7.10.4.3 Procedure

Heat the sample to 75°C in a water bath and maintain at this temperature for 1
After heat treatment, make any dilutions necessary as described in section 7.4

Add the 50 mL and 10 mL test volumes to equal volumes of double-strength me
the 1 mL volumes to 25 mL of single-strength medium.

Top-up with single-strength medium so as to leave a minimum amount of air

**A considerable amount of gas may be produced in this test especially for th
volumes; the use of stout glass or plastic bottles is advisable. Each of these s
placed inside its own plastic bag in order to contain any breakage or spillag
incubation.**

Incubate the bottles at 37°C for 48 hours. A positive reaction is shown by blackeni
medium caused by reduction of sulphite and the precipitation of iron(II) sulphide.
made at this stage represents the MPN of spores of sulphite-reducing clostridia

7.10.4.4 Confirmation of Cl. perfringens

Transfer a loopful (0.02 mL) from each bottle showing a positive reaction to a
LMM or CMM which has been freshly steamed, to expel dissolved oxygen, and
Growth is improved in the litmus milk by adding to each tube a nail or a short le
iron wire sterilized by heating to redness immediately before inoculation. Altern
the tubes may be incubated in an anaerobic jar or cabinet.

Incubate at 37°C for 48 hours. Tubes containing *Cl. perfringens* will show a 'storm
reaction, in which, as a result of lactose fermentation, the milk is acidified and coag
and the clot is disrupted by gas and often blown up the tube.

From the number of tubes showing a positive reaction, calculate the MPN
perfringens spores in 100 mL of the sample by reference to the appropriate ta
Appendix C.

7.11 Colony Count

7.11.1 Introduction

The colony count of heterotrophic bacteria may be enumerated by the pour or spread
methods using Yeast Extract Agar (YEA) or R2A Agar (Reasoner and Geldreich
Plates are incubated at 22°C and 37°C as detailed in section 7.11.6. The significance
results obtained is discussed in section 3.6.1.

The most useful application of the colony count is to detect change, especially su
change, in the bacterial content of waters. Comparisons between results for a given
can only be made if the same method is used each time. The method should be stated
report. Colony counts at 37°C are useful to assess the quality of relatively unpol
groundwaters and can provide an early indication of more serious pollution.

7.11.2 Definition

In the context of these methods, colony forming units (CFU) comprise bacteria, yeasts
moulds capable of growth under the conditions specified.

7.11.3 Choice of medium

The pour plate method using YEA is used in the UK for compliance monitoring.

For special investigations such as complaints of taste or odour it may be advantageou
use R2A Agar, or the spread plate method using either medium. R2A Agar is a
nutrient medium which enhances the recovery of disinfectant-damaged organisms
those organisms with a low nutrient requirement which may be inhibited by the hig
concentrations of nutrient in YEA. The spread plate method will give better recovery t
the pour plate method as organisms are not heat shocked by the addition of molten ag
Furthermore, subculture from colonies is facilitated by use of the spread plate meth

7.11.4 Colony count by the pour plate method

7.11.4.1 Principle

Mixing of test volumes of the water sample with molten YEA or R2A Agar in Petri dishes. Incubation under the conditions specified and a count of the colonies that develop.

7.11.4.2 Procedure

Prepare the sample as described in section 7.4, making any necessary dilutions. Starting with the most dilute sample solution, pipette a 1 mL volume of each dilution (and the original sample) into separate, empty sterile Petri dishes.

Pour about 20 mL of molten agar, pre-cooled to 45–50°C, into each Petri dish within 20 minutes of dispensing the 1 mL sample volumes and cover the dish with a lid. If the medium has been cooled in a waterbath, wipe off excess water from the container prior to pouring so as to avoid contamination from the side of the container. Mix the sample and medium by rapid but gentle to and fro circular movements for approximately 10 seconds. Lift the lid slightly during mixing so that no solution adheres to the lid and keep the Petri dish flat on the bench. Allow the solution to solidify and incubate in an inverted position.

7.11.5 Colony count by the spread plate method

7.11.5.1 Principle

Spreading of test volumes of sample onto the surface of a pre-dried agar medium in Petri dishes. Incubation under specified conditions and a count of the colonies that develop.

7.11.5.2 Procedure

Prepare the sample as described in section 7.4, making any necessary dilutions. Pipette a volume of 0.1 mL on the surface of a pre-dried agar medium and spread with a sterile bent glass or plastic rod. Alternatively, spread by rotating the dish.

Allow the inoculum to soak into the agar, and incubate in the inverted position.

7.11.6 Incubation and counting of colonies

For YEA, incubate one set of plates at 22°C ± 1°C for 72 ± 3 hours and another set at 37°C for 24 ± 3 hours. Plates incubated at 37°C may be incubated for up to 48 hours if further information is required.

For R2A Agar, incubate one set of plates at 22°C ± 1°C for 5–7 days and another set at 30°C for 3 days.

Count the colonies as soon as the plates are removed from the incubator. Count the colonies on the plate containing the original sample. If the count is greater than 300 then count the colonies from a diluted sample containing between 30–300 colonies. If all plates carry more than 300 colonies record this as greater than 300 at the highest dilution plated and express the sample count in terms of this. Do not use the term 'too numerous to count' without further qualification (see section 6.3.5).

Spreading colonies can interfere with counts and the following gives guidance on what should be counted a single CFU.

(i) A chain of colonies that appears to be caused by disintegration of a clump of organisms.

(ii) A spreading growth that develops as a film at the bottom of the Petri dish.

(iii) A colony that forms in a film of water at the edge or over the surface of the agar.

If the colonies cannot be counted immediately then keep the plates at 4°C for no longer than 24 hours.

7.12 Acridine Orange Direct Count

7.12.1 Introduction

The acridine orange direct count (AODC) produces a count of all viable and microbial cells in the sample. Consequently the AODC may be higher than count (section 7.11) as the latter method will only give a count of viable micro- The main value of the AODC in water microbiology is to give a rapid estimate microbial biomass, living and dead. All reagents should be free of bacteria should be used to check on background levels.

7.12.2 Definition

The AODC is a count of all viable and non-viable bacteria, yeasts and moulds sample (Hobbie, Daley and Jasper 1977, APHA 1989).

7.12.3 Principle

Fixing the sample for storage, staining with acridine orange followed by vacuum on to a non-fluorescing polycarbonate membrane and enumeration using an ep cence microscope.

7.12.4 Apparatus

The following apparatus can be used.

Blender or vortex mixer.
Filtration units suitable for use with 25 mm diameter membrane filters.
Test tubes, glass screwcapped, 13 × 125 mm.
Syringes, 3 mL disposable with disposable syringe filters of 0.2 µm pore siz
Membrane filters, non-fluorescent, polycarbonate 25 mm diameter, 0.2 µm po also cellulose 25 mm diameter 5 µm pore size.

7.12.5 Microscopy

A suitable microscope with appropriate filters.

Details of the practical aspects of fluorescent microscopy can be found in 'Immunofl cence: antigen-detection techniques in diagnostic microbiology' (PHLS 1992).

7.12.6 Reagents

Phosphate buffer. Dissolve 13.6 g potassium dihydrogen phosphate (KH_2PO_4) in v and dilute to 1 litre. Adjust the pH to 7.2 and filter through a 0.2 µm membrane fil

Fixative solution. A 5.0% m/V glutaraldehyde solution in phosphate buffer. Prepare d

Staining solution. An 0.1% m/V acridine orange solution in phosphate buffer.

Immersion oil, low fluorescing.

7.12.7 Procedure

7.12.7.1 Fixation

Prepare the sample as described in section 7.4. Transfer 9 volumes of the sample to a te tube containing 1 volume of fixative solution. The fixed sample can be stored for up to weeks at 4°C.

7.12.7.2 Staining

Prior to staining mix the fixed sample using a blender or vortex mixer and if necessary make ten-fold dilutions in phosphate buffer, pH 7.2. Place 1 volume of the fixed sample in a small clean container and add 1 volume of acridine orange solution with a sterile disposable syringe filter. Leave for 2 minutes and then transfer to a non-fluorescent polycarbonate filter placed on a cellulose membrane filter in a filter holder. Alternatively

mix the fixed sample and acridine orange on a non-fluorescent filter, leave for 2 minutes and add approximately 3 mL of filtered phosphate buffer to give an even distribution of cells on the membrane.

