The Microbiology of Recreational and Environmental Waters

1 Introduction

The recreational and environmental use of water is increasing in terms of both the nature of the activities being undertaken, and the number of participants becoming involved. Recreational and environmental waters include marine and fresh waters, as well as bathing, spa and leisure pool waters which are frequently indoors and which often exhibit high bathing densities. Whilst some activities involve little direct contact with the water, most involve some degree of immersion and possible ingestion of water.

The relationship between illness and contact with, or ingestion of, water that contains micro-organisms has been investigated many times, and various guidelines involving indicator organisms and bathing densities have been reported. Although plausible associations between recreational exposure and minor diseases have been reported, it is difficult to correlate these illnesses to the microbiological quality of the water.

Water, as rain, typically contains very few micro-organisms. However, it acquires a range of micro-organisms and chemicals whilst passing across, or through, terrain before eventually forming streams or lakes. Diffuse and spot (ie localised) sources of pollution are responsible for the contamination of water. Micro-organisms also gain access to water as a result of activity from animals, birds and humans. Where any of these sources introduce sufficient numbers of viable, pathogenic micro-organisms into the water, the possibility of illness arising as a result of recreational activity should be considered.

It has been reported[1] that risks to public health from sea bathing are minimal, except where water is aesthetically unacceptable. The European Union (EU) Bathing Water Directive[2] includes mandatory and guideline microbiological levels. The standards apply to waters where bathing is not prohibited and is traditionally practised by large numbers of bathers. The directive is currently under review and is further discussed in section 3. Regular monitoring of recreational water is important to establish baseline data of water quality, and such monitoring should be undertaken at sites where recreational activity is regularly practised. This should enable water quality data to be collated over a period of time and permit some degree of risk assessment to be undertaken in relation to the types of activity being performed. In addition, it may (i) identify the need for remedial action to be taken to improve water quality; (ii) quantify what degree of action is required; and (iii) ultimately serve to demonstrate the efficacy of that action towards the improvement of the water quality.

Recent epidemiological studies[3] relating to coastal sites in Britain have not shown a correlation between serious illnesses and bathing, but have suggested an association between minor illnesses and the degree of exposure to coastal waters. Some correlation was reported between faecal indicator organisms in the water and some subsequent minor illnesses.

In this booklet, attention has been directed towards the quality of swimming pool, hydrotherapy pool and whirlpool waters. In addition, the methods and quality assurance described in this booklet are intended to apply to surface and pool waters. This booklet outlines the principles of microbiological examination of recreational and environmental waters, and describes techniques for sampling and examination to ensure proficiency of laboratory practice and comparability of results.

Some of the methods given in this booklet are tentative and, where laboratories adopt these procedures, comparison should be made with existing methods to ensure that the recovery of specific micro-organisms is satisfactorily demonstrated.

2 Epidemiology and epidemiological studies

2.1 Epidemiology

The microbiological content of marine and fresh surface waters will reflect the quantity and quality of inputs from many sources, including sewage effluents, birds, animals and industrial and agricultural discharges. Micro-organisms, used as indicators of faecal pollution, may originate from sewage, animal faeces or industrial processes, and include coliform organisms, faecal coliform organisms *(Escherichia coli)*, enterococci/faecal streptococci, sulphite-reducing clostridia and bacteriophages.

Micro-organisms in recreational and environmental waters that are potentially pathogenic to humans are, in many cases, spread by direct person-to-person transmission. These pathogens belong to many classes of micro-organisms and those most likely to be important in the United Kingdom (UK) are mentioned below. Although gastrointestinal disease is the most common disease encountered, skin, eye and neurological symptoms have also been related to recreational and environmental water contact.

Bacterial pathogens causing mainly gastrointestinal disease include *Shigella* species, *Salmonella* species, *Campylobacter* species, and *Escherichia coli* (for example, *Escherichia coli* O157:H7). *Leptospira* species are present in the environment and humans become infected through cuts to the skin or via mucous membranes. Protozoa that cause gastrointestinal disease include *Cryptosporidium parvum* and *Giardia duodenalis*. Other parasites include amoebae such as *Acanthamoeba* species, *Hartmanella vermiformis* and *Naegleria fowleri*. Gastrointestinal symptoms are also caused by small round structured viruses (SRSV), astroviruses, caliciviruses, adenovirus 40/41 and rotaviruses. The SRSV appear to be the most important cause of viral gastrointestinal disease associated with recreational water contact. Of the other human enteric viruses found in surface waters used for recreation, hepatitis A causes disease of the liver. However, the enteroviruses and culturable adenoviruses have, to date, not been linked to specific symptoms.

