Appendix B: Methods

30 Jan 2009
Duncan Rouch & Vennessa Fleming

INTRODUCTION

This appendix describes the methods that were developed for use in this project. All methods were validated and results were quality controlled using reference organisms. This collection was assembled from individual method files, for which each method was independently developed, using the best sources of information in each case. Collectively, these methods form the practical manual in our laboratory for analysis of biosolids.

ACKNOWLEDGEMENTS

The recipes for media together with the background information, instructions for use and references were taken directly from the Oxoid website, given below. These sections appear in parenthesis and are in blue print.

www.oxoid.com/au/blue/index.asp

TABLE OF CONTENTS

1. Isolation of *Clostridium perfringens* from biosolids

30-10-07b
From Stephen Smith, 7-07, modified by Duncan Rouch 30-10-07

This method was limited to detecting cells of *Clostridium perfringens*, not spores. (To detect spores the sample should put to 70 ^{o}C for 20 minutes prior to filtration).

Materials & Equipment

- Tryptone Sulphite Cycloserine (TSC) Agar in 55 mm Petri dishes
- Maximum Recovery Diluent (MRD)
- Phosphate Buffered Saline (PBS)
- 0.45 µm 47 mm nitro-cellulose grid filters [Millipore EZHAWG474 with Ez-Pak membrane dispenser]
- Triple-head filtration apparatus: Sartorius [with steel funnels], or Millipore [with disposable Microfil V funnels]
- 1 L sterile RO water (for washing funnels between samples)
- Seward Stomacher 400 and stomacher bags
- Anaerojar (Merck or Oxoid)
- Incubator at 37 ^{o}C, for 2-3 h
- Incubator at 44 ^{o}C, for 48 h
- Test tubes (16 mm), caps, and racks

QC Bacteria

TSC Medium

Positive controls:	**Expected results**
Clostridium perfringens ATCC 13124	Good growth; black coloured colonies.
Negative control:	
Escherichia coli ATCC 25922 *	Inhibited.

(0) **Samples**
Collected samples kept on ice during transit, then stored at 4 ^{o}C until analysis. Analysis performed within 72 h of sample reception.

Membrane filtration method

1. Add 10 g of soil or sludge to 90 mL of Maximum Recovery Diluent (MRD) and
    a. in a stomacher plastic bag, and place in the stomacher machine for 2 minutes at 230 rpm, or
    b. with 10 g of glass beads (4 mm in diameter) in a 250 mL Schott bottle. Shake the bottle on a rotary shaker for 4 minutes at 230 rpm.
2. Straight after mixing, put 10 mL of the diluted sample into a test-tube. Make further 10-fold serial dilutions (10^{-2}, 10^{-3}, 10^{-4}), by taking 1 mL of the mixed initial dilution and place in 9 mL MRD in a test-tube. Mix with a vortex mixer for 5 s. For filtration use only three dilutions: 10^{-1}, 10^{-3} and 10^{-4}.

3. **Filtration**
    2a Use the Sartorius stainless-steel apparatus.
        i. With the funnels on the filter bases, but no filters, add about 20 mL RO water, then open the valves to suck through the water.
        ii. Remove the funnels and flame the filter beds for a few seconds each.
        iii. Aseptically put sterile filters (47 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.

iv. Flame the funnels, by putting the wide end over a Bunsen burner flame for a few seconds, and remember not to put any of your hand over the small end hole, then put back on the filters.
v. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
vi. Apply the vacuum at each head in turn, for a few seconds, then stop the vacuum, again in turn. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness.
vii. Remove the funnels and place each filter onto a 55 mm plate containing TSC Agar.

2b Alternatively use the Millipore apparatus with disposable Microfil V funnels

viii. Place a Microfil V funnel/filter on each head
ix. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
x. Apply a slight vacuum at each head in turn, for a few seconds. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness. If more than a couple of drops of solution remain on a funnel, remove it aseptically and tip the remaining solution onto the filter. Then stop the vacuum, again in turn.
xi. Aseptically remove the funnels and place each filter onto a 55 mm plate containing TSC agar).
xii. Aseptically put sterile filters (47 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.
xiii. Repeat steps ix, x and xi
xiv. For the subsequent lower dilution repeat steps xii, ix, x and xi.

Incubation

1. **Resuscitation:** Place all plates into an Anaerojar (Merck or Oxoid). Open an envelope of Anaerogen and quickly place the sachet into the Anaerojar. Incubate at 37°C for 2-3 hours.
2. **Growth:** Transfer plates to an incubator at 44 °C for 48 hours.

Enumeration

Colonies should be enumerated by counting the black colonies after 48 hours incubation. If many colonies are not black, then count all (as there may have been poor reactions).

Media

TSC agar

- Make up the required amount of Perfringens agar base (Oxoid CM0587) following the manufacturer's instructions and autoclave for 10 minutes at 121 °C.
- When the medium has cooled to 50 °C, add a vial of TSC supplement (Oxoid SR088E) for every 500 mL of medium.
- Pour into sterile plastic 55 mm triple vented Petri dishes.

QC strains

Positive control	
Clostridium perfringens ATCC 13124	Good growth: black coloured growth
Negative control	
Escherichia coli ATCC 25922	Inhibited

Mode of Action

Sodium metabisulphite and ferric ammonium citrate are used as an indicator of sulphite reduction by *Clostridium perfringens*, which produces black colonies in TSC media.

2. Confirmatory tests for *Clostridium*, following isolation on TSC agar

Compiled from UK and Australian standard methods (7, 8).

30-10-07, Duncan Rouch

Confirmation tests

Clostridium perfringens is confirmed by the following reactions:

(i) Non-motile - growth not spread through buffered nitrate-motility medium.
(ii) Nitrate reducing - red colour on addition of nitrate test reagents A and B to buffered nitrate-motility medium.
(iii) Lactose fermenting - yellow colouration of lactose-gelatin medium.
(iv) Gelatin liquefying - contents of the lactose-gelatin medium tube become liquefied.

Depending on the intended purpose of the analysis and the required accuracy, subculture a suitable number of black colonies (however faint). If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be subcultured if more than ten are present.

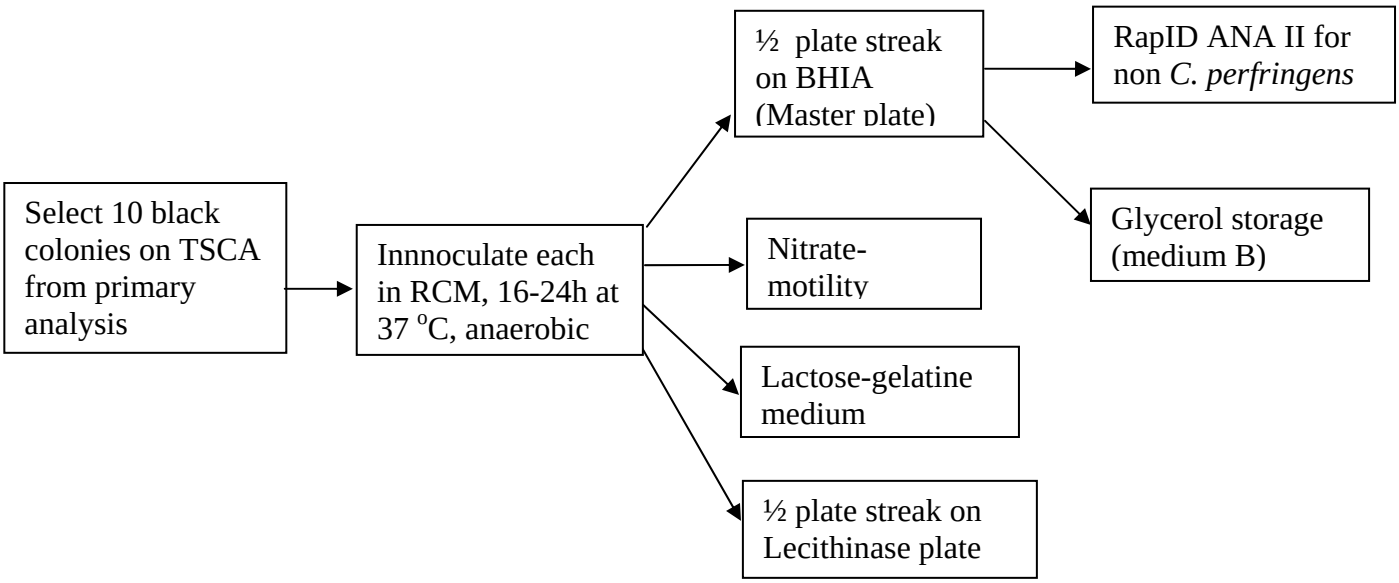
Media

1) Nitrate-motility
2) Lactose-gelatin
3) Lecithinase

Non-C.p. sulphite reducers on TSC

- Remel Inc, RapID ANA II (Oxoid)

Flow chart of confirmation analysis procedure



Media

1) Nitrate-motility

Buffered nitrate-motility medium[6]

- Beef extract (BBL 212303) 3 g
- Bacteriological Peptone (Oxoid LP0037) 5 g
- Potassium nitrate (Sigma 221295) 5 g
- D-Galactose (Sigma G0625) 5 g
- Glycerol 5 g
- Disodium hydrogen phosphate 2.5 g
- Agar 3 g
- Distilled, deionised or similar grade water 1 litre

1. Dissolve the ingredients in the water, adjust pH to 7.3 ± 0.1, and dispense in 10 ml aliquots in 16 mm test tubes.
2. Sterilise the medium by autoclaving at 121 °C for 15 minutes. (The final pH of the medium should be 7.3 ± 0.2)
3. Prepared tubes should be stored at a temperature between 2 - 8 °C for up to one month if protected against dehydration.

Nitrate reduction test reagents

Reagent A

- Sulphanilic acid (Sigma S5263) 1.0 g
- 5N acetic acid 125 mL

Warm gently to aid dissolving.

Reagent B

- N, N-dimethyl α-naphthylamine (or 1-Napthylamine, Sigma N9005) 0.25 g
- 5N acetic acid. 200 mL

To make 200 mL of 5N acetic acid, add 57.5 mL of glacial acetic acid to 142.5 mL distilled water. Dissolve the amine in the acetic acid solution.

The reagents should be stored at a temperature between 2 - 8 °C, protected from light.

2) Lactose/gelatine

Lactose-gelatin medium[4]

- Tryptose (Oxoid LP0047) 15 g
- Yeast extract (Oxoid (LP0021) 10 g
- Disodium hydrogen phosphate 5 g
- Gelatin (Oxoid LP0008) 120 g
- Lactose (Univar, AR grade) 10 g
- Phenol red (Sigma, P4633) (0.4 % m/v solution in ethanol) 25 ml
- Distilled, deionised or similar grade water 1 litre

1. Dissolve the tryptose, yeast extract and disodium hydrogen phosphate in half-volume water at room temperature. Then dissolve the gelatine in half-volume boiled water, stirring continuously with a kitchen wire Wisk or spoon.
2. Mix the two solutions in a beaker and adjust the pH to 7.5 ± 0.2, with 5 M NaOH.
3. Add the phenol red.
4. Dispense in 10 ml aliquots in McCartney bottles, add Durham tubes, and sterilise the medium at 121 °C for 15 minutes. The final pH should be 7.5 ± 0.2 (Phenol Red pKa =7.9).
5. Prepared media may be stored at a temperature between 2 - 8 °C for up to one month, if protected against dehydration.

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, UK.
2. 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, UK.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
4. Enumeration of food-borne *Clostridium perfringens* in egg yolk free tryptosesulphite-cycloserine agar. *Applied Microbiology*, Hauschild, A. H. W. & Hillsheimer, R., 1974, **27**, 521-526.
5. Membrane filtration enumeration of faecal clostridia and *Clostridium perfringens* in water. *Water Research*, Sartory, D. P., 1986, **20**, 1255-1260.
6. Media for confirming *Clostridium perfringens* from food and faeces. *Journal of Food Protection*, Harmon, S. M. & Kautter, D. A., 1978, **41**, 626-630.
7. Standing Committee of Analysts, Microbiology of Drinking Water (2002) – section B, Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and Clostridium perfringens by membrane filtration, Environment Agency, UK.
8. Australian Standards (2000) Water Microbiology. Method 17.1: Spores of sulfite-reducung anaerobes (clostridia) including *Clostridium perfringens* –Membrane filtration method. AS/ANZ 4276.17.1

3) Lecithinase medium

"PERFRINGENS AGAR BASE (TSC AND SFP)

Oxoid: CM0587. A basal medium for use with selective agents to make either TSC agar or SFP agar for the presumptive identification and enumeration of *Clostridium perfringens*.

Formula	gm/litre
Tryptose	15.0
Soya peptone	5.0
Yeast extract	5.0
Sodium metabisulphite	1.0
Ferric ammonium citrate	1.0
Agar	19.0
Final pH 7.6 ± 0.2	

To Prepare Tryptose Sulphite Cycloserine Agar (TSC Agar)
To 500 ml of Agar base cooled to 50°C add the rehydrated contents of 1 vial of TSC supplement, SR0088 and 25 ml of egg yolk emulsion, SR0047. Mix well and pour into sterile Petri dishes".

"EGG YOLK EMULSION

Oxoid: SR0047. A stabilised emulsion of egg yolk for use in culture media. It may be added directly to nutrient media for the identification of *Clostridium, Bacillus* and *Staphylococcus* species by their lipase activity".

"Examination of Bacteria for Lecithinase
After incubation for up to 5 days at 35°C, lecithinase-producers render the broth opalescent, whilst, on the solid medium, their colonies are surrounded by zones of opacity. Egg Yolk Emulsion SR0047 is recommended for use in the preparation of the medium.

References
1 Willis A. T. and Hobbs G. (1959) *J. Path. Bact*. 77. 511-521.
2 Willis A. T. (1977) *Anaerobic Bacteriology 3rd Edn. Butterworths, London*."

Reference: www.oxoid.com/au/blue/index.asp

Additional Media

- Tryptone Water (Oxoid, CM0087)
- Brain Heart Infusion Agar (Oxoid, CM0375)

Methods
1) Subculturing with confirmation tests

1. Sub-culture each black colony to be tested from the membrane filter to one tube of tryptone water (TW), and incubate for 4 h at 37 °C in anaerobic condition.

2. Sub-culture from the TW tube to;
    a. One plate of BHIA, Master Plate, (Each ½ plate streaked for purity test/ RapID ANA II analysis/ glycerol storage).

    b. A tube of nitrate-motility medium, using a thin wire, taking care not to break the agar, and incubate anaerobically at 37 °C for 24 hours. To test for nitrate reduction, add 0.5 mL of nitrate test reagent A and 0.2 mL of nitrate test reagent B to each tube. A red colour indicates nitrate reduction to nitrite.

    c. A tube of lactose-gelatin medium, using a thick or thin wire, and incubate anaerobically at 37 °C for 24-48 hours.
        i. Lactose fermentation is shown by colour change of the indicator from red to yellow, and gas production.
        ii. To assess gelatin liquefaction, after the incubation, place the tubes in a refrigerator for at least one hour. Then observe whether gelatin liquefaction has occurred, shown by flow of the media when tipped.

    d. A plate of TSCA with egg yolk emulsion, with ½ plate streaks, for lecithinase testing and incubate anaerobically at 37 °C for 24 hours. Colonies of lecithinase-producers should be surrounded by zones of opacity within the media.