For both methods apply a vacuum (about 13 kPa, 100 mm of mercury), wash with 2 mL phosphate buffer and refilter. Remove the filter using forceps and allow to air dry for 1–2 minutes.

7.12.7.3 Microscopic examination

Place a drop of immersion oil on a clean glass microscope slide and then place the filter on the immersion oil. Add another drop of immersion oil to the surface of the filter and place a coverslip over the filter.

Examine using a x100 oil immersion lens. Carry out a preliminary examination of at least 10 random fields of view to ensure that distribution of cells is uniform and that cells can be counted (preferably 10–50 per field). Count the number of cells observed in at least 20 squares, preferably 50, of a calibrated counting graticule.

7.12.7.4 Calculation of the acridine orange direct count

Calculate the average number of cells per square and the area of the filter through which the sample was filtered using the specifications of the filter unit. Calculate the number of cells per mL thus:-

$$\text{Total cells per mL} = \frac{abc}{d}$$

where a = average number of cells per square
b = total number of squares per filter
c = the dilution factor, if applicable
d = the sample volume, in mL

7.13 Test for *Pseudomonas aeruginosa*

7.13.1 Introduction

Pseudomonas aeruginosa should not be used as an indicator of faecal pollution. However, in the manufacture of food, drink and pharmaceutical products and in hospitals, it is desirable that the water should be free from this organism. In these circumstances, and for the examination of bottled waters, the following methods of detection and enumeration may be used.

7.13.2 Definition

In the context of the methods, *Ps. aeruginosa* is an aerobic, Gram-negative non-sporing bacillus possessing oxidase and catalase, grows at 42°C but not at 4°C and shows oxidative metabolism in the test of Hugh and Leifson (1953). It reduces nitrates and nitrites, produces ammonia from the breakdown of acetamide, liquifies gelatin and hydrolyses casein but not starch. One of the most important characters is the production of the blue-green pigment pyocyanin, and/or the fluorescent pigment fluorescein.

7.13.3 Count of *Pseudomonas aeruginosa* by membrane filtration

7.13.3.1 Principle

Filtration of a test volume of the sample through a membrane. Incubation on an absorbent pad saturated with broth containing ethanol and cetrimide, followed by a count of the pigment-producing colonies. Alternatively, a solidified version of the medium containing selective agents may be used. Subculture to a confirmatory medium to show growth at 42°C with casein hydrolysis and the production of pyocyanin and/or fluorescein.

7.13.3.2 Choice of medium

Modified King's A Broth (MKB) (Medium 19 of Drake 1966) is recommended for isolation or a solidified version supplemented with cephaloridine, fucidin and cetrimide

(Mead and Adams 1977). This medium, referred to as Pseudomonas Ag
available commercially. Milk Agar with Cetrimide (MAC) (Brown and Fost
recommended as the confirmatory medium.

7.13.3.3 Procedure

After preparing the sample and making any necessary dilutions (see section 7.4
test volumes as in section 7.5 and place each membrane on a pad saturated with
Petri dish or on the surface of a well-dried plate of PA. Incubate at 37°C for 48 h
closed container if MKB is used). Count all colonies which produce a gree
reddish-brown pigment and those which fluoresce under UV light. To detect fluo
it is advisable to use within a darkened room a UV light source (wavelength 350
with a dark (Woods' glass) filter to eliminate visible light. Exposure of the colo
for a short time enhances the pigmentation and thus facilitates counting.

7.13.3.4 Confirmation of Pseudomonas aeruginosa

Regard those colonies which clearly produce pyocyanin on the membrane
aeruginosa. To confirm other colonies, subculture each colony to be tested (see
MAC and incubate at 42°C as described for the multiple tube method (section 7

7.13.4 Count of *Pseudomonas aeruginosa* by multiple tube

For most waters where it is necessary to enumerate *Ps. aeruginosa*, membrane filtr
likely to be more suitable than the multiple tube method. The latter should be us
when membrane filtration is difficult or impossible because of the presence of
particulate matter. Since such waters are likely to be polluted, the use of test v
greater than 10 mL should rarely be necessary.

7.13.4.1 Principle

Culture of test volumes of the sample in a liquid medium containing asparagin
ethanol. Examination for growth and pigment formation at 38–39°C. This tempera
used to inhibit the growth of other pseudomonads which are common in water and so
which grow better at a lower temperature. Subculture to a confirmatory medium to
growth at 42°C with pigment formation, casein hydrolysis and cetrimide resistanc

7.13.4.2 Choice of medium

Asparagine Broth and Ethanol (ABE) (Drake 1966) is recommended for isolation;
test volume of the sample is usually added to four times its volume of med
Alternatively, for test volumes of 1 mL, Repli-dishes instead of tubes may be used
the more concentrated form of the medium. Repli-dishes are Petri dishes which
divided into 25 square compartments each of 5 mL capacity. For confirmation, MA
recommended.

7.13.4.3 Procedure

After preparing the sample and making any necessary dilutions as described in sec
7.6.2, add 10 mL test volumes to 40 mL of medium in screw-capped bottles, and 1
volumes to 4 mL of medium in tubes or to 1 mL of concentrated medium in Repli-dish

Incubate at 38–39°C and examine after 48 hours for growth and fluorescence. This is b
carried out in a darkened room under UV light of wavelength 350 ± 20 nm; it is import
to exclude visible light from the UV source by means of a dark (Woods' glass) filt
Examine after 96 hours for green, blue or red pigments in the medium. Regard growth a
pigment production as presumptive evidence of the presence of *Ps. aeruginosa*.

7.13.4.4 Confirmation of Ps. aeruginosa

Subculture from each tube, bottle or Repli-dish showing fluorescence or pigmentation to
plate of MAC.

However, some vibrios may also give similar reactions. The identific
confirmed by testing in order to show;

(i) fermentation of glucose in the test of Hugh and Leifson (1953),

(ii) fermentation of mannitol,

(iii) hydrolysis of arginine in the test of Thornley (1960),

(iv) growth in 1% tryptone water without added sodium chloride, and

(v) inhibition of growth in 1% tryptone water containing 6% sodium chl

All tests are incubated for 24 hours at 30°C.

The single tube confirmation medium of Kaper et al (1979) or commercial id
kits may also be used for identification, although some less halophilic *Vib*
particularly *V. fluvialis* and *V. furnissii* may still be mis-identified as aeromon

Incubate at 42°C for 24 hours. Examine for growth, pigment formation and for casein hydrolysis which is shown by clearing of the medium around the colonies. *Ps. aeruginosa* grows at 42°C, hydrolyses casein and produces pyocyanin and/or fluorescein. Occasionally, non-pigmented variants of *Ps. aeruginosa* may occur.

From the number of tubes giving a positive reaction, calculate the MPN of *Ps. aeruginosa* per 100 mL of the sample by reference to the appropriate tables in Appendix C.

If only a qualitative result is required, report *Ps. aeruginosa* present or absent from the volume of water examined.

7.14 Test for Motile *Aeromonas* Species (Tentative)

7.14.1 Introduction

The taxonomy of the genus *Aeromonas* is still the subject of considerable research. At least 13 species can be identified by molecular genetic techniques. Using simple biochemical tests it is not possible to identify these species accurately but most isolates can be broadly divided into three groups corresponding to *A. hydrophila*, *A. caviae* and *A. veronii* biotype sobria. At present all the aeromonads may be considered equally significant and so for practical purposes it is not necessary for the water microbiologist to identify aeromonads beyond the level of the genus.

7.14.2 Definition

For the purposes of the method strains are identified as *Aeromonas* if they are oxidase-positive, resistant to ampicillin (10 μgmL^{-1}) and ferment dextrin and mannitol. In addition, they are Gram-negative, fermentative in the test of Hugh and Leifson (1953), able to grow in 1% tryptone water containing no added sodium chloride but not in tryptone water containing 6% sodium chloride, resistant to 50 μgmL^{-1} 0/129 phosphate (2,4-diamino-6,7-diisopropyl pteridine phosphate) and able to hydrolyse arginine in the test of Thornley (1960).

