the purpose for which it was taken. As an example the pre-printed label or bar-code should provide the following information;

(i) a reference number;

(ii) the date and time of sampling;

(iii) the reason for taking the sample;

(iv) the specific sample point of the supply;

(v) the type of water, for example raw, filtered, treated etc; and

(vi) for consumers, the location of the sampling point.

Any additional information or amendments to the above should be recorded at the time of sampling.

Residual chlorine should always be recorded. Additional information may be of value when interpreting the results of microbiological analyses and this may be provided on a separate sheet which identifies the sample reference numbers and locations. Such information might include whether the sample tap was properly disinfected and observations on weather conditions, possible sources of pollution or unusual features observed or encountered during sampling. All relevant data should be recorded and given in sufficient detail to allow the exact location to be readily identified, if a repeat sample is required.

6.4.3.2 Non-routine samples

Non-routine samples include consumer complaints, investigational, operational and any other samples which have not been pre-scheduled. Any comments relevant to the sample should also be recorded.

6.4.4 Sample locations

The prime objective is to obtain a sample which is representative of the water being examined. Care must be taken to avoid accidental contamination during sample collection and its subsequent handling and transport to the laboratory. Warning markers should be available and used for problem samples, for example those that might be contaminated with sewage.

6.4.5 Sampling from raw waters

Raw water samples should be taken at the inlet to the works and should be representative of the incoming water quality. Such samples could be taken from inlet channels or from specially fitted raw water taps, which need not be disinfected before samples are taken. Where samples have to be taken from surface waters they should not be taken too near a bank or too far from the point of abstraction. Areas of stagnation in streams and reservoirs should be avoided. Routine samples should be taken as closely as possible from the same sampling location.

Ideally, raw water and water at each stage of treatment should be piped through dedicated sample lines, which must be made from approved materials (Colbourne 1985, BSI 1988), and should be as short as possible. It must be possible to sample without shutting off pumps or disinfection, and 'dead water' must be flushed to waste on each sampling occasion. In practice it may not always be possible to provide dedicated sampling points for all waters and it is then necessary to select sites which provide samples which are as representative as possible of the water to be examined. In certain circumstances it may be necessary to take dip samples. However, stringent precautions should be taken to avoid contamination. See 6.4.12.

Where samples have to be taken from a river or stream, sites should be selected preferably where marked quality changes are likely to occur or where there are important river users causing major discharges or abstractions. Weirs or small discharges which are only very

local in effect should be avoided, unless of course, it is established that the effec
local. Sites should preferably be chosen where flow data are available. When sampl
banks of rivers and reservoirs should be avoided wherever possible and use n
convenient bridges or gantries to take representative samples from the body of the

If sampling is intended to monitor the effect of discharges it should be carried ou
careful consideration to the mixing of the discharge and receiving water and its eff
the river or stream, above as well as below the discharge point.

When sampling from boreholes or wells, the sample should be collected from a previ
disinfected tap fitted to the rising main before the water passes into any reservoir or ci
Particular attention should be paid to the cleanliness of the tap. The depth selecte
sampling should be determined from hydrogeological and site knowledge. The influ
of casings where biofilms may form should also be considered. If the well is fitted w
hand pump, the pump should be cleaned, the mouth flamed and the pump operated f
least five minutes before taking the sample. If the only method of collection available i
means of a pail or can, the sample should be collected in a sterile weighted bottle.

Where adequate sample taps are not available, or where a more comprehensive study
the water quality is required, samples should be collected at a point where water enter
reservoir or lake, and from sampling points representative of the water body. General
this will involve the selection of a number of fixed buoy locations, samples being collect
from different depths at each point. Sampling from the banks should be avoided because
the likelihood of unrepresentative samples due to disturbance of sediment.

6.4.6 Sampling treated water

6.4.6.1 Outlets from water treatment works

To enable representative samples to be taken for compliance purposes all works should be
fitted with proper metal sampling taps which comply with BS 6920, or stainless steel taps,
with diaphragm valve and swan neck. No attachments or inserts should be fitted to these
taps. Taps should be provided at each outlet main of the works and all taps within the
works should be correctly and prominently labelled with the type of sample and flushing
time.

The delivery pipework to the sample tap should be as short as possible and in good
condition. There should be adequate pressure to obtain the samples. Ideally the sample
point should be above ground and there should be adequate drainage to prevent the
accumulation of water during flushing. Sample taps should be clean, free from slimes,
grease, cleansing agents etc, any of which may affect the microbiological sample.
Particular attention should be paid to disinfecting the taps. Constantly running taps and
plastic or mixer taps should not be used at sample sites for microbiological compliance
monitoring purposes.

6.4.6.2 Service reservoirs and water towers

Covered service reservoirs represent point sources where bacterial ingress can and does
happen to varying degrees. For operational purposes, the water undertaker should consider
all units with compartmented or multi-reservoir sites as separate reservoirs for sampling
purposes unless they are directly interconnected. However, for compliance purposes,
water undertakers should be confident that samples taken are representative of all the
water leaving each surface reservoir and should take account of such factors as flow
patterns, joint inlet/outlet arrangements, common inlet/outlet arrangements. If two or more
structures share a common outlet main and have no other outlets then the Drinking Water
Inspectorate advise that a suitable single compliance sampling point on the common outlet
main will be acceptable for regulatory purposes (DWI 1994). If the reservoir has been out
of service for a prolonged period or because of contamination, consideration should be
given to the analysis of other indicator organisms, enteroviruses and parasites. Guidance
on reservoir inspection, cleaning and maintenance should be followed (WAA 1988).

All service reservoirs should have metal sampling taps with washers, conforming to BS 6920, fitted to each compartment of the tank at points representative of water entering and leaving the reservoir. Sample taps should have no attachments or inserts, should not be exposed to contamination, and should be protected, as far as possible, from freezing and vandalism. Where it is impossible to provide a tap on site, a tap should be provided on the outlet main at the nearest possible point to the reservoir. The delivery pipework should be short and in good condition and it should be possible to flush the sample tap at full bore for the requisite flushing time. Taps should be labelled with a flushing time. When the reservoir is below ground, monitoring of the water temperature during flushing may be useful in indicating when the water from the reservoir is being withdrawn. As an interim measure, pending provision of a proper sampling point, or in the event of lack of access, a good quality consumer tap at the nearest property fed directly off the outlet main could be used. A register of these alternative sites should be maintained and the properties inspected.

6.4.6.3 Sampling taps at consumers' premises

Care should always be exercised over the choice of taps for sampling. Sampling taps should always be clean, free from all attachments and from slime, grease, cleaning agents and other extraneous matter. The taps should be in good repair, and should supply water from a service pipe directly connected to the main. Uniflow mixer taps and taps from a cistern or storage tank should not be used. For microbiological sampling in particular, a knowledge of tap types and the problems associated with them is essential in order to to be able to choose the correct method of disinfection.

6.4.6.3.1 Fixed addresses Samples taken from fixed points may comprise no more than 50% of regulatory microbiological samples. Fixed points for compliance testing should be selected such that they are equivalent to a domestic situation. Hydrants should not be used as fixed sampling sites. Ensure at all times that adequate flushing and disinfection is carried out.

