ENVIRONMENT AGENCY

The Microbiology of Sewage Sludge (2004) - Part 4 -Methods for the detection, isolation and enumeration of *Salmonellae*

Methods for the Examination of Waters and Associated Materials

The Microbiology of Sewage Sludge (2004) - Part 4 - Methods for the detection, isolation and enumeration of *Salmonella*

Methods for the Examination of Waters and Associated Materials

This booklet contains three methods for the detection and enumeration of *Salmonella*.

A The detection of *Salmonella spp*. by use of a presence/absence technique.
B The detection and enumeration of *Salmonella spp*. by a most probable number technique.
C The enumeration of *Salmonella spp*. by a membrane filtration technique with resuscitation and culture on a chromogenic medium.

This booklet also replaces and supercedes "The Microbiology of Sewage Sludge (2003) - Part 4 - Methods for the detection, isolation and enumeration of *Salmonella*".

Within this series there are separate booklets dealing with different topics concerning the microbiology of sewage sludge. Other booklets include

The Microbiology of Sewage Sludge (2003) -
Part 1 - An Overview of the Treatment and Use of Sewage Sludge and its impact on the environment and public health.
Part 2 - Practices and procedures for sampling and sample preparation
Part 3 - Methods for the isolation and enumeration of *Escherichia coli*

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. An index of methods and details on how to obtain copies are available from the Agency's internet web-page (www.environment-agency.gov.uk/nls) or from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

May 2004

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

A The detection of *Salmonella* species using a presence/absence technique

A1 Introduction

Many different serotypes of *Salmonella* species are present, to varying extents, in the intestinal tracts of humans, animals and birds. All members of the genus are potentially pathogenic. Due to their origin *Salmonella* species occur widely in sewage and the environment and may be present in significant numbers in sewage sludge. Hence there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Salmonella infections give rise to symptoms of diarrhoea and vomiting, the incubation period varies between 12 - 72 hours and symptoms usually persist for 2 - 3 days. Most cases of infection occur via the consumption of raw, or undercooked, food, particularly poultry and foods containing eggs. The significance of *Salmonella* species is described in more detail elsewhere(1) in this series.

A2 Scope

This method is suitable for the examination of untreated, conventionally treated and enhanced treated sludges. Enhanced treated sludges may include sludge derived from treatment processes such as pasteurisation, thermophilic digestion and lime stabilisation. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere(2) in this series.

Users wishing to employ this method should verify its performance under their own laboratory conditions(3).

A3 Definitions

In the context of this method, organisms that form characteristic colonies on selective media after culture in enrichment media, and which produce the serological and biochemical reactions described are regarded as *Salmonella* species.

Salmonella species normally conform to the general definition of the family Enterobacteriaceae(4) and can be further differentiated, biochemically, into four subgroups, subgenus I to IV. The bacteria of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

The usual biochemical reactions include production of hydrogen sulphide and utilisation of citrate as a source of carbon, indole and urease not being produced, and lysine and ornithine being decarboxylated. Phenylalanine and tryptophan are not oxidatively de-aminated, and sucrose, salicin, inositol and amygdalin are not fermented.

A4 Principle

A sample of sludge is diluted with either maximum recovery diluent or buffered peptone water and homogenised. This is followed by a pre-enrichment procedure, involving incubation in a non-selective medium (to recover environmentally stressed organisms) and then selective enrichment

with subculture to a selective agar containing xylose and additional indicators of acidity and hydrogen sulphide production. Characteristic colonies are confirmed by biochemical tests and serological tests based on slide agglutination.

A5 Limitations

This method may not be suitable for sludges likely to contain high concentrations of toxic or inhibitory substances, or where samples contain high numbers of competing or non-target organisms that may inhibit the growth or detection of target organisms. The need to dilute and homogenise, and in some cases, neutralise samples (for example lime-treated sludges) may dictate the use of double strength pre-enrichment medium, or limit the quantity of sludge sample that can be used in a single test.

The method is not suitable for the recovery of *Salmonella typhi*, *Salmonella paratyphi* or *Salmonella* species that do not exhibit characteristic growth on xylose lysine desoxycholate agar.

A6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible(5).

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations(6) and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere(3) in this series.

Salmonella species are classified as “Hazard Group 2”(7) and due to the pathogenic nature of these organisms, extra care should be taken in their isolation and identification. Staff should be adequately trained and supervised. Work involving subculture and handling of cultures should be performed in a separate designated area. Adequate facilities should be in place for the disposal and sterilisation of test materials.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere(3) in this series. Principally, fan convection incubators and circulating water baths are required. Other items include:

A7.1 Suitable sample containers(2) of appropriate volume.

A7.2 Incubators or waterbaths capable of maintaining temperatures of $37 \pm 1^{o}C$ and $41.5 \pm 0.5\ ^{o}C$.

A8 Media and reagents

Commercial formulations of these media and reagents are available but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior

to their use in the method. Variations in the preparation and storage of media should also be verified.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

A8.1 *Buffered peptone water*[8]

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution (typically, 90 ml) into suitable screw-capped tubes, or bottles, and sterilise by autoclaving at 121 ºC for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Double-strength medium can be prepared by dissolving twice the above quantities of ingredients in the same volume of water, and treated as above. Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

A8.2 *Rappaport Vassiliadis enrichment broth*[9, 10]

Solution A

Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Distilled, deionised or similar grade water	800 ml

Solution B

Magnesium chloride anhydrous	13.6 g
Distilled, deionised or similar grade water	100 ml

Solution C

Malachite green	36 mg
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients of solution A in the 800 ml of water. To achieve this, it may be necessary to heat to boiling. Prepare this solution on the day of use. To this solution add 100 ml of solution B and 100 ml of solution C. Mix thoroughly. Dispense the resulting solution (typically, 10 ml) into suitable capped containers and sterilise by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 5.2 ± 0.2. Autoclaved media may be stored between 2 - 8 ºC, protected from dehydration and used within one month.

A8.3 *Xylose lysine desoxycholate agar*[11]

Basal medium

Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5.0 g
Sodium chloride	5.0 g

Yeast extract	3.0 g
Phenol red (0.4 % m/v aqueous solution)	20 ml
Agar	12.0 g
Distilled, deionised or similar grade water	1 litre

Solution A.

Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Distilled, deionised or similar grade water	100 ml

Solution B.

Sodium desoxycholate	10.0 g
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients of the basal medium in the water. This will require gentle heating. Dispense the resulting solution in appropriate volumes into suitable screw-capped bottles and sterilise by autoclaving at 115 ºC for 10 minutes. The basal medium may be stored in the dark at room temperature and used within one month.