7.14.3 Count of *Aeromonas* by membrane filtration

7.14.3.1 Principle

Filtration of the test volume of sample through a membrane and isolation of the organisms by placing the membrane on an agar medium containing ampicillin as a selective agent, dextrin as a fermentable carbohydrate and bromothymol blue as an indicator of acidity. Subculture of presumptive isolates to confirm identity by the oxidase test and fermentation of mannitol.

7.14.3.2 Choice of medium

A variety of media has been developed for the isolation of aeromonads in recent years. Many of them contain ampicillin as the selective agent. Ampicillin-dextrin agar (ADA) of Havelaar, During and Versteeght (1987) is recommended. This is the same medium of Rippey and Cabelli (1979) except that it has been made more specific by the substitution of dextrin for trehalose. Some *Vibrio* species can also grow on ADA with similar reactions to *Aeromonas*. If the samples are likely to contain strains of *Vibrio*, as would be the case for estuarine waters, then ADA can be made more selective by the inclusion of the O/129 phosphate at a concentration of 50 mgL^{-1}.

7.14.3.3 Procedure

After preparing the sample and making any required dilutions as described in section 7.4, filter the test volume as in section 7.5 and place each membrane on the agar surface in a Petri dish containing ADA. Incubate aerobically at 29–31°C for 24 hours. Count all yellow colonies.

7.14.3.4 Confirmation

Subculture all or a representative number of colonies to NA. After overnight incubation at 30°C test colonies for oxidase. Oxidase-positive colonies are presumptive *Aeromonas*.

Chapter 8: Examination for Pathogenic Organisms

8.1 Introduction

The direct search for pathogenic bacteria has no place in the routine microbiological examination of water supplies. There may be occasions, however, when investigations for faecal pathogens will be necessary. Technical difficulties are considerable in that any pathogens present in the water are likely to be greatly outnumbered by normal faecal flora or by putrefactive organisms derived from sewage. The detection of particular pathogens therefore necessitates the examination of large volumes of water by means of concentration techniques and selective enrichment media. This should only be undertaken in a containment level 3 laboratory by trained and experienced staff under accepted codes of safe practice (NWC 1983, HSAC 1991a, 1991b).

All the methods in this chapter are to be regarded as tentative as they have not been fully validated and subject to formal inter-laboratory evaluation. It certain cases it may be possible to adapt the methods to make a quantitative estimate of the number of organisms present.

8.2 Concentration Methods

The most effective way of examining large volumes of water for pathogenic bacteria is by membrane filtration. However, because membranes readily become blocked by suspended solids, this method is applicable only to waters of low turbidity. In some cases it may be necessary to filter the water in small amounts and to replace the membranes when they become blocked.

When filtration through membranes is impracticable, a suitable filter-aid, for example diatomaceous earth should be used which should be of a sufficient particle size to retain most of the bacteria without causing a blockage of the filter. In addition to concentrating the number of organisms, the method also separates bacteria from any toxic agents which may be present in solution.

8.3 Membrane Filtration

8.3.1 Qualitative examination

Carry out the filtration as in section 7.5. For volumes larger than 100 mL the use of 500 mL funnels is recommended. After filtration place the membrane in a suitable enrichment medium for incubation.

8.3.2 Quantitative examination

Filter separate volumes of the sample through separate membranes. One × 500 mL volume and five × 100 mL volumes will give a measurable MPN count if there are less than 18 organisms per litre, (that is, at least one of the volumes shows no growth). If all volumes show growth, the estimate will be >18 per litre.

After filtration, place each membrane in a suitable enrichment medium for incubation.

8.4 Use of Filter-aid

The usual membrane filtration apparatus may be used, but with a sterile absorbent pad in place of the membrane to act as a supporting base for the filter-aid. A small amount of sterile aqueous suspension of the filter-aid (see Appendix B) is first filtered to form an initial layer on the absorbent pad. Further suspension is then mixed with the sample and filtered. By this means the filter-aid, intimately mixed with the bacteria and debris from the water, is continuously deposited on the initial layer. This should retain more than 90 per cent of the bacteria present in the sample, although for dirty waters, greater proportions of filter-aid may be required.

8.4.1 Qualitative examination

Place a sterile absorbent pad in the membrane filtration apparatus. Take a bottle containing 15 mL of sterile filter-aid suspension, shake well and pour into a funnel. Apply suction and

filter to form an initial layer. To each litre of sample, add 15 mL of well-shaken Mix well and filter the suspension in 100 mL aliquots. Mix well to re-suspend th before each portion of the sample is added to the funnel.

After filtration is complete, remove the funnel carefully and transfer the abso with the layer of filter-aid to a pre-enrichment medium.

With the same medium, rinse any filter-aid adhering to the funnel into the cultu

8.4.2 Quantitative examination

Filter a measured volume of sample as above. Add the pad with the filter-aid to 10 pre-enrichment medium in a sterile flask or wide-mouthed container. Wash adhering filter-aid into the container with the medium from the flask.

Mix well, and for the MPN estimation, pipette 5 × 10 mL volumes into sterile t wide-mouthed containers whilst keeping the suspension well mixed. Keep the rer 50 mL as a separate culture. If high counts are expected, pipette from the 50 mL vo × 1 mL, 5 × 0.1 mL volumes into separate tubes, each containing 10 mL of t enrichment medium.

Incubate at the temperature and time specified for the particular organism being s

Subculture from the pre-enrichment cultures to selective enrichment media and in again at the temperature and time specified.

Subculture to selective solid media to permit recognition of the particular pathoge

After confirmation, determine the MPN of the pathogen in the original volume exan

8.5 Sewer—Swab Technique

A useful qualitative method for the detection of pathogenic bacteria in polluted wat the swab technique described by Moore (1948, 1950), Moore, Perry and Chard (1 Gauze swabs (SCA 1983) suitably anchored by wire or other means, are immersed i flow of water for several days. Organisms are entrapped and thus concentrated in the s which can then be treated in the same manner as the filter-aided concentrate. In addi with heavily polluted waters, the liquid from the swab, and/or dilutions prepared fro can be plated out directly on to agar media, as well as into enrichment medium.

8.6 *Campylobacter*

8.6.1 Introduction

The genus *Campylobacter* comprises two main groups according to growth temperat The first group will grow at 42°C and are referred to as thermophilic campylobacters. T second group will grow at 25°C but not at 42°C. Thermophilic campylobacters include important human pathogens *C. jejuni* and *C. coli* and details of methods for their isolat are given below.

8.6.2 Principle

Isolation of organisms on a membrane followed by enrichment and subculture to selective agar. The broth and agar are both incubated micro-aerobically at 42°C Confirmation of thermophilic campylobacters is by cell morphology, Gram stain an oxidase test.

8.6.3 Definition

Bacteria in the genus *Campylobacter* are Gram-negative spirally shaped rods which often resemble gulls' wings. They are motile by means of a single polar flagellum giving a characteristic darting motility. Members of the genus are micro-aerobic, requiring an oxygen tension of around 5% and a carbon dioxide tension of around 10%, are oxidase-positive, reduce nitrates but do not produce acid from carbohydrates.

8.6.4 Choice of media for isolation

The Preston Enrichment Broth, (PEB) (Bolton and Robertson 1982, Bolton et al 1983), Preston Selective Agar (PSA) (Bolton and Robertson 1982), and the blood-free modification of the selective agar (Bolton, Hutchinson and Coates 1986) are recommended for the isolation of thermophilic campylobacters from water. The agar media can be obtained commercially as dehydrated products.

8.6.5 Practical considerations

Membrane filtration is recommended for the concentration of campylobacters from water. Concentration by certain filter aids is not effective in removing all organisms of this type from the sample and in this instance is not recommended.

Campylobacters will only grow in a micro-aerobic environment. For enrichment cultures it is necessary to include the micro-aerobic supplement described by George et al (1978). The volume of enrichment broth used should be adjusted so that any container used is completely filled after inoculation. The lid should be sealed tightly prior to incubation.