3. Colonies from the master plate should be sub-cultured and stored in glycerol.

Expected results for *Clostridium perfringens*
1) Nitrate-motility (+, -)
2) Lactose-gelatin (+,+)
3) Lecithinase (+)

2) Analysis of Non-C.p. sulphite reducers detected on TSC Agar

Remel Inc, RapID ANA II (Oxoid)
With also (a) ANA II reagent and (b) spot indole reagent.

Reference
1. Allen S.D. et al., (2003) Clostridium, in Manual of Clinical Microbiology (2003) Ed Murray P.R,. V1, 54: 835-856."

3. Enumeration of *E. coli* in biosolids

Based on The Environment Agency, UK, Methods for Examination of Waters and Associated Material - The Microbiology of Drinking Water, (2002) part 4, Method B, and The Microbiology of Sewage Sludge (2003) Part 3, Method A. Last updated 28-4-08, Duncan Rouch.

Definitions
In the context of this method, organisms which produce acid from lactose, and produce β-glucuronidase forming green colonies on membrane filters after incubation for 4 hours at 30 °C followed by 14 hours at 44 °C are regarded as *E. coli* bacteria.

For the purposes of the examination of water and associated materials, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce β-glucuronidase.

Principle
A sample of sludge is homogenised, serially diluted with maximum recovery diluent and filtered through a membrane filter. The membrane filter is placed on an agar medium and *E. coli* are enumerated on the filter after incubation for 4 hours at 30 °C followed by 14 hours at 44 °C. The agar medium contains lactose, phenol red (as an indicator of acidity) and the chromogenic substrate,
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) either as the cyclohexylammonium salt or the sodium salt, which when hydrolysed, indicates the presence of β-glucuronidase. Colonies that are β-glucuronidase-positive and ferment lactose are regarded as *E. coli*. No further confirmation should be required. If necessary, confirmation tests demonstrating the production of acid from lactose, the formation of indole from tryptophan at 44 °C and an oxidase-negative reaction may be carried out.

Limitations
Enumeration of colonies by this method will exclude a proportion of strains of *E. coli* that are unable to grow at 44 °C, or that fail to ferment lactose. A small number of strains of *E. coli* do not express β-glucuronidase activity on primary isolation or are β-glucuronidase-negative.
This method is not suitable for sludge samples that have been lime-treated or where enhanced microbial reduction is expected. These samples should be examined using an appropriate multiple tube most probable number (MPN) technique. 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 the target organisms. This will limit the level at which *E. coli* will be detected and enumerated. The maximum number of colonies that should be counted on a single membrane filter is approximately 100.

Materials & Equipment
- Membrane lactose glucuronide agar (MLGA)
- Maximum Recovery Diluent (MRD)
- Phosphate Buffered Saline (PBS)
- 0.45 µm 47 mm nitro-cellulose grid filters [Millipore EZHAWG474 with Ez-Pak membrane dispenser]
- Triple-head filtration apparatus: Sartorius [steel funnels], or Millipore [disposable Microfil V funnels]
- 1 L sterile RO water (for washing funnels between samples)
- Seward Stomacher 400 and stomacher bags
- Incubator at 30 ^{o}C, for 2-3 h
- Incubator at 44 ^{o}C, for 14 h
- Test tubes (16 mm), caps, and racks

QC bacteria

Positive controls	**Expected results**
Escherichia coli ATCC® 25922	Good growth; green coloured colonies.
Enterobacter aerogenes ATCC®13048	Good growth; yellow coloured colonies.
Pseudomonas aeruginosa ATCC®27853	Good growth; pink coloured colonies.
Negative control	
Bacillus subtilis ATCC®6633	Inhibited

(1) **Samples**
Collected samples kept on ice during transit, then stored at 4 ^{o}C until analysis. Analysis performed within 72 h of sample reception.

(2) Prepare samples for analysis

a. Using a spoon sterilized in alcohol and flamed, weigh about 10 g of sewage sample into a stomacher bag, and record the actual weight, minimizing movements with the sample.
b. Add 90 mL of Maximum Recovery Diluent (MRD), to provide a 10^{-1} dilution of the sample.
    i. Stomacher processing of wet samples:
        1. Wet samples, as from digestors and drying-pans, can be processed immediately.
        2. Wet solid samples, as in Winter of stockpiles, soak for 30 mins before further processing.
        3. Mix by stomaching at 230 rpm for 2 minutes. Can process up to 4 samples at a time (maximum total volume is 400 mL).
    ii. Glass bead method for dry samples:
    first spray weighing boats with 70% ethanol and leave to dry on a rack.
    As in Summer time of dry stockpiles, break up with 10 g of 4 mm glass beads in a 250 mL Schott bottle. Shake the bottle on a rotary shaker for 4 minutes at 230rpm (Position 4 on Ratek shaker).
c. Straight after mixing, put 10 mL of the diluted sample into a test-tube. Make further 10-fold serial dilutions (10^{-2}, 10^{-3}), by taking 1 mL of the mixed initial dilution and place in 9 mL MRD in a test-tube. Mix with a vortex mixer for 5 s.
d. For filtration use all dilutions: 10^{-1}, 10^{-2} and 10^{-3}.

(3) **Filtration**

2a Use the Sartorius stainless-steel apparatus.

i. With the funnels on the filter bases, but no filters, add about 20 mL RO water, then open the valves to suck through the water.
ii. Remove the funnels and flame the filter beds for a few seconds each.
iii. Aseptically put sterile filters (47 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.
iv. Flame the funnels, by putting the wide end over a Bunsen burner flame for a few seconds, and remember not to put any of your hand over the small end hole, then put back on the filters.
v. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
vi. Apply the vacuum at each head in turn, for a few seconds, then stop the vacuum, again in turn. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness.
vii. Remove the funnels and place each filter onto a 55 mm plate containing membrane lactose glucuronide agar (MLGA).

2b Alternatively use the Millipore apparatus with disposable Microfil V funnels

viii. Place a Microfil V funnel/filter on each head
ix. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
x. Apply a slight vacuum at each head in turn, for a few seconds. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness. If more than a couple of drops of solution remain on a funnel, remove it aseptically and tip the remaining solution onto the filter. Then stop the vacuum, again in turn.
xi. Aseptically remove the funnels and place each filter onto a 55 mm plate containing membrane lactose glucuronide agar (MLGA).
xii. Aseptically put sterile filters (47 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.
xiii. Repeat steps ix, x and xi
xiv. For the subsequent lower dilution repeat steps xii, ix, x and xi.

(4) Incubation

a. Incubate plates at 30.0 ^{o}C for 2-3 h, followed by 44.0 ^{o}C for 14 h.

Enumeration: Reading of results

- After the total incubation period of 18 - 24 hours, examine the membrane filters under good light, if necessary with a hand lens.
- Count all green colonies (however faint) irrespective of size within 15 minutes of being removed from the incubator, as the colouration of the colonies may change on cooling and standing. All green colonies are regarded as *E. coli*.
- It is important to note the relative number of yellow colonies (i.e. non-*E. coli*, coliform bacteria) and pink colonies (i.e. non-target organisms) present on the membrane filter, as these may interfere with the growth and detection of E. coli.
- In addition, any blue colonies (i.e. possibly lactose-negative *E. coli*) should be regarded as *E. coli*.
- The combined count of yellow and green colonies can be regarded as the number of coliform bacteria.
- The specificity of the reactions within the medium means the likelihood of green colonies on MLGA being *Escherichia coli* is very high.
- Following suitable confirmation of performance within the laboratory, confirmation of green colonies may not be needed. Isolation of presumptive colonies is followed by confirmation tests for the production of acid from lactose, negative oxidase reaction and indole formation [1].
- Calculate the number of colonies per 100g dry solids (DS), taking into account the dilutions filtered and the percent dry solids analysis.

Media

"MLGA (Oxoid CM1031)

Formula	g / L
Peptone	40.0
Yeast extract	6.0
Lactose	30.0
Phenol red	0.2
Sodium lauryl sulphate	1.0
Sodium pyruvate	0.5
Agar	10.0
X-Glucuronide (BCIG)	0.2
pH 7.4± 0.2 "	

Make up according to manufacturer's instructions (Oxoid Manual, 9th ed, 2006).

Mode of Action

- The medium contains lauryl sulphate to inhibit Gram-positive organisms.
- Identification of *Escherichia coli* and coliforms is facilitated through two biochemical reactions within the medium:
    - Lactose fermentation is detected by the dye phenol red which gives yellow colonies when acid is produced.
    - The chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (BCIG) is cleaved by the enzyme glucuronidase and produces a blue chromophore which builds up in the bacterial cells.
- Coliforms are lactose-positive so colonies will be yellow; *Escherichia coli* is both lactose-positive and possesses glucuronidase so will appear as green colonies.

References

1. The Environment Agency, UK, Methods for Examination of Waters and Associated Material - The Microbiology of Drinking Water, part 4 (2002) -Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7).
2. The Environment Agency, UK, The Microbiology of Sewage Sludge (2003) - Part 3 - Methods for the isolation and enumeration of Escherichia coli, including verocytotoxigenic *Escherichia coli.*

4a. Confirmation of *Escherichia coli*

Refer to, The Microbiology of Drinking Water (2002) – Section B, Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including E. coli O157:H7). Updated, *Duncan Rouch, 25-2-08*

Introduction: confirmation tests

For the purpose of this analysis and the required accuracy, subculture a suitable number of green colonies (however faint). All green colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be sub-cultured if more are present. The specificity of the green colonies on membrane lactose glucuronide agar being *E. coli* is very high. Occasionally, green (presumptive *E. coli*) colonies may not confirm as *E. coli* but may, nevertheless, confirm as coliform bacteria.

Petri dishes may be stored at 4 °C prior to sub-culturing to allow retention of colour and identification of colony types.

Media

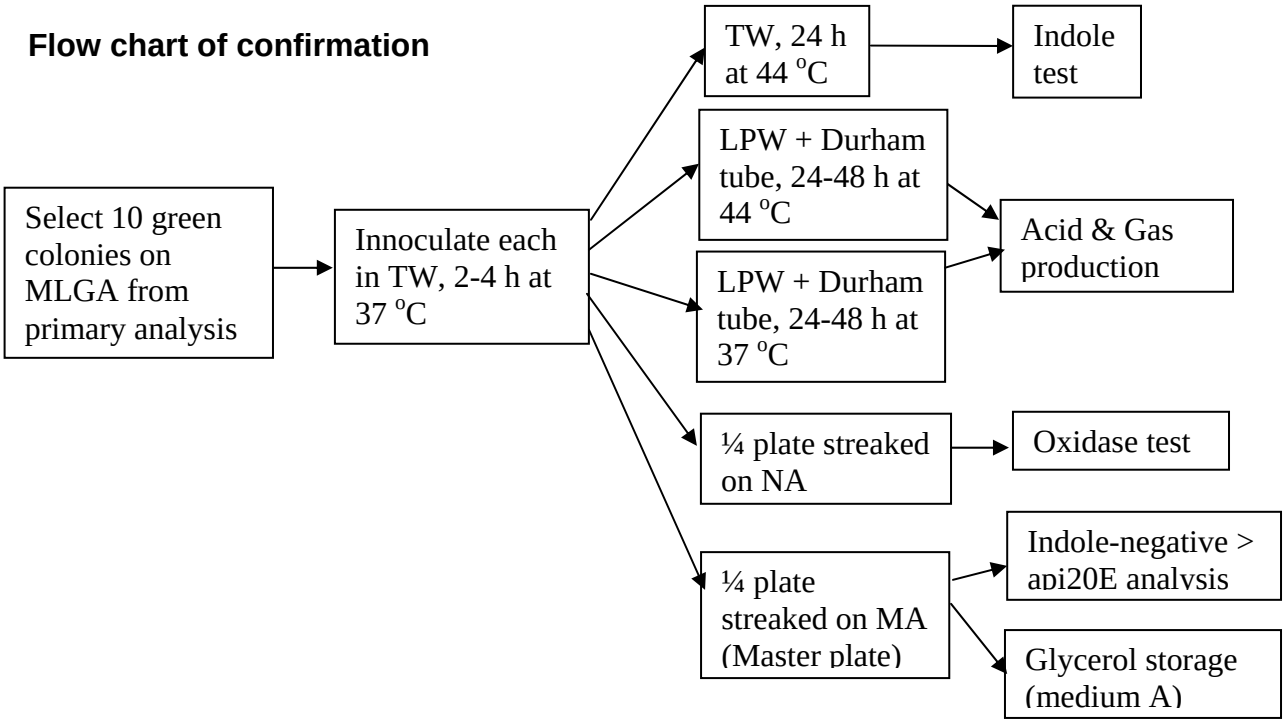
1. Tryptone Water: (for Kovac's Indole test)
2. Lactose Peptone Water (test for acid and gas production)
3. MacConkey Agar
4. Nutrient Agar: (for oxidase test)

Reagents

1. Kovacs' reagent for indole test.
2. Oxidase reagent

Other enterics (MLGA green Non-E. coli isolates) to be identified by api20E (BioMerieux Australia)

Flow chart of confirmation



Where confirmation is deemed necessary, the number of confirmed *E. coli* is calculated as the count of colonies (whether green, yellow or blue) regarded as presumptive *E. coli* multiplied by the proportion of the isolates that confirm. In this case, confirmed colonies are those that are lactose-positive in lactose peptone water at 44 °C, positive for the production of indole in tryptone water at 44 °C, and oxidase-negative.

However, some indole-negative isolates may also prove to be *E. coli*, for analysis by the API 20E method.

Also, on rare occasions, a significant number of isolates from the 37°C incubation may confirm as *E. coli* and the count calculated may be higher that that calculated for the 44 °C incubation. For these examples, the higher count from the 37°C incubation should be reported.

Conversely, the variation in phenotypic properties of confirmed *E .coli* strains can be used to catalogue strains to different biotypes, see Table 1.

Table 1: Properties of different *E. coli* biotype strains from confirmation analysis

TW 44 °C	LPW 37 °C		LPW 44 °C		Identity
(Indole)	Acid	Gas	Acid	Gas	
+	NA	NA	+	+	Presumptive *E. coli* Biotype 1
-	+	+	+	+	Biotype 2
-	+	-	+	-	Biotype 3
+	+	+	-	-	Biotype 4
+	+	-	+	-	Biotype 5

Media

1) Tryptone water for the indole test or (Oxoid CM0087)

"The use of certain peptones that give satisfactory results in tests carried at 37 °C may not be satisfactory for the indole test at 44 °C(6). Care should, therefore, be taken in the appropriate selection of reagents.

- Tryptone 20 g
- Sodium chloride 5 g
- Distilled, deionised or similar grade water 1 litre

Dissolve the ingredients in the water and adjust the pH before autoclaving to 7.5 ± 0.1. Distribute in 5 ml volumes into 16 mm capped test tubes, and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

Quality Control

Positive control:	**Expected result**
Escherichia coli ATCC® 25922*	Turbid growth; indole positive.
Negative control:	
Enterobacter aerogenes ATCC® 13048*	Turbid growth; indole negative.