Properly managed and disinfected swimming pool waters, spa waters and other pool waters should not contain viable micro-organisms. However, *Cryptosporidium* and *Giardia* are of concern since they are more resistant to disinfection than other micro-organisms referred to above. In addition, *Pseudomonas aeruginosa* has been associated with eye, skin and ear infections after contact with spa pool waters or "hot-tub" waters. Also, *Mycobacterium marinum* has been associated with skin infections and pool water contact, and adenovirus conjunctivitis has been linked with swimming pool water. The abrasive non-slip flooring that often surrounds pool waters has been associated with *Trichophyton* (athletes' foot) and the wart virus (verruca).

2.2 Epidemiological studies

Three reviews[4, 5, 6] report a range of epidemiological studies and their different approaches towards identifying and quantifying risks. After contact with poor microbiological quality marine and fresh water, it has been shown that gastrointestinal, eye and respiratory symptoms are likely to occur. The most common, significant indicator organisms detected[7] are enterococci, although no correlation has been shown with symptoms experienced.

Investigations associated with recreational and environmental waters have been reviewed[8,9], where it was noted that *Leptospira* and the amoeba, *Naegleria*, had caused illnesses as a result of contact with surface water. In addition, *Pseudomonas aeruginosa* had caused infections as a result of contact with pool water, and *Cryptosporidium parvum* and *Legionella* have caused illnesses after contact with swimming pool water. SRSV caused diarrhoea and vomiting after contact with river water[10].

3 Current standards for recreational and environmental waters

Current legislation for environmental waters within the UK is derived from various EU Directives. These directives set standards for specific uses of water, rather than general environmental purposes, and include standards for physical, chemical and microbiological parameters.

Monitoring compliance with these directives is the responsibility of a number of regulatory authorities within the UK. The Environment Agency (EA), the Scottish Environment Protection Agency (SEPA) and the Department of the Environment (Northern Ireland) are the respective authorities within England and Wales, Scotland and Northern Ireland.

The Bathing Water Directive[2] was enacted in England and Wales as The Bathing Waters (Classification) Regulations 1991[11]. The directive includes five microbiological parameters, for which standards are prescribed. These are shown in Table 3.1. In addition, a defined bathing season is prescribed for sampling and analysis undertaken in individual countries at designated bathing sites.

Table 3.1 Microbiological parameters contained in the Bathing Water Directive[2]

Parameter	Guide value	Imperative value
Total coliform organisms	500 / 100 ml	10000 / 100 ml
Faecal coliform organisms	100 / 100 ml	2000 / 100 ml
Enterococci	100 / 100 ml	–
Salmonella	–	0 / litre
Enteroviruses	–	0 / 10 litres

Other EU Directives that set microbiological standards for environmental waters include the Surface Water Abstraction Directive[12] and the Shellfish Water Directive[13].

Swimming pool waters and spa pool waters, including whirlpool waters and hydrotherapy pool waters, are not covered by the Bathing Water Directive[2]. In addition, there are no legislative or regulatory standards in the UK for these types of water. Guidance on these waters is given in the Pool Water Guide[14]. Table 3.2 tabulates microbiological levels which are based on the principle that most waters of this type have been subjected to some form of treatment and disinfection. A properly maintained pool water with the correct treatment regime, pH and level of disinfectant should satisfy these microbiological levels. Further guidance on water quality and sampling is given in section 6.2.

Table 3.2 Microbiological levels for swimming pool waters and spa pool waters[14]

Parameter	Recommended value*
Coliform organisms	0 / 100 ml
Faecal coliform organisms	0 / 100 ml
Total viable count (37°C)**	0 - 10 cfuml^{-1}
Total viable count (22°C)	0 - 100 cfuml^{-1}
Pseudomonas aeruginosa	0 / 100 ml

* cfuml^{-1} = colony forming units per millilitre.
** Pool waters with a heavy bathing load may have colony counts in the range 10 - 100 cfuml^{-1}. Values above 100 cfuml^{-1} would indicate that operating conditions are unsatisfactory.