Selective agars should be incubated in a suitable micro-aerobic atmosphere containing a mixture of 5% oxygen, 10% carbon dioxide and 85% nitrogen. This can be obtained by using commercially available gas generating kits. Since hydrogen is produced, jars should not be opened adjacent to a source of ignition.

8.6.6 Procedure

8.6.6.1 Membrane filtration

Filter the sample as described in section 7.5, using a membrane of pore size 0.2 µm. It may be necessary to use several membranes for each sample if the water is turbid.

8.6.6.2 Enrichment

Place the membrane(s) in a suitable screw-capped container filled almost completely with 50–100 mL of PEB. Seal tightly and incubate at 42°C for 48 hours.

8.6.6.3 Isolation and identification

Subculture from the enrichment broth to PSA or the blood-free modification of this medium. Incubate in a gas jar containing a micro-aerobic gas generating kit at 42°C for 48 hours. Examine the plates for characteristic colonies, using a hand lens if necessary. *Campylobacter* colonies vary in size but are typically flat and watery with an entire edge and may be circular or ovoid. They have a tendency to spread along the lines of inoculation. Carry out an oxidase test on suspect colonies. Variation in colonial morphology is not uncommon so it is advisable to test several colonies for oxidase production. Carry out a Gram-stain on oxidase-positive colonies using 1:20 carbol fuchsin as the counterstain because campylobacters stain weakly with neutral red. Campylobacters produce Gram-negative curved rods which resemble gulls' wings, S-shapes or short spirals. For most practical purposes colonial and cellular morphology and the oxidase test are adequate for identification. If required, species and sub-species differentiation can be carried out using the bio-typing scheme proposed by Skirrow and Benjamin (1980) and described in detail by Lander and Gill (1985).

8.7 *Escherichia coli* 0157:H7

8.7.1 Introduction

E. coli 0157:H7 is a recognised cause of illness characterised by bloody diarrhoea, severe abdominal pain and haemolytic uraemic syndrome sometimes followed by renal failure. The organism is common in cattle and outbreaks related to beef and milk have been recorded. Isolation is based on enrichment in modified buffered peptone water (MBPW) followed by sub-culture to modified sorbitol MacConkey agar (MSMA) and selection of non-sorbitol fermenting colonies. The method described was developed for milk analysis and has not been fully tested and validated for water.

8.7.2 Principle

The method described is suitable for detecting *E. coli* 0157:H7 from water materials by membrane filtration and isolation into an enrichment medium. broths are inoculated onto MSMA and non-sorbitol fermenting colonies subc biochemical and serological testing.

8.7.3 Definition

E. coli is a Gram-negative, oxidase negative, non sporing bacillus. Most thermotolerant and can grow at 44°C but *E. coli* 0157:H7 grows only poo temperature in non-selective media and will not grow above 41°C in selective fails to ferment sorbitol at 37°C and MSMA can therefore be used as a scre differentiation of 0157:H7 from other *E. coli* strains.

8.7.4 Procedure

8.7.4.1 Membrane filtration

Filter suitable volumes of the sample as described in section 7.5 using memb nominal pore size 0.45 µm. It may be necessary to use several membranes with ve waters.

8.7.4.2 Enrichment

Place membranes into universal containers with 20 mL of the enrichment broth (M Incubate at 37°C for 24 hours.

8.7.4.3 Isolation and identification

Subculture the enrichment broth onto MSMA and incubate for 24 ± 1 hours at 37°C 24 hours examine the membrane for typical colonies which are 2–3 mm in diamet pale orange in colour. Colonies are smooth, entire and circular. Other coliforms pr pink colonies from the fermentation of sorbitol.

Note: Recovery of *E. coli* 0157:H7 from enrichment broth is improved by immunomagnetic separation with antibody-coated magnetic beads, Wright, Chapma Siddons (1994). Non-specific binding of other organisms is reduced by washing with phosphate buffered saline containing 0.002–0.05 % tween 80.

8.7.4.4 Confirmation

Subculture sorbitol-negative colonies on suitable non-selective NA and incubate at for 24 hours. Examine plates for purity and if cultures are not pure, subculture a selec of colonies on fresh NA for a further 24 hours at 37°C. Check that isolates are oxi negative.

Isolates may be confirmed by slide agglutination using a specific commercially prepa latex suspension. Separately, place two drops of saline or phosphate-buffered saline o microscope slide. Emulsify the same colony of the suspected culture in each drop to giv smooth, fairly dense suspension. Check to ensure that the suspension is smooth and t auto-agglutination has not occurred. Mix one loopful of latex reagent to one suspensi and rock the slide gently. Examine for evidence of agglutination. Repeat the test with t appropriate positive and negative controls. Isolates showing strong positive agglutinati when compared with the positive control should be considered positive.

Isolates should be confirmed as *E. coli* by appropriate biochemical tests (Cowan 1993) use of a suitable test kit.

8.8 *Salmonella*

8.8.1 Introduction

Many different serotypes of salmonellas are present in wild and domestic animals an agricultural livestock and any of these sources may act as reservoirs of human salm

onellosis. Salmonellas in the environment are derived from acute and convalescent cases, from chronic carriers and symptomless faecal excreters in human and animal populations.

Industrial effluents from abattoirs and animal processing factories contribute salmonellas to sewage and thence to the local environment and water courses.

8.8.2 Principle

Concentration of organisms on a membrane filter or by means of a filter-aid followed by culture in a non-selective pre-enrichment medium. Subculture to a selective enrichment broth followed by plating on selective agar and biochemical and serological confirmation of characteristic colonies.

8.8.3 Definition

Salmonellas normally conform to the general definition of the Family Enterobacteriaceae (Le Minor, Rohde and Taylor 1970) and can be further differentiated, biochemically, into four groups, subgenus I to IV. Those of subgenus I, the largest group and the only ones considered to be pathogenic for humans are ß-galactosidase-negative.

The usual biochemical reactions include production of gas from glucose, and hydrogen sulphide production. Indole and urease are not produced. Citrate is utilised as a sole carbon source; lysine and ornithine are decarboxylated. Phenylalanine and tryptophan are not oxidatively deaminated; sucrose, salicin, inositol and amygdalin are not fermented.

Salmonellas are subdivided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. They may be further differentiated by bacteriophage and plasmid typing.

8.8.4 Choice of media

Enrichment media either suppress the growth of other enterobacteria, allowing salmonellas to thrive, or they enhance the growth of salmonellas, allowing them to predominate in a mixed culture. Media selective for *Salmonella* species contain inhibitory substances such as selenium salts, brilliant green or malachite green. After incubation, the selective enrichment cultures are plated on differential agar media on which salmonellas form colonies with a characteristic appearance and morphology. Representative colonies are subcultured for biochemical and serological testing.

The following media may be used in these methods:

(i) Buffered Peptone Water (BPW) (Edel and Kampelmacher 1973) for pre-enrichment.

(ii) Selenite Broth (SB) (Hobbs and Allison 1945a,1945b) for the selective enrichment of *Salmonella typhi* and *S. paratyphi B*.

(iii) Rappaport Vassiliadis Broth (RVB) (Vassiliadis et al 1976, Vassiliadis 1983) for the selective enrichment of other salmonellas. This medium and a modification containing soy peptone instead of tryptone and a lower concentration of magnesium chloride are commercially available as dehydrated products.

(iv) Xylose Lysine Desoxycholate Agar (XLDA) (Taylor and Harris 1965) for selective isolation of salmonellas.

(v) Bismuth Sulphite Agar (BSA), modified from Wilson and Blair (1927).

(vi) Brilliant Green Agar (BGA) (ICMSF 1978). A modified version of this medium is commercially available as a dehydrated product. BGA is not recommended for the isolation of *S. typhi*.

(vii) Nutrient Agar Slopes.

(viii) Lysine Iron Agar slopes (LIAS) (Edwards and Fife 1961) or Triple Sugar Iron Agar slopes (TSIAS) and Urea Broth (UB), for preliminary biochemical identification.

8.8.5 Safety

Selenium salts are toxic if inhaled or ingested and there is a possible risk of teratogenic in pregnant laboratory workers.

In laboratories of a containment level inappropriate for the chance isolation of *Salmone typhi*, (see section 6.1 and especially sections 6.1.2 and 6.1.3) media containing selen should not be included in the range of selective enrichment media.