2) Lactose peptone water*

- Peptone 10 g
- Sodium chloride 5 g
- Lactose 10 g
- Bromocresol Purple (2 % m/v in ethanol) 1.0 mL
- Distilled, deionised or similar grade water 1 litre

Dissolve the ingredients in the water and adjust the pH to 7.25 ± 0.05 (Bromocresol Purple, pKa 6.3). Distribute in 3.5 mL volumes into Bijoux bottles, including Durham tubes. Cap the bottles. Autoclave the bottles at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

***Refer to Microbiology Techniques Manual, pg 121.*

3) “MacConkey Agar (Oxoid CM0007)

Formula	gm/litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4 ± 0.2	

Directions

Suspend 52g in 1 litre of distilled water. Bring to the boil to dissolve completely (Neutral red, pKa 6.8). Sterilise by autoclaving at 121°C for 15 minutes. Dry the surface of the gel before inoculation.

Colonial Characteristics

After 24 hours at 35-37°C typical colonies are as follows:

Organism	Colour	Remarks
Escherichia coli	red	non-mucoid
Aerobacter aerogenes	pink	mucoid
Enterococcus species	red	minute, round
Staphylococci	pale pink	opaque
Pseudomonas aeruginosa	green-brown	fluorescent growth

Quality control

Positive controls:	Expected results
Escherichia coli ATCC® 25922 *	Good growth; red coloured colonies.
Staphylococcus aureus ATCC® 25923 *	Good growth; pale pink coloured colonies.
Negative control:	
Uninoculated medium.	No change “

Reference: www.oxoid.com/au/blue/index.asp

4) "Nutrient Agar
Oxoid CM0003

Formula	gm/litre
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.4 □ 0.2 @ 25°C	

Directions
Powder: Suspend 28g in 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 minutes.

Quality Control

Positive controls:	Expected results
Staphylococcus aureus ATCC® 25923 *	Good growth; straw/white colonies
Escherichia coli ATCC® 25922 *	Good growth; straw colonies
Negative control:	
Uninoculated medium	No change"

"

Reference: www.oxoid.com/au/blue/index.asp

Reagents

1) Kovacs' reagent for the indole test[7] or (Sigma 60983)

- *p*-Dimethylaminobenzaldehyde 5.0 g
- Amyl alcohol (3-methylbutan-1-ol) 75 ml
- (analytical grade reagent free from organic bases)
- Hydrochloric acid (concentrated) 25 ml

Dissolve the aldehyde in the amyl alcohol and slowly add the acid. Protect from light and store at 2 - 8 °C. The reagent should be pale-yellow or straw-coloured after preparation. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

2) Oxidase reagent
N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma T3134, stored at RT in dark), made up as a 1% solution of in water.

Methods

Sub-culturing for Indole and Oxidase Tests

Sub-culture each green colony to be tested from the membrane filter to one tube of tryptone water (TW) for 2-4 h at 37 °C, then vortex before sub-sampling.

Add 100 μL aliquots of the sub-culture to;

a. two tubes of LPW (both with Durham tubes),
b. one tube of TW,
c. ¼ plate streaked on one MA petri dish (for purity test/master plate), and
d. ¼ plate streaked on one NA petri dish (for purity test/ the oxidase test).

Incubate one of the LPW tubes and the TW tube at 44 °C for 24 hours. The other LPW tube is incubated at 37 °C for 24 h, and the three plates placed overnight at 37 °C.

Of the two tubes incubated at 44 ^{o}C after 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube.

Examine the 37 °C LPW after 24 hours for acid production, and if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow.

Incubate the MA and NA at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate.

Colonies from the master plate should be sub-cultured and stored in glycerol.

Typically, *E. coli* colonies are oxidase-negative, produce acid in LPW at 37 °C and at 44 °C, and indole in TW at 44 °C. Tests for β-glucuronidase may assist in the early confirmation of *E. coli* (9, Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

Indole test

After incubation of the TW tubes at 44 °C add 250 µL of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

Oxidase test[11]

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37 °C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction.

The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA.

1. Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the treated filter paper.
2. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

On each occasion where oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example, *E. coli*).

api20E analysis[12]

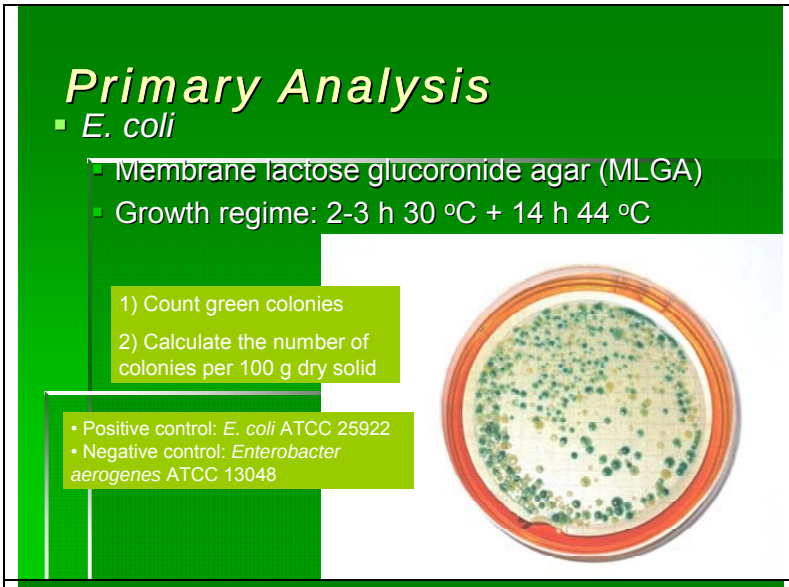
Any isolate that shows green colour on MGLA, but is indole-negative, or negative for acid production at 44 ^{o}C, should be analysed by the api20E commercial method (BioMerieux Australia), following the manufacturers instructions.

Numbered References

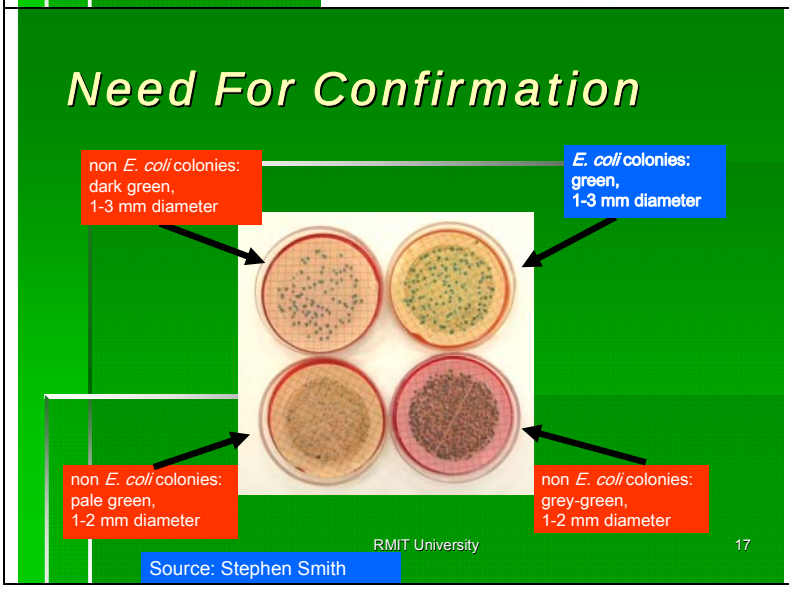
1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
4. A medium detecting □-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water. *Letters in Applied Microbiology*, Sartory, D.P. & Howard, L., 1992, **15**, 273-276.
5. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998*. Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
6. The standardisation and selection of bile salt and peptone for culture media used in the bacteriological examination of water. *Proceedings of the Society for Water Treatment and Examination*, Burman, N.P., 1955, **4**, 10-26.
7. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *Zeitschrift fur Immunitatsforschung und experimentelle Therapie*, Kovàcs, N., 1928, **55**, 311-315.
8. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, Barrow G.I. & Feltham R.K.A.). London, Cambridge University Press, 1993.
9. Fluorogenic assay for immediate confirmation of *Escherichia coli. Applied and Environmental Microbiology*, Feng, P.C.S. & Hartman, P.A., 1982, **43**, 1320-1329.
10. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, Kampfer, P., Rauhoff, O. & Dott, W., 1991, **29**, 2877-2879.
11. Microbiology Techniques Manual, pg 140.
12. Humphrey, N. (1999). A survey of *E. coli* in UK Sludges, report to UK Water Research Limited.

4b. Efficiency of the primary method in detecting *E coli*

Assessment of the selection effectiveness of the Primary *E. coli* method and reduced numbers of *E. coli* biotypes during treatment processes.



Primary *E. coli* assay
The standard primary *E. coli* method is based on the membrane lactose glucuronide agar (MLGA)



Confirmation of *E. coli*
Due to the possible presence of other coliform bacteria confirmation methods were used to assess up to 10 colonies from each sample, as per the UK blue book (2003). The confirmation methods consisted of a range of biochemical assays, using classic assays and api20E commercial method.

Of 184 colonies tested from MLGA, 79% were confirmed as presumptive *E. coli*. Of the 43 atypicals 79% were confirmed as *E. coli* by api20E (bioMerieux), 5% were probable *E. coli*/ coliform and 16% coliform, the latter mostly *Citrobacter* species. Interestingly, the majority of coliforms were detected in a stockpile sample (E7), in which no *E. coli* strains were detected, due to the high removal of pathogen indicators during treatment. Thus, when *E. coli* strains were present they were easily detected by the primary method, but when absent, what was left was the low 'noise' of a few related coliform bacteria.

Calculation of the percentage of green colonies that were shown to be E. coli by confirmatory tests.
Only 7 of 184 isolates were not *E. coli*, indicating 96% confidence that green colonies were correctly identified as *E. coli*. Nevertheless, confirmation should be continued for low-count samples (where no *E. coli* are expected) and for other colonies showing weak green colouration or for WWTPs not previously examined.

Removal Versus Biotype

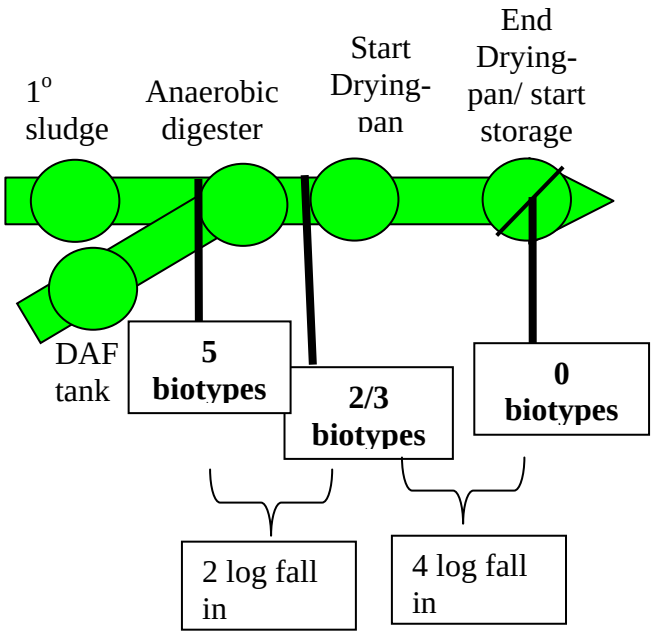
An unforseen advantage of classic confirmation assessment was the detection of *E. coli* biotypes. The classic confirmation included four main methods (a) growth in tryptone water at 44 ^{o}C, (b) acid in lactose peptone water at 37^{o}C, (c) acid in lactose peptone water at 44 ^{o}C, and (d) gas at 44 ^{o}C (Appendix E). Variation in these results provided observation of 8 biotypes, different strains of *E. coli*, see Table B1. These biotype strains were confirmed to be *E. coli*, by api20E (bioMerieux).

E. coli biotypes

TW 44 Indole	LPW 37 Acid 37	LPW 44 Acid 44	Gas 44	Biotypes
+	+	+	+	1
-	+	+	+	2
-	+	+	-	3
+	+	-	-	4
+	+	+	-	5
-	+	-	-	6
+	-	-	-	7
-	-	-	-	8

At Mount Martha five biotypes were observed in primary sludge and DAF samples, on the two occasions of sampling (run 2 13-11-07 and run 3, 3-12-07), while downstream of the anaerobic digester (pump output and drying pan), only two or three biotypes were observed. Given that the anaerobic digester treatment reduced the presence of *E. coli* by about 2 logs, these results suggest that there is variation in the potential survival between different biotypes of *E. coli*, so that a number of biotypes were more sensitive to the stress by the anaerobic digester treatment than others. Thus, the presence of these stress-sensitive strains was reduced more than others. We may conclude that the reduction in number of biotypes is a measure of the removal of *E.coli* by the anaerobic digester.

In addition the number of detectable biotypes at the end of drying-pan treatment must fall to zero, as no *E. coli* are detected at this time. The results are summarized in the diagram below.



***E. coli* removal versus biotype numbers at Mt Martha WWTP**

5. Enumeration of *E. coli* bacteriophages (coliphages)

From Stephen Smith. Modified in line with Souter,Ashbolt and Roser (2000) Methods for Assaying Bacteriophages of Faecal Indicator Bacteria in Environmental Water, Duncan Rouch 7-2-08.

While this method can be used to specifically detect F-specific RNA bacteriophage, in practice it was widened to detect the range of coliphages, due to the low number of bacteriophages observed in biosolids.

Contents

0) Materials and equipment

Strains

- Host strain: *E. coli HS(pFamp)R*
- Positive control sample: Bacteriophage MS2
- Negative control: *Escherichia coli* NCTC 11560

Media

TYGA

Tryptone	10 g
Yeast Extract	1 g
NaCl	8 g
Distilled H_20	1 L
Agar	14 g

Adjust pH to 7.2 +/- 0.1. Autoclave 121 ^oC for 15 mins.

ssTYGA

As for TYGA, except agar 7 g L^{-1} [200 mL to 400 mL aliquots]
After sterilization or re-melting add, per 100 mL media;

Ingredients	200 mL	300 mL	400 mL
1.0 mL of glucose-calcium chloride solution	2.0	3.0	4.0
1.0 mL magnesium sulphate solution	2.0	3.0	4.0
0.40 mL Nalidixic acid solution	0.8	1.2	1.6
1.0 mL Ampicillin solution	2.0	3.0	4.0

TYGB

As for TYGA, except leave out agar

Auxiliary solutions

Glucose – Calcium Chloride Solution

$CaCl_2.2H_20$ 3.0 g
Glucose 10 g
Distillled H_20 100 mL
When dissolved, filter sterilize (0.22 µm).

Magnesium –Sulphate Solution

$MgSO_4.7H_20$ 0.30 g
Distillled H_20 10 mL
When dissolved, filter sterilize (0.22 µm).

Nalidixic acid solution (25 mg mL^{-1})

Nalidixic acid 250 mg
1M NaOH 2mL
Distilled H_20 8 mL
Dissolve the Nalidixic acid in the NaOH solution, add the distilled H_20, and mix well.
Store at 4 ^oC 1 week, or -20 ^oC for 6 months.
Use at 0.4 mL per 100 mL ssTYGA post-autoclaving
Media containing Nalidixic acid can be kept up to 1 month.

Ampicillin solution (15 mg mL^{-1})

Ampicillin 150 mg
1M NaOH 2 mL
Distilled H_20 8 mL
Dissolve the Ampicillin in the NaOH solution, add the distilled H_20, and mix well.
Store at 4 ^oC 1 week, or -20 ^oC for 6 months.
Use at 1.0 mL per 100 mL ssTYGA post-autoclaving.
Media containing Ampicillin can be kept up to 1 week.