6.4.6.3.2 Random addresses Samples taken from randomly selected addresses must comprise at least 50% of the regulatory samples in any zone in one calendar year. Samples must be taken from taps fed by the rising main. Where the selected premises are not accessible, neighbouring premises should be chosen and labels and log sheets suitably amended. The use of random sampling points may require the sampling of taps which would be considered unsuitable under normal circumstances. Such taps may contain external fittings and may be made of materials which render them impossible to disinfect. Mixer taps where the hot and cold water mix together in the spout should not be sampled. Dual flow "mixer" taps may be sampled. Taps which leak between the spindle and the gland when the tap is turned on should not be sampled. A random sample should not be taken from any tap where the water has been fed through an in-line device such as a water filter or softener. If an unsuitable tap has to be sampled, details of any cleaning or disinfection procedure, together with any comments should be recorded on the label or log sheet.

It should be emphasised to staff taking samples that the exact randomness of a sample is less important than the suitability of the tap from where the sample is taken. It is more appropriate to sample a property (containing a suitable tap) adjacent to or in the proximity of an address which is pre-scheduled and chosen at random but which contains a tap considered unsuitable.

6.4.7 Order of taking samples

When sampling from fixed sites, such as raw water taps, treatment works taps, taps on service reservoirs or fixed points in distribution, or when sampling from a random site for regulatory purposes or in response to a consumer enquiry, the order of sampling should be as follows:

1 Samples that are required to be taken before flushing.

2 Flush for a specified length of time.

3 On-site chemical and physical tests, for example chlorine residual, temperature, p

4 Physical and chemical samples required to be taken after flushing, and biologi samples, including invertebrate samples if required.

5 Disinfect the tap (section 6.4.8).

6 Bacteriological sample.

Swab samples, if required, can be taken first or last according to the reason for sampli (see section 6.4.8.4).

6.4.8 Sampling procedure—sampling from taps

All treated water sample taps should be disinfected before being sampled for micr biological analysis, except when investigating a consumer complaint (see section 4.6.2.1 when a pre-disinfection sample may also be taken, if there is justification.

6.4.8.1 Disinfection by flaming

This procedure can be carried out on all metal taps. Turn the tap on and leave to run at uniform rate for 2–3 minutes. Turn the tap off and flame thoroughly starting at the nozzl and working back to the body of the tap until the water held in the spout boils. Care shoul be taken that very hot water, which may spurt out of the tap during flaming, does not caus injury. If the spout drains dry when the tap is turned off, heat the full length of the spou such that the first issue of water boils when the tap is turned back on. A propane or butan burner can be used for flaming as it should produce a tight controllable flame. Methylate spirit flames should not be used as they are not hot enough and are difficult to control Ensure that flammable or heat-sensitive items, such as curtains, are moved away from the fire zone. After flaming, run the tap to waste until the water is cool.

6.4.8.2 Disinfection by using sodium hypochlorite solution

This procedure can be carried out on plastic taps, mixer taps and metal taps where flaming is not appropriate. Remove all external fittings. Accumulations of grease and slime should be removed from the tap with a clean sterile swab. Turn the tap on and leave to run at a uniform rate for 2–3 minutes. Disinfection can be carried out by swabbing the outside of the tap, and as much of the inside as possible, with a 10% solution of commercial sodium hypochlorite solution (to give 1% available chlorine). Alternatively, use a wash bottle or similar device filled with a 1.0% solution of sodium hypochlorite to spray outside the tap and inject inside the tap spout. Disinfection swabs should fit snugly within the spout to ensure maximum contact with all surfaces. **Sodium hypochlorite is highly corrosive and must be handled with great care. If contact with skin or clothes occurs, wash immediately with copious amounts of water.** Leave for 2–3 minutes to allow the sodium hypochlorite to disinfect the tap. Run the water to waste for a sufficient period to ensure that all the sodium hypochlorite is removed before taking the sample.

6.4.8.3 Taking the sample

Take the sample in a sterile bottle. Hold the bottle in one hand and remove the stopper or cap with the other. The sample bottle should never be rinsed out or the closure put on any surface. Fill the bottle, leaving a small air gap, from a gentle stream and avoid splashing. Do not change or alter the flow rate of the water leaving the tap as the bottle is being filled. To do so may cause debris or other material to become dislodged from the system and enter the sample bottle. Replace the stopper or cap immediately. Care must always be taken not to touch the top of the bottle during removal or replacement of the cap. Secure the cap and shake the sample before returning it to a cool box. If accidental contamination is suspected, the sample must be discarded and a fresh, sterile bottle used to take a further sample.

6.4.8.4 Bacteriological swabs

Additional information about the microbiological state of the tap can sometimes be obtained by the use of a sterile swab. It can be helpful to swab sample random addresses

and all customer complaints; the swab samples may be taken before and/or after all other sampling has been completed. After removal from its container, the swab should be rubbed around as much of the inside surface of the tap as possible and then carefully replaced in its container. The container should be clearly identified and labelled with appropriate details.

6.4.9 Sampling from hydrants

Regulatory or compliance samples must not be taken from hydrants or standpipes. Remove the hydrant box lid and remove any debris from the area of the connection. Bail out any accumulated water to well below the bottom of the threads. Unscrew the hydrant cover and open the hydrant valve a small amount, sufficient to flush any accumulated debris from the hydrant. Run to waste approximately 5 litres of water.

Unwrap the standpipe, which should be transported in a clean plastic bag, and connect it to the hydrant. Open the standpipe tap smoothly; abrupt or rapid opening can cause turbulence in the main and stir up sediment. Flush water through the standpipe until the water runs clear. Turn off the water. If net samples for animals or similar samples are required, they should be taken at this point.

Remove the standpipe and bail out 200 mL of water from the hydrant bowl. Add 100 mL of a 1.0% solution of hypochlorite. Care should be taken and any safety requirements observed. See section 6.4.8.2. Replace the standpipe and open the valve slightly, until chlorinated water first discharges. Turn the water off. Wait for 10 minutes before flushing the chlorinated water out of the hydrant. Flush for one minute and, without turning off the water, measure the chlorine content of the water. Continue flushing until the chlorine content is below 0.5 mgL^{-1} or until the level is no higher than the chlorine level in the mains water. Without turning the water off, take a sample of the water, avoiding either the bottle or the top touching any surface. Turn off the water, remove the standpipe and replace the hydrant cap and the box lid.

6.4.10 Sampling from bowsers

All taps and hatches should be checked to ensure no damage has occurred due to vandalism and that the bowser contains water. Check the free and total residual chlorine from the bowser. Run the tap to waste for about 10 seconds. Disinfect the tap as described in 6.4.8, flush and sample.

6.4.11 Sampling new mains, renovated mains and repaired mains

If applicable, follow the procedures in section 6.4.9 (sampling from hydrants). Other details can be found in 'Operational Guidelines for the protection of Drinking Water Supplies' (WAA 1988).

6.4.12 Sampling for investigational purposes—dip sampling

Dip sampling should only be carried out for investigational purposes and not on a routine basis or for regulatory purposes. A dip sample may be taken for microbiological analysis using a sterile dip bottle. Sterile dip sample bottles can be prepared by using a wide mouthed 500 mL sample bottle with a wire attached. The sample bottle can be wrapped in suitable material and autoclaved with the lid wrapped in tin foil. Clean string can be attached to the wire and then the outer wrapping from the bottle removed. The wrapped lid can be held until the bottle has been dipped and filled. The lid is then unwrapped and carefully placed on the bottle. Dip samples may also be taken using a sterile weighted sampling device or a sample bottle attached to a long pole.

6.4.13 Drinks vending machines

See section 5.4.2 for details of how to take samples from drinks vending machines.