4 Quality control and quality assurance

4.1 Laboratory safety

A microbiology laboratory should adopt levels of safety and quality appropriate to the analysis required. In the UK, the Health and Safety at Work etc Act (1974) and legislation under the Act, for example The Control of Substances Hazardous to Health Regulations 1999 and The Management of Health and Safety at Work Regulations 1992, place specific duties on management with regard to safety in laboratories. A laboratory should have a policy statement on health and safety matters, and provide specific laboratory safety manuals. In addition, any specific hazards associated with equipment, or particular methods, should be highlighted and a safe system of work described.

Staff should be aware of the potential hazards likely to be present in a microbiology laboratory, particularly the presence of those micro-organisms capable of causing disease. Staff should also be trained in fundamental practices, such as aseptic techniques, and be informed of the consequences of employing poor practices. Periodic reviews may need to be carried out in light of new developments and techniques, and properly documented training records are an essential part of good laboratory practice. Access to microbiology laboratories should be limited to authorised persons, all of whom should be provided with suitable protective clothing. Particular care should be taken on the handling and disposal of contaminated material in laboratories.

4.2 Good laboratory practice

All microbiological procedures should be subjected to the following considerations:

(i) the assessment of risks to health of staff arising from work, and the precautions that need to be taken;

(ii) the introduction of appropriate measures to prevent or control risks or perceived risks;
(iii) the assurance that appropriate control measures are used and that procedures are properly observed and equipment maintained;
(iv) monitoring, where appropriate, the potential exposure of workers, and undertaking appropriate health surveillance;
(v) informing, instructing and training employees about the risks and precautions to be taken.

4.3 Quality assurance

A programme of quality assurance is considered essential for microbiological testing. This programme should cover the whole process from sample collection to the reporting of results. The laboratory should have appropriate facilities, in terms of accommodation, staff, equipment, techniques, appropriate and safe working practices, efficient and timely reporting, and effective management procedures to ensure a safe and efficient laboratory. It should operate a system of internal quality control and, where necessary, take part in appropriate external quality control schemes.

4.4 Equipment

All microbiological laboratories will require certain basic items of equipment, for example microscopes and autoclaves. Such equipment will need regular cleaning and maintenance and, where appropriate, checking for accuracy. Where appropriate, major pieces of equipment should be serviced at regular intervals and relevant documentation retained.

4.4.1 Temperatures

Temperature control is of particular importance in water microbiology. Temperature checks should be made regularly, for example at the beginning and end of each working day. Thermometers or other temperature measuring instruments should be checked regularly for accuracy. Temperatures should be capable of being recorded to tolerances of 0.5°C or better.

4.4.2 pH meters

The pH of media is critical, and should be tested and recorded. All pH meters should be regularly checked and standardised against appropriate buffer solutions.

4.4.3 Balances

Balances should possess sensitivities appropriate to the substance being weighed and its intended use. They should also be calibrated and serviced regularly. In addition, balances should be checked frequently against certified reference weights and details recorded.

4.4.4 Reagents and media

See also Appendix B. Water used for the preparation of media should be of a suitable quality. Distilled or de-ionised water has been found to be satisfactory. Reagents used in the preparation of solutions or media should be of analytical grade quality. Reagents and media may be available commercially and should be prepared and used according to the manufacturer's instructions. Prepared media can be stored in tightly sealed bottles and kept in the dark at room temperature. Ideally, media should be used as soon as possible after quality control tests have been satisfactorily demonstrated. Where media are used less frequently, quantities should be prepared in small volumes to minimise waste. Petri dishes containing media should be stored at between 2-8°C. Where media are stored for long periods before use, it should be validated that the storage periods do not adversely affect the performance of the media in the ability to recover target organisms, whilst at the same time retain inhibitory properties towards non-target organisms.

4.4.5 Sterilizing equipment

4.4.5.1 Autoclaves

Autoclaves are used to sterilize items of laboratory equipment, reagents, media, etc and to render infectious material safe for disposal. In order to ensure that the correct temperature is attained in all parts of the autoclave, it should be serviced and checked regularly. In addition, each operational cycle of the autoclave should have a record on which the sterilization cycles are clearly identified. Laboratory media and reagents that have been sterilized should be identifiable, for example marked with a batch number. Temperature tolerances for the autoclaving cycle should be ± 2°C or better.

4.4.5.2 Hot air ovens

Hot air ovens also require regular monitoring of the time-temperature relationship for each operating cycle. They should ensure an even distribution of heat to all parts of the load, and materials sterilized should be identifiable.