RNase (40 µg mL^{-1})

Consumables

- 90 mm petri dishes
- Microfuge tubes
- Tubes: short or full test tubes

Equipment

- Spectrophotometer & plastic cuvettes
- Shaking incubator at 37 ^oC, use the shaking platform in the 37 ^oC room.
- Water bath at 50 ^oC
- Water bath at 60-100 ^oC

Quality Control Bacteria

Strain	Expected results
Positive control	
Escherichia coli NZRM 4027 *HS(pFamp)R*	F-specific plaques (and non f-specific plaques)
Negative control	
Escherichia coli NCTC 11560	No F-specific plaques ('somatic' host) but other type plaques for *E. coli*

Explaining specificity of bacteriophage detection

The F-specific bacteriophages can only cause plaques on NZRM 4027, while somatic bacteriophages that attach to the cell membrane can cause plaques on both NZRM 4027 and NCTC 11560, see diagram below.

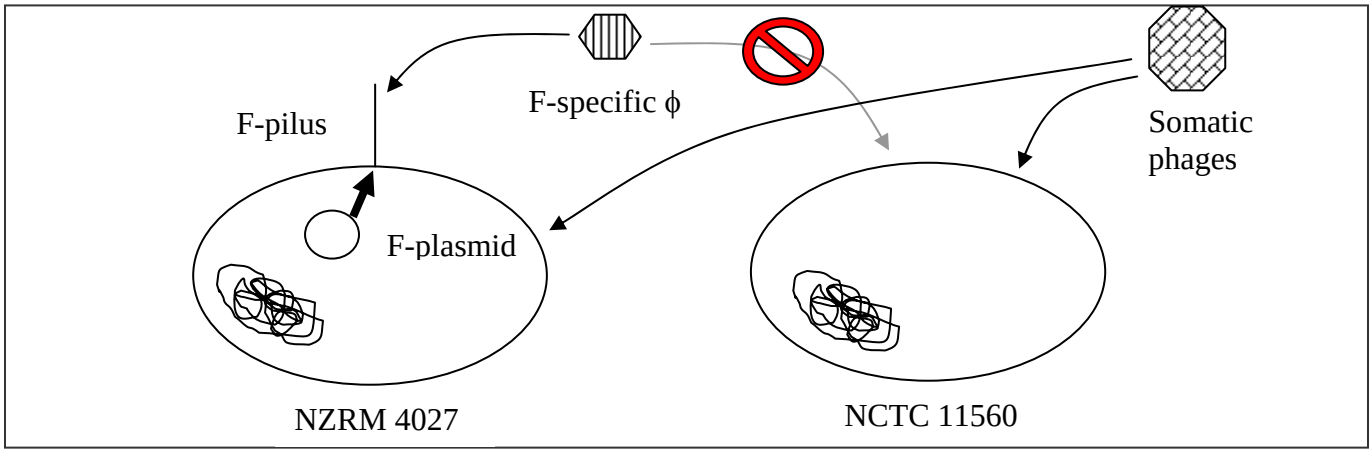


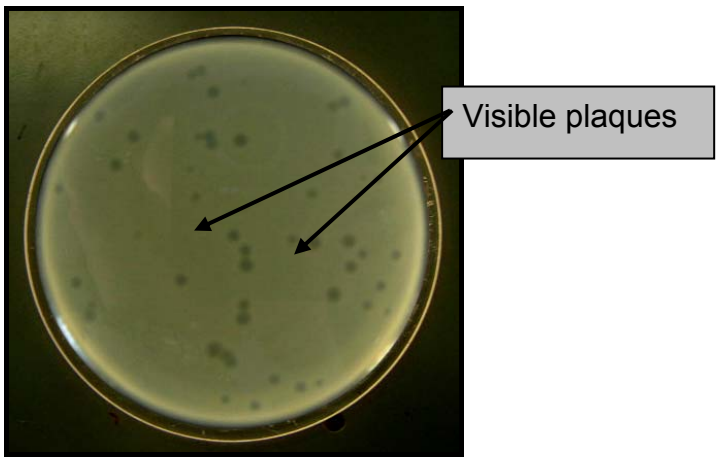
Figure 1. Host ranges of F-specific and somatic coliphages.

1) Standard procedure for phage enumeration (soft agar/host overlay)

- Inoculate an overnight culture in 5 mL TYGB, at 37 ^{o}C.
- Add 0.5 mL of the overnight culture to 50 mL of TYGB in a 500 mL flask. Incubate the culture with shaking at 37 ^{o}C $\pm$ 1.0 ^{o}C for 2 h $\pm$ 1 h.
- Dry the TYGA plates for 20-30 mins in the laminar flow hood, then label with sample name and dilution number.
- After 1 h growth, withdraw aliquots at 30 minute intervals and measure the turbidity at OD600nm.
- Place the culture on melting ice when the turbidity corresponds to a cell density of approximately 4 x 10^{+8}, i.e., OD600 = 0.40 (Figure 1).
- Melt the ssTYGA (0.7% agar) stock at 90-100 ^{o}C in a water bath, add the appropriate amounts of the glucose-calcium solution and magnesium sulphate solution, then reduce the temperature to 60 ^{o}C before making aliquots. Hand dispense 3.5 ml aliquots of ssTYGA, using a 10 mL pipettor (note 5 mL pipettors substantially lose accuracy at high temperatures), into test tubes held in racks in the water bath. When filled, transfer racks, one at a time, to the water bath held at 50 ^{o}C.
- Remove samples and dilutions from the fridge, and allow them to warm up to room temperature.
- Arrange equipment for efficient processing, e.g. put the vortex mixer next to the 50 ^{o}C waterbath, and have a separate pipettor for each solution.
- Perform the following 4 steps rapidly, processing up to 9 tubes at a time,
    - Add antibiotics: 20 uL Nalidixic acid solution, and 48 uL Ampicillin solution.
    - Add 1 mL of each sample (or sample dilution) to triplicate tubes,
    - Add an aliquot of 0.25 mL of host strain to each tube.

- For each tube vortex it briefly, and gently pour the contents over dry 90 mm TYGA plates: move the end of the tube over the plate while pouring at a near horizontal angle, to avoid any bubbles transferring to the plate.

- When the layers have solidified invert the plates and incubate at 37 ^{o}C $\pm$ 1.0 ^{o}C.
- Enumerate zones of clearing (plaques) on the plates after incubation periods of 4 h and 18 h $\pm$ 2 h (Plate 3.6). Native flora from samples may overgrow plaques, so in this case a reading at 4 h is important, see below.



Enumeration of typical zones of clearing (plaques) in samples examined using the standard protocol for F-specific RNA bacteriophage

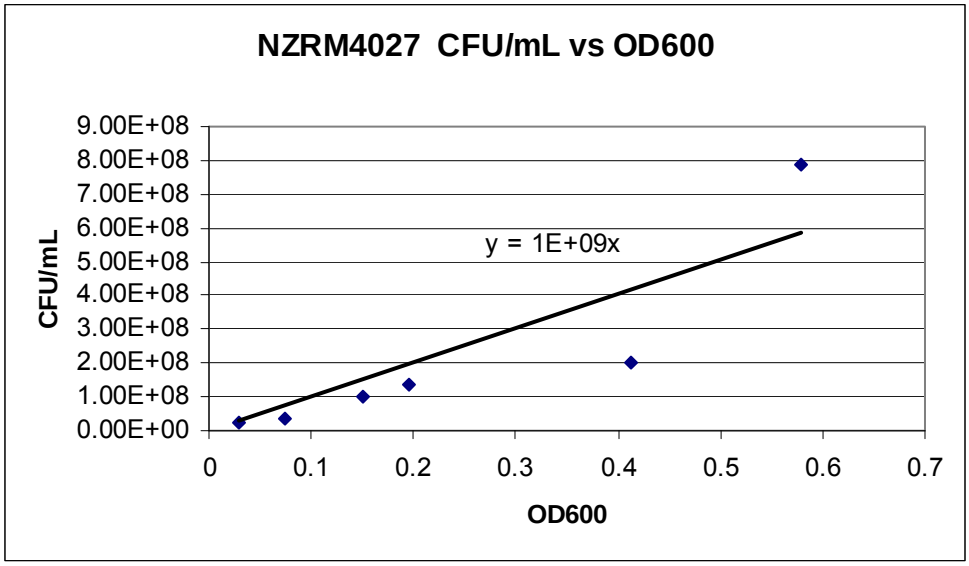
2) Growth of host bacterium

- *E. coli* NZRM 4027 *HS(pFamp)R*

- Culturing and maintenance of strains involved a number of sequential steps:
  - Make a glycerol stock, and keep at -20 ^{o}C (Microbiology Techniques Manual, p 57)
  - Streak on a MAC plate to obtain single colonies, and incubate at 37 ^{o}C overnight, at some time the week before using the phage enumeration method. The culture plate will keep for 1-2 weeks.
  - The night before, select a few lactose-positive colonies from the MAC plate to make an overnight culture in TYGB, 5 mL at 37 ^{o}C.

Calibration of host turbidity measurements

- Set up an overnight culture
- Inoculate a 0.5 mL aliquot into 50 ml TYGB and incubate at 37 ^{o}C $\pm$ 1.0 ^{o}C for 3 h $\pm$ 1 h in a shaking water bath.
- Withdraw samples at intervals of 30 minutes for 3 h and measure turbidity in a 1 mL plastic cuvette with a 10 mm path length using a spectrophotometer set at a wavelength of 600 nm.
- First, adjust the spectrophotometer to zero with a blank sample of sterile TYGB.
- Take a further 1 ml aliquot of the culture and dilute to 10^{-6} cfu ml^{-1} in MRD.
- Enumerate the diluted suspensions by spreading 0.1 ml volumes of 10^{-4} to 10^{-6} dilutions onto tryptone yeast extract glucose agar TYGA plates in triplicate.
- Invert and incubate the plates at 37 ^{o}C $\pm$ 1.0 ^{o}C for 24 h $\pm$ 2 h.
- After incubation, calculate the measure of the host strain as colony forming particles per mL (cfp mL^{-1}).
- Use linear regression of the data to plot the relationship between turbidity and colony counts (Figure 1).
- Subsequently, the standard procedure for inoculum culture will be based on turbidity measurements.



Growth of host strain determined by the relationship between colony number and turbidity

Quality Control of host strain

- At time zero (T0) and after 3 h (T3), perform the enumeration procedure (Section 3.3.5.2) on MAC
- Enumerate the number of lactose-positive and lactose-negative colonies. Calculate the proportion of lactose-negative colonies relative to the total number of colonies recorded.
- Test antibiotic susceptibility using a 0.1 ml aliquot of the 10^{-2} dilution spread onto MAC. Add discs with appropriate antibiotics [For *E. coli HS(pFamp)R*: Amp+, Nal+].
- Incubate plates at 37 ^{o}C $\pm$ 1.0 ^{o}C for 24 h $\pm$ 2 h and measure the inhibition zones surrounding the antibiotic discs.

3) Preparing stocks of F-specific RNA bacteriophage

Prepare a high titre stock culture of bacteriophage MS2 (NCTC 12487) (Adams, 1959).

- Thaw a 0.5 ml aliquot of host strain (working culture) and transfer to 50 ml TYGB and
- Incubate at 37 ^{o}C $\pm$ 1.0 ^{o}C for 18 h $\pm$ 2 h in a shaking water bath.
- Transfer a 10 - 20 µl aliquot of the host culture to sterile culture tubes containing 2.5 mL of semi-solid TYGA (Appendix A.2; A.3) in a water bath set at 45 ^{o}C.
- Overlay the ssTYGA (0.7% agar) onto the surface of dry TYGA plates and allowed to harden.
- Re-hydrate the freeze-dried culture of MS2 with 1 ml of TYGB
- Prepare serial decimal dilutions by transferring 0.1 ml aliquots into 0.9 ml of TYGB.
- Spot each dilution onto the surface of the overlayed TYGA plates and allow them to dry.
- Following incubation at 37 ^{o}C overnight, add 10 - 20 ml of TYGB to the plate of the highest dilution showing confluent lysis.
- Scrape of the soft agar layer with a sterile spreader
- Centrifuge the agar/broth mixture at 1,000 rpm for 25 minutes to sediment cellular debris and agar. Pass the supernatant through a 0.22 µm filter (25 mm disposable syringe filter, Nalgene® Fisher Scientific, Loughborough, UK) into a sterile container

Add glycerol to the suspension and distribute the mixture in 1.2 ml aliquots into microcentrifuge tubes and store -20 ^{o}C $\pm$ 5.0 ^{o}C (long term) or 4 ^{o}C $\pm$ 2.0 ^{o}C (short term).

4) Controlling interferences from background bacterial flora

Typical, operationally produced biosolids, in particular conventionally treated products, contain large populations of background microorganisms.

- Suppress the growth of organisms, which may interfere with the formation of phage plaques, was suppressed by adding 0.2 ml of nalidixic acid solution (Appendix A.2; A.3) to 50 ml of ssTYGA before transfer to the culture tubes.

This procedure was compared with the standard protocol (Section 3.3.5.5) using samples of liquid raw sludge (LRS), DMAD and soil. Nalidixic acid effectively suppressed the background flora and improved the recovery of phage (Table 3.6). Therefore, nalidixic acid was included routinely as part of the standard method for samples examined in the research programme.

Enumeration of F-specific RNA bacteriophage in soil and biosolids using a standard protocol and an adapted method incorporating nalidixic acid to suppress high background flora

	Soil **pfp g^{-1} (log_{10})**	**LRS** **pfp g^{-1} (log_{10})**	**DMAD** **pfp g^{-1} (log_{10})**
4 h incubation			
Standard protocol	<1.00	1.30	<1.00
Nalidixic acid	<1.00	2.08	2.00
18 h incubation			
Standard protocol	<1.00	2.39	1.30
Nalidixic acid	<1.00	2.64	2.00

5) Confirmatory test, quality assurance and expression of results for the standard protocol

In parallel with the standard procedure for each sample, replicate samples were prepared in ssTYGA containing a final concentration of 40 µg mL^{-1} of RNase-solution to inhibit the infection process. For quality assurance, a procedural blank (sterile diluent) and a standard preparation of MS2 were also examined simultaneously.

Plaque forming particles of F-specific RNA bacteriophage in 1 ml samples were calculated using the following equation:

$$C_{pfp} = \frac{(N - N_{RNase}) \times F}{n}$$

C_{pfp} = confirmed number of F-specific RNA bacteriophages per ml

N = total number of plaques counted on host plates

N_{RNase} = total number of plaques counted on host plates with Rnase

F = dilution factor

n = number of replicates

Acknowledgement

We thank Linda Joyce, Microbiology Department, St Vincents Hospital, for antibiotic-resistance profiling of the *E. coli* host strain.

6a. Primary analysis of *Salmonella* by tetrathionate agar/Rambach agar

Brown, K & Keevil, W. (2000) Methods for the detection and enumeration of pathogens in biosolids, UK Water Industry Research Limited, London. Communicated by S. R. Smith, Imperial College, 14-5-07. Modified by Duncan Rouch, 29-1-08.

Materials and Equipment

- Tetrathionate Broth agar
- Rambach agar
- MRD diluent
- PBS diluent
- Sterile RO water
- 0.45 µm 45 mm nitro-cellulose grid filters
- Filtration apparatus
- Seward Stomacher 400 and stomacher bags
- Incubator at 37 ^{o}C, for 24 h + 24 h

Membrane filtration method

(5) **Samples**

Collected samples kept on ice during transit, then stored at 4 ^{o}C until analysis. Analysis performed within 48 h of sample reception.