8.8.6 Practical considerations

Undue delay between the time the sample is taken and processed in the laboratory w reduce the chances of isolating salmonellas.

Selective media may be too inhibitory for organisms stressed in the environment and p enrichment in a non-inhibitory medium is necessary before transfer to liquid selecti enrichment media.

Salmonella isolation may be improved if several small sub-samples are examined rat than one large portion.

Dilution of the material reduces the effect of substances toxic to salmonellas.

Competition from other organisms, particularly the *Pseudomonas* group, may redu isolation efficiency. The technique of incorporating a final concentration of 5 mgL^{-1} cefsulodin in the pre-enrichment stage will eliminate the *Pseudomonas* species *Serratia* species may flourish (Harmon, Stuart and Mawer 1991).

In any microbiological method, quality control is important to ensure that the cult media, both liquid and solid, are able to support the growth of low numbers of the mic organisms under investigation. The use of old stock culture solutions of *Salmonella* is recommended for the quality control of salmonella plating media. Old stock solutions te not to produce typical colonial appearances.

8.8.7 Pre-enrichment procedure

8.8.7.1 Membrane filtration

Filter the sample as described in section 7.5, using membranes of pore size 0.45 µm. Pla each membrane in approximately 100 mL of enrichment medium in a wide-mouth screw-capped container.

8.8.7.2 Filtration with filter-aid

8.8.7.2.1 For qualitative examination Filter the sample as described in sections and 8.4 and transfer the absorbent pad with filter-aid to approximately 100 mL enrichment medium in a wide-mouthed container. Any filter-aid adhering to the funne rinsed into the container with the medium.

8.8.7.2.2 For quantitative examination Filter a measured volume of sample described in section 8.4. Place the filter pad with filter-aid in 100 mL of enrichme medium. Rinse all the filter-aid into the medium. Mix well and transfer 5 separate volum of 10 mL to sterile tubes, keeping the remaining 50 mL volume as a separate culture. high counts are expected, pipette 5 × 1 mL and 5 × 0.1 mL volumes from the 50 m volume to separate tubes each containing 10 mL of enrichment medium.

8.8.8 Enrichment procedure

8.8.8.1 Isolation of salmonella species other than S. typhi and S. paratyphi

8.8.8.1.1 Incubation of buffered peptone water. Incubate the containers of BPW w membranes or filter-aid for a resuscitation period of 24 ± 2 hours at 37°C.

8.8.8.1.2 For qualitative examination. Subculture 0.1 mL from 100 mL BPW pre-enrichment culture to 10 mL of RVB. Incubate RVB at 41°C for up to 48 hours.

8.8.8.1.3 For quantitative examination. Subculture 0.1 mL from each BPW pre-enrichment culture to 10 mL of RVB. Incubate RVB as above.

8.8.8.2 Isolation of S. typhi and S. paratyphi B

Incubate the containers of SB with membranes or filter-aid at 37°C for up to 48 hours.

8.8.9 Subculture of enrichment media to selective agars

After incubation for 24 and 48 hours subculture the RVB or SB to XLDA and to either BGA or BSA. BGA should not be used for the isolation of *S. typhi*.

Incubate the selective agars at 37°C for 18 to 24 hours.

8.8.10 Isolation and identification on selective agar

Examine the plates for characteristic colonies.

8.8.10.1 Xylose Lysine Desoxycholate Agar

Salmonellas produce red colonies, usually with a black centre. Xylose-fermenting coliforms produce yellow colonies. *Pseudomonas* species produce either red colonies or yellow colonies with grey-black centres. Shigellas produce small pink-red colonies. *Proteus* produces irregular pink colonies with small black centres.

Salmonella colonies may be obscured by overgrowth and this medium should be used in conjunction with BGA or BSA.

8.8.10.2 Brilliant Green Agar

Salmonellas produce red colonies and lactose- and sucrose-fermenting coliforms produce yellow/green colonies. The growth of *Proteus* species is usually inhibited, but when it does occur, colonies are red. Similarly the growth of *Pseudomonas* species is usually inhibited but small crenated colonies may be produced.

8.8.10.3 Bismuth Sulphite Agar

Salmonellas produce black-centred colonies with a clear periphery. The ratio of black centre to clear edge varies and the colony may be surrounded by a metallic sheen (McCoy and Spain 1969). Some strains of *Salmonella* produce a green colony with a darker green centre. Coliforms produce large brown colonies with no clear periphery. *Proteus* species produce irregular green-brown colonies and *Pseudomonas* species produce small green colonies with a sticky consistency.

8.8.11 Confirmation

8.8.11.1 Biochemical confirmation

Subculture characteristic colonies from each plate, using a straight wire, to confirmatory media such as LIAS or TSIAS, together with UB and NA as a check for purity. The straight wire carrying the organisms from the colony 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 must remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be blown out of the tube. Incubate at 37°C for 18–24 hours. Regard cultures which give characteristic reactions in these confirmatory media as presumptive salmonellas (see Tables 5 and 6). Alternatively one of the commercially available identification systems may be used.

Table 5 Reactions in Lysine Iron Agar

Genus	Slope	Butt	H_2S
Arizona	Alk	Alk	+
Salmonella	Alk	Alk	+
Proteus	Red	A	+ or –
Providencia	Red	A	–
Citrobacter	Alk	A	+
Escherichia	Alk	A or NC	–
Shigella	Alk	A	–
Klebsiella	Alk	Alk	–
Enterobacter	Alk	A	–

A = Acid (yellow)
Alk = Alkaline (purple)
NC = No change
H_2S+ = Blackening

Table 6 Reactions in Triple Sugar Iron Agar and Urea Broth

Genus	Slope	Butt	H_2S	Urea B
Klebsiella	A	AG	–	+ or –
Enterobacter	A	AG	–	–
Escherichia	A	AG	–	–
Proteus vulgaris	A	AG	+	+
Proteus morganii	NC or Alk	A or AG	–	+
Shigella	NC or Alk	A	–	–
S. typhi	NC or Alk	A	+ (weak)	–
Salmonella (other)	NC or Alk	AG	+	–

A = Acid (yellow)
AG = Acid (yellow) and gas formation
NC = No change
Alk = Alkaline (red)
H_2S + = Blackening – =No change
Urea Broth + = Alkaline (red) – =No change

8.8.11.2 Serological confirmation

Subculture characteristic colonies on to moist NA slopes.

For optimum flagella formation, it is essential that fluid is present in the tube and s broth should be added if required. Incubate overnight at 37°C. Carry out a agglutination as follows:

Place 3 separate drops (0.02 mL) of saline onto a clean microscope slide, using a wire or pipette. Emulsify growth from the moist butt of the slope in each separate dr produce homogeneous suspensions.

Mix a loopful of *salmonella* Polyvalent 'O' (PSO) antiserum with the first dr suspension and a loopful of *salmonella* Polyvalent 'H' (PSH) serum with the se Gently rock the slide back and forth and examine for agglutination against a background under a bench lamp. The third drop containing no antiserum indicates wh or not the culture auto-agglutinates. These strains should be re-plated on XLDA, BG BSA and dry smooth colonies dealt with as previously described.

Organisms which agglutinate with PSO and PSH antisera or strains which agglutinate PSH serum only can be presumptively regarded as members of the *Salmonella* group advisable to check colonially characteristic PSO-negative, PSH-positive or PSH-neg

strains for agglutination with Salmonella Vi antiserum to exclude *Salmonella typhi* as the Vi antigen may mask the 'O' and 'H' antigens.

Cultures should be submitted to a reference centre for final confirmation and serotyping.

8.9 *Shigella*

8.9.1 Introduction

Members of the genus *Shigella* are intestinal pathogens of humans, causing dysentery or shigellosis, and may also be carried asymptomatically in the intestinal tract.

There are four species, *S. dysenteriae*, *S. sonnei*, *S. flexneri* and *S. boydii* all of which cause gastrointestinal disease. The commonest species isolated in the United Kingdom is *S. sonnei*, although the most severe disease is caused by *S. dysenteriae*, type 1 which produces a potent exotoxin (Shiga toxin).

