample size
- $p =$ proportion of samples positive
- $q =$ proportion of samples negative $= 1 - p$

$n_1 = n_2 = n_3 = n_4 = n_5 = 50$

$p_1 = \frac{26}{50} = 0.52 \quad q_1 = 0.48$

$p_2 = \frac{29}{50} = 0.58 \quad q_2 = 0.42$

$p_3 = \frac{32}{50} = 0.64 \quad q_3 = 0.36$

$p_4 = \frac{34}{50} = 0.68 \quad q_4 = 0.32$

$p_5 = \frac{34}{50} = 0.68 \quad q_5 = 0.32$
\[ p = \frac{\sum x_i}{\sum n_i} \]
\[ = \frac{(26 + 29 + 32 + 34 + 34)}{5 \times 50} \]
\[ = \frac{155}{250} \]
\[ = 0.62 \]

\[ q = 1 - 0.62 \]
\[ = 0.38 \]

\[ X^2 = \sum \left( \frac{(x_i - np)^2}{n_ipq} \right) \]
\[ = \frac{(26 - 50 \times 0.62)^2 + (29 - 50 \times 0.62)^2 + (32 - 50 \times 0.62)^2 + (34 - 50 \times 0.62)^2 + (34 - 50 \times 0.62)^2}{50 \times 0.62 \times 0.38} \]
\[ = 4.07 \]

\[ X^2_{0.05;4} = 9.488 \]

\[ X^2 < X^2_{0.05;4} \]

Therefore do not reject \( H_0 \)