6.4.14 Transport and storage of samples

A review of published studies shows that indicator organisms from different water sources can behave differently in transit (Tillett and Benton 1993). Therefore samples should be

delivered to the laboratory and analysed as quickly as possible after collection. particularly applies to bacteriological samples where every effort should be made to examination within six hours of collection.

Samples for microbiological analysis should be kept cool (2–10°C) during transport. Consideration should be given to the manufacture of specially designed containers, provision of mechanically refrigerated compartments in sampling vans. Containers for transporting microbiological samples should not be used for other purposes but i is unavoidable, care must be taken not to contaminate the samples. Containers mu cleaned and disinfected regularly. Where logistics do not allow examination withi hours, storage may be prolonged and samples may be examined up to twenty four h after collection provided that samples are kept in the dark and cool (2–10°C) (Guid Document). This procedure should be regarded as a 'last resort' and should onl undertaken in exceptional circumstances. If a sample is examined after a much lo storage period and is found to contain undesirable organisms, for example a fa coliform, this is a significant finding since the organism must have been present ea However, failure to find an organism after an extended storage period does not rule ou possibility that the organism was present at an earlier stage. For this reason emphasised that all samples should be examined as soon as practicable after collec This advice has been confirmed by the findings of a research contract funded by (PECD 7/7/413). This research showed that for raw waters a statistically signifi reduction occurred in faecal colony counts after twenty four hours storage compared to analysis after six hours storage.

Chapter 7: Bacteriological Methods

7.1 Introduction

This chapter describes in detail the various procedures for the microbiological examination of water. The purpose of this detailed description is not to prescribe a rigid set of rules from which any deviation is to be deprecated, but rather to specify techniques which are likely to be accompanied by acceptable experimental error yet give reproducible results. Changes to the methods must be fully documented and evaluated in order to establish whether the performances of the modified methods remain acceptable. The importance of proper quality assurance has already been emphasised. The methods described in this section of the Report have been used widely for many years in the assessment of the wholesomeness of drinking water, and are accepted as appropriate for compliance monitoring in the UK. Changes in technique and the introduction of new methods can only be justified by the demonstration that the changes provide equivalent or better performance in terms of sensitivity and specificity.

Details of the preparation and sterilization of necessary glassware are described in Appendix A and methods for the preparation of media and reagents in Appendix B. These methods should be adhered to if uniform results are to be obtained. Whilst the use of certain standard commercial preparations is occasionally recommended, no endorsement of any particular manufacturer is suggested. The need for regular monitoring of the performance and accuracy of laboratory equipment and for testing the quality of reagents and media is, however, greatly emphasized. Particular attention should be paid to the control of operating temperatures of incubators and water baths.

7.2 Laboratory Hygiene

In water examination, particular significance is attached to the presence of very small numbers of coliform organisms and *E. coli*. As these organisms are very common in humans and their environment, and are cultivated routinely in the laboratory, special precautions are needed to avoid accidental contamination of samples. These have been discussed earlier, and especially in Chapter 6, but the need to conform to good laboratory practice at all times is stressed.

7.3 Methods for the Detection and Enumeration of Indicator Organisms

Two procedures, membrane filtration, and the multiple tube test, also known as the most probable number (MPN) or dilution method, are available. Both these techniques can be used to detect and enumerate indicator organisms, the media and incubation conditions differing according to the organism sought. Each method is therefore described in detail together with its application to the test for coliform organisms and *E. coli*; the variations necessary for the other indicator organisms are described in later sections. The preparation and dilution of samples are the same for every test and these are described first.

7.4 Preparation of Samples

To facilitate mixing, an air space should be present in the sample bottle. Invert the sample bottle rapidly several times. Hold the bottle in one hand and remove the stopper or cap with the other but at the same time retaining it. Flame the mouth of the bottle. Make any dilutions required at this stage.

The sample volume should be sufficient to ensure all routine examinations can be carried out. Any excess sample not required may be stored in a refrigerator until the initial examination has been completed. This sample can then be discarded, or used for further tests in the event of an unexpected high count or other mishap.

7.4.1 Diluent

Use sterile quarter-strength Ringer's solution for all dilutions, see page 119.

7.4.2 Making the dilutions

Measure out a known volume, for example 90 or 9 mL of diluent into sterile dilution bottles or tubes. Alternatively, use volumes of diluent pre-sterilized in screw-capped

bottles. In this case it should be recognised that some bottles may lose diluent on st
Volumes should be checked and any bottles obviously containing incorrect amount
be discarded.

Make one, or more, ten-fold dilutions by transferring one volume of sample to
volumes of diluent.

Using a fresh, sterile pipette each time, repeat the process as often as necessary to e
the correct dilution range has been covered. Carefully mix each prepared dilution b
further dilution or before use. Prepare sufficient quantities of each dilution to enab
tests to be carried out.

7.5 Membrane Filtration Method

7.5.1 Principle

In the membrane filtration method a measured volume of the sample is filtered thro
membrane composed of cellulose esters or equivalent. The pore size is such tha
organisms to be enumerated are retained on or near the surface of the membrane wh
then placed, normally face upward, on a differential medium selective for the indi
organisms sought. This may be either an agar medium or an absorbent pad saturated
broth. After a specified incubation period, it is assumed that the indicator organ
retained by the membrane will, depending on the medium used, form coloni
characteristic morphology and colour. Other organisms are either inhibited or ca
distinguished by their colonial appearance. The colonies of the organism sough
counted and the result, taking into account any dilutions made, is expressed as the nu
per 100 mL of the sample. This presumptive count is then confirmed by subculturing
a representative number of colonies formed.

7.5.2 Filtration apparatus

The membrane filtration apparatus consists of a base supporting a porous disc. The
funnel, which should be graduated, is secured to the base by means of screw-thre
clamps or a magnet. The filtration apparatus is connected to a vacuum source. For
examination of large numbers of samples, multiple filtration units may be used.
filtration apparatus should be sterilized on a regular basis between batches of analy
Spare funnels as required can be disinfected by immersion in boiling or nearly boi
distilled water for at least one minute between samples. After disinfection, each fu
should be placed in a stand and allowed to cool before use. Alternatively, a fresh
sterilised funnel may be used for each sample.

7.5.3 Membranes

Membrane filters, for example 47 mm in diameter, with a rated nominal pore-size
0.45 µm or membranes which have equivalent filtration characteristics, are rec
mended; these will retain almost all the bacteria mentioned in this Report. A pore siz
0.2 µm is, however, recommended for the isolation of campylobacters (section 8.6.6
The use of membranes with grid-marks is recommended to facilitate counting. I
necessary to check periodically that the membranes used are suitable for the organi
sought in the waters examined. Quality assurance is important and membranes must
free from toxic substances. When membranes with grid-marks are used, bacterial gro
should not be inhibited or stimulated along the lines.

Membranes should be obtained pre-sterilized and should not be re-used. Membra
should not be used beyond their shelf life date.

7.5.4 Absorbent pads

Absorbent pads of at least the same diameter as the membranes and approximately 1 m
thick should be used. The pads should be made of high quality paper fibres, and
uniformly absorbent and free from any toxic substances which could inhibit bacte
growth. Absorbent pads are available pre-sterilized or they can be sterilized, preferably
121°C for 20 minutes, either in containers or wrapped in waterproof paper or foil.

Place each individual pad with aseptic precautions in a sterile Petri dish.