8.9.2 Principle

Isolation of organisms on a membrane followed by enrichment in a selective medium and plating onto a selective agar. Characteristic colonies are confirmed by slide agglutination and may be sub-cultured onto a non-selective agar for further biochemical tests or serotyping.

8.9.3 Definition

Bacteria in the genus *Shigella* are Gram-negative non-motile rods which are oxidase-negative and catalase-positive (with the exception of *S. dysenteriae* type 1, which is catalase-negative). Citrate cannot be used as a sole carbon source, and sugars are fermented without gas production. (A few strains however produce gas).

8.9.4 Choice of media

The following media are recommended for isolation of shigella from water:

(i) A modification of Hajna GN Enrichment Broth (HEB).

(ii) A modification of Desoxycholate Citrate Agar (MDCA).

(iii) Hektoen Agar (HA) (King and Metzger 1968).

8.9.5 Procedure

8.9.5.1 Membrane filtration

Filter the sample as described in section 7.5, using a membrane of pore size 0.45 µm. It may be necessary to use several membranes if the water is turbid.

8.9.5.2 Enrichment

Place the membrane(s) in 100 mL of HEB in a suitable screw-capped container. Shake the broth and incubate at 37°C for 6–8 hours.

8.9.5.3 Isolation and identification on selective agar

Shake the enrichment broth before subculturing to HA and MDCA. Incubate the agar plates at 37°C for 18–24 hours. Characteristic non-lactose-fermenting colonies are subcultured on a non-selective agar such as NA and incubated at 37°C for 18–24 hours.

Note: Those working on the isolation of shigellas should familiarise themselves with the type of growth and colonial morphology of these organisms on HA and MDCA agars (see Tables 7 and 8). Plating on selective agar must produce isolated colonies. Failure to do so may result in 'false negatives' since typical colonies may be overgrown. Where this occurs or colonial identification is uncertain, a subculture to fresh agar should be performed.

Table 7 Colonial appearance on HA after incubation at 37°C for 18–24 hou

Organism	Characteristic Appearance
Shigella	Moist green colonies, 2–4 mm diameter. (*Shigella son* may produce larger irregularly shaped colonies).
Salmonella	Blue-green colonies, with or without black centres.
Pseudomonas	Large rough textured green colonies.
Enterobacter, Citrobacter, Escherichia, Klebsiella	Yellow colonies. The medium around the colonies ofte turns salmon pink.
Proteus	Pale green or ochre yellow colonies.

Table 8 Colonial appearance on MDCA after incubation at 37°C for 18–24 h

Organism	Characteristic Appearance
Shigella	Small raised cream coloured colonies.
Salmonella	Large black colonies with a thin white periphery.
Pseudomonas	Very small flat cream coloured colonies.
Proteus	Cream colonies with small black centre.
Coliforms	Pink mucoid colonies with raised centres.
Escherichia	Pale pink colonies with grey centres.

8.9.6 Confirmation

8.9.6.1 Biochemical confirmation

Non-lactose-fermenting colonies which have been subcultured onto a non-selective may be further identified using one of the commercially available multi-test biochen identification kits.

8.9.6.2 Serological confirmation

Colonies which identify biochemically as shigellas must be confirmed serologically, u polyvalent anti sera and single-factor sera.

Place two separate drops (0.02 mL) of saline on a glass slide. Emulsify suspected cu with a loop into each drop of saline to give a smooth, fairly dense suspension. Add drop of undiluted serum to one suspension and mix. Rock the slide gently and observ agglutination using indirect lighting over a dark background. Agglutination shoul strong and clearly visible within one minute. Non-specific agglutination due to n antigenic reactions may occur and these sometimes are usually slow to appear. This however be apparent in the second suspension, which is used as a control.

8.10 Staphylococci

8.10.1 Introduction

Staphylococci, particularly *Staphylococcus aureus*, are of concern in the hospital envi ment because of their potential to cause infections. They have also been advocate indicators of the quality of bathing water including swimming pools, hydrotherapy p and sea water. They are not normally present in drinking water supplies but their dete may be required in food and pharmaceutical manufacture and in hospitals.

8.10.2 Definition

Members of the genus *Staphylococcus* are Gram-positive cocci, facultatively anaero non-motile, do not form spores and are usually catalase-positive and sensitiv lysostaphin. In the context of the method, staphylococci are defined as ferme mannitol and able to grow in the presence of 7.5% sodium chloride and 0.005% sod azide.

8.10.3 Principle

Filtration of the test volume of sample and incubation on a selective agar medium containing sodium azide, sodium chloride, mannitol and phenol red. Presumptive isolates should be subcultured to confirm identity.

8.10.4 Choice of medium

The method described utilises the medium M-5LSMA developed by Stengren and Starzyk (1984). This medium is lipovitellin-salt-mannitol agar modified by the inclusion of 0.005% sodium azide (see section 7.9.3) to suppress the growth of Gram-positive bacilli. The method is primarily directed at detecting *Staph aureus*. A few species, for example *Staph epidermidis* and *Staph hominis* do not ferment mannitol and are not readily detected. The medium also contains egg yolk to enable lipase activity to be detected by the production of an opaque zone around the colony. This is difficult to see under a membrane and can therefore be omitted when the medium is used for membrane filtration alone.

8.10.5 Procedure

After preparing the sample and making any required dilutions as described in section 7.4, filter the test volume as in section 7.5 and place each membrane on the agar surface in a Petri dish containing M-5LSMA. Incubate the plates aerobically at 29–30°C for 48 hours. Count all colonies that are yellow, opaque off-white or pale yellow, and record as presumptive staphylococci.

8.10.6 Confirmation

To confirm strains as *Staphylococcus* species, subculture each colony to be tested (see 6.3.3) to NA. After incubation for 18–24 hours at 30–37°C check growth for purity and carry out a Gram-stain. Further identification to species level may be performed using an appropriate commercial identification kit. To confirm isolates as *Staph aureus* it is necessary to test either for coagulase production or to re-streak on M-5LSMA. Opaque zones around colonies after 24 hours indicate lipovitellin-lipase activity which correlates well with coagulase production. Yellow zones around the colonies after 24–48 hours indicate mannitol fermentation. *Staph aureus* ferments mannitol and also produces lipovitellin-lipase and coagulase.

8.11 *Vibrio cholerae* and Other *Vibrio* Species

8.11.1 Introduction

The species *Vibrio cholerae* can be subdivided into over 80 O-serovars. The organisms that usually cause epidemic cholera are toxin producing strains of the O1 Serovar; however, recently a new Serovar, 0139, has been identified as having epidemic potential. Strains of other serovars (non-O1 *V.cholerae*) can also cause diarrhoea either by means of cholera toxin or, more commonly, by other enteropathogenic mechanisms. Other species of *Vibrio*, in particular *V. parahaemolyticus*, *V. fluvialis* and *V. mimicus*, can also cause diarrhoea. All *Vibrio* species are natural inhabitants of brackish, estuarine or coastal water. All require salt for optimum growth and survival, and the degree of requirement reflects the ionic conditions of their natural ecological niche; *V. cholerae* has the least requirement and *V. parahaemolyticus*, being of coastal origin, has the greatest. Potable water can be a vehicle for *V. cholerae* and carriage of other *Vibrio* species is possible. No species of *Vibrio* should be present in potable water.

8.11.2 Definition

Members of the Genus *Vibrio* species are Gram negative curved or comma-shaped rods, actively motile, aerobic, facultatively anaerobic, oxidase-positive, able to grow at pH 8.6 and inhibited by 0/129 phosphate.

8.11.3 Principle

Vibrio cholerae and most other pathogenic *Vibrio* species are tolerant of alkaline conditions and are able to grow rapidly at 37°C in simple peptone water containing sufficient sodium chloride to encourage growth. These properties are utilised for the

enrichment of the organisms in alkaline peptone water (1% peptone, 1% sodium chloride and pH 8.6). A concentration of 1% sodium chloride gives good growth of all species likely to be encountered, although 0.5% sodium chloride is adequate for *V. cholerae*.