(6) Prepare samples for analysis

a. Using a spoon sterilized in alcohol and flamed, weigh about 10 g of sewage sample into a stomacher bag, and record the actual weight, minimizing movements with the sample.
b. Add 90 mL of Maximum Recovery Diluent (MRD), to provide a 10-fold dilution of the sample.
    i. Stomacher processing of wet samples:
        1. Wet samples, as from digesters and lagoons, can be processed immediately.
        2. Wet solid samples, as in summer time of stockpiles, soak for 30 mins before further processing.
        3. Mix by stomaching at 230 rpm for 2 minutes. Can process up to 4 samples at a time (maximum total volume is 400 mL).
    ii. Glass bead method for dry samples:
    as in Summer time of dry stockpiles, break up with 2.5-3.5 mm glass beads in a 250ml Schott bottle. Shake the bottle on a rotary shaker for 4 minutes at 230rpm. Centrifuge the soil/sludge solution for 1 minute at 200-300*g*.
c. Make further 10-fold serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}), by taking 1 mL of the mixed initial dilution and place in 9 mL PBS in a glass test-tube with plastic cap. Mix with a vortex mixer for 5 s.

(7) **Filtration**

Use the 3-head 45 mm filter stainless-steel apparatus.

i. With the funnels on the filter bases, but no filters, add about 20 mL RO water, then open the valves to suck through the water.
ii. Remove the funnels and flame the filter beds for a few seconds each.
iii. Aseptically put sterile filters (45 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.
iv. Flame the funnels, by putting the wide end over a Bunsen burner flame for a few seconds, and remember not to put any of your hand over the small end hole, then put back on the filters.

v. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
vi. Apply the vacuum at each head in turn, for a few seconds, then stop the vacuum, again in turn. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness.
vii. Aseptically transfer the filter to a plate of Tetrathionate broth agar.

Incubation

1. Incubate the Tetrathionate agar plates in aerobic condition for 24 hours at 37 °C.
2. Aseptically transfer the membrane filters to fresh plates of Rambach agar.
3. Incubate the filters in aerobic condition for a further 24 hours at 37 °C.

Enumeration

Colonies should be enumerated by counting the pink-red colonies on Rambach agar after 24 hours incubation.

Media

Special requirements

- Water bath at 100 °C
- Water bath at 50 °C

Tetrathionate Broth agar

- Add 46 g of Tetrathionate Broth USA (Oxoid CM671B) and 15 g of bacteriological agar to 1 litre of water and bring to the boil while stirring.
- When the medium has cooled to 50 °C, add novobiocin to a final concentration of 40 mg novobiocin per litre of medium.
- Pour into sterile plastic 55mm triple vented Petri dishes.

Rambach agar (Merck 1.07500.).

- If Merck 1.07500.0001 add 1 vial of liquid mix to 250 mL water in a Schott bottle (if Merck 1.07500.0002, add 1 vial to 1,000 mL)
- Add one vial of nutrient powder and mix by swirling until completely suspended.
- Heat the bottle in a boiling water bath, while carefully shaking the bottle from time to time. The medium is totally dissolved when no visual particles stick to the glass of the bottle: taking 20-25 minutes (Merck 1.07500.0001), or 35-40 minutes (Merck 1.07500.0002).
- When the medium has cooled to 50 °C, add novobiocin to a final concentration of 40 mg novobiocin per litre of medium.
- Pour into sterile plastic 55mm triple vented Petri dishes.

6b. Primary analysis of *Salmonella* by RVS agar/XLD agar

Based on The Blue Book, 'The Microbiology of Drinking Water (2002) - Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques". *Modified by Duncan Rouch, 18-4-08.*

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.

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.

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* or *Salmonella paratyphi*.

Materials and Equipment

- Rappaport-Vassiliadis Soya Peptone Broth Agar (RVSA) (Oxoid CM0866, plus agar)
- Agar (Oxoid LP0011)
- Xylose Lysine Desoxycholate (XLD) Agar (Oxoid CM0469)
- MRD diluent
- PBS diluent
- Sterile RO water
- 0.45 µm 45 mm nitro-cellulose grid filters
- Filtration apparatus
- Seward Stomacher 400 and stomacher bags
- Incubators of 37 ± 1 ^{o}C and 41.5 ± 0.5 ^{o}C.

Membrane filtration method

(8) **Samples**

Collected samples kept on ice during transit, then stored at 4 ^{o}C until analysis. Analysis

performed within 72 h of sample reception.

(9) Prepare samples for analysis

a. Using a spoon sterilized in alcohol and flamed, weigh about 10 g of sewage sample into a stomacher bag, and record the actual weight, minimizing movements with the sample.
b. Add 90 mL of Maximum Recovery Diluent (MRD), to provide a 10-fold dilution of the sample.
    i. Stomacher processing of wet samples:
        1. Wet samples, as from digestors and drying-pans, can be processed immediately.
        2. Wet solid samples, as in summer time of stockpiles, soak for 30 mins before further processing.
        3. Mix by stomaching at 230 rpm for 2 minutes. Can process up to 4 samples at a time (maximum total volume is 400 mL).
    ii. Glass bead method for dry samples:
    as in Summer time of dry stockpiles, break up with 2.5-3.5 mm glass beads in a 250ml Schott bottle. Shake the bottle on a rotary shaker for 4 minutes at 230 rpm. Centrifuge the soil/sludge solution for 1 minute at 200-300 *g*.
c. Make further 10-fold serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}), by taking 1 mL of the mixed initial dilution and place in 9 mL PBS in a glass test-tube with plastic cap. Mix with a vortex mixer for 5 s.

(10) **Filtration**

Use the 3-head 45 mm filter stainless-steel apparatus.

i. With the funnels on the filter bases, but no filters, add about 20 mL RO water, then open the valves to suck through the water.
ii. Remove the funnels and flame the filter beds for a few seconds each.
iii. Aseptically put sterile filters (45 mm 0.45 µm, gridded, cellulose nitrate) on the filter beds, using forceps flamed in alcohol.
iv. Flame the funnels, by putting the wide end over a Bunsen burner flame for a few seconds, and remember not to put any of your hand over the small end hole, then put back on the filters.
v. For each appropriate dilution add 1.0 mL to each of the three filter heads, and add 20 mL of sterile PBS to improve dispersion of samples, starting with the most dilute dilution.
vi. Apply the vacuum at each head in turn, for a few seconds, then stop the vacuum, again in turn. Samples with high particulate content (such as 10^{-1} dilutions) may need to be filtered longer to dryness.
vii. Aseptically transfer the filter to a plate of Rappaport-Vassiliadis Soya (RVS) Peptone Broth Agar.

Incubation

Incubate the Rappaport-Vassiliadis Soya Peptone Broth Agar (RVSA) plates in aerobic condition for 2-4 h at 37 °C, then 18-24 h at 41.5 °C.

Aseptically transfer the membrane filters to fresh plates of X. L. D. agar (XLD).

Incubate the filters in aerobic condition for a further 18-24 hours at 37 °C.

Enumeration

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:

Media

Special requirements

- Hot plate or Water bath at 100 °C
- Water bath at 50 °C

1a. "Rappaport-Vassiliadis Soya Peptone Broth Agar (RVSA)
Oxoid Code: CM0866

Formula	gm/litre	gm/500 mL
Soya peptone	4.5	2.25
Sodium chloride	7.2	3.6
Potassium dihydrogen phosphate	1.26	1.13
Di-potassium hydrogen phosphate	0.18	0.09
Magnesium chloride (anhydrous)	13.58	6.79
Malachite green	0.036	0.018
Agar (LP0011)	12.5	6.25

pH 5.2 ± 0.2 @ 25°C

Directions

- Suspend 26.75 grams in 1 litre of distilled water and heat gently to dissolve, if necessary.
- Assess pH value.
- To 1 litre add 12.5 g Agar.
- Sterilise by autoclaving at 115°C for 15 minutes (special run).

Description

Rappaport-Vassiliadis Soya (RVS) Peptone Broth is recommended as a selective enrichment medium for the isolation of salmonellae from food and environmental specimens. RVS Broth shares with the original formulation[1], the ability to exploit the full characteristics of *Salmonella* species when compared with other Enterobacteriaceae. These are:

1. The ability to survive at relatively high osmotic pressure.
2. To multiply at relatively low pH values.
3. To be relatively more resistant to malachite green.
4. To have relatively less demanding nutritional requirements.

RVS Broth is based on the revised formulation described by van Schothorst *et al.*[2], and is recommended as the selective enrichment medium for the isolation of *salmonellae* from food and environmental specimens. It can also be used to isolate salmonellae from human faeces without the need for pre-enrichment.

RVS Broth is a modification of the Rappaport Vassiliadis (RV) Enrichment Broth described earlier by van Schothorst and Renauld[3]. The modifications to their earlier formula are:

1. The addition of di-potassium hydrogen phosphate to buffer the medium so that the pH is maintained during storage of the prepared broth.
2. Clarifying the optimum concentration of magnesium chloride $6H_2O$.

The two modifications are said to enhance the reliability of the enrichment broth[1]. Peterz *et al.*[4] have also highlighted the importance of the concentration of magnesium chloride in the final medium.

Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.
Store the prepared medium at 2-8°C.
Prepare RVS or RVSA medium has a recommended shelf life of 4 weeks at 4 °C

Appearance

Dehydrated medium: Pale green coloured, free-flowing powder.
Prepared medium: Blue coloured solution.

Quality control

Positive control:	**Expected results**
Salmonella typhimurium ATCC® 14028*	Good growth
Negative control:	
Escherichia coli ATCC® 25922 *	Inhibited"

Performance of RVS broth*

Organism	**Growth [Log(10) increase]**	**Death [Log(10) decrease]**
Salmonella enteritidis ATCC13076	8	-
Salmonella typhimurium ATCC14028	7	-
Salmonella virchow NCTC5742	7	-
Salmonella Poona NCTC4840	8	-
Salmonella abony NCTC6017	7	-
Escherichia coli ATCC25922	-	2
Enterococcus faecalis ATCC29212	-	3

* aerobic incubation at 41 °C for 24 h +/- 3 h (Oxoid, certificate of analysis 605417)

"Precautions

RVS Broth should not be used if *Salmonella typhi* is suspected. In order to achieve optimum recovery it is recommended that the enrichment broth is incubated at 42 ± 1°C.

References

1. Rappaport F., Konforti N. and Navon B. (1956) *J. Clin. Path* 9. 261-266.
2. van Schothorst M., Renauld A. and van Beek C. (1987) *Food Microbiology* 4. 11-18.
3. van Schothorst M. and Renauld A. (1983) *J. Appl. Bact*. 54. 209-215.
4. Peterz M., Wiberg C. and Norberg P. (1989) *J. Appl. Bact*. 66. 523-528".
5. BS EN ISO 6579:2002 Microbiology of food and animal feeding stuffs Horizontal Method for the detection of *Salmonella* species.

***Or* 1b. Rappaport Vassiliadis enrichment broth (9, 10)**

S*olution 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.

2a. "Xylose-Lysine-Desoxycholate Agar (XLD)
Oxoid Code: CM0469

Formula	gm/litre
Yeast extract	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium desoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5
pH 7.4 ± 0.2 @ 25°C	

Directions
Suspend 53g in 1 litre of distilled water. Heat with frequent agitation until the medium boils. **DO NOT OVERHEAT**. Transfer immediately to a water bath at 50 °C. Pour into sterile Petri dishes as soon as the medium has cooled.
It is important to avoid preparing large volumes which will cause prolonged heating.

Description
Xylose-Lysine-Desoxycholate Agar was originally formulated by Taylor[1] for the isolation and identification of *shigellae* from stool specimens. It has since been found to be a satisfactory medium for the isolation and presumptive identification of both *salmonellae* and *shigellae*[2]. It relies on xylose fermentation, lysine decarboxylation and production of hydrogen sulphide for the primary differentiation of *shigellae* and *salmonellae* from non-pathogenic bacteria.
Rapid xylose fermentation is almost universal amongst enteric bacteria, except for members of the *Shigella, Providencia* and *Edwardsiella* genera[1]. Xylose is thus included in the medium so that *Shigella* spp. may be identified by a negative reaction.
Salmonella spp. are differentiated from non-pathogenic xylose fermenters by the incorporation of lysine in the medium. *Salmonellae* exhaust the xylose and decarboxylate the lysine, thus altering the pH to alkaline and mimicking the *Shigella* reaction. However, the presence of *Salmonella* and *Edwardsiella* spp. is differentiated from that of *shigellae* by a hydrogen sulphide indicator.
The high acid level produced by fermentation of lactose and sucrose, prevents lysine-positive coliforms from reverting the pH to an alkaline value, and non-pathogenic hydrogen sulphide producers do not decarboxylate lysine. The acid level also prevents blackening by these micro-organisms until after the 18-24 hour examination for pathogens.
Sodium desoxycholate is incorporated as an inhibitor in the medium. The concentration used allows for the inhibition of coliforms without decreasing the ability to support *shigellae* and *salmonellae*.
The recovery of *Shigella* spp. has previously been neglected despite the high incidence of shigellosis. This has been due to inadequate isolation media[3]. The sensitivity and selectivity of

X.L.D. Agar exceeds that of the traditional plating media e.g. Eosin Methylene Blue, Salmonella-Shigella and Bismuth Sulphite agars, which tend to suppress the growth of shigellae. Many favourable comparisons between X.L.D. Agar and these other media have been recorded in the literature[4,2,5,6,7,8,9,10].

The recovery of salmonellae and shigellae is not obscured by profuse growth of other species[3] therefore X.L.D. Agar is ideal for the screening of samples containing mixed flora and suspected of harbouring enteric pathogens e.g. medical specimens or food products. Chadwick, Delisle and Byer[11] recommended the use of this medium as a diagnostic aid in the identification of Enterobacteriaceae.

X.L.D. Agar, in conjunction with MLCB Agar, is specified for use following enrichment culture in Modified Semi-Solid Rappaport Medium (MSRV) when examining faeces for *Salmonella* spp[12]. It is also used for the isolation of *Salmonella* from food and animal feedstuffs (ISO: 6579: 2002)[13].

Incubate the plates at 35-37°C for 18-24 hours.

Colonial Appearances

Organism	Appearance
Salmonella, Edwardsiella	Red colonies with black centres
Shigella, Providencia, H_2S-negative *Salmonella* (e.g. *S. paratyphi A*)	Red colonies
Escherichia, Enterobacter, Klebsiella, Citrobacter, Proteus, Serratia	Yellow, opaque colonies

Note: Red colonies may occur with some *Proteus* and *Pseudomonas* species.

Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.

Store the prepared medium at 2-8°C.

Prepared XLD Agar has a recommended shelf life of 4 weeks at 4 °C.

Appearance

Dehydrated medium: Straw-pink coloured, free-flowing powder.

Prepared medium: Red coloured gel.

Quality Control

Positive control:	Expected Results (48 hours)
Salmonella typhimurium ATCC® 14028 *	Good growth; red colonies with black centre
Negative control:	
Escherichia coli ATCC® 25922 *	No growth.