Dissolve the ingredients of solutions A and B in the respective amounts of water and, separately, pasteurise the individual solutions by heating at approximately 60 ºC for 1 hour. To prepare the complete medium, melt 100 ml of the basal medium and cool to approximately 50 ºC. To this solution add, aseptically, 2.0 ml of solution A and 2.5 ml of solution B. Mix thoroughly. The pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Pour the complete medium into sterile Petri dishes and allow the medium to solidify.

A8.4 *Triple sugar iron agar*[12]

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15.0 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients (except the phenol red solution) in the water. To achieve this, it will be necessary to heat to boiling. Add the phenol red solution and mix well. Dispense the resulting solution in small volumes (typically, 5 - 10 ml) in suitable containers. Sterilise the solution by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored between 2 - 8 ºC, protected from dehydration and used within one month.

A8.5 *Urea broth*

Broth base	
Peptone	1.0 g
Glucose	1.0 g
Disodium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5.0 g
Phenol red (0.4 % m/v aqueous solution)	1.0 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water, and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution (typically, 95 ml) into suitable screw-capped bottles and sterilise by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. Prepared base medium may be stored in the dark at room temperature and used within one month. Prior to use, to 95 ml of the broth base add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea and aseptically dispense in 2-3 ml volumes into sterile capped containers.

A8.6 *Other media*

Standard and commercial formulations of other media and reagents used in this method include maximum recovery diluent, brilliant green agar(13, 14), Rambach agar(15), nutrient agar, nutrient broth, saline solution and anti-sera.

A9 Analytical procedure

A9.1 *Sample preparation*

An appropriate weight of the wet sample, typically 10 g, is initially suspended in 90 ml of buffered peptone water. If a specified quantity of dried sludge is required for analysis, for example for regulatory requirements, a weight of wet sludge equivalent to the required weight of dried sludge can be taken. The amount of wet sludge to take is calculated with reference to the dry solids content(16) of the sludge sample. For example, where 2 g of dried sludge are required for analysis and the wet sludge sample contains a dry solids content of 30 %, then the amount of wet sludge, W, equivalent to 2 g of dried sludge is calculated from

$$W = \frac{100 \times 2}{30} \text{ g}$$

$$= 6.7 \text{ g}$$

A suspension of the sample is then homogenised and processed, depending on the characteristics of the matrix as described elsewhere(2) in this series.

For lime-treated sludges, where initial dilution in maximum recovery diluent may be required prior to homogenisation and neutralisation(2) then the homogenised and neutralised suspension, typically 100 ml, should be added to an equal volume of double-strength buffered peptone water.

A9.2 *Pre-enrichment*

Incubate the homogenised suspension in buffered peptone water at 37 °C for 21 ± 3 hours.

A9.3 *Enrichment and subculture to selective media*

After incubation, mix well and subculture 0.1 ml of the buffered peptone water into 10 ml of Rappaport Vassiliadis enrichment broth and incubate at 41.5 °C for 24 ± 3 hours. After incubation, plate out a loopful (10 µl) of the Rappaport Vassiliadis enrichment broth onto a Petri dish of xylose lysine desoxycholate agar. Immediately, return the Rappaport enrichment broth to the incubator at 41.5°C for 24 hours. Incubate the xylose lysine desoxycholate agar at 37°C for 21 ± 3 hours. After incubation, examine the selective agar. If, on the selective agar, no growth is observed, then plate out a further loopful (10 µl) of the Rappaport Vassiliadis enrichment broth onto a second Petri dish of xylose lysine desoxycholate agar and incubate at 37°C for 21 ± 3 hours. On rare occasions, it may be advantageous to use an additional selective medium not dependent on the demonstration of the production of hydrogen sulphide. For example, brilliant green agar(13, 14) or Rambach agar(15) may be used.

A9.4 *Reading of results*

After incubation, examine the Petri dishes of selective agar under good light, using a magnifier, if required. Colonies observed on xylose lysine desoxycholate agar are differentiated as follows:

Colonial appearance on xylose lysine desoxycholate agar

Organism	Characteristic appearance
Salmonella species	Smooth red colony 2-3 mm in diameter, typically, with black centre, or wholly black colony.
Xylose-fermenting coliform bacteria	Yellow colony.
Pseudomonas species	Red or yellow colony with grey-black centre.
Shigella species	Small pink-red colony.
Proteus species	Red colony that is irregular and may have small black centre.

Where isolates are overgrown, it may be necessary to sub-culture to a fresh Petri dish of xylose lysine desoxycholate agar. This facilitates the production of pure cultures and enables typical colonial morphology to be observed.

A9.5 *Confirmation tests*

A9.5.1 *Biochemical confirmation*

Using a straight wire, sub-culture characteristic colonies from each Petri dish to triple sugar iron agar and urea broth, and to nutrient agar as a check for purity. For triple sugar iron agar, the wire should be stabbed into the butt, and streaked across the agar slant as it is withdrawn. Avoid stabbing through the butt to the bottom of the tube. The end of the wire should remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be ejected from the tube. Incubate the inoculated media at 37 °C for 18 - 24 hours. Regard cultures that give characteristic reactions in these confirmatory media as presumptive salmonellas.

Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth[3]
	Slope[1]	Butt[1]	H_2S production[2]	
Salmonella typhi	NC or Alk	Acid	+ve (weak)	-ve
Other *Salmonella*	NC or Alk	Acid + Gas	+ve	-ve
Shigella	NC or Alk	Acid	-ve	-ve
Proteus morganii	NC or Alk	Acid ± Gas	-ve	+ve
Proteus vulgaris	Acid	Acid + Gas	+ve	+ve
Escherichia	Acid	Acid + Gas	-ve	-ve
Citrobacter	Acid	Acid + Gas	+ve	+ve or –ve
Klebsiella	Acid	Acid + Gas	-ve	+ve or –ve
Enterobacter	Acid	Acid + Gas	-ve	-ve

[1] Alkaline (Alk) reaction is red, acid reaction is yellow or no change (NC)
[2] +ve = blackening of the medium, -ve = no blackening
[3] +ve = alkaline reaction (red), -ve = no change in colour

Alternatively, commercially available identification systems may be used, following appropriate performance verification within the laboratory.

A9.5.2 *Serological confirmation*

Sub-culture characteristic colonies from each Petri dish of selective agar to moist nutrient agar slopes. For optimum flagellar formation, it is essential that fluid is present in the tube and sterile nutrient broth should be added, if required. Incubate overnight at 37 °C. A slide agglutination test should then be carried out. For example, using a wire loop or pipette, place 3 separate drops (each approximately 0.02 ml) of sterile saline solution onto a clean microscope slide. Emulsify growth from the moist butt of the nutrient agar slope in each of the separate drops, to produce homogeneous suspensions. Mix a loopful of *Salmonella* polyvalent 'O' (PSO) antiserum with the first drop of suspension, and a loopful (10 µl) of *Salmonella* polyvalent 'H' (PSH) serum into the second drop. The third drop containing no anti-serum indicates whether or not the culture auto-agglutinates. Gently rock the slide back and forth for about 30 seconds, being careful not to allow the 3 drops to come into contact. Examine the drops for agglutination against a black background. Auto-agglutinating strains should be re-plated onto xylose lysine desoxycholate agar and incubated as before, and dry smooth colonies selected and treated as previously described.