After overnight enrichment at 37°C, broths are subcultured to the selective medium, thiosulphate-citrate-bile salt-sucrose agar (TCBSA) (Kobayashi et al 1963). This medium was developed for the isolation of *V. parahaemolyticus* but has proved useful for the isolation of most other vibrios, especially *V. cholerae*. It has achieved wide acceptance as the medium of choice for the isolation of vibrios because it is highly selective, readily available commercially, easy to prepare (requiring no autoclaving) and permits the preliminary differentiation of the sucrose-fermenting (yellow colonies) species, such as *V. cholerae* and *V. fluvialis,* from the non-sucrose-fermenting species (blue green colonies), such as *V. parahaemolyticus* and *V. mimicus*.

8.11.4 Procedure

Add at least 100 mL of sample to an equal volume of double-strength alkaline peptone water. Incubate at 25°C for 2 hours followed by 12–16 hours at 37°C.

After incubation, subculture from the top of the enrichment broth to a plate of TCBSA and incubate the plate at 37°C for 16–24 hours.

Examine the plates for vibrio-like colonies and subculture at least three of each colony type to NA for a purity check, oxidase test and further identification. Colonies of vibrios will usually be of 1–3 mm diameter and either yellow (sucrose-fermenting) or blue-green (non-sucrose-fermenting).

Strains that are oxidase-positive require further identification. If epidemic strains of *V. cholerae* 01 are sought, then colonies that are both sucrose-fermenting (yellow on TCBSA) and oxidase-positive can be checked for agglutination with *V. cholerae* O1 antiserum. A strain that agglutinates can be identified as a presumptive isolate of *V. cholerae* O1 and should be sent to the appropriate reference laboratory for confirmation.

8.11.5 Enumeration

If enumeration is required, the multiple tube method can be used. Inoculate 1×50 mL and 5×10 mL amounts of sample into equal volumes of double-strength alkaline peptone water and proceed as above. Volumes of 1 mL or less should be added to 20 mL volumes of single-strength alkaline peptone water.

8.11.6 Membrane filtration

Direct enumeration by filtration and incubation of the filter on the surface of a TCBSA plate is not satisfactory due to the overgrowth of the membranes by other bacteria. Filtration can be used as a means of concentration by placing the filter in single strength alkaline peptone water for enrichment and proceeding as above.

8.11.7 Confirmation

Commercial identification kits can be used to identify oxidase-positive colonies.

8.12 *Yersinia*

8.12.1 Introduction

The isolation procedure described gives good recovery of *Yersinia* species from raw waters.

8.12.2 Definition

Yersinias are Gram-negative rods, non-motile at 37°C (but some species are motile at 22°C), catalase-positive, oxidase-negative and facultatively anaerobic. Sugars are attacked fermentatively with occasional gas production. Virulent strains are aesculin-negative and can bind crystal violet dye.

In the context of the method, organisms are regarded as yersinias which, after enrichment and alkali treatment, give characteristic colonies on Cefsulodin Irgasan Novobiocin agar (CINA) (Mair and Fox 1986, based on Schiemann 1982), and which then give positive reactions with TSIAS and Urea Broth, and are non-motile at 37°C after 4–6 hours. Further biochemical and serological identification should be carried out to speciate the isolates.

8.12.3 Principle

Concentration of organisms on a membrane filter followed by enrichment. Incubation and alkali treatment of enrichment broth, followed by plating on CINA. Biochemical and serological confirmation of characteristic colonies.

8.12.4 Choice of media for isolation

Yersinia organisms are poor competitors, and the method employed for enrichment may require adjustment depending upon the degree of contamination of the sample, and the timescale regarded as acceptable for isolation.

The enrichment procedure uses Tris-buffered peptone which is incubated at 9°C for up to two weeks. The first choice of a selective agar is CINA which is available as a commercially prepared dehydrated product.

8.12.5 Identification media

The following media may be used:

(i) Triple Sugar Iron agar slopes (TSIAS);

(ii) Urea Broth (UB);

(iii) MacConkey Agar (MA).

8.12.6 Procedure (Greenwood 1993)

8.12.6.1 Enrichment

After filtration of the sample as described in section 7.5, place membrane(s) in 100 mL of tris-buffered peptone in screw-capped containers and incubate at 9°C for two weeks.

8.12.6.2 Alkali treatment

Prepare 0.5% m/V potassium hydroxide in 0.5% saline. To 9 volumes of this, add 1 volume of the incubated enrichment broth, subculture to CINA and incubate at 30°C for 24 hours.

8.12.6.3 Isolation on selective agar

Subculture typical 'bullseye' colonies isolated on CINA to each of the following media and incubate at 30°C for the specified times:

(i) TSIAS—24 hours;

(ii) UB—1 to 3 days;

(iii) MA—24 hours;

Cultures from the MA plate which are urease-positive and produce acid but no gas and which do not produce H_2S on TSIAS are subcultured from the MA plate to NB.

8.12.6.4 Motility test

Incubate the nutrient broth cultures at 37°C for 4–6 hours. Cultures which do not exhibit motility are assumed to be *Yersinia* species. Yersinias are motile at temperatures below 28°C.

8.12.6.5 Speciation

Speciation can be carried out using commercially available identification kits.

APPENDICES

Appendix A: Laboratory Glassware and Plastic ware:

Specifications:

Sample bottles

See section 6.4.1.

Pipettes

Glass pipettes should conform to BS (BSI 1976:700). The 1 mL and 10 mL sizes should be the straight-sided, total delivery type. Alternatively, appropriately calibrated automatic pipettes with sterile disposable tips may be used. Plastic disposable pipettes may also be useful.

Glassware for liquid media

The 50 mL volumes of double-strength medium required for the multiple tube method should be distributed in screw-capped bottles of at least 125 mL capacity, and the 10 mL volumes in test-tubes with a capacity of about 30 mL. For the 5 mL volumes of single-strength medium and for most other liquid media, tubes with a capacity of about 22 mL or screw-capped bottles are suitable. When inner (Durham) fermentation tubes are required, suitable sizes for the 5 and 10 mL volumes, and for the 50 mL volumes of medium are 35 × 8 mm and 75 × 10 mm respectively. When media have to be stored for several weeks, screw-capped containers should be used.

Petri dishes

It is important that Petri dishes used for colony counts should be of a standard size to ensure that the surface area and depth of the medium are always constant.

Cleaning and Preparation

Where applicable, after use, pipettes should be placed in jars of disinfectant, for example hypochlorite solution containing 0.1% of free chlorine, left for one hour and rinsed several times. After a final rinse in distilled water, pipettes should be dried and packed in metal canisters for sterilization. Plugging pipettes with non-absorbent cotton wool may help to reduce cross-contamination. It is advisable to clean glass pipettes periodically with a non-toxic detergent or acid and then rinse thoroughly.

Test tubes, bottles and flasks should be autoclaved after use and cleaned with a brush, washed in water with a non-toxic detergent, rinsed in clean water and finally in distilled water.

Test tubes and flasks should be plugged with non-absorbent cotton wool or covered with closely-fitting aluminium or polypropylene caps. The necks and stoppers of bottles should be covered with paper or foil to prevent contamination.

Sterilization of Glassware

Glassware may be sterilized in hot-air ovens at 160°C for 1 hour. If this temperature is exceeded, the exposure time may be reduced proportionately. A temperature above 170°C should be avoided since organic matter tends to char and cotton wool is rendered friable. Alternatively, bottles and tubes may be autoclaved at 121°C for 20 minutes. If bottles with ground-glass stoppers are used, a strip of paper or foil approximately 75 × 10 mm should be inserted between the stopper and the neck of the bottle before sterilization. This prevents jamming of the stopper and cracking of the glass on cooling.

Appendix B: Media and Reagents:

Choice of Constituents:

Peptone

In the preparation of media, unless a particular grade is specified, any microbiological peptone may be used which forms a clear solution and does not precipitate when alkali is added to adjust the pH.

Agar

In the instructions which follow, a satisfactory gel strength with the concentration of agar in the medium is assumed. This may vary according to the quality of the agar available. It is also assumed that the agar forms a solution which remains sufficiently clear for filtration to be unnecessary.

Bile salts

The term bile salts includes sodium taurocholate and sodium tauroglycocholate. Different preparations of bile salts vary in their inhibitory properties; each new batch should be tested against a known satisfactory product and the concentration adjusted accordingly.