7.5.5 Media

Details of media recommended are given in Appendix B. The media used with membrane filters differ in composition from those for the multiple tube method because membranes selectively absorb certain substances; there is also a differential absorbent capacity between the nutrient pad and the membrane. Dehydrated media should be prepared according to the manufacturers' instructions. Alternatively, the media may be prepared in the laboratory from approved high quality constituents. Sterile broth is dispensed aseptically on to each filter pad in sterile Petri dishes or similar containers; this may be done conveniently with an automatic pipette. The pad should be saturated with broth. When agar medium is used, the surface of the agar should be completely smooth and dry.

7.5.6 Incubators and water baths

Accurate temperature control of incubators is essential, see section 6.2.4.9. When resuscitation techniques involving pre-incubation at lower temperatures are required, manual transfer between different incubators can be avoided if an incubator with fan-assisted air circulation and fitted with a time control to give automatic switching between the temperatures is used. Incubators of this type function adequately only when the lower temperature is at least 5°C above ambient. When incubators are required to operate in an environment where the ambient temperature may approach within 5°C of the lower temperature, the use of a cooled incubator is essential. Instead of incubators, dual time-temperature controlled water baths with circulation may be used. With water baths, it is essential to use suitable water-tight immersible containers to hold the Petri dishes.

7.5.7 Procedure

7.5.7.1 Preparation of samples

The volumes and dilutions of samples should be chosen so that the number of colonies to be counted on the membrane lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter different volumes so that the number of colonies on one of the membranes is likely to fall within this range. For treated waters, filter 100 mL of the sample; for polluted waters either filter smaller volumes or dilute the sample before filtration. When the volume to be filtered is less than 10 mL, add at least 20 mL of sterile diluent to the funnel before addition of the sample to aid uniform dispersion of the bacteria over the entire surface of the membrane during filtration.

7.5.7.2 Filtration procedure

Place the disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off.

Remove the funnel and place a sterile membrane, grid-side upwards, on the porous disc of the filter base. Grasp only the edge of the membrane filter with flat-ended sterile forceps. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum of about 65 kPa (500 mm of mercury) and filter the water slowly through the membrane. Close the stopcock as soon as the sample has been filtered so that as little air as possible is drawn through the membrane.

Remove the funnel to a boiling water bath if it is to be re-used and transfer the membrane carefully either to a pad saturated with medium or to a well-dried agar plate. Ensure that no air-bubbles are trapped between the membrane and the medium.

Pour off any excess medium from the saturated pad, either before or after the membrane is placed in position. If this is not done, confluent growth may result.

For different volumes of the same sample, the funnel may be re-used without boiling provided that the smallest volumes of sample are filtered first.

For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow to cool and repeat the filtration process.

After filtration of each sample, disinfect the funnel by immersion in boiling distilled wa for at least one minute. During the filtration of a series of samples the filter base need be sterilized unless it is contaminated or a membrane is damaged.

Do not alternate the filtration of known polluted samples with those of treated samp through the same apparatus.

Filter all samples of chlorinated water and those expected to give negative results fi followed by those known or suspected of being polluted. Alternatively, a separ membrane filtration apparatus can be reserved for all chlorinated samples and another polluted samples.

When funnels are not in use they should be covered with a sterile lid or a sterile Petri d lid.

The time between the end of the filtration step and the beginning of the incubation st should be as short as possible and no longer than 2 hours.

7.5.7.3 Incubation and examination

When using pads soaked with liquid medium, place the Petri dishes with the membra inside a container with a tightly fitted lid to prevent drying out. Alternatively, a polyth bag may be used and carefully folded over, tied or sealed. For anaerobic organisms, spe incubation conditions are required.

Incubate the membranes at the appropriate temperature and for the time specified for organism sought.

After incubation, count the characteristic colonies on the membrane, if necessary wit hand lens. Make a note of the numbers of each morphological type. As colours are liabl change on cooling and standing, count the colonies within a few minutes of removal fr the incubator or water bath.

Express the result as the number of presumptive indicator organism per 100 mL of sam (see section 6.3.5).

7.5.8 Advantages and limitations of the membrane filtration method

7.5.8.1 Advantages

The outstanding advantage of the membrane filtration technique is the speed with wh results can be obtained as direct counts. For example, presumptive coliform and *E. c* counts may be available within 18 hours. This enables a quicker response to be made w respect to the remedial action that may be required. Individual colonies can be availa for confirmatory testing after 18 hours.

There is considerable saving in technical labour and in the amount of media and glassw required compared to other techniques.

The conditions of incubation can be varied easily to encourage the growth of attenuated slow-growing organisms.

False-positive reactions that may occur with some media in the multiple-tube test coliform organisms due to the growth of aerobic and anaerobic spore-bearing organis or to mixtures of organisms, are unlikely to occur with membranes.

7.5.8.2 Limitations

In the membrane filtration method, gas production, is not detected. This however is l critical in view of the recognised change in the importance of gas production in relation the definition of coliforms, see section 3.2, but may be a consideration with respect to ot organisms.

Membranes are unsuitable for use with waters of high turbidity. In these circumstances the membrane will become blocked before sufficient water can be filtered, while the accumulated deposit on the membrane may inhibit the growth of indicator organisms.

The membrane method is also unsuitable for water containing only small numbers of the indicator organisms sought in the presence of many other bacteria capable of growth on the media used (see, for example section 7.7.5).

7.6 Multiple Tube Method

7.6.1 Principle

In the multiple tube method measured volumes of sample, or of one or more dilutions, are added to a series of tubes containing a liquid differential medium. It is assumed that on incubation each tube which received one or more test organisms in the inoculum will show growth, and characteristic change produced by the organism sought when growing in the medium used. Provided that negative results occur in some of the tubes, the most probable number of organisms in 100 mL of the sample can be estimated from the number and distribution of tubes showing positive reactions. Confirmation that positive reactions are due to the growth of the specific indicator organism sought can be obtained by subculture to tubes of confirmatory media, some of which may need to be incubated at higher temperatures, depending on the organism sought. Positive reactions in the confirmatory tests yield further information about the types of organism present. The presumptive and confirmed counts are calculated by reference to probability tables (see section 6.3).

The multiple tube method is particularly suitable for the examination of sludges and waters containing sediment.

7.6.2 Procedure

7.6.2.1 Choice of volumes for inoculation

With waters expected to be of good quality, add 1 × 50 mL volume and 5 × 10 mL volumes of the sample to equal volumes of double strength medium.

With waters of doubtful or unknown quality, use 1 × 50 mL volume, 5 × 10 mL and 5 × 1 mL volumes; add the 1 mL volumes to 5 mL of single-strength medium.

With more polluted waters include also 5 × 0.1 mL volumes (5 × 1 mL volumes of a 1 in 10 dilution) and omit the 50 mL volume of the sample.

With heavily polluted waters, additional dilutions of a hundred-fold, a thousand-fold or even higher, may be needed to give some negative reactions necessary for the MPN estimation.

7.6.2.2 Inoculation of the culture medium

With a sterile pipette add 1 mL volume of sample to a tube containing 5 mL of single strength medium, and 10 mL volume of sample to a tube containing 10 mL of double strength medium. Measure 50 mL volume of sample into a screw-capped bottle containing 50 mL of double-strength medium.

7.6.2.3 Incubation and examination of the cultures

Incubate the inoculated tubes at the specified temperature, and examine them at set intervals, according to the indicator organism being sought.

Count the number of tubes of each volume showing a positive reaction and by reference to the appropriate tables in Appendix C, calculate the MPN of organisms present in 100 mL of the sample. For example, in a 15-tube test with 5 × 10 mL, 5 × 1 mL and 5 × 0.1 mL volumes, if the number of tubes showing a positive reaction in each set of five tubes is 3, 2 and 0 respectively, then the MPN is 13 organisms per 100 mL. At this stage carry out any confirmatory tests that may be required.