Organisms that agglutinate with PSO and PSH anti-sera, or strains that agglutinate with PSH serum only can be regarded as *Salmonella* species.

A10 Calculations

The results indicate the presence or absence of *Salmonella* species in the sample examined.

A11 Expression of results

The results for confirmed *Salmonella* species should be reported as being detected or not detected, in the wet or dry weight of sample examined.

Where examinations are carried out for regulatory purposes, results are, typically, reported as *Salmonella* species detected or not detected in 2 g dry weight of sludge.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Salmonella poona*) and non-target bacteria (for example, *Escherichia coli*) and incubated under appropriate conditions. Positive and negative controls should also be inoculated and incubated with each batch of samples tested to ensure the validity of the tests being carried out.

A13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

4. Nomenclature des Salmonella. Annals Institut Pasteur. *Microbiologie*, L Le Minor, R Rhode and J Taylor, 1970, **119**, pp206-210.

5. This text is based on Resolution 74 by CEN TC 292 - Wastes - Working Group 5, the agreed text of which was adopted by CEN TC 308 - Characterisation of sludges - for the section on "General Hazards" associated with sludge material and waste.

6. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

7. Categorisation of Pathogens According to Hazard and Categories of Containment. Fourth edition, 1995, supplement 1998. Advisory Committee on Dangerous Pathogens.

8. Comparative studies on the isolation of "sub-lethally injured" Salmonellae in nine European laboratories. *Bulletin of the World Health Organisation*, W Edel and E H Kampelmacher, 1973, **48**, pp167-174.

9. Salmonella isolation using RVS broth and MCLB agar. *Food Microbiology*. N van Schothorst, A Renaud and C van Beek, 1987, **4**, pp11-18.

10. The effect of incubation temperature and magnesium chloride concentration on the growth of salmonella in home-made and in commercially available Rappaport-Vassiliadis broths. *Journal of Applied Bacteriology*, M Peterz, C Wilberg and P Norberg, 1989, **66**, pp523-528.

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12. Report of Enterobacteriaceae Subcommittee and the Nomenclature Committee of the International Association of Microbiological Societies. *International Bulletin of Bacterial Nomenclature and Taxonomy*, 1958, **8**, pp25-70.

13. *Micro-organisms in Food 1 – Their Significance and Methods of Enumeration.* 2nd edition. International Commission on Microbiological Specifications for foods. 1978, Toronto, University of Toronto Press.

14. A modification of the brilliant green agar for improved isolation of *Salmonella. Journal of Applied Bacteriology*, D C Watson and A P Walker, 1978, **45**, pp195-204.

15. New plate medium for facilitated differentiation of Salmonella spp. from Proteus spp. and other enteric bacteria. *Applied and Environmental Microbiology*, A Rambach, 1990, **56**, pp301-303.

16. Standing Committee of Analysts, The Conditionability, Filterability, Settleability and Solids Content of Sludges (1984) - A Compendium of Methods. *Methods for the Examination of Waters and Associated Materials*, in this series, London, ISBN 0117517879.

B The isolation and enumeration of *Salmonella* species by a multiple tube most probable number technique

B1 Introduction

Many different serotypes of *Salmonella* species are present, to varying extents, in the intestinal tracts of humans, animals and birds. All members of the genus are potentially pathogenic. Due to their origin these organisms occur widely in sewage and the environment and may be present in significant numbers in sewage sludge. Hence there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Salmonella infections give rise to symptoms of diarrhoea and vomiting, the incubation period varies between 12 - 72 hours and symptoms usually persist for 2 - 3 days. Most cases of infection occur via the consumption of raw, or undercooked, food, particularly poultry and food containing eggs. The significance of *Salmonella* species is described in more detail elsewhere (1) in this series.

B2 Scope

This method is suitable for the examination of untreated and conventionally treated sludges, including crude, primary settled, lagoon-stored, thickened, caked, composted and mesophilic anaerobic digested sludge where limited microbial reduction is expected. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere(2) in this series.

Users wishing to employ this method should verify its performance under their own laboratory conditions(3).

B3 Definitions

In the context of this method, organisms that form characteristic colonies on selective media after culture in enrichment media, and which produce the serological and biochemical reactions described are regarded as *Salmonella* speices.

Salmonella species normally conform to the general definition of the family Enterobacteriaceae(4), and can be further differentiated, biochemically, into four subgroups, subgenus I to IV. Those bacteria of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

The usual biochemical reactions include production of hydrogen sulphide, indole and urease not being produced, citrate is utilised as a carbon source, lysine and ornithine being decarboxylated. Phenylalanine and tryptphan are not oxidatively de-aminated, and sucrose, salicin, inositol and amygdalin are not fermented.

B4 Principle

Isolation and enumeration is based on appropriate homogenisation of sludge, followed by multiple tube pre-enrichment involving incubation in a non-selective medium (to recover environmentally

stressed organisms) and selective enrichment with sub-culture to selective agar containing lactose and an indicator of acidity. Characteristic colonies are confirmed by biochemical tests and by slide agglutination. The most probable number of organisms in the sample is estimated from appropriate probability tables.

B5 Limitations

This method may not suitable for sludges likely to contain high concentrations of toxic or inhibitory substances or where the growth of high numbers of competing organisms inhibit the growth or detection of the target organisms.

The recovery or organisms in sludge of a thick, lumpy or fibrous character will depend on the effectiveness of the homogenisation pre-treatment. Sludges containing extraneous matter of a sharp or aggressive nature may be very difficult to homogenise.

The method is not suitable for the recovery of *Salmonella typhi*, *Salmonella paratyphi* or *Salmonella* species that do not exhibit characteristic growth on xylose lysine desoxycholate agar.

B6 Health and safety

Sewage and sewage sludge samples can contain hazardous and flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible[(5)].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[(6)] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[(3)] in this series.

Salmonella species are classified as "Hazard Group 2"[(7)]. Extra care should be taken in the isolation and identification of salmonellas due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised. Work involving sub-culture and handling of cultures should be performed in a separate designated area. Adequate facilities should be in place for disposal and sterilisation of test materials.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[(3)] in this series. Principally, fan convection incubators and circulating water baths are required. Other items include:

B7.1 Suitable sample containers[(2)] of appropriate volume.

B7.2 Incubators or waterbaths capable of maintaining temperatures of $37 \pm 1^{o}C$ and $41.5 \pm 0.5\ ^{o}C$.