Distilled water

Where distilled water is specified, a glass still should be used; alternatively, deionised water may also be used.

Dehydrated media

Some of the media described are available commercially in dehydrated form and should be reconstituted according to the manufacturers' instructions.

Sterilization of media

A time-temperature combination of 121°C for 15 minutes is usually specified for many microbiological purposes. However, a temperature of 115°C for a minimum of 10 minutes is recommended for most media used in water examination. The heating profile of the autoclave should be established for typical loads. This information may then be used to specify the sterilization regime for different volumes of media.

Storage of media

In general, after sterilization most media in sealed containers may be stored safely for several months at room temperature provided they are kept in the dark. Media dispensed aseptically may be kept at 4–10°C for up to one month; before use, media should be inspected carefully for any untoward signs such as contamination or excessive evaporation. Most reagents are best kept at 4°C.

Preparation of Media and Reagents

pH adjustment

The pH of media is of vital importance. Measurement and, where necessary, adjustment of pH is part of the preparation of most media. Where a method of adjustment is not specified, general guidance can be found in 'Collins and Lyne's Microbiological Methods' (Collins 1989).

BASIC MEDIA

Nutrient Broth

Meat extract	10 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	1 litre

Add the ingredients to the water and heat to dissolve. Adjust the pH to about 8.2 with a solution of molar sodium hydroxide and boil for 10 minutes. Clarify by filtration and adjust pH to between 7.2–7.4. Dispense in bottles or tubes and autoclave at 115°C for 10 minutes.

Nutrient Agar

Nutrient Broth gelled by the addition of agar, usually in the order of 1–2 % m/V.

Blood Agar

Nutrient Agar with the addition of 5% (V/V) sterile horse blood.

MEDIA FOR COLIFORM ORGANISMS

Membrane Lauryl Sulphate Broth (PHLS/SCA 1980a)

Peptone	40 g
Yeast Extract	6 g
Lactose	30 g
Phenol red (0.4% m/V aqueous solution)	50 mL
Sodium lauryl sulphate—specially pure	1 g
Distilled water	1 litre

Add the ingredients to the water and mix gently to avoid froth. The final pH of the medium should be 7.4–7.5 and it may be necessary to adjust the pH to about 7.6 before sterilization to achieve this. Distribute in screw-capped bottles and autoclave at 115°C for 10 minutes. (The media may need to be removed from the autoclave as soon as possible to avoid breakdown of the lactose and a reduced pH).

Improved Formate Lactose Glutamate Medium (PHLS 1969, modified from Gray 1964)

This is commercially available in dehydrated form as Minerals Modified Glutamate Medium, which may require pH adjustment.
Double-strength medium:

Lactose	20.0 g
L(+) Glutamic acid sodium salt	12.7 g
L(+) Arginine monohydrochloride	40 mg
L(−) Aspartic acid	48 mg
L(−) Cystine	40 mg
Sodium formate	500 mg
Dipotassium hydrogen phosphate	1.8 g
Ammonium chloride	5.0 g
Magnesium sulphate heptahydrate	200 mg
Calcium chloride dihydrate	20 mg
Iron(III) citrate scales	20 mg
Thiamine (Aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1% m/V ethanolic solution)	2 mL
Distilled water to	1 litre

The medium is most conveniently prepared in quantities of 10 litres or more. If it is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing. Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

SOLUTION 1

L(+) Arginine monohydrochloride	400 mg
L(–) Aspartic acid	480 mg
Distilled water	50 mL

Heat to dissolve.

SOLUTION 2

L(–) Cystine	400 mg
5M Sodium hydroxide	10 mL
Distilled water	90 mL

Heat to dissolve.

SOLUTION 3

Nicotinic acid	20 mg
Pantothenic acid	20 mg
Distilled water	5 mL

Dissolve in the cold.

SOLUTION 4

Iron(III) citrate scales	200 mg
Distilled water	10 mL

Heat to dissolve.

SOLUTION 5

Calcium chloride dihydrate	5 g
Distilled water	100 mL
Concentrated hydrochloric acid	0.1 mL

Dissolve in the cold and sterilize at 121°C for 20 minutes.
Keep as a stock solution.

SOLUTION 6
Sterile 0.1% solution of thiamine in distilled water. Add the contents of an ampoule (100 mg) to 99 mL of sterile distilled water. The solution should be kept at 4°C and any remaining discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L(+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate in 9 litres of hot distilled water. Add the whole of solutions 1,2,3 and 4, and 4 mL of solution 5. Adjust the pH to 6.8 or higher if necessary, so that the final pH, after completion and sterilization, is 6.7.

After adjustment of pH, add 20 mL of 1% ethanolic solution of bromocresol purple. Make up the final volume to 10 litres. This should require about another 810 mL of distilled water. If the bulk of the medium is not required for immediate use, store in 500 mL volumes and autoclave at 115°C for 10 minutes. For use, add the necessary amount of lactose and thiamine (solution 6), allow to dissolve and distribute in 10 mL and 50 mL volumes. Each tube or bottle should contain an inverted (Durham) fermentation tube. Sterilize at 115°C for 10 minutes or steam at 100°C for 30 minutes on three successive days.

Single-strength medium:

Prepare single-strength medium by diluting the double-strength medium with an equal volume of distilled water and distribute in 5 mL volumes in tubes containing an inverted fermentation (Durham) tube. Sterilize at 115°C for 10 minutes or steam at 100°C for 30 minutes on three successive days.

Lauryl Tryptose Lactose Broth (APHA 1989)

Double-strength medium:

Tryptose	40.0 g
Lactose	10.0 g
Sodium chloride	10.0 g
Dipotassium hydrogen phosphate	5.5 g
Potassium dihydrogen phosphate	5.5 g
Sodium lauryl sulphate—specially pure	200 mg
Distilled water	1 litre

Add the tryptose, sodium chloride, lactose and phosphates to the water and warm to dissolve. Add the sodium lauryl sulphate and mix gently to avoid froth. Adjust to pH 6.8. Prepare single-strength medium by dilution of the double-strength medium with an equal volume of distilled water.

Distribute single-strength medium in 5 mL volumes and double-strength medium in 10 mL and 50 mL volumes. Each tube or bottle should contain an inverted fermentation (Durham) tube. Autoclave at 115°C for 10 minutes.

Lauryl Tryptose Mannitol Broth with Tryptophan (PHLS/SCA 1980c)

Tryptose	20 g
D(–) Mannitol	5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2750 mg
Potassium dihydrogen phosphate	2750 mg
Sodium lauryl sulphate	100 mg
L(–) Tryptophan	200 mg
Distilled water	1 litre

Add the tryptose, sodium chloride, mannitol, phosphates and tryptophan to the water and warm to dissolve. Add the sodium lauryl sulphate and mix gently to avoid froth. Adjust to pH 6.8. Distribute in 5 mL volumes in tubes containing an inverted fermentation (Durham) tube. Autoclave at 115°C for 10 minutes.

Brilliant Green Lactose Bile Broth

Peptone	10 g
Lactose	10 g
Ox bile (dehydrated)	20 g
Brilliant green (0.1% m/V aqueous solution)	13 mL
Distilled water to	1 litre

Dissolve the peptone in 500 mL distilled water. Add the dehydrated ox bile dissolved in 200 mL of distilled water; this solution should have a pH between 7.0–7.5. Make up with distilled water to approximately 975 mL. Add the lactose and adjust the pH to 7.4. Add the brilliant green solution and make up the volume with distilled water to 1 litre.

Distribute 5 mL volumes in test tubes containing inverted fermentation (Durham) tubes and autoclave at 115°C for 10 minutes.

Tryptone Water for Indole Reaction

Certain peptones which give satisfactory results in tests at 37°C are not satisfactory for the indole test at 44°C (Burman 1955). Care should be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled water	1 litre

Dissolve the ingredients in the water and adjust to pH 7.5. Distribute in 5 mL volumes and autoclave at 115°C for 10 minutes.

MacConkey Agar

Bile salts	5 g
Peptone	20 g
Lactose	10 g
Sodium chloride	5 g
Agar	12 g
Neutral red (1% m/V aqueous solution)	5 mL
Distilled water	1 litre