4.4.6 Refrigerators and cold rooms

Refrigerators should have their operating temperature checked frequently and details recorded. They should be de-frosted at regular intervals. Cold rooms should be equipped with continuous recording devices. Materials should not be stored beyond their storage life.

4.4.7 Incubators and water baths

Water baths should be appropriately filled and the level checked frequently. Water baths should be cleaned regularly. Different loading patterns of incubators may give rise to different temperature attainment patterns in various parts of the incubator. In addition, the stacking of containers and "overloading" with too many Petri dishes may contribute to a thermal lag. This may give rise to misleading results. Water baths and incubators should, ideally, be provided with continuous recording devices.

4.4.8 Membrane filter holders

Membrane filter holders require checking for leaks at regular intervals. They should be sterilized before use and cleaned after use.

4.4.9 Microscopes

The optics and stage of microscopes should be cleaned after use, for example with lens tissue. Particular attention should be paid to oil immersion lenses, and microscopes should be covered when not in use. Microscopes should be serviced at regular intervals and details recorded. Where immuno-fluorescence microscopes are used, bulbs should be replaced at required intervals. Where measurements are taken, the eye-piece graticule should be calibrated at regular intervals.

4.4.10 Materials

All glassware used in the preparation of media, or in the handling of samples, should be of suitable quality. It should be free of inhibitory substances and adequately cleaned after use and sterilized before use. Chemicals and other substances used in the preparation of media and reagents should be of the appropriate analytical quality.

4.5 Internal quality control

To ensure that a laboratory is, and remains, capable of isolating, accurately identifying and enumerating micro-organisms in a sample, it is essential for the laboratory to operate an internal quality control system. A similar system should also operate to ensure that samples are not contaminated with extraneous micro-organisms. These systems should include the submission of sterile samples, and samples known to contain specific micro-organisms, to the isolation procedure. These quality control samples should be analysed in parallel with each batch of "real" samples examined. Control organisms may be obtained from natural surface waters or, alternatively, freeze-dried reference strains may be used. Reference strains should be subcultured to prepare strains which may be freeze-dried, or frozen, and then used to prepare working suspensions.

A prepared medium should be checked for its sterility and its ability to grow the targeted organisms being isolated. If a selective medium is used, it should also be checked for its ability to inhibit the growth of unwanted or non-target organisms. A medium should be incubated under the appropriate conditions of time and temperature as described in the method and, ideally, should not be used until it has been shown to be satisfactory. Under ideal circumstances, such tests should be quantitative, and should challenge the medium with a known number of organisms and require the recovery of a defined percentage of the organism. In practice, it is difficult to provide suspensions of micro-organisms which will remain stable for sufficient periods of time to be of use. However, commercial suspensions of dried organisms are now becoming available. The data derived from the use of such suspensions can assist in the construction of quality control charts and facilitate laboratories to establish levels of recovery. Laboratories should, however, define the remedial action that is required should these levels not be achieved. All media should be stored under conditions

that prevent deterioration and should be discarded if deterioration is evident, or if the expiry date has been exceeded.

4.6 External quality control

Laboratories should participate in appropriate external quality control schemes. Examination of micro-organisms should be undertaken as part of routine laboratory practice. Results of the individual laboratory can then be compared with those of other participating laboratories, and also provide an independent check on the quality of the laboratory's continuing performance. In addition, such schemes may be used to provide a means of assessing newly trained staff, and where appropriate, the comparable recovery efficiency of new methods against existing methods.

5 Statistical considerations

5.1 Introduction

Water samples from recreational and environmental waters are examined for the enumeration, or the presence or absence, of pathogens or relevant indicator organisms. When the enumeration of organisms is undertaken, it is important to consider the reliability of the results obtained, and for this there are three factors that should be considered. Firstly, there is the representative nature of the sample being examined with respect to the bulk of the original water source sampled. The variation in the number of organisms (counts) reported for samples obtained from a single water source can be significant, especially over time as well as between sampling locations. Secondly, there is the inevitable imprecision introduced by laboratory procedures when the result is based on an aliquot, or subsample, of the original sample submitted and not on the entire sample. Similar imprecision applies when the count is estimated from a multiple tube method. A sample bottle brought to the laboratory should be thoroughly mixed before it is processed. However, the organisms may not be distributed evenly or uniformly. Under the most favourable circumstances, the organisms will be spread randomly throughout the sample of water. Hence, by chance, different aliquots, or subsamples, will contain different numbers of organisms. This random variation plays a significant part in all quantitative microbiological examinations[15]. Thirdly, there is "uncertainty of measurement" introduced by fluctuations in the performance of staff, methods, equipment and materials in the laboratory. It is likely that the variation due to the representative nature of the sample will be the more dominant[16].