When using dilutions, choose a consecutive series of three ten-fold dilutions wh some positive and some negative reactions and multiply the MPN by the ap dilution factor as described in Appendix C.

7.7 Count of Total Coliforms and *Escherichia coli* by Membrane Filtration

7.7.1 Introduction

Tests for coliform organisms and *E. coli* are the most important routine microbi examinations carried out for drinking water as they provide the most sensitive me assessing the effectiveness of disinfection and for detecting faecal contaminatio

7.7.2 Principle

Isolation of organisms on a membrane placed on an absorbent pad saturated wit containing lactose and phenol red as an indicator of acidity, with subsequent confi of acid production from lactose and, where necessary, oxidase reaction and formation.

7.7.3 Definitions

In the context of the method, organisms which are oxidase negative produce aci lactose and form all shades and sizes of yellow colonies on membranes after incubat 4 hours at 30°C followed by 14 hours at 37°C are regarded as total coliform orga

These are considered to be members of genera or species within the Family Ente teriaceae, capable of growth at 37°C, that possess ß-galactosidase. This definitio include anaerogenic strains. Coliforms are, by definition oxidase negative. The foll genera have been isolated in routine practice:

Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia, Yersinia.

Organisms which produce acid from lactose after incubation for 4 hours at 30°C fol by 14 hours at 44°C are presumptive faecal coliform bacteria, of which the majo isolates may subsequently confirm as *E. coli.*

The term "faecal coliform" is not precise and is used to denote a coliform of faecal o it is used in this sense in the Directive and in the Regulations. The Directive implie the Guidance Document states) that faecal coliforms are those capable of growth at ie are thermotolerant. In some documents thermotolerant coliforms are referred faecal coliforms. However, as will be noted not all thermotolerant coliforms are of origin nor are all faecal coliforms thermotolerant. Thermotolerant coliforms s therefore be regarded as "presumptive faecal coliforms".

For the purposes of water examination *E. coli* has historically been regarded as a me of the Family Enterobacteriaceae which ferments lactose or mannitol at 44°C wit production of acid and usually gas within 24 hours, and which produces indole tryptophan. Most strains produce ß-glucuronidase. Strains which possess these chara istics at 37°C but do not express them at 44°C and strains which are anaerogenic (d produce gas) may also be *E. coli*. When identified as *E. coli* they have the same san and operational significance with regard to their faecal origin, and, in order to re some confusion in terminology, they are regarded in this Report as faecal coliforms. Drinking Water Inspectorate advised the committee responsible for revising this m script that for UK regulatory purposes, confirmed *E. coli* can be regarded as fa coliforms. For practical purposes, examination for organisms satisfying the definitio coliforms and producing indole from tryptophan at 44°C can be considered as examin for *E. coli.*

Under certain circumstances, *Aeromonas* spp., may grow under the conditions of the for total coliforms, but may be recognised because they are oxidase positive. The oxi test is essential for the confirmation of coliforms. Confirmed total coliforms and *E. col* oxidase negative.

7.7.4 Choice of medium for isolation and enumeration

Membrane lauryl sulphate broth (MLSB) (PHLS/SCA 1980a) is recommended for the isolation and enumeration of total coliforms and *E. coli*. This is available commercially as a complete dehydrated medium.

7.7.5 Procedure

For each sample, place an absorbent pad into each of two empty sterile Petri dishes. Add sufficient MLSB to saturate the pad, allow to soak in and pour off any excess fluid.

Prepare any necessary dilutions and filter as described in section 7.5.7. Set up two membranes for each sample and place on the pads soaked in MLSB.

Incubate both membranes at 30°C for 4 hours then transfer one membrane to 37°C for total coliforms and the other to 44°C for *E. coli*. Use incubators or waterbaths for incubation as described in section 7.5.6. Accurate temperature control and even temperature distribution are essential, especially for *E. coli* at 44°C. False positive results will be obtained at temperatures below that recommended and some strains will fail to multiply at higher temperatures. Incubate the membranes at 37°C or 44°C for 14 hours to give a total incubation time of 18 hours. If an early indication of a result is required urgently, the membranes may be examined for presumptive positive results after a total incubation time of 12 hours but must be returned to the incubator for the full period of 18 hours before results can be regarded as negative. From a water treatment point of view it may be convenient to incubate a single membrane at 37°C. In this case an immediate operational response should be made to **any** presumptive positive result and should be treated as *E. coli* until the confirmatory tests for coliforms and *E. coli* have been completed. It should be assumed that any presumptive indication is treated as *E. coli* and remedial action taken.

After incubation, examine the membranes under good light, if necessary with a hand lens. Count all yellow colonies (however faint) irrespective of size within a few minutes of being removed from the incubator. Colours are liable to change on cooling and standing. It is important to note whether pink colonies are present in numbers which may interfere with the growth of coliforms. The detection of acid production is influenced by the pH of the medium, thus it is important that the medium is of the correct pH. If the growth of pink colonies is considered to be such that they may be obscuring lactose-fermenting colonies, a further sample should be taken and examined by membrane filtration; appropriate dilution and presence-absence methods should be considered (section 7.8.8).

7.7.6 Confirmatory techniques

7.7.6.1 Colonies chosen for subculture

With raw waters, it is not essential to confirm presumptive total coliform and *E. coli* results except in special circumstances, for example borehole waters.

For treated waters, subculture a suitable number of yellow colonies (however faint) depending on the purpose intended and accuracy required (see section 6.3.3). If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be examined if fewer than ten are present, and at least ten colonies if more are present. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be examined. Where a number of colonies is clearly distinguishable a note of the number of each morphological type should also be made. The number of confirmed colonies on the membrane can then be interpreted. If confluent growth is obtained, any sample retained in the refrigerator (section 7.4) may be re-examined using an appropriate dilution to enable isolated colonies to develop.

Whenever colonies are subcultured for confirmation, they should be tested for confirmation both as total coliforms and as *E. coli*, whether initially isolated at 37°C or 44°C. This is important because presumptive coliform colonies at 37°C may confirm as *E. coli*, and presumptive *E. coli* colonies isolated at 44°C may not confirm as *E. coli* but may nevertheless confirm as coliforms. It is particularly important when colonies are present on

7.8.3 Choice of medium for isolation and enumeration.

Minerals Modified Glutamate Medium (MMGM) (PHLS 1969) is recommended for the isolation of coliforms from waters and a Durham tube can be included in each bottle or tube of medium for the detection of gas production, if the information concerning gas production is required or considered relevant. This medium has been found to be slightly superior to Lauryl Tryptose Lactose Broth (LTLB) (APHA 1989) especially with chlorinated waters and for samples with coliform counts below 50 per 100 mL of water (PHLS/SCA 1980b). LTLB is, however, an acceptable alternative medium to MMGM.

The use of MacConkey broth is not recommended because of variations in the inhibitory properties of different batches of bile salts.

7.8.4 Practical considerations

The method was originally developed for the detection of coliforms on the basis of acid **and** gas production from lactose in a suitable isolation medium. In the context of the definition given in sections 3.2, 7.7.3 and 7.8.2 the production of acid from lactose serves as the primary indicator for the detection of coliforms. The determination for gas may however be useful in the characterisation of these organisms. As some non-coliform organisms can also produce acid and gas it may be necessary, for further information to be obtained, to subculture to more selective media such as LTLB or Brilliant Green Lactose Bile Broth (BGLBB). These media do not contain pH indicators and fermentation of lactose is demonstrated **only** by the production of gas. Most coliforms produce gas from lactose and can be detected using LTLB or BGLBB. Anaerogenic (non gas producing) strains cannot be detected using these media and subculture to MA is necessary. Subculture from cultures showing acid production in MMGM to MA is likely to give rise to mixed growth whereas subculture to LTLB or BGLBB followed by incubation and subculture to MA will give better selection of coliforms.