References

1. Taylor W.I. (1965) *Am. J. Clin. Path*. 44. 471-475.

2. McCarthy M.D. (1966) *N.Z. J. Med. Lab. Technol*. 20. 127-131.

3. Isenberg H.D., Kominos S. and Sigeal M. (1969) *Appl. Microbiol*. 18. 656-659.

4. Taylor W. I. and Harris B. (1965) *Am. J. Clin. Path*. 44. 476-479.

5. Taylor W. I. and Harris B. (1967) *Am. J. Clin. Path*. 48. 350-355.
6. Taylor W. I. and Schelhart D. (1967) *Am. J. Clin. Path*. 48. 356-362.
7. Taylor W.I. and Schelhart D. (1966) *Appl. Microbiol*. 16. 1387-1392.
8. Rollender M.A., Beckford O., Belsky R.D. and Kostroff B. (1969) *Am. J. Clin. Path*. 51. 284-286.
9. Taylor W. I. and Schelhart D. (1969) *Appl. Microbiol*. 18. 393-395.
10. Dunn C. and Martin W.J. (1971) *Appl. Microbiol*. 22. 17-22.
11. Chadwick P., Delisle G.H. and Byer M. (1974) *Can. J. Microbiol*. 20. 1653-1664.
12. Aspinall S.T., Hindle M.A. and Hutchinson D.N. (1992) *Eur. J. Clin. Microbiol. Inf. Dis*. 11. 936-939.
13. Anon. Microbiology of Food and Animal Feeding Stuffs Horizontal Method for the detection of Salmonella species BS : EN : ISO 6579:2002
14. Weissman J.B., Gangarosa E.J., Schmerler A., Marier R.L. and Lewis J.N. (1975) *Lancet I.* 1898, 88-90“.

Reference: www.oxoid.com/au/blue/index.asp

Or 2b. 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.

Colonial appearance on xylose lysine desoxycholate agar

Organism	**Recovery (%)***	**Characteristic appearance**
Salmonella species (*S. abony* NCTC6017, *S. Arizonae* ATCC13314, *S. enteritidis* ATCC13076, *S. poona* NCTC4840, *S. typhimurium* ATCC14028, *S. Virchow* NCTC5742)	72-100	Smooth red colony 2-3 mm in diameter, typically, with black centre, or wholly black colony.
Edswardsiella	ND	Red colony with black centre
Citrobacter freundii ATCC8090[#]	72	Yellow colony.
Enterococcus faecalis ATCC29212	0	No growth.
Escherichia coli ATCC25922, ATCC8739	0, 2	Yellow, opaque colony.
Enterobacter	ND	Yellow, opaque colony.
Klebsiella pneumoniae ATCC29665	55	Yellow, mucoid colony.
Proteus mirabilis ATCC29906, ATCC12453^	87, 89	Orange/red colony that is irregular and may have small black centre.
Pseudomonas aeruginosa ATCC9027^	89	Red/yellow colony with grey-black centre.
Serratia marcescens ATCC8100	90	Orange/yellow, opaque colony.
Shigella species (*S. Sonnei* ATCC25931, *S. Flexneri* ATCC12022)	74, 83	Small irregular pink-red colony.
Providencia, H_2S-negative *Salmonella (e.g., S. paratyphi A)*	ND	Red colony
Staphylococcus aureus ATCC6538	0	No growth

*, After aerobic incubation at 37 ^{o}C for 24 h (Oxoid, certificate of analysis 562159).
^, Red colonies may occur with some *Proteus* and *Pseudomonas* species.
#, Some *Citrobacter* species may also produce yellow colonies with black centres, according to the Manual of Clinical Microbiology.

Colonies should be enumerated by counting the red colonies with black centres on X. L. D. agar after 18-24 hours incubation.

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.

The desoxycholate inhibits gram-positive organisms. Ferric-ammonium sulphate (indicator) and sodium thiosulfate (sulphur source) allow identification of organisms that produce H_2S, giving the appearance of colonies with a black centre.

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.
11. Isolation of Shigellae I. Xylose lysine agars. New media for isolation and enteric pathogens. *American Journal of Clinical Pathology*, W I Taylor and B Harris, 1965, **44**, pp471-475.
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."

7. Confirmatory tests for *Salmonella* spp

Reference: The Blue Book, 'The Microbiology of Drinking Water (2002) - Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques,' and 'The Microbiology of Sewage Sludge (2004) - Part 4 - Methods for the detection, isolation and enumeration of *Salmonella'. Modified by Duncan Rouch, 25-2-08.*

Salmonella

The presence of salmonella species is confirmed by three biochemical tests.

Media

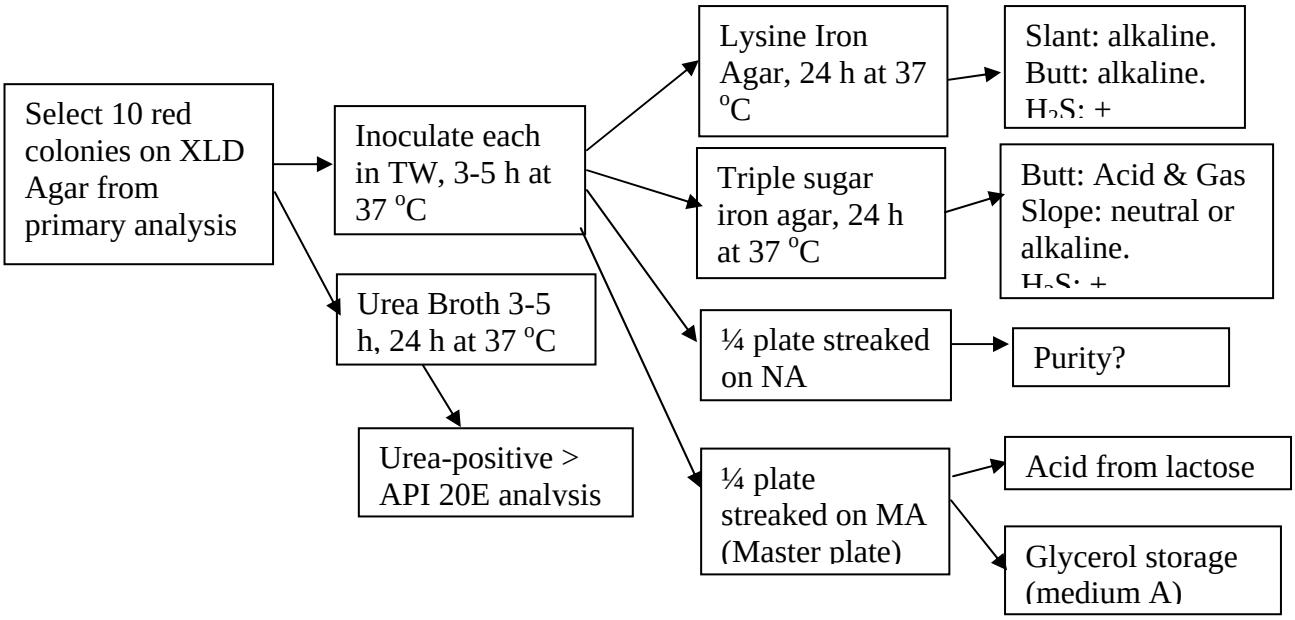
1) Lysine iron agar, (alkaline, Gas +)
2) triple sugar iron agar, (complex +)
3) urea broth (-)

For tube agars (Lysine iron agar, triple sugar iron agar) use test tubes or 15 mL plastic screw-cap tubes.

Other enterics (non-Salmonella sps)

API 20E (BioMerieux Australia)

Flow chart of confirmation analysis



1. “Lysine iron agar

Oxoid CM0381

Formula	gm/litre
Bacteriological peptone	5.0
Yeast extract	3.0
Glucose	1.0
L-lysine	10.0
Ferric ammonium citrate	0.5
Sodium thiosulphate	0.04
Bromocresol purple	0.02
Agar	14.5
pH 6.7 ± 0.2	

Directions
Suspend 34 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Dispense into tubes and sterilise by autoclaving at 121° C for 15 minutes. Cool the tubes in an inclined position to form slants with deep butts.

Description
Lysine Iron Agar CM0381 is a differential medium which detects salmonellae (including lactose fermenting *Salmonella arizonae*) by lysine decarboxylase activity and H_2S production. Edwards & Fife[1] developed the medium to detect lactose-fermenting salmonellae which will produce pink colonies on lactose-containing media e.g. DCA and BGA. In the usual examination for enteric pathogens these organisms would be overlooked. Further, many of these cultures, when transferred to Triple Sugar Iron (TSI) Agar slants, produced acid conditions in the medium so quickly that the expected positive reaction for hydrogen sulphide was suppressed. Since *Salmonella arizonae* strains which ferment lactose rapidly are found occasionally in outbreaks of food infection, it is important to determine their occurrence.
The only recognised groups of Enterobacteriaceae which regularly decarboxylate lysine rapidly and which produce large amounts of hydrogen sulphide, are the salmonellae[2,3].
Lysine Iron Agar is therefore a sensitive medium for the detection of lactose-fermenting and non lactose-fermenting *salmonellae*.

Technique
The medium is tubed, sterilised and slanted so that a short slant and deep butt are formed. It is inoculated with a straight needle by stabbing to the base of the butt and streaking the slant. The caps of the tubes must be replaced loosely so that aerobic conditions prevail on the slant. Incubate at 35°C overnight.

Thatcher & Clark[4] described a procedure for the isolation of *salmonellae* from foods in which suspect colonies from selective agar plates were purified and then inoculated into Lysine Iron Agar and Triple Sugar Iron Agar. Using this combination of media a greater discrimination can be made between the coliform organisms, e.g. *Escherichia* and *Shigella*.

Timms[5] described the techniques of isolation and identification of salmonellae infection in turkeys, using Lysine Iron Agar.

Lysine Iron Agar

Cultures which rapidly produce lysine decarboxylase cause an alkaline reaction (purple colour) throughout the medium. Those organisms that do not decarboxylate lysine produce an alkaline slant and an acid butt (yellow colour).

Cultures which produce hydrogen sulphide cause an intense blackening in the medium.
Due to deamination of the lysine, *Proteus* and *Providencia* cultures produce a red slant over an acid butt.

Reactions

Cultures	**Slant**	**Butt**	**H_2S**
Salmonella	Alkaline	Alkaline	**+**
Proteus	Red	Acid	**-**
Providencia	Red	Acid	**-**
Citrobacter	Alkaline	Acid	**+**
Escherichia	Alkaline	Acid or neutral	**-**
Shigella	Alkaline	Acid	**-**
Klebsiella	Alkaline	Alkaline	**-**

“

Quality control

Positive controls:	**Expected results**
Lysine decarboxylation	
Enterobacter aerogenes ATCC® 13048	Slant: Alkaline. Butt: Alkaline. H_2S: Negative.
Deamination	
Proteus mirabilis ATCC 29906	Slant: Red. Butt: Acid. H_2S: Positive.
Negative control:	
Enterobacter cloacae ATCC® 23355	Slant: Alkaline. Butt: Acid. H_2S: Negative.

Precautions

Salmonella paratyphi A does not produce lysine decarboxylase and therefore will give an alkaline slant and an acid butt.
H_2S-producing *Proteus* species do not blacken this medium[6].

References

1. Edwards P. R. and Fife Mary A. (1961) *Appl. Microbiol*. 9. 478-480.
2.Moeller V. (1954) *Acta. Pathol. Microbiol. Scand.* 355. 259-277.
3. Ewing W. H., Davis B. R. and Edwards P. R. (1960) *Pub. Hlth Labs*. 18. 77-83.
4. Thatcher F. S. and Clark D. S. (1968) *University of Toronto Press,* p.100.

5. Timms L. (1971) *Med. Lab. Tachn.* 28. 150-156.
6. Finegold S. M. & Martin W. J. (1982) *Bailey & Scott's Diagnostic Microbiology. 6th Edn. C. V. Mosby. St. Louis*. p.63l".

Reference: www.oxoid.com/au/blue/index.asp

2. “Triple sugar iron agar
Oxoid CM0277

Formula	gm/litre
`Lab-Lemco’ powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
pH 7.4 ± 0.2	

Directions
Suspend 65 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Mix well and distribute. Sterilise by autoclaving at 121°C for 15 minutes. Allow the medium to set in sloped form with a butt about 1 in.deep.

Description
A composite medium for the differentiation of Enterobacteriaceae according to their ability to ferment lactose, sucrose and glucose, and to produce hydrogen sulphide.
Not only does this medium perform most of the functions of Kligler Iron Agar but, in addition, its sucrose content permits the recognition and exclusion of sucrose-fermenting species. These organisms may ferment lactose slowly or not at all during the incubation period, but they attack sucrose readily. Some *Proteus* and other species may give similar reactions to *salmonellae* and *shigellae* and it is necessary to distinguish them by their ability to hydrolyse urea. For this reason Triple Sugar Iron Agar should be used in parallel with Urea Broth or Urea Agar.
This medium was formerly considered to be interchangeable with Kligler medium for the detection of hydrogen sulphide producing Enterobacteriaceae. It is now thought that Triple Sugar Iron Agar is not suitable for the detection of hydrogen sulphide production by sucrose-fermenting organisms, such as some *Citrobacter* and *Proteus* species, in which the sucrose fermentation masks the hydrogen sulphide indicator in the medium †[1].
Triple Sugar Iron Agar is recommended for the presumptive identification of colonies or sub-cultures from plating media such as Salmonella Shigella Agar (Modified) CM0533, Bismuth Sulphite Agar CM0201, Brilliant Green Agar CM0263, MacConkey Agar No.3 CM0115, or Desoxycholate Citrate Agar (Hynes) CM0227.

Triple Sugar Iron Agar
For samples without urea hydrolysis, examine the Triple Sugar Iron Agar tubes after 18 hours and 48 hours. The following are typical reactions:

Organism	Butt	Slope	H_2S
Enterobacter aerogenes	AG	A	-

Enterobacter cloacae	AG	A	-
Escherichia coli	AG	A	-
Proteus vulgaris	AG	A	+
Morganella morganii	A or AG	NC or ALK	-
Shigella dysenteriae	A	NC or ALK	-
Shigella sonnei	A	NC or ALK	-
Salmonella typhi	A	NC or ALK	+
Salmonella paratyphi	AG	NC or ALK	-
Salmonella enteritidis	AG	NC or ALK	+
Salmonella typhimurium	AG	NC or ALK	+

AG = acid (yellow) and gas formation, **A** = acid (yellow), **NC** = no change, **ALK** = alkaline (red)
+ = hydrogen sulphide (black) †, - = no hydrogen sulphide (no black)
†The presumptive evidence so obtained may be confirmed serologically after sub-culturing the organism from the Triple Sugar Iron Agar slope in Nutrient Broth No.2 CM0067.

Storage conditions and Shelf life

Store the dehydrated medium at 10-30°C and use before the expiry date on the label.
Store the prepared medium at 2-8°C.

Quality Control

Typical reactions of organisms in Triple Sugar Iron Agar.

Organism	**Slant**	**Butt**	**Gas**	**H_2S**
Escherichia coli ATCC® 8739	A	A	+	-
Proteus hauseri ATCC® 13315	A	A	+	+
Pseudomonas aeruginosa ATCC® 9027	ALK	ALK	-	-
Salmonella enteritidis ATCC® 13076	ALK	ALK	+	+

References

1. Bulmash J. M. and Fulton M. (1966) *J. Bact*. 88. 1813.
2. American Public Health Association (1976) *Compendium of Methods for the Microbiological Examination of Foods. APHA Inc. Washington DC.*
3. Edwards P. R. and Ewing W. H. (1972) *Identification of Enterobacteriacea. 3rd Edn. Burgess Publishing Co. Minneapolis. USA*".