Statistical methods can be used to estimate some, but not all, of these sources of variation. Hence, a final result should not be qualified with a statement of confidence. This cannot be readily calculated, and would lead to some degree of confusion since it would not address all aspects of accuracy and precision.

5.2 Variation between samples

Recreational and environmental waters will vary enormously in the numbers of relevant organisms present, and an individual water source will display large variations over time (often related to season). In addition, there will probably be large differences between locations at any one site[17]. A recent study[18] has demonstrated that samples collected at the same time at sites only one metre apart show large variations in bacterial quality. These variations are far higher than those that would occur randomly. Hence, the amount of information, and its interpretation, from a single sample is very limited. Multiple samples, over both time and place, are required. A situation in which a single sample gives an estimate of the whole bacterial profile occurs only when organisms are distributed randomly. In this case, the Poisson distribution is used. However, such randomness has yet to be reported for recreational or environmental waters and, indeed, would not be expected. An estimate of the likely range of counts should, therefore, be based on information from a number of samples, and the greater the natural variation in the water source, the greater the number of samples. The importance of variation over time and place should, therefore, be appreciated by those responsible for monitoring recreational or environmental waters.

5.2.1 Monitoring trends

Recreational or environmental waters which are monitored for microbiological quality should have pre-determined sampling sites. It may be prudent to organise a pilot study to determine the most practicable and informative locations. The results for each site should be kept and examined at regular intervals, alongside results from adjacent sites. Changes to water quality need to be recognised either as those that are brought about by contamination or as those that are caused by the natural variation in water quality, ie "background" variations.

If changes in water quality occur then considerations may need to be directed towards the recreational activities being practised and the investigations needed to be carried out in order to determine the cause of the changes. In theory, it should be possible to construct a model, using past data, which describes systematic variation such as seasonal and tidal changes. This model may predict likely ranges of future counts. If the observed counts are reported outside these ranges more frequently than is usually expected, then changes may need to be postulated. Often the variations are so complex that a model cannot be regarded as definitive, but is merely a useful tool for screening for possible changes.

5.3 Accuracy of laboratory methods

Some laboratory procedures inevitably lead to variation in reported results even though the procedures are correctly performed. This may lead to confusion with the natural variation due to "sampling error". Hence, a result that is reported with an estimated 95% confidence interval may be misunderstood. While it may be assumed that the confidence interval applies to the bacterial density of the water source, it could refer to the confidence interval resulting from the laboratory procedures, which, as already stated in section 5.1, are generally much smaller.

Errors due to faulty sampling or laboratory procedures and equipment, etc (ie uncertainty of measurement) are not fully discussed in this booklet. Errors of this sort should be detected by the use of good quality assurance programmes, including quality control checks and participation in external quality assurance schemes. See also section 4.

5.3.1 Potential imprecision due to dilution of the sample

Samples of contaminated water may require dilution before examination so that a reliable count can be obtained. If a multiple tube method is used, then some tubes, but not all, should show growth. If the membrane filtration method is used, the aim is to achieve a count with good "statistical information". In practice, the membrane filter should not be overcrowded since there is the potential for growth inhibition and merging of colonies. Ideally, there should be a reasonably high number of colonies on the membrane filter to enable a satisfactory count to be made. This is, generally, in the order of 20 - 80 colonies.

Depending upon the regulatory requirement, it is normal practice to report bacterial counts of indicator organisms as the number of organisms per 100 ml. With clean, undiluted samples, approximately 100 ml of the sample are examined. In the multiple tube method involving an 11-tube series, this will be 105 ml. If the sample needs to be diluted (prior to any additional dilution inherent in the multiple tube method) and this dilution is, say for example, 10-fold, then only approximately 10 ml of the original sample will be examined. The count obtained is then multiplied by the appropriate dilution factor to give an estimate of the number of colonies contained in 100 ml of sample. The original sample volume (100 ml) should be thoroughly mixed to achieve a random distribution of the organisms. Therefore, the count from the diluted aliquot, or subsample, can be used to estimate the likely count in the original 100 ml and confidence intervals calculated using random distribution theory [19]. Some examples are shown in Table 5.1.