B7.3 Stomacher.

B8 Media and reagents

Commercial formulations of these media and reagents are available but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method. Variations in the preparation and storage of media should also be verified.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

B8.1 *Buffered peptone water*[8]

Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution (typically, 90 ml) into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121 ºC for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Double strength medium can be prepared by dissolving twice the quantities of ingredients in the same volume of water, and treated as above. Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

B8.2 *Maximum recovery diluent*

Bacteriological peptone	1 g
Sodium chloride	8.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The sterilised diluent should be stored at room temperature in the dark and used within one month.

B8.3 *Rappaport Vassiliadis enrichment broth*[9, 10]

Solution A	
Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Distilled, deionised or similar grade water	800 ml
Solution B	
Magnesium chloride anhydrous	13.6 g
Distilled, deionised or similar grade water	100 ml
Solution C	
Malachite green	36 mg
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients of solution A in the 800 ml of water. To achieve this, it may be necessary to heat to boiling. Prepare this solution on the day of use. To this solution add 100 ml of solution B and 100 ml of solution C. Mix thoroughly. Dispense the resulting solution (typically, 10 ml) into suitable capped containers and sterilise by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 5.2 ± 0.2. Autoclaved media may be stored between 4 - 8 ºC, protected from dehydration, and used within one month.

B8.4 *Xylose lysine desoxycholate agar*[11]

Basal medium.

Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5.0 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red (0.4 % m/v aqueous solution)	20 ml
Agar	12.0 g
Distilled, deionised or similar grade water	1 litre

Solution A.

Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron (III) citrate	4.0 g
Distilled, deionised or similar grade water	100 ml

Solution B.

Sodium desoxycholate	10.0 g
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients of the basal medium in the water. This will require gentle heating. Dispense the resulting solution in appropriate volumes into suitable screw capped bottles and sterilise by autoclaving at 115 ºC for 10 minutes. The basal medium may be stored in the dark at room temperature and used within one month.

Dissolve the ingredients of solution A and solution B in the respective amounts of water and separately pasteurise the individual solutions by heating at approximately 60 ºC for 1 hour. To prepare the complete medium, melt 100 ml of the basal medium and cool to approximately 50 ºC. To this solution add, aseptically, 2.0 ml of solution A and 2.5 ml of solution B. Mix thoroughly. The pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Pour the complete medium into sterile Petri dishes and allow the medium to solidify.

B8.5 *Triple sugar iron agar*[12]

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron (III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg

Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15.0 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients (except the phenol red) in the water. To achieve this, it will be necessary to heat to boiling. Add the phenol red solution and mix well. Dispense the resulting solution in small volumes (typically, 5 - 10 ml) in suitable containers. Sterilise the solution by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored between 2 - 8 ºC, protected from dehydration, and used within one month.

B8.6 *Urea broth*

Peptone	1.0 g
Glucose	1.0 g
Disodium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5.0 g
Phenol red (0.4 % m/v aqueous solution)	1.0 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water, and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution (typically, 95 ml) into suitable screw-capped bottles and sterilise by autoclaving at 115 ºC for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. Prepared base medium may be stored in the dark at room temperature and used within one month. Prior to use, to 95 ml of the broth base add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea and aseptically dispense in 2-3 ml volumes into sterile capped containers.

B8.7 *Other media*

Standard and commercial formulations of other media and reagents that may be used in this method include brilliant green agar[13, 14], Rambach agar[15], nutrient agar, nutrient broth, saline solution and anti-sera.

B9 Analytical procedure

B9.1 *Sample preparation*

Using a sterile pipette, spatula or other suitable device appropriate to the consistency of the sludge, prepare an initial dilution of the sample by weighing 20 g of well-mixed wet sludge sample aseptically into an appropriate container. Add approximately 100 ml of buffered peptone water and transfer to a sterile stomacher bag. For certain sludges, for example if there is a danger that the bag may be pierced or ruptured by sharp material in the sludge, it may be necessary to place this bag inside a second bag. Homogenise the suspension for 2 minutes. Quantitatively transfer the homogenised suspension back to the original container. It may be necessary to wash and rinse out the stomacher bag with the buffered peptone water. Ensure the total volume of buffered peptone water used to prepare the homogenised suspension is 180 ml. Thoroughly mix the homogenised suspension but avoid excessive foaming.

If enumeration is required on lime-treated sludge samples, the homogenised suspension should be prepared in maximum recovery diluent and the lime neutralised after homogenisation. In these cases, an equal volume of double-strength buffered peptone water should be added to the

homogenised and neutralised suspension and the resulting suspension thoroughly mixed. Sample preparation techniques are described in more detail elsewhere[2] in this series.

B9.2 *Pre-enrichment*

The homogenised suspension is used to prepare a series of dilutions in tubes or bottles such that some of the tubes, or bottles, exhibit characteristic growth within the medium and others do not, i.e. some of the tubes are regarded as positive for *Salmonella* and others are regarded as negative. Volumes of well-mixed suspension are aseptically transferred to sterile tubes or bottles as appropriate to the series. Typically, an 11-tube series may be analysed as 1 x 50 ml (equivalent to 5 g of original sample), 5 x 10 ml and 5 x 1 ml. For sludges containing high numbers of *Salmonella* organisms it may be necessary to include more dilutions, for example 5 x 0.1 ml and 5 x 0.01 ml etc. In these cases, additional dilution with buffered peptone water will be necessary. For lime-treated sludges where the initial homogenised suspension might be more dilute after homogenisation and neutralisation, the volumes transferred should be adjusted to achieve an equivalent wet weight of original sample of 1 x 5 g, 5 x 1g, 5 x 0.1 g etc. This will facilitate the most probable number calculation. Once transferred to the tubes, the volume may be made up to 20 ml with sterile buffered peptone water, if required. All tubes or bottles should then be incubated at 37 °C for 21 ± 3 hours.

B9.3 *Enrichment and subculture to selective media*

After incubation, the tubes are carefully shaken and 0.1 ml of the buffered peptone water transferred from each tube or bottle into separate containers containing 10 ml of Rappaport Vassiliadis enrichment broth and incubate at 41.5 °C for 21 ± 3 hours. After incubation, plate out a loopful (10 µl) of the Rappaport Vassiliadis enrichment broth onto a Petri dish of xylose lysine desoxycholate agar. Immediately, return the Rappaport Vassiliades enrichment broth to the incubator at 41.5 °C for 24 hours. Incubate the xylose lysine desoxycholate agar at 37 °C for 21 ± 3 hours. After incubation, examine the selective agar. If, on the selective agar, no growth is observed, then plate out a further loopful (10 µl) of the Rappaport Vassiliadis enrichment broth onto a second Petri dish of xylose lysine desoxycholate agar and incubate at 37 °C for 21 ± 3 hours. On rare occasions, it may be advantageous to use an additional selective medium not dependent on the demonstration of the production of hydrogen sulphide. For example, brilliant green agar[13, 14] or Rambach agar[15] may be used.