7.8.5 Procedure

Prepare the sample as described in section 7.4. Inoculate bottles and tubes of MMGM as described in section 7.6.2.2.

Incubate the bottles and tubes at 37°C and examine for acid (and gas production if this information is required or considered relevant) after 18–24 hours and after a further 24 hours. Retain all positive cultures as they may be needed for repeat subcultures.

Subculture all cultures in MMGM showing acid, with or without gas, to two tubes of either LTLB or BGLBB containing Durham tubes. Make a note of those tubes subcultured from primary cultures in MMGM which did not produce gas.

Incubate one tube at 37°C for 48 hours and the other at 44°C for 24 hours. The production of gas at 37°C can help in the characterisation of coliforms and gas at 44°C the characterisation of *E. coli*.

Examine tubes of LTLB or BGLBB subcultured from anaerogenic cultures in MMGM for growth as indicated by the presence of turbidity. If growth is present, subculture to MA and incubate at 37°C for 18–24 hours. The appearance of coliforms including *E. coli* on this medium is described in section 7.8.7.3.

7.8.6 Confirmation of total coliforms

For each specimen, subculture from at least one tube of LTLB or BGLBB at 37°C to MA and NA. If all primary cultures in MMGM were anaerogenic then subculture from at least one tube of LTLB or BGLBB showing growth.

Incubate the plates of MA and NA at 37°C for 18–24 hours. The colonial morphology of typical coliform organisms is described in section 7.8.7.3. If a pure culture is obtained on NA then perform the oxidase test as described in section 7.8.7.2. If there is any doubt about the purity of the culture then subculture at least one typical coliform colony from MA to NA, incubate at 37°C for 18–24 hours and carry out the oxidase test. Confirmed coliform organisms are oxidase negative.

7.8.7 Confirmation of *E. coli*

Subculture from all MMGM tubes showing acid, with or without gas to TW and incu at 44°C for 24 hours. The presence of *E. coli* can be demonstrated by growth production of gas in LTLB or BGLBB and indole in TW (see section 7.8.7.1). La Tryptose Mannitol Broth with added Tryptophan (PHLS/SCA 1980c) can be a sui alternative single tube medium for the detection of *E. coli* on the basis of gas and in production at 44°C. Single tube media may occasionally give false negative results i indole test and it is essential that a negative indole reaction is repeated by subculture the culture in MMGM to TW. Anaerogenic indole positive isolates should be tested fu for confirmation as *E. coli* after subculture to MA.

7.8.7.1 Indole test

Incubate TW at 44°C for 24 hours and add 0.2–0.3 mL of Kovacs' reagent (Kovacs 19 Indole production is demonstrated by the rapid appearance of a deep red colour in upper layer.

7.8.7.2 Oxidase test

Some bacteria found in water may conform to the definition of coliform organisms in respects, but are able to produce gas from lactose only at temperatures below 3 *Aeromonas* species, which occur naturally in water, have an optimum growth tempera in the range 30–35°C but may nevertheless produce acid, with or without gas, from lac at 37°C. They are of uncertain public health significance and are distinguishable from coliform group by a positive oxidase reaction. The oxidase test is carried out with cultures of lactose-fermenting organisms grown on NA as follows:

Place 2–3 drops of freshly prepared oxidase reagent on to a filter paper in a Petri

With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, sm some of the growth on the prepared filter paper.

Regard the appearance of a deep blue-purple colour within 10 seconds as a posi reaction.

Commercial test kits for oxidase testing are available and should be used in accorda with manufacturers' instructions.

On each occasion that oxidase reagent is used, conduct control tests with an organ known to give a positive reaction (for example *Ps. aeruginosa*) and one known to gi negative reaction (for example *E. coli*).

7.8.7.3 Growth on MacConkey agar

Colonies of coliform organisms are usually circular in shape, convex or low convex wi smooth surface and an entire edge. They are red but the depth of colour varies considera and colonial difference cannot be relied upon for differentiation within the group.

7.8.8 Presence-absence tests for total coliform organisms

7.8.8.1 Principle

The presence-absence (P-A) test is a simple modification of the multiple tube proced simplified in that it incorporates one large volume of medium (100 mL) instead of a se of tubes of different volumes. The procedure is based on the principle that colifo should be absent from 100 mL of sample, and a presumptive positive result theref indicates that coliforms may be present and that appropriate further action must be tak

Presence-absence test kits for total coliforms are an acceptable method for testing w leaving a treatment works where daily sampling is required. At the time of publicatio this Report a report on the use of presence-absence test kits is expected within the D (PECD 7/7/424) and at present, advice is that these kits should not be used on more t

7.8.3 Choice of medium for isolation and enumeration.

Minerals Modified Glutamate Medium (MMGM) (PHLS 1969) is recommended for the isolation of coliforms from waters and a Durham tube can be included in each bottle or tube of medium for the detection of gas production, if the information concerning gas production is required or considered relevant. This medium has been found to be slightly superior to Lauryl Tryptose Lactose Broth (LTLB) (APHA 1989) especially with chlorinated waters and for samples with coliform counts below 50 per 100 mL of water (PHLS/SCA 1980b). LTLB is, however, an acceptable alternative medium to MMGM.

The use of MacConkey broth is not recommended because of variations in the inhibitory properties of different batches of bile salts.

7.8.4 Practical considerations

The method was originally developed for the detection of coliforms on the basis of acid **and** gas production from lactose in a suitable isolation medium. In the context of the definition given in sections 3.2, 7.7.3 and 7.8.2 the production of acid from lactose serves as the primary indicator for the detection of coliforms. The determination for gas may however be useful in the characterisation of these organisms. As some non-coliform organisms can also produce acid and gas it may be necessary, for further information to be obtained, to subculture to more selective media such as LTLB or Brilliant Green Lactose Bile Broth (BGLBB). These media do not contain pH indicators and fermentation of lactose is demonstrated **only** by the production of gas. Most coliforms produce gas from lactose and can be detected using LTLB or BGLBB. Anaerogenic (non gas producing) strains cannot be detected using these media and subculture to MA is necessary. Subculture from cultures showing acid production in MMGM to MA is likely to give rise to mixed growth whereas subculture to LTLB or BGLBB followed by incubation and subculture to MA will give better selection of coliforms.

7.8.5 Procedure

Prepare the sample as described in section 7.4. Inoculate bottles and tubes of MMGM as described in section 7.6.2.2.

Incubate the bottles and tubes at 37°C and examine for acid (and gas production if this information is required or considered relevant) after 18–24 hours and after a further 24 hours. Retain all positive cultures as they may be needed for repeat subcultures.

Subculture all cultures in MMGM showing acid, with or without gas, to two tubes of either LTLB or BGLBB containing Durham tubes. Make a note of those tubes subcultured from primary cultures in MMGM which did not produce gas.

Incubate one tube at 37°C for 48 hours and the other at 44°C for 24 hours. The production of gas at 37°C can help in the characterisation of coliforms and gas at 44°C the characterisation of *E. coli*.