Table 5.1 Estimated count and 95% confidence intervals for the number of organisms in a 100 ml sample, where, after dilution, a subsample is examined

Organisms observed in the subsample	10-fold dilution		100-fold dilution	
	EC	CI	EC	CI
10	100	50-180	1000	480-1830
50	500	380-650	5000	3750-6640
100	1000	820-1200	10000	8190-12200

EC = estimated count.
CI = 95% confidence interval.

The imprecision introduced by dilution is likely to be relatively small compared with the variability of bacterial density in the water source. The confidence intervals shown in Table 5.1 should not be stated when results are reported. Quoting such ranges may be misunderstood and taken to be a statement about the likely bacterial density in the water

source. The examples in Table 5.1 are meant to illustrate the additional imprecision, other than that due to water source sampling, which is introduced when aliquots, or subsamples, of the original sample submitted are examined.

5.3.2 Other imprecision of laboratory methods

5.3.2.1 Multiple tube method

In the multiple tube method, a series of subsamples is taken from the original sample, and processed to ascertain which subsamples show the presence of the organisms under investigation. A mathematical formula based on laws of probability is then used to estimate the most probable number (MPN) of organisms present, given the number of tubes in the series which show growth[20, 21, 22]. These various mathematical approaches have been reviewed[23] and the principles involved in the estimation of bacterial densities by dilution methods described[22]. Tables have been developed[24, 25] which give a greater number of combinations of positive and negative results, some of which in practice should only occur very rarely[26, 27]. A confidence interval was often published with the MPN that demonstrated the uncertainty, as estimated from multiple tubes, of the bacterial count for the sample, not for the water source, although it has often been confused with the latter. For this reason, the confidence interval should not be reported.

Modern computers now enable the determination of the probability of counts associated with each dilution series to be quantified exactly[28, 29]. While the latest calculations of the most probable numbers show little discrepancy with previously published values, these new calculations have highlighted two issues. Firstly, the imprecision of previously published confidence intervals must be considered. Secondly, for moderate or high bacterial density, the multiple tube method does not give a clear “most probable number”. There is a “most probable range” (MPR) of counts, which is almost equally likely to be as correct as the MPN. All calculations are based on the assumption that the organisms present in the water are evenly distributed, and the importance of thorough mixing of the sample cannot be over-emphasised. Although the multiple tube method is very sensitive for the detection of a small number of indicator organisms, the MPN is not a precise value. Apparent differences between results should, therefore, be interpreted with caution. It should also be appreciated that variations in bacterial numbers in the water source may be very much greater than any imprecision introduced by the multiple tube method.

Appendix A shows tables of the MPN and the appropriate MPR for a 6-tube series (1x50 ml; 5x10 ml), an 11-tube series (1x50 ml; 5x10 ml; 5x1 ml) and a 15-tube series (5x10 ml; 5x1 ml; 5x0.1 ml). Results should be reported with care, and it may be appropriate to quote the MPR rather than the MPN in order to illustrate the imprecision of the method. However, it should be clearly stated that the range applies to the sample and not to the water source. When plotting results for trends, it is often more practicable to use the MPN.

5.3.2.2 Membrane filtration method

Counts on membrane filters are subject to statistical variation, and replicate tests on subsamples from the same bulk sample are unlikely to give exactly the same number of colonies. If 100 ml of sample are filtered and incubated, and then every relevant colony on the membrane counted, and every colony confirmed, then the presumptive and confirmed counts are as precise as the method allows. No statistical imprecision need be considered. If the sample is diluted prior to filtration, then the count becomes an estimate of the density in the undiluted sample, as already described. Similarly, if the presumptive count of the undiluted, filtered sample is taken from a portion or segment of the membrane filter then imprecision is introduced comparable to that introduced by dilution of the sample. This assumes that the segment of the filter chosen is typical and representative of the whole filter.

5.3.2.3 Confirmation

Confirmatory tests of the presumptive colonies present on a membrane filter should be carried out. When multiple colonies are present, different approaches can be adopted when consideration is given to the number of colonies that should be tested for confirmation. If the aim is to estimate the count of the relevant colonies, then consideration should be given to the imprecision introduced if a fraction of the presumptive colonies on the filter are subject to confirmatory tests, or whether all the colonies should be used. The number of colonies tested for confirmation should be chosen to give a sufficient level of accuracy. This may require subculturing all the presumptive colonies on a membrane filter when fewer than ten colonies are present.

If the aim is to demonstrate the presence or absence of the organism, then a different