B9.4 *Reading of results*

After incubation, examine the Petri dishes of selective agar under good light, if necessary using a magnifier. Colonies observed on xylose lysine desoxycholate agar are differentiated as follows:

Colonial appearance on xylose lysine desoxycholate agar

Organism	Characteristic appearance
Salmonella species	Smooth red colony 2-3 mm in diameter, typically, with black centre or wholly black colony
Xylose-fermenting coliform bacteria	Yellow colony
Pseudomonas species	Red or yellow colony with grey-black centre.
Shigella species	Small pink-red colony
Proteus species	Red colony that is irregular and may have small black centre.

Where isolates are overgrown, then subculture to a fresh Petri dish of xylose lysine desoxycholate agar. This facilitates the production of pure cultures and enables typical colonial morphology to be observed.

B9.5 *Confirmation tests*

B9.5.1 *Biochemical confirmation*

Using a straight wire, subculture characteristic colonies from each Petri dish to triple sugar iron agar and urea broth, and nutrient agar as a check for purity. For triple sugar iron agar, the wire should be stabbed into the butt, and streaked along the slant as it is withdrawn. Avoid stabbing through the butt to the bottom of the tube. The end of the wire should remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be ejected from the tube. Incubate the inoculated media at 37 °C for 18 - 24 hours. Regard cultures that give characteristic reactions in these confirmatory media, i.e. lysine iron agar, triple sugar iron agar and urea broth, as presumptive salmonellas.

Alternatively, a commercially available identification system may be used, following appropriate performance verification within the laboratory.

Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth[3]
	Slope[1]	Butt[1]	H_2S production[2]	
Salmonella typhi	NC or Alk	Acid	+ve (weak)	-ve
Other *Salmonella*	NC or Alk	Acid + Gas	+ve	-ve
Shigella	NC or Alk	Acid	-ve	-ve
Proteus morganii	NC or Alk	Acid ± Gas	-ve	+ve
Proteus vulgaris	Acid	Acid + Gas	+ve	+ve
Escherichia	Acid	Acid + Gas	-ve	-ve
Citrobacter	Acid	Acid + Gas	+ve	+ve or –ve
Klebsiella	Acid	Acid + Gas	-ve	+ve or –ve
Enterobacter	Acid	Acid + Gas	-ve	-ve

[1] Alkaline (Alk) reaction is red, acid reaction is yellow or no change (NC)
[2] +ve = blackening of the medium, -ve = no blackening
[3] +ve = alkaline reaction (red), -ve = no change in colour

B9.5.2 *Serological confirmation*

Subculture characteristic colonies from each Petri dish of selective agar to moist nutrient agar slopes. For optimum flagellar formation, it is essential that fluid is present in the tube and sterile nutrient broth should be added if required. Incubate overnight at 37 °C. A slide agglutination test should then be carried out. For example, using a wire loop or pipette, place 3 separate drops (each approximately 0.02 ml) of sterile saline solution onto a clean microscope slide. Emulsify growth from the moist butt of the nutrient agar slope in each of the separate drops to produce homogeneous suspensions. Mix a loopful of *Salmonella* polyvalent 'O' (PSO) anti-serum with the first drop of suspension, and a loopful of *Salmonella* polyvalent 'H' (PSH) serum with the second drop. Gently rock the slide back and forth for about 30 seconds, being careful not to allow the 3 drops to come into contact. Examine for agglutination against a black background. The third drop containing no anti-serum indicates whether or not the culture auto-agglutinates. Auto-agglutinating strains should

be re-plated onto xylose lysine desoxycholate agar and incubated as before, and dry smooth colonies treated as previously described.

Organisms that agglutinate with PSO and PSH anti-sera, or strains that agglutinate with PSH serum only, can be considered as *Salmonella* species.

B10 Calculations

Record the number of tubes or bottles that exhibit characteristic growth on xylose lysine desoxycholate agar or other selective medium, if used, and which confirm as containing *Salmonellae*. By reference to the appropriate probability tables, see appendix B1, determine the most probable number (MPN) of *Salmonellae* in the sample examined. For example, if in an 11-tube series comprising 1 x 5 g, 5 x 1 g and 5 x 0.1 g, the number of tubes confirming positive for *Salmonellae* in a consecutive series is 1, 3 and 0 respectively, then from table B1, the MPN is 8 *Salmonellae* per 10 g of sample.

If smaller quantities are examined, account should be taken of dilution factors. For the most probable number of organisms per g of dried sludge, the calculation needs to account of the percent dry solids content[16].

For the most probable number of organisms, C_d, per g dry weight of sample, i.e. count per g of dried sludge:

$$C_d = \frac{C_w \times 100}{e}$$

Where C_w is the most probable number of *Salmonella* organisms in 1 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

B11 Expression of results

The most probable number of *Salmonella* organisms present in sludges may be expressed on a wet or dry weight basis. Typically, results are reported as the most probable number of *Salmonella* organisms per g, or 10 g, of dried sludge.

B12 Quality assurance

Positive and negative controls should be set up for new batches of media and each batch of samples tested using appropriate reference strains of target bacteria (for example, *Salmonella poona*) and non-target bacteria (for example, *Escherichia coli*) to ensure the validity of analysis carried out.

For larger batches of samples, or for monitoring routine performance, it may be appropriate to examine one or more samples in duplicate. A comparison of the counts obtained can then be undertaken.

B13 References

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Appendix B1 Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 10 g of original (wet) sludge. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values. Approximate 95 % confidence intervals, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published(17). A procedure for estimating these confidence intervals for other dilution series has also been published(18). These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source(19). The MPR in Tables B1 and B2 illustrate those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to the tubes of medium.

Tables B1 and B2 give the MPN (and where applicable the MPR) for an 11-tube series with 1 x 5g, 5 x 1 g and 5 x 0.1 g and a 15-tube series with 5 x 1 g, 5 x 0.1 g and 5 x 0.01 g of original (wet) sludges respectively. Only those values of the more likely combinations of positive and negative reactions are given. For example, in a 15-tube series positive reactions in the 0.01 g tubes would not be expected if all of the tubes containing 1 g and 0.1 g were negative. Hence, MPN and MPR values for a combination of results, like for instance, 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct(17, 20, 21). For example, the organisms may not have been uniformly distributed throughout the sample, or toxic or inhibitory substances may have been present.

Calculation of MPN values

The number of positive reactions for each series of tubes is recorded and, from the relevant table, the MPN of organisms present in 10 g of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in Table B3.