3. “Urea broth
Oxoid CM0071

Also Require UREA 40%, Oxoid SR0020

Formula	gm/litre
Peptone	1.0
Glucose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.004
pH 6.8 ± 0.2	

Directions
Add 0.9 g to 95 ml of distilled water. Sterilise by autoclaving at 115°C for 20 minutes. Cool to 55°C and aseptically introduce 5 ml of sterile 40% Urea Solution SR0020. Mix well and distribute 10 ml amounts into sterile containers.

Description
This is a liquid modification of Christensen medium[1]. The modification is suitable for the differentiation of urease- producing organisms from members of the *Salmonella* and *Shigella* groups, during the routine examination of rectal swabs and faeces. Maslen noted that in the routine examination of faeces for *Salmonella* and *Shigella* organisms many non- lactose-fermenting colonies isolated were later found to belong to the urease-positive Proteae. He evolved this medium as a means whereby the latter organisms could be rapidly detected and eliminated - thus saving a considerable amount of time and media. Maslen claimed that the advantages of the fluid medium were:
1. A Pasteur pipette could be used to inoculate other diagnostic media.
2. Rapid growth ensued and it was possible to discern a clear-cut positive reaction within two to five hours at 35°C.
3. It was easier to detect any contamination during storage.

Storage conditions and Shelf Life
Store the dehydrated medium at 10-30°C and use before the expiry date on the label.
Store the prepared medium at 2-8°C.

Quality control

Positive controls:	Expected results
Proteus mirabilis ATCC® 29906	Pink broth; urease positive.
Negative control:	
Escherichia coli ATCC® 25922 *	No colour change; urease negative.

Precautions

It is preferable that the medium be used on the day of preparation. If not, examine the tubes carefully to ensure sterility.

After overnight incubation other members of the Enterobacteriaceae may show alkaline reactions.

Reference

1. Maslen L. G. C. (1952) *Brit. Med. J*. 2. 545-546."

Reference: www.oxoid.com/au/blue/index.asp

Additional Media

Tryptone Water (Oxoid, CM0087)

Methods

Sub-culturing with biochemical tests

Sub-culture each red colony to be tested from the membrane filter to one tube of tryptone water (TW), and one tube of Urea Broth, for 3-5 h at 37 °C. Regard all organisms producing a pink coloration in the Urea Broth (i.e. due to the alkalinity caused by urea hydrolysis) as not belonging to the *Salmonella* or *Shigella* groups, and keep for identification by API 20E (see below). Continue to incubate the Urea Broth Tube, overnight.

Using a straight wire, sub-culture from the TW tube to;

1. lysine iron agar,
2. triple sugar iron agar. For both lysine iron agar and 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 blown out of the tube.
3. Also inoculate one plate of MA (streaked on one for the lactose metabolism/ purity test, and spotted on one as the master plate) and streaked on one plate of NA (for purity test).

Incubate the inoculated media at 37 °C for 18 - 24 hours. Regard cultures that give characteristic reactions (see above) in these confirmatory media, i.e., lysine iron agar and triple sugar iron agar, as presumptive salmonellas.

Colonies from the master plate should be sub-cultured and stored in glycerol.

Other enterics (non-*Salmonella* spp)

API 20E (BioMerieux Australia)

8. Regrowth of bacteria after re-wetting of biosolids

Duncan Rouch, updated 9-5-08

Aim

To establish

(1) Whether *E. coli* or *Salmonella* can regrow in stockpiles, which are wetted by rain
   a. Without added bacteria (test samples for both species)
   b. With added bacteria, separately
      i. *E. coli*
      ii. *Salmonella*

(2) Establish survival of *E. coli* and *Salmonella* in dried stockpile samples
   a. *E. coli*
   b. *Salmonella*

Methods

Preparing moist and saturated biosolids, seeding with bacterial cultures and performing bacterial counts

Materials

- 120 mL sterile red-capped screw-capped plastic tubes

- Strains: from the environment
  - In stationary phase, to promote survival, 16-24 h growth.
  - *Escherichia coli* M34
  - *Salmonella* Dublin

- 20 ^{o}C Room

Bacterial Analysis [see *E. coli* and *Salmonella* analysis methods for details.]

- 0.45 µm 47 mm nitro-cellulose grid filters [Millipore EZHAWG474 with Ez-Pak membrane dispenser]
- Triple-head filtration apparatus: Sartorius [steel funnels], or Millipore [disposable Microfil V funnels]
- 1 L sterile RO water (for washing funnels between samples)
- Seward Stomacher 400 and stomacher bags
- Incubator at 30 ^{o}C, *E. coli* for 2-3 h
- Incubator at 41.5 ^{o}C for 2-3 h for *Salmonella*: then 44 ^{o}C for 14 h for *E. coli*
- Incubator at 37 ^{o}C for *Salmonella* for 18-24 h
- Test tubes (16 mm), caps, and racks

Media

- Nutrient Broth No. 2 (NB)
- Nutrient Agar (NA)
- Maximum Recovery Diluent (MRD)
- Phosphate Buffered Saline (PBS)
- *E.coli* medium: Membrane lactose glucuronide agar (MLGA)
- *Salmonella* media: Rappaport-Vassiliadis Soya Peptone Broth Agar (RVSA) and Xylose Lysine Desoxycholate Agar (XLD)

Samples

- Stockpiles of 1 y only: Composite samples at 3 depths (0-0.2 m, 0.4-0.6 m, 0.9-1.1 m)
- Drying-Pan samples

2 Sites: ETP and Mt Martha (visited Feb 08)

Number of biosolid field samples = 6.

Bacterial Growth

- Set up an overnight culture of each strain in NB, 20 mL scale, from master plates.
- The next day dilute each culture in fresh NB to 5 x 10^8 cfu/mL, by measuring the OD600 values, previously calibrated to plate counts on NA.
    - For *E. coli* M34, adjust to OD600 = 0.20, by diluting by about 0.12 (1.2 / 10).
    - For *S. Dublin*, adjust to OD600 = 0.44, by diluting about 0.3 (3 / 10).
- Dilute the bacterial 1/500 into the biosolid, as required, i.e. 0.12 mL per 60 g biosolid.
- Add 0.12 mL sterile RO water to each sample that is without added bacteria.

Sample Processing

Two moisture conditions are set up per biosolid sample, dried, about 5% MC, and moist, 50±10% MC.

1. Firstly the biosolids are dried at 37 ^oC for about 4 days, about 500 g in sterilized 2 L ice-cream containers.
2. Take 10 g of each biosolid for determining moisture content.
3. For each biosolid measure out 6 x 15 g aliquots to 120 mL sterile red cap containers.
4. Three of these will remain the dry aliquots
5. For moist aliquots: to three of the aliquots add 3 mL sterile RO water and mix.

Six mixtures of aliquots are set up per biosolid: 3 dry and 3 moist,
The three mixtures for both dry and moist biosolids are:

1. Without added bacteria
2. Amended with *Escherichia coli* at 10^6 cfu/ g biosolids:
3. Amended with *Salmonella dublin* at 10^6 cfu/ g biosolids

Mixtures of 2 biosolids are set up per day, so the setup of 6 biosolids is staggered over 3 days. This results in 12 samples to be analysed per day, over 3 days.

Mixtures are held for 4 weeks at 20 ^oC.

Sample Analysis

5 g samples are aseptically taken at 0, 2, and 4 weeks.

1. Samples without added bacteria will be analysed by both the *E. coli* and Salmonella primary analysis methods, including break up by glass beads, 10 g in 90 mL MRD, and triplicate plates at dilutions of 10^{-1}, 10^{-2} +/- 10^{-3} (9 mL MRD diluent, 6 to 9 plates per sample).
2. In contrast, samples with added *E. coli* (mix 2) or Salmonella (mix 3) will analysed by the each specific method, at dilutions 10^{-2} , 10^{-3} , 10^{-4} , then 10^{-1}, 10^{-2} , 10^{-3} (9 plates per sample).

See Xcel sheet for detailed planning.

Number of samples to be analysed per week is 36, to be split over 3 days (12 per day). This is near the maximum rate possible in the current lab, and will require an additional pair of hands, masters and/or third year undergraduate project students.

On week 4, additional 5 g samples may be taken to determine moisture content.

9. Laboratory simulation of N-mineralisation

Based on Harding & Ross (1964), Smith, Woods & Evan (1998), a presentation by Stephen Smith 27-7-07, and additional information from Stephen Smith 19-9-07.
Duncan Rouch, 9-5-08

Purpose

To measure the phyto-available nitrogen and phosphate components of biosolids.

Materials

- Fresh Soil (kept sealed until use at RT, and used within 48 hr of collection), about 900 g per test
- Biosolid samples (to be assayed),
    - For Bioliquids 100 mL per test
    - For Biosolids: 10 g per test, mixed with 90 mL sterile DW.
        - For dried cake (with about 25 % moisture): redissolve 10 g in 90 mL sterile RO water, in a sterile 250 mL Shott bottle, or 120 mL sterile plastic container. Shake on a rotating platform for 4 mins.
        - For highly dried stockpile sample: crush with a mortar and pestle*, to fine particles. Weight 10 g, using a weighing boat*. Spread the 10 g of the particles over the soil, briefly mix with a sterilized spoon*, then add the 90 mL water over the mixture.
- Sterile 2L icecream containers, 1 per soil mix
- Ammonium chloride solution (1% in DW, 100 mL per run)
- Sodium nitrate solution (1% in DW, 100 mL per run)
- SDW, about 90 mL per biosolid sample, and 70 mL per soil aliquot, with 120 mL sterile marked container for measuring.
- 120 mL screw-capped sample containers, 18 per test
- 10-15L storage containers, about 1 per sampling Day
- 10 L white buckets with lids (for soil collection)
- 2 x 1L yoghurt containers, to wash the blades of the blender

* first spray with 70 % ethanol, wipe with a tissue and air dry before use

Equipment

- Measuring spoons and spatulas
- 2 L plastic ice-cream containers, sterilized by autoclaving, 1 per soil mix.
- Balance, 1 kg
- Hand blender, Wizz Mix (Breville, Australia)
- Constant temperature room, 20 ^{o}C
- Freezer, -20 ^{o}C

Soil component

Fresh soil is collected to a depth of 15 cm, to a weight of about 10 kg, kept at RT in the dark, and used within 48 hr of collection. The water holding capacity (WHC) of the soil is determined, and the moisture status adjusted to 40 % of the WHC (see separate method).

One soil type to be used, obtained from Surbiton Park (Western Water), Victoria: from an unfertilized section. The amount of soil required, with moisture of 40 % of the WHC, is 900 g per test.

Experimental testing

Set up an Excel spreadsheet for samples versus days, to help calculate the number of sample containers needed and to number them.

Bioliquids and biosolids are thoroughly but gently mixed with fresh soil to preserve soil structure, with the soil previously amended to a moisture content up to 40 % of the WHC, using a hand blender. Blend at speed 1, to preserve soil globules but do not break them up -so no dry soil is left, about 1 min.
Setup two containers with hot DW to sequentially wash the blades, and replace the liquid in the last container for each mixture. Leave the blades in the first container to soak for 1 min, before spinning briefly, then moving to the second container to spin briefly, the remove and spin again for a few seconds to dry.

Controls

1) Soil but no sludge + 100 mL SDW,
    - (1 x full sampling per soil: 3 replicates x 6 time points =18 per experiment)
2) Soil + ammonium chloride 1%, 100 mL
    - (1x full sampling per soil: 3 replicates x 6 time points =18 per experiment)
3) Soil + sodium nitrate 1 %, 100 mL
    - (1 x full sampling per soil: 3 replicates x 6 time points =18 per experiment)
4) Sludge only, (only 0 day replicates, for each sludge = 13 x 3 replicates = 39 per site)

    - Mineral N treatments, NH4Cl and NaNO3, are applied at a rate equivalent to 200 kg N ha-1. Rates of incorporation are calculated on the assumption that the soils have a bulk density of 1 and the cultivation depth is 10 cm.

The mixtures are separated into 50 g aliquots in 120 mL sterile disposable screw-capped containers, x 3 (number of replicates) x 6 (number of time points: 0, 5, 10, 20 ,40, 60 days) and incubated at 20 ^{o}C in the dark.

- This time period is chosen as NO_3-N production is expected to reach a maximum by approximately 50 days as observed in previous incubation experiments with biosolids at similar temperatures (Smith et al., 1998; Smith and Durham, 2002; Breedon et al., 2003).

At each given removal time point, the triplicate samples will be frozen at -20 ^{O}C, to stop the N mineralization process. Samples will be extracted, using the KCl method for N analysis and Olsen P analysis (see separately), at the end of the experimental sampling period. Extracts to be stored frozen at -20 ^{O}C until ready to be sent for analysis All samples, 10 mL each, will be sent together, at RT, by courier to Adrian Beech, CSIRO Land & Water, Urrbrae, Adelaide, the analytical service. The service will measure content of total N, ammonia, nitrate, biocarbonate extractable P and organic matter content.

References

Breedon, T.J., Bellett-Travers, D.M., & Smith, R.S. (2003). A Laboratory Incubation Study Of Nitrogen Transformations In Biosolids-Amended Agricultural Soil. Report for the EPSRC, the Environmental Agency, Severn Trent Water, Thames Water, Yorkshire Water, and Scottish Water.

Harding, D.E. & Ross, D.J. (1964) Some factors in low-temperature storage influencing the mineralisable-nitrogen sources. J Sci Fd Agric 15: 829-833.

Smith SR, Durham E (2002) Nitrogen release and fertiliser value of thermally-dried biosolids Journal Of The Chartered Institution Of Water And Environmental Management 16 (2): 121-126.

Smith, S.R., Woods, V. & Evans T.D. (1998) Nitrate dynamics in biosolids-treated soils. I. Influence of biosolids type and soil type. Bioresource Technology 66: 139-149.

Biosolids requirement

Biosolid samples are added to fresh soil at a ratio of 1:9 for liquid samples, and 1:99 for solid samples. As the purpose of the biosolid is to provide nitrogen sources, the samples can be frozen prior to use.

These ratios are equivalent to normal rates applied by the Thames Water's 'TERRA ECO-SYSTEMS' recycling to land operation, for 100 m^3 ha^{-1} for liquids (Smith et al., 1998). That is, 1 ha = 10^4 m^2 area * 0.1 m (10 cm) depth = 10^3 m^3. Addition for liquid sludge of 100 m^3 ha^{-1} is 10% volume of 1 ha, thus equivalent to 100 mL liquid sludge per kg soil.

Fresh sludge is added at 50 t ha^{-1} for (Smith et al., 1998). Mechanically dewatered sludges have about 25% DS (dry solids), so at 50 t ha^{-1} for fresh sludge is equivalent to 12.5 t DS ha^{-1}. Round this down to a rate of 10 t DS ha^{-1}, which is equivalent to 10 g DS kg^{-1} soil. Also on this basis, assuming density=1, 1 ha = 10^6 kg, and I t = 10^3 kg, so 10 t = 10^4 kg, which is 1% of the weight of 1 ha.