Examine tubes of LTLB or BGLBB subcultured from anaerogenic cultures in MMGM for growth as indicated by the presence of turbidity. If growth is present, subculture to MA and incubate at 37°C for 18–24 hours. The appearance of coliforms including *E. coli* on this medium is described in section 7.8.7.3.

7.8.6 Confirmation of total coliforms

For each specimen, subculture from at least one tube of LTLB or BGLBB at 37°C to MA and NA. If all primary cultures in MMGM were anaerogenic then subculture from at least one tube of LTLB or BGLBB showing growth.

Incubate the plates of MA and NA at 37°C for 18–24 hours. The colonial morphology of typical coliform organisms is described in section 7.8.7.3. If a pure culture is obtained on NA then perform the oxidase test as described in section 7.8.7.2. If there is any doubt about the purity of the culture then subculture at least one typical coliform colony from MA to NA, incubate at 37°C for 18–24 hours and carry out the oxidase test. Confirmed coliform organisms are oxidase negative.

two sampling occasions in any one week. They may also be useful for non-routine samples taken in distribution systems, for example after a mains repair.

These test kits should be disposed of safely in accordance with documented procedures.

7.8.8.2 Choice of medium

As with the multiple tube method, MMGM is the recommended medium.

7.8.8.3 Procedure

Volumes of 100 mL of double strength medium are decanted into screw-capped bottles of at least 250 mL capacity, with, if necessary, each bottle containing a large inverted Durham tube. The bottles are then sterilized by heating at 115°C for 10 minutes. The caps should be tightened when the cooled bottles are removed from the autoclave. P-A kits may be stored at room temperature in the dark for up to one month.

Volumes of 100 mL of sample are added to each bottle, which, for convenience, can be marked at the 200 mL level. The sample can be poured directly into the bottle, following the procedures given in section 6.4.8. After replacing the cap, the bottle should be inverted to remove any gas present in the Durham tube which may have been added to the bottle. The addition of chlorine-neutralising agents is unnecessary.

7.8.8.4 Incubation and interpretation of results

The bottles should be incubated at 37°C and examined after 18 to 24 hours. The presence of acid should be regarded as a presumptive positive and appropriate action should be taken as necessary. Under these circumstances a 'safety first' principle should be adopted and any test showing the presence of acid only should be regarded as presumptive until confirmatory tests have established otherwise.

If a negative result is obtained after 18–24 hours, the bottles should be incubated for a further 24 hours to obtain a final result. The presence of acid indicates a positive result, and tests should be carried out to confirm the presence of coliforms, *E. coli* and any other organisms as deemed necessary by the microbiologist.

A positive result from a treated water sample should initiate an immediate re-sample for analysis by a quantitative method in a microbiology laboratory.

7.9 Tests for Faecal Streptococci

7.9.1 Introduction

In the United Kingdom faecal streptococci are regarded as secondary indicators of faecal pollution, and the main use of the faecal streptococcus test is to assess the significance of coliform organisms in a sample in the absence of *E. coli*. Occasionally, identification of the species of streptococci present may help to distinguish between human and animal pollution (section 3.5).

7.9.2 Definitions

In the context of these methods, faecal streptococci are Gram-positive cocci which form pairs or chains and possess Lancefield's Group D antigen. They can grow in the presence of bile salts, in concentrations of sodium azide inhibitory to coliform organisms and most other Gram-negative bacteria, and at a temperature of 44° ± 0.25°C. They also hydrolyse aesculin and are catalase-negative. Some species are resistant to heating at 60°C for 30 minutes, grow in nutrient broth containing 6.5% sodium chloride, and to pH 9.6.

Enterococcus faecalis and some related species reduce 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble red dye formazan. Some species are resistant to heating at 60°C for 30 minutes, to pH 9.6, and can grow in nutrient broth containing 6.5% sodium chloride.

7.9.3 Toxicity of sodium azide

Many of the media described in this section contain sodium azide. As this sub highly toxic, great care should be taken in preparing these media, especial dehydrated ingredients in powder form are used. Sodium azide also has the pro forming explosive compounds with metals, especially copper and lead. Waste containing sodium azide must therefore be discarded into drains with care, pr through plastic pipes. Azide compounds should be decomposed and rendered sa excess of sodium nitrite before disposal.

7.9.4 Count of faecal streptococci by membrane filtration

7.9.4.1 Principle

Isolation of organisms on a membrane placed on the surface of an agar medium con TTC. Faecal streptococci usually produce pink, maroon or red colonies as a result formation of formazan.

7.9.4.2 Definition

In the context of this method, presumptive faecal streptococci usually reduce T insoluble red formazan to produce red, maroon or pink colonies on Membrane E coccus Agar (MEA) (Slanetz and Bartley 1957) after incubation at an appro temperature. Some strains may produce pale or colourless colonies. Confirmation is basis of aesculin hydrolysis on Bile Aesculin Agar (BAA) or Kanamycin Aesculin Agar (KAAA) incubated at 44°C for 18 hours. Further tests as detailed in section may also be carried out.

7.9.4.3 Choice of medium for isolation and enumeration

The medium recommended for the isolation and enumeration of faecal streptococ MEA. Complete dehydrated medium is available commercially and should be prep exactly as described by the manufacturer, with special emphasis on the avoidanc overheating. If the medium is orange or pink when cooled to 50°C, then considera should be given as to whether or not it should be discarded. Proper QC validatio particularly important in these cases.

7.9.4.4 Practical considerations

Growth of faecal streptococci on MEA is better at 37°C although some organis resembling faecal streptococci may grow which cannot be confirmed as such. Selectiv is better at 44°C although lower counts of faecal streptococci may be obtained. It therefore recommended that membranes from samples of potable water are incubated 37°C and from untreated waters at 44°C. Although an incubation temperature of 45°C more selective than 44°C, inhibition of the more temperature-sensitive faecal streptoco may result from a rise even of as little as less than 0.5°C.

7.9.4.5 Procedure

Prepare and filter the sample as described in section 7.5, making any necessary dilution Place the membrane on the surface of a well-dried plate of MEA.

For potable waters, incubate at 37°C for 48 hours and for untreated waters incubate a 37°C for 4 hours followed by 44°C for 44 hours.

After incubation, count all red, maroon or pink colonies which are smooth and convex in shape. These are presumptive faecal streptococci. Some types of faecal streptococci may produce pale or colourless colonies. Colonial size is variable but is usually not less than 0.5 mm. *Bacillus* species may produce pink colonies but these are often rough, flat and sometimes spreading. Depending on (i) whether the aim is to estimate the count or demonstrate the presence/absence of organisms, and (ii) the number of colonies present on the membrane, subculture a suitable number of red, maroon, pink or colourless colonies from the membrane. See section 6.3.3.

7.9.4.6 Confirmation of faecal streptococci

Subculture to BAA and incubate at 44°C for 18 hours. Faecal streptococci should produce discrete colonies surrounded by a brown or black halo from aesculin hydrolysis. The development of this colour is usually evident within a few hours and will give rapid confirmation. *Bacillus* species may produce some discolouration around the original inoculum site but should not develop discrete colonies. KAAA may be used instead of BAA. The use of Glucose Azide Broth (GAB) at 44°C is not recommended as a confirmatory test as false positive results may be obtained with other Gram-positive organisms. Also the transfer of membranes from the primary isolation medium to KAAA is not recommended because of the spreading of the black colour to aesculin-negative colonies.

7.9.5 Count of faecal streptococci by multiple tube

7.9.5.1 Principle

Culture of the sample in a liquid enrichment medium containing sodium azide, glucose and bromocresol purple as indicator. Growth and acid production indicate a positive result and therefore the presumptive presence of faecal streptococci. Subculture to confirmatory media to demonstrate growth at 44°C and hydrolysis of aesculin.