(i) Use only three consecutive series of dilutions for calculating the MPN.

(ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in Table B3).

(iii) If less than three series of dilutions give positive results, begin with the series containing the largest volume of sample (see example (d) in Table B3).

(iv) If only one series of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in Table B3).

Table B1. MPN and MPR per 10 g of original (wet) sludge for an 11 tube series containing 1 x 5 g, 5 x 1 g, and 5 x 0.1 g test sample.

Number of tubes giving a positive reaction			MPN per 10 g	MPR* per 10g
1 x 5 g	5 x 1g	5 x 0.1 g		
0	0	0	None found	
0	0	1	1	
0	1	0	1	
0	1	1	2	
0	2	0	2	
0	2	1	3	
0	3	0	3	
1	0	0	1	
1	0	1	2	
1	1	0	2	
1	1	1	4	
1	1	2	6	
1	2	0	4	4-5
1	2	1	7	6-7
1	2	2	9	9-10
1	3	0	8	7-9
1	3	1	10	10-11
1	3	2	13	12-15
1	3	3	17	15-18
1	4	0	12	11-14
1	4	1	16	15-19
1	4	2	21	19-24
1	4	3	27	24-30
1	4	4	33	30-38
1	5	0	23	20-27
1	5	1	33	29-40
1	5	2	53	44-65
1	5	3	91	75-110
1	5	4	160	134-190
1	5	5	>180**	

*MPR gives counts that are at least 95% as probable as the MPN in being the correct number.

**There is no discrimination when all the tubes show growth; the theoretical MPN is infinity. The true count is likely to exceed 180.

Table B2. MPN and MPR per 10 g of original (wet) sludge for a 15 tube series containing 5 x 1 g, 5 x 0.1 g, and 5 x 0.01 g test sample.

Number of tubes giving a positive reaction			MPN per 10 g	MPR* per 10g
5 x 1 g	5 x 0.1g	5 x 0.01 g		
0	0	0	None found	
0	0	1	2	
0	1	0	2	
1	0	0	2	
1	0	1	4	
1	1	0	4	
1	2	0	5	
2	0	0	4	
2	0	1	5	
2	1	0	5	
2	1	1	7	
2	2	0	7	7-9
2	3	0	11	
3	0	0	7	
3	0	1	9	
3	1	0	9	
3	1	1	13	
3	2	0	13	
3	2	1	16	14-16
3	3	0	16	14-16
4	0	0	11	11-13
4	0	1	14	14-16
4	1	0	16	14-16
4	1	1	20	18-20
4	2	0	20	18-22
4	2	1	25	23-27
4	3	0	25	23-27
4	3	1	31	29-34
4	4	0	32	29-34
4	4	1	38	34-41
5	0	0	22	20-23
5	0	1	29	25-34
5	0	2	41	36-50
5	1	0	31	27-36
5	1	1	43	36-50
5	1	2	60	50-70
5	1	3	85	70-95
5	2	0	50	40-55
5	2	1	70	60-80
5	2	2	95	80-110
5	2	3	120	105-135
5	3	0	75	65-90
5	3	1	110	90-125
5	3	2	140	120-160
5	3	3	175	155-200
5	3	4	210	185-240
5	4	0	130	110-150
5	4	1	170	150-200
5	4	2	220	190-250
5	4	3	280	240-320
5	4	4	345	300-390
5	5	0	240	200-280
5	5	1	350	290-420
5	5	2	540	450-600
5	5	3	910	750-1100
5	5	4	1600	1350-1900
5	5	5	>1800**	

*MPR gives counts that are at least 95% as probable as the MPN in being the correct number.

**There is no discrimination when all the tubes show growth; the theoretical MPN is infinity. The true count is likely to exceed 1800.

Table B3 Examples of the derivation of the MPN from the numbers of positive reactions in a 15-tube series of dilutions*

Example in text	Wet weight of sample (g)					MPN per 10 g
	1	0.1	0.01	0.001	0.0001	
(a)	***5***	***3***	***2***	0		140
(b)	5	***5***	***3***	***2***	0	1400
(c)	5	***5***	***2***	***0***	0	500
(d)	***3***	***1***	***0***	0		9
(e)	***0***	***1***	***0***	0		2

* Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

C The enumeration of *Salmonella* species by membrane filtration with a chromogenic detection medium

Details of this method are included for information purposes only, as an example of the defined substrate techniques that are available. Information on the routine use of this method, or similar methods, would be welcomed to assess their capabilities.

C1 Introduction

Many different serotypes of *Salmonella* species are present, to varying extents, in the intestinal tracts of humans, animals and birds. All members of the genus are potentially pathogenic. Due to their origin they occur widely in sewage and the environment and may be present in significant numbers in sewage sludge. Hence there is a risk, albeit small, that the use of such sludge in agriculture may cause outbreaks of disease due to the transmission of these organisms through the food chain. For these reasons, there is a need to monitor the efficacy of sludge treatment processes and storage practices to control the levels of pathogens in sludge and its application to agricultural land.

Salmonella infections give rise to symptoms of diarrhoea and vomiting, the incubation period varies between 12 - 72 hours and symptoms usually persist for 2 - 3 days. Most cases of infection occur from consumption of raw, or undercooked, food, particularly poultry and food containing eggs. The significance of *Salmonella* species is described in more detail elsewhere(1) in this series.

C2 Scope

This method is an example of the defined substrate techniques that are available and is suitable for the examination of untreated, conventionally treated and enhanced treated sludges. Samples of conventionally treated sludge may include lagoon stored, thickened and mesophilic anaerobic digested sludge. Enhanced treated sludges may include sludge derived from treatment processes such as pasteurisation, thermophilic digestion, lime-stabilisation and composting. Depending on the sludge matrix, different preparative techniques may be required prior to using this method. These techniques are described elsewhere(2) in this series.

Users wishing to employ this method should verify its performance under their own laboratory conditions(3).

C3 Definitions

In the context of this method *Salmonella* species exhibit characteristic growth on chromogenic medium at 36 °C, following a resuscitation stage using tetrathionate broth, and do not produce the enzyme β-galactosidase. These organisms are regarded as presumptive *Salmonella*. They are characterised by the production of bright red colonies due to their ability to ferment propylene glycol with the production of acid and the *in situ* demonstration of C_8-esterase enzyme activity on Rambach agar. *Salmonella* species can also be confirmed using biochemical and serological reactions.

Salmonella species normally conform to the general definition of the family Enterobacteriaceae, and can be further differentiated, biochemically, into four subgroups, subgenus I to IV. Those bacteria of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific

combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

The usual biochemical reactions include production of hydrogen sulphide; indole and urease not being produced; citrate is utilised as a carbon source; lysine and ornithine being decarboxylated. Phenylalanine and tryptophan are not oxidatively de-aminated, and sucrose, salicin, inositol and amygdalin are not fermented.