Samples

Sludges to be sampled (according to the work plan for the project) at:

1. Entry to the drying pan (L)
2. In pans of different aged drying process;
    a. 1 month (L)
    b. 3 months (L)
    c. At harvesting (4-9 months) (L)
3. In stockpiles of different storage ages, with samples at three depths;
    a. 1 year (S x 3)
    b. 2 year (S x 3)
    c. 3 year (S x 3)

Total of 13 samples per site. Two sites to be sampled.
Thus, total number of sludge samples to be analysed: 26

Total number of mixture samples per site 13 x biosolids x 6 time points x 3 replicates = 234, for two sites = 468.

Total control samples = 93 per site/experiment. Thus total samples per site, 234 + 93 = 327

Soil requirement

Total sample volume calculation per sludge example:
18 x main biosolids: 50 g x 3 replicates x 6 time points = 900 g sludge and soil (B 9 g: S 891 g)
8 x main bioliquids: 50 g x 3 replicates x 6 time points = 900 g sludge and soil (L 90 mL : S 910 g)
1 x Controls 1, 2, 3: as above, each 900 g soil only
26 x Control 4: 50 g x 3 = 150 g sludge only

For biosolids: each require 9 g, and total of 18 x 891 g soil = 16,038 g soil
For bioliquids: each require 90 mL, and total of 8 x 910 g soil = 7,280 g soil
For controls require 900 g x 3 controls = 2,700 g soil

Thus, the total soil requirement is 26.018 kg

10. Extraction with potassium chloride for determination of mineral nitrogen (ammonium-N, nitrate+nitrite-N and nitrite-N)

Sources: http://www.ecn.nl/docs/society/horizontal/hor16_annex3.pdf
http://pangea.stanford.edu/research/matsonlab/Protocol/KCLProtocol120700.pdf.

Updated by Duncan Rouch, 9-5-08.

Preparation

1. Materials

- 70 mL white screw cap polypropylene containers (Techno-Plas, P5744UU)
- 15 mL polypropylene centrifuge tubes (Iwaki, 2323-015)
- Sharpie labelling pen
- Reagent grade potassium chloride A.C.S. crystal
- E-pure (or similar) water supply
- 10 mL plastic syringes and 5 µM syringe filters (Millipore, Millex-SV, PVDF, 5.0 µM, SLSV 025 LS)
- Disposable gloves
- Data book

All disposable units used at 1 per sample.

2. Make up 2N potassium chloride in water. This is used at 50 mL per sample.

Table for making 2N KCl

Final vol.	500 mL	1.0 L	2.0 L	5.0 L
KCl (mw 74.56)	74.56 g	149.12 g		745.6 g
Initial vol. milli-Q water	300 mL	600 mL	1.2 L	3.0 L
No. samples	10	20	40	100

Dissolve KCl in the initial volume with a stir bar and magnetic stirrer, then make up to final volume.

Procedure:

1. Remove plant material and rocks greater than 2mm with gloved hand.
    a. Mix well. If the soil is dry, crush clumps and mix. If soil is wet, brake up clumps by hand and mix well.
    b. Always wear a clean glove on the hand used for mixing, and change glove between samples.
    c. After mixing, close container to prevent moisture loss prior to % moisture sub-sampling and weighing for soil wet wt.

2. Label 70 mL (white cap) and 15 mL (mauve cap) containers with sample ID.

3. Record sample number in data book. Always include date, wet wt. of soil (make data table in notebook with 6 columns, leaving space for dry weights in notebook), incubations, time incubated cups need to be extracted (usually 30 min later).

4. Tare 70 mL (white cap) container and weigh out 10 g (+/- 0.1 g) sub-sample of soil, and record exact weight. Change spoon (wash with 70% alcohol) between sample type, treatments and position.

5. Weigh out another 10 g sample into a labelled crucible to determine dry weight (see auxiliary protocol).

6. Add 50 mL of KCL to soil container. Take care the lid is on tightly. Shake 30-60 mins, position 6 on Ratek shaker.

- Measure 50 mL of KCL into two fresh containers, cap immediately and label with date and "Blank".
- Leave containers undisturbed on the bench for 30-60 mins, for most particles to settle.

7. 10 mL of each sample is taken, using a 10 mL syringe and 5.0 µM filter, and put into a 15 mL tube. Tightly cap vials as soon as they are filled. Store samples immediately at -20 ^{o}C until ready for analysis.

8. Pour off leftover KCl from each cup into the KCl waste container (e.g., 2 L flask) and throw the used containers and lids in a yellow autoclave bag. The waste container will also be autoclaved.

9. Analyze KCl extracts for ammonium-N (NH4-N), nitrate-N + nitrite-N (NO3-N+NO2-N), biocarbonate soluble phosphate, by external service. Courier samples overnight on ice to CSIRO Adelaide for analysis.

11. Olsen phosphate extraction method

Duncan Rouch, 9-5-08

Purpose: to extract both organic and inorganic phosphate.

Materials

- 120 mL sterile red cap polypropylene containers
- 15 mL polypropylene centrifuge tubes (Iwaki, 2323-015)
- E-pure (or similar) water supply
- 10 mL plastic syringes
- 5 µM syringe filters (Millipore, Millex-SV, PVDF, 5.0 µM, SLSV 025 LS)
- Disposable gloves
- Data book
- Sharpie labelling pen

All disposable units used at 1 per sample.

Reagent

0.5 M $NAHCO_3$, pH 8.50 (the correct pH is critical to reproducible extraction)
Mw 84.01, so;

- use 42.0 g^{-1} L,
- dissolve in 800 mL, then
- adjust to pH 8.50, with 10 M NaOH solution,
- make up volume to 1.0 L.

Method

1. Break up soil to about 2 mm size grains
2. Weigh out 5 g of soil into a 120 mL (red cap) tube, and record exact weight, to one dp.
3. Add 100 mL reagent, and screw lid on tight
4. Place container on shaker, speed 6, at RT for 30 mins.
5. Settle 30 mins
6. Filter 10mL into a 15 mL (mauve cap) centrifuge tube, using a sterile 10 mL syringe and Millex-SV filter.
7. Store tubes in freezer until being sent for analysis.
8. Pour off leftover KCl from each cup into the KCl waste container (e.g., 2 L flask) and throw the used containers and lids in a yellow autoclave bag. The waste container will also be autoclaved.
9. Analyze bicarbonate soluble phosphate, by external service. Courier samples overnight on ice to CSIRO Adelaide for analysis.

Limitations

The use of the $NaHCO_3$ extractant should be limited to soils of pH between 5.5 and 8.5 containing less than 3 percent of organic matter (http://vric.ucdavis.edu/veginfo/topics/fertilizer/soiltests.pdf).

Reference

Olsen et al., 1954. S.R. Olsen, C.V. Cole, F.S. Watanabe and L.A. Dean, Estimation of Available Phosphorus in Soils by Extraction with Sodium Bicarbonate. United States Department of Agriculture, Circular 939, United States Government Printing Office, Washington, DC, USA (1954).

12. Analysis of biosolids extracts for NH_4-N, NO_3-N and NO_2-N

Julie Smith, CSIRO.

12th June 2008

Generally the methods are those described in the methods manual 'Standard methods for the examination of water and wastewater, 21st ed' published jointly in 2005 by the American Public Health Assoc., the American Water Works Assoc. and the Water Environment Federation. These are described below as APHA with a specific method number.

- Ammonia-Nitrogen [Water quality – determination of ammonium nitrogen by flow analysis (CFA and FIA) and spectrometric detection (1997) International Standard ISO 11732]. The sample is buffered to pH 12 and reacted with sodium salicylate and sodium nitroprusside before reaction with sodium dichloroisocyanurate solution (DCIC) in the automated segmented flow analyser (Alpkem Flow Solution 3). After reaction at 37°C, the intensity of the colour is measured at 640nm and the NH_4-N concentration calculated from a set of calibration standards measured at the same time.

- Nitrate (plus nitrite) nitrogen [APHA method 4500-NO_3^- F]. In this segmented flow analysis method nitrate is reduced to nitrite in an open tubular cadmium column in an atmosphere of helium then reacted with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD) in phosphoric acid. The pink colour formed is determined colorimetrically using the automated segmented flow analyser (Alpkem Flow Solution 3) at 540nm. The NO_3-N (also referred to as NO_X-N indicating the sum of nitrite and nitrate nitrogen) concentration is calculated from a set of calibration standards measured at the same time.

- Nitrite nitrogen [APHA method 4500-NO_2^-B] uses the same method as for NOx-N but without the cadmium reducing column.

13. Analytical method for phosphate presence

Smith, Julie (CLW, Urrbrae)

Dissolved reactive phosphorus [APHA method 4500-P F]. This is often referred to as molybdate reactive phosphorus. This is also a segmented flow method (Alpkem Flow Solution 3). The sample reacts with ammonium molybdate and potassium antimony tartrate at pH 1 and reduced with ascorbic acid solution then heated to 37°C to produce a blue colour measured at 880nm. The phosphate concentration is calculated from a set of calibration standards measured at the same time.

14. Dry weight determination of biosolids

Based on soil dry matter content analysis method:
http://www.rothamsted.ac.uk/aen/smbweb1/methods.php?id=896
Duncan Rouch, 2-10-07

Materials & Equipment

- Ceramic crucibles of 25 mL size or 30 mL, (3 per sample)
- Metal oven trays
- Maker pen
- 2 Measuring spoons
- Balance (to measure weights to 2 decimal places)
- Oven set at 105 ^{o}C
- Tray containing orange silica gel

Method

1) Label each crucible with marker pen (minimum 3 replicates per sample) and weigh to at least 2 decimal places. Record the weight (crucible weight).

2) Fill each crucible with fresh biosolids. You must weigh out at least 10 g, with filling up to 1 cm from the top. Do this in triplicate for each sample.

3) Wipe the outside of each crucible with tissue paper, ensuring that it is entirely free of sample, tare then weight (sample wet weight).

Place the crucibles in a metal tray, and put them in an oven pre-heated to 105 ^{o}C, along with a tray of some orange silica gel. Heat the soils at 105 °C for 24 h. Then switch the heat off, leaving the crucibles in the oven until they are near room temperature (about 1 h), and record the weights (total dry weight = crucible plus sample dry weight).

Calculations

4) Assemble values of variables.

- Fresh weight of sample = fresh biosolids weight
- Dry weight of sample = Total dry weight (weight of crucible + dry biosolids) – weight of crucible

5) Sample dry matter content (DM) is calculated from:
DM% = (Dry weight of sample / fresh weight of sample) x 100%

6) Sample moisture content (MC) is calculated from:
MC% =100 – DM%

15. Water holding capacity

According to Harding and Ross (1964). Communicated by Stephen Smith 19-9-07. Version 24-1-08, Duncan Rouch.

Background

Moisture content and water holding capacity of biosolids

The moisture content of biosolids is defined as the actual water percentage of the total weight. In contrast, the water holding capacity (WHC) of biosolids is defined as the maximum water content that biosolids can carry while remaining in solid form. Biosolids tend to have significantly higher WHC values than soil, so that in land application biosolids may provide improved WHC of the soil, to support growth of crops and horticultural plants under drying weather and climatic conditions.

Moisture content

Moisture content of samples from pans and stockpiles (%)

	Pans	Stockpiles
Mean	90.0	37.5
Median	94.1	37.6
Min	63.1	6.2
Max	98.5	73.2
No. samples	23	17

The average moisture content of stockpile samples in this project was 37.5% (mean) or 37.6% (median), with moisture in samples ranging from 6.2% to 73.2%.

Water Holding Capacity

Water holding capacity of biosolids averaged 83.0%, ranging from 29.0%, for a surface sample of a 2006 stockpile (E8), to 257.0% for a pre-harvest pan sample (M5a). In comparison, soil samples exhibited a water holding capacity of about 30%. This suggests that the water holding capacity of stockpiles may reduce during storage, especially on the surface layer.

Water Holding Capacity (%)

Average	83.0
min	29.0
max	257.0
No. samples	9

In application to land these results indicate the importance of biosolids for holding water for plants and crops.

Aim

Assay of water holding capacity of biosolids/soils, in particular for providing percentage water content of biosolids/soils in N-mineralization analysis, and regrowth analysis.

Materials & Equipment

- Glass funnel, with end stopper, 1 per soil sample
- Clamp stand, to hole funnel
- 100 mL beaker, to collect water drained from funnel
- Plastic wrap
- Whatman 150 mm No. 1 filter paper, 1 per soil sample
- Crucibles, 6 per soil sample
- Drying oven set at 105 ^{o}C

Procedure

1. Samples of approximately 50g of soil, with stones over 2 mm removed, are placed in a small funnel, with the end blocked, and lined with a Whatman 150 mm no. 1 filter paper. Fill the funnel with water until the level is higher than the level of the soil to allow the soil to become saturated.
2. The saturated soil samples are left to stand for 30 mins to achieve equilibrium.
3. The funnel is unblocked, to allow the water to drain. Continue draining for 30 mins, covered with plastic wrap to avoid evaporation.
4. Three 10 g sub-samples of saturated soil (record as mass of drained soil) are oven dried overnight at 105 ^{o}C for 24 hours, to constant weight (record as mass of oven dried soil).
5. If you plan to alter the moisture content of the soil, also directly weigh three 10 g sub-samples, 'as is', with stones over 2 mm removed, and without added water, to determine the baseline for changing the moisture status.

Calculations

The water holding capacity is calculated as follows:

WHC (%) = (mass of drained soil - mass of oven dried soil)/ (mass of oven dried soil)
Determine Mean and Standard Deviation of triplicate WHC values.

Example calculations are shown in the Appendix.

Application Note

Water is added to fresh soils to reach approximately 40 % of water holding capacity, which is within the standard range used for laboratory incubation experiments (Harding and Ross, 1964; Smith et al., 1998). See Appendix A for how to calculate the amount of water to add.

References

Harding, D.E. & Ross, D.J. (1964) Some factors in low-temperature storage influencing the mineralisable-nitrogen sources. J Sci Fd Agric 15: 829-833.

Smith, S.R., Woods, V. & Evans T.D. (1998) Nitrate dynamics in biosolids-treated soils. I. Influence of biosolids type and soil type. Bioresource Technology 66: 139-149.

Example calculations

Table 2. Dry Weight Analysis

	Sample No.	Crucible No.	Weight (g)	Sample Weight (g)	Total Dry weight	Sample Dry Weight	DM%	MC%	WHC%
Wet	a	11	28.39	10.47	36	7.61	72.7	27.3	37.6
	b	12	31.22	11.12	39.26	8.04	72.3	27.7	38.3
	c	13	31.06	10.49	38.7	7.64	72.8	27.2	37.3
	Ave		30.22	10.69	37.99	7.76	72.61	27.39	37.73
As is	a	14	31.3	10.02	40.64	9.34	93.2	6.8	7.3
	b	15	32.33	10.07	41.72	9.39	93.2	6.8	7.2
	c	16	33.58	10.18	43.08	9.5	93.3	6.7	7.2
	Ave		32.40	10.09	41.81	9.41	93.26	6.74	7.23

The WHC is 37.73%
Adjust the moisture status to 40% of the WHC
(0.4*.3773*100):
15.09 % WHC
That is, 15.09 g water per 100 g total
Soil already has 7.2 g water per 100 g total
So we
add 7.86 g water per 100 g total
That is, 7.86 g
water + 92.14 g soil