7.9.5.2 Choice of medium

GAB (Mallman and Seligmann 1950) is recommended as the liquid enrichment medium.

7.9.5.3 Procedure

After preparation of the sample and making any necessary dilutions as described in section 7.6.2, inoculate the test volumes into tubes of single or double strength GAB as described in section 7.6.2.1.

Incubate the tubes at 37°C and examine after 24 and 48 hours. After 48 hours regard any trace of acidity as a positive reaction.

7.9.5.4 Confirmatory test

From each tube giving a positive reaction, place one drop (about 0.02 mL) or loopful of growth onto the surface of a BAA plate and incubate at 44°C. Development of a black or brown colour in or around the inoculum within a few hours confirms the presence of faecal streptococci. If the inocula are well spaced out, numerous cultures can be tested on one plate. Incubate for up to 18 hours.

From the number of tubes giving a positive confirmatory reaction, calculate the MPN of faecal streptococci in 100 mL of the sample by reference to the tables in Appendix C.

The catalase test is used to distinguish streptococci, which are catalase-negative, from other Gram-positive cocci. Since this test can only be done with pure cultures, its application in the MPN method requires subculture to solid media.

7.9.6 Additional confirmatory and differential tests for faecal streptococci

Although the possession of Lancefield's Group D antigen is referred to in the definition, serological methods of confirmation present many practical difficulties and their use is therefore not recommended in routine water examination.

Tolerance of 40% bile is also characteristic of faecal streptococci, but in practice this test is not usually necessary and growth on MA can be used instead. Further tests with subcultures may be done if necessary, partly as an aid to species differentiation. However, full identification depends on the demonstration of biochemical and other characteristics as described by Cowan (1993) or by means of one of the multi-test differential systems now available.

Enterococcus species are differentiated from other streptococci by their ab
Nutrient Broth (NB) containing 6.5% sodium chloride and in Glucose P
Broth (GPB) (Clarke 1953) at pH 9.6.

7.9.6.1 Catalase test

Emulsify some of the culture to be tested in a few drops (about 0.1 mL) of qu
Ringer's solution in a small screw-capped bottle. Add 0.05 mL of 3% hydro
solution and replace the cap. The appearance of bubbles (of oxygen) indic
activity. An alternative method is to add the hydrogen peroxide to an overni
the organisms in a tube of either NA or NB. The test should preferably not be
a slide because of the risk of aerosol formation.

7.9.6.2 Bile tolerance

Subculture to a plate or tube of Bile Agar (BiA) (40%) and incubate at 37°
hours. Growth on this medium indicates tolerance of bile salts. Alternatively
show growth in the presence of bile salts. Faecal streptococci form small deep
on this medium.

7.9.6.3 Heat resistance

Transfer 1 mL of a 24 hour broth culture to a small test tube, and place in a wa
60°C for 30 minutes. Cool the tube rapidly and incubate at 37°C for 24 hours.
to a Blood Agar (BlA) plate or other non-selective medium, incubate and ex
growth.

7.9.6.4 Growth at pH 9.6

Inoculate a tube of GPB and incubate at 37°C for 24 hours. Tolerance of
indicated by heavy growth and de-colourization of the medium.

7.9.6.5 Salt tolerance

Inoculate a tube of NB containing 6.5% of sodium chloride (Salt Broth) and inc
37°C for 24–48 hours. Examine for growth.

7.10 Tests for Sulphite-reducing Clostridia and *Clostridium perfringens*

7.10.1 Introduction

The tests for sulphite-reducing clostridia play only a subsidiary role in water exam
These organisms form spores which are resistant to heating compared with veg
cells and advantage is taken of this for the detection of clostridia in water. *Cl. perfr*
an important member of this group, is associated with faecal contamination. If fou
time when other faecal indicator organisms are no longer detectable, it can indicate
or intermittent pollution.

7.10.2 Definitions

In the context of the methods, sulphite-reducing clostridia are Gram-positive ana
spore-forming rods which reduce sulphites to sulphides. *Cl. perfringens* forms a s
clot in Litmus Milk Medium (LMM) or Crossley's Milk Medium (CMM) (Cro
1941).

7.10.3 Count of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration (Tentative)

7.10.3.1 Principle

This method has not been fully validated and several media are described. A
preliminary treatment to destroy vegetative bacteria, filtration of a volume of the sam
through a membrane, followed by anaerobic incubation on a sulphite-containing
medium. Count all black colonies that develop. Subculture to a confirmatory mediu
demonstrate the presence of *Cl. perfringens*.

7.10.3.2 Choice of media

Several variations of sulphite-containing media and culture methods for anaerobic growth are available for the detection of sulphite reducing clostridia. The following media are recommended on a tentative basis:

(i) Tryptose-Sulphite-Cycloserine Agar (TSCA) containing egg yolk (Harmon, Kautter and Peeler 1971) or without egg yolk (Hauschild and Hillsheimer 1974, Sartory 1986);

(ii) Shahidi—Ferguson Perfringens Agar (SFPA) (Shahidi and Ferguson 1971);

(iii) Oleandomycin—Polymyxin—Sulphadiazine Perfringens Agar (OPSPA) (Handford 1974).

These are available as commercially dehydrated media and should be assessed for recovery in parallel with Differential Re-inforced Clostridial Medium (DRCM) (section 7.10.4) (Gibbs and Freame 1965).

7.10.3.3 Practical consideration

Funnels to be used for clostridia should be sterilized by autoclaving as spores may not be inactivated by boiling.

7.10.3.4 Procedure

Heat the sample to 75°C in a water bath and maintain at this temperature for 10 minutes. The time needed to reach this temperature can be determined with a similar bottle containing the same volume of water and a thermometer.

After heat treatment, prepare and filter the sample as described in section 7.5, making any necessary dilutions.

Place the membrane face-upwards or inverted on the agar and carefully pour 15–20 mL of agar, pre-cooled to 50°C over the membrane and allow to set. The agar should be poured in such a manner to avoid the washing-off of bacterial spores. Overlaying the agar is optional but may give better results; this should be assessed in individual laboratories.

Incubate in an anaerobic jar or cabinet at 37°C. Examine the plates after 24 and 48 hours and count all black colonies.

7.10.3.5 Confirmation of Cl. perfringens

Using a straight wire, subculture each colony to be tested (see 6.3.3) into a tube or bottle of freshly steamed and cooled LMM or CMM. For colonies on inverted membranes it will be necessary to stab through the membrane. Incubate the milk media as described in section 7.10.4.4 below and regard a stormy clot reaction as confirmation of *Cl. perfringens*. Black colonies should also be subcultured to two nutrient agar plates, one to be incubated aerobically at 37°C for 48 hours to exclude *Bacillus* species, which are facultative anaerobes, and the other anaerobically as a check for purity.

7.10.4 Count of sulphite-reducing clostridia and *Clostridium perfringens* by multiple tube

7.10.4.1 Principle

After preliminary treatment to destroy vegetative bacteria, test volumes of the sample are cultured in bottles of a liquid medium containing sodium sulphite and iron(III) citrate. All bottles showing blackening are subcultured to a confirmatory medium.

7.10.4.2 Choice of medium

DRCM is recommended for isolation. It should be distributed in screw-capped bottles instead of tubes, with sufficient depth of medium to ensure adequate anaerobiosis during incubation. LMM and CMM are recommended for confirmation.