C4 Principle

A sample of sludge is initially homogenised[2] and then serially diluted. The diluted sludge is filtered through a membrane filter and incubated at a temperature of 36 °C on a sterile glass fibre filter saturated with resuscitation medium comprising tetrathionate broth. After incubating for 24 hours, the membrane filter is further incubated at 36 °C on chromogenic medium (Rambach agar). The membrane filters are then examined after 24 and 48 hours and positive colonies enumerated. Incubation for 48 hours is particularly important for the recovery of some species such as *Salmonella dublin*. The presence of *Salmonella* is indicated by the presence of bright red colonies resulting from the fermentation of propylene glycol. Colonies of other members of the Enterobacteriaceae appear blue, green, violet or colourless due to their inability to ferment propylene glycol. Some produce ß-galactosidase which hydrolyses 5-bromo-4-chloro-3-indolyl-ß-glucuronide in the medium to produce a blue chromophore[4, 5].

Salmonella species may be distinguished from occasional false-positive results (for example given by *Citrobacter* species) and by tests for C_8-esterase enzyme activity using a fluorogenic substrate carried out *in situ* on presumptive colonies on Rambach agar[4, 5]. *Salmonella* colonies exhibit fluorescence when exposed to ultra-violet light at 366 nm.

C5 Limitations

Sludges with high solids content (greater than 20 % m/v) tend to block the membrane filter at minimal dilutions or may mask or inhibit the growth of target organisms. This will limit the level at which *Salmonella* species can be detected and enumerated. The growth of high numbers of non-target bacteria on the membrane filter may inhibit or obscure the growth of salmonellas[6]. There is some evidence to suggest that some *Salmonella* sub-species do not exhibit characteristic growth and that some sub-species could not be distinguished from *E. coli*.

C6 Health and safety

Sewage and sewage sludge samples can contain hazardous or flammable substances. They may also contain pathogenic organisms and are liable to undergo biological action. Consequently, these samples should be handled with care. Gases that can be produced by microbiological activity are potentially flammable and once generated within the sample container will cause the container to become pressurised. Infectious material and/or pathogenic aerosols will, therefore, be of concern and may be potentially hazardous if containers explode. Glass bottles should not be used, wherever possible[7].

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[8] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

Salmonella species are classified as "Hazard Group 2"[9] Extra care should be taken in the isolation and identification of salmonellas due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised. Work involving subculture and handling of cultures should be performed in a separate designated area. Adequate facilities should be in place for disposal and sterilisation of test materials. Ethanolic solutions are highly flammable and when sprayed should be used under local exha4ust ventilation and inhalation of spray avoided.

When ultra-violet lamps are utilised, appropriate eye protection should be used.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

C7.1 Incubator capable of maintaining a temperature of 36.0 ± 1.0 °C.

C7.2 Stomacher and stomacher bags with or without integral mesh filter.

C7.3 Centrifuge capable of maintaining 200 - 300 g for 1 minute.

C7.4 Sterilsed membrane filters, for example 47 mm diameter, cellulose nitrate, 0.45 μm nominal pore size.

C7.5 Course glass fibre filters, for example 47 mm diameter, 2.7 μm nominal pore size.

C7.6 Sterilised filter-housing units and traps.

C7.7 Plate microscope.

C7.8 Blue-daylight filter.

C7.9 Ultraviolet observation lamp or chamber (366 nm).

C7.10 Nebuliser spray.

C8 Media and reagents

Commercial formulations of these media and reagents are available. The performance of all media and reagents should be verified prior to their use.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

C8.1 *Rambach agar* (CHROMagar® Microbiology)[4, 5]

Opaque agar	20 g
Propylene glycol	10.4g
Peptone/Yeast extract	8 g
Chromogenic and selective supplements	2.7 g
Distilled, deionised or similar grade water to	1000 ml

Disperse the agar in the water and heat to 100 °C at atmospheric pressure, stirring until fully

dissolved. Cool to 45 °C prior to pouring plates. Store in the dark between 2 - 8 °C until required.

C8.2 *Modified tryptone soya broth*

Tryptone soya broth base	17 g
Tryptose	3 g
Dextrose	2.5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Bile salts No 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Distilled, deionised or similar grade water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 0.2 and dispense into suitable containers. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Store between 2 - 8 °C until required.

Novobiocin solution

Novobiocin (sodium salt)	1 g
Deionized water	10 ml

Dissolve the novobiocin in the water. Filter-sterilise, using a 0.2 µm filter and syringe. Store between 2 - 8 °C in the dark and use within 4 weeks.

Addition of sterile novobiocin solution (to a final concentration of 40 mg l^{-1}) should be carried out immediately prior to use.

C8.3 *Tetrathionate broth*

'Lab-Lemco' powder	0.9 g
Peptone	4.5 g
Yeast extract	1.8 g
Sodium chloride	4.5 g
Calcium carbonate	25 g
Sodium thiosulphate pentahydrate	40.7 g
Distilled, deionised or similar grade water	1000 ml

Add the ingredients to the water and bring to the boil to dissolve. Cool slowly to 45 °C. This solution may be stored at 2 - 8 °C until required. When ready to use, add 20 ml of iodine-iodide solution:

Iodine-iodide solution

Iodine	6 g
Potassium iodide	5 g
Distilled, deionised or similar grade water	20 ml

Mix well, and add sterile novobiocin to achieve a final concentration of 40 mg l^{-1} immediately before use.

C8.4 *C_8-esterase solution*

4-methylumbelliferyl caprylate in ethanol	1 % (v/v) solution

C8.5 *Phosphate buffered saline solution*

Sodium chloride	8 g
Disodium hydrogen phosphate	1.44g
Potassium dihydrogen phosphate	0.24g
Potassium chloride	0.2g
Distilled, deionised or similar grade water to	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.4 ± 0.2. Dispense the solution in suitable volumes into suitable containers, and cap. Sterilise the solution by autoclaving at 121 °C for 15 minutes. Store between 2 - 8 °C, until required.

C8.6 O*ther media*

Standard and commercial formulations of other media and reagents used in this method include 2N hydrochloric acid.

C9 Analytical procedure

C9.1 *Sample preparation*

Add 225 ml of novobiocin-supplemented modified tryptone soya broth to 25 g of sewage sludge in a suitable container. Mix thoroughly. Alternatively, add 225 ml of phosphate buffered saline solution to 25 g of sewage sludge in a suitable container. Mix thoroughly.

Homogenise the sample using an appropriate technique based on the characteristics of the sludge matrix(2).

After homogenising the diluted sample, lime-treated sludges should be adjusted to a pH value of 7.0 ± 0.2 by the addition of 2N hydrochloric acid(2).

Conventionally treated sludges may be homogenised by stomaching in a stomacher bag. Transfer the diluted sample into a sterile stomacher bag and homogenise.

Transfer the homogenised sample to suitable sterile centrifuge tubes and centrifuge at 200-300 g for 1 minute. Decant the supernatant liquid from the centrifuge tubes and filter through a course glass-fibre filter to remove sediment material.

C9.2 *Sample processing*

The volume of dilutions of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80 colonies. Typically, the filtrate should be serially diluted 10-fold to 1000-fold with novobiocin-supplemented modified tryptone soya broth. This enables the enumeration of up to 100000 (i.e. 10^5) *Salmonellae* per g of original (wet) sludge. Higher levels will require additional dilutions of the filtrate.

Serially dilute the filtrate 10-fold to 1000-fold with novobiocin-supplemented modified tryptone soya broth. Prepare the relevant number of sterile bottles according to the number of selected

dilutions. Add 90 ml of sterile novobiocin-supplemented modified tryptone soya broth to each bottle. Using a sterile pipette, transfer 10 ml of the filtrate to the first bottle containing 90 ml of broth and mix thoroughly. Using a fresh pipette, transfer 10 ml of this diluted filtrate to a second bottle containing 90 ml of broth and mix thoroughly. Continue this process until all appropriate dilutions have been prepared.

C9.3 *Membrane filtration and resuscitation*

For each prepared dilution, filter 10 ml of the diluted sample through a membrane filter housed in a sterile filter-housing unit. Using sterile tweezers, remove each membrane filter from the housing unit and place the membrane filter onto the surface of a glass fibre filter, contained in a Petri dish, saturated with tetrathionate broth. Ensure that no air bubbles are trapped between the membrane filter and the media-saturated glass fibre filter. "Rolling" the membrane filter onto the medium reduces the likelihood of air bubbles becoming trapped. Place a lid on the Petri dish.

Incubate the Petri dishes at 36 ºC for 16 - 24 hours. From a practical point of view, overnight incubation is not detrimental, and enables samples to be processed within a normal working day.

After incubation, the membrane filter is carefully removed, using sterile tweezers, from the Petri dish and transferred to the agar surface of a Petri dish containing Rambach agar. "Rolling" the membrane filter onto the medium reduces the likelihood of air bubbles becoming trapped. Cover the membrane filter with a Petri dish lid. These Petri dishes should then be incubated at 36 ºC for 21 ± 3 hours. After incubation, the membrane filters are examined and colonies counted. The Petri dishes are then returned to the incubator and incubated for a further 21 ± 3 hours. After incubation, the Petri dishes are re-examined. Colonies of some *Salmonellae*, such as *Salmonella dublin*, may only be evident after 48 hours incubation.

C9.4 *Reading of results*

Using a plate microscope, the number of bright red colonies on the membrane filter is counted and recorded.

C9.5 *Confirmation tests*

Confirmation of *Salmonella* against false-positive results (for example, *Citrobacter* species) can be carried out by spraying the membrane filters with 1 % v/v 4-methylumbelliferyl caprylate dissolved in absolute ethanol, and examining the filters under ultra-violet light at 366 nm. Colonies fluorescing within 1 minute are confirmed as salmonellas (demonstrating the presence of C_8-esterase enzyme activity).

Alternative confirmation of *Salmonellae* can also be achieved using biochemical tests, with commercially available test-kits, or serological agglutination tests for somatic and flagellar antigens. In both cases, manufacturer's instructions should be followed.

C10 Calculations

The number of confirmed *Salmonella* at a specific dilution is used to calculate the number of *Salmonella* present per g of original (wet) sludge or per g of dried sludge, as required. The calculation takes into account the volume and dilutions filtered and, for the number of *Salmonella* per g of dried sludge, the percent dry solids content[10]. The following equations may be used:

a) For count, C_w, per g of original (wet) sludge

$$C_w = \frac{N \times b \times d}{a}$$

Where C_w is the number of *Salmonella* in 1 g of the original (wet) sludge;
N is the number of bright red, fluorescing, colonies counted on the membrane filter;
a is volume of sample filtered through the membrane filter (typically, 10 ml);
b is initial dilution factor for the sludge in supplemented medium (in this case, 10); and
d is the dilution factor for the serial dilutions in supplemented medium.

b) For count, C_d, per g dry solids, i.e. count per g of dried sludge:

$$C_d = \frac{C_w \times 100}{e}$$

Where C_d is the number of *Salmonella* in 1 g of the dried sludge;
C_w is the number of *Salmonella* in 1 g of the original (wet) sludge; and
e is the percent dry solids content of the original (wet) sludge.

For example, if 25 g of original sludge is initially diluted 10-fold, and a further two 10-fold dilutions made, and 10 ml of the final dilution taken for filtration, and 32 characteristic colonies counted on the membrane filter, then

$$C_w = \frac{32 \times 10 \times 10 \times 10}{10} = 3200$$

If the percent dry solids content of the original (wet) sludge is 7.5 %, then

$$C_d = \frac{C_w \times 100}{7.5} = 42700$$

C11 Expression of results

The number of *Salmonella* present in sludge can be expressed on a wet weight or dry weight basis. Typically, results are reported as the number of *Salmonella* per g of dried sludge.

C12 Quality assurance

Positive and negative controls should be set up for new batches of media and each batch of samples tested using appropriate reference strains of target bacteria (for example, *Salmonella poona*) and non-target bacteria (for example, *Escherichia coli*) to ensure the validity of analysis carried out.

For larger batches of samples or for monitoring of routine performance it may be appropriate to analyse one or more samples, in duplicate. A comparison of results can then be undertaken.

C13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

3. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

4. Methods for the detection of pathogens in biosolids. UK Water Industry Research, Report No 00/SL/06/05, 2000.

5. New plate media for facilitated differentiation of *Salmonella* species from *Proteus* species and other enteric bacteria. *Applied Environmental Microbiology*, A Rambach, 1990, **56** pp301-303.

6. Evaluation of Rambach agar for detecting *Salmonella* sub-species I to VI. *Applied Environmental Microbiology*, J Kuhn, B Wonde, W Rabsch and R Ressbrodt, 1994, **60**, 2, pp749-751.

7. This text is based on Resolution 74 by CEN TC 292 - Wastes - Working Group 5, the agreed text of which was adopted by CEN TC 30 8 - Characterisation of sludges - for the section on "General Hazards" associated with sludge material and waste.

8. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

9. Categorisation of Pathogens According to Hazard and Categories of Containment. Advisory Committee on Dangerous Pathogens 1995.

10. Standing Committee of Analysts, The Conditionability, Filterability, Settleability and Solids Content of Sludges (1984) A Compendium of Methods and Tests, *Methods for the Examination of Waters and Associated Materials*, in this series, ISBN 0117517879.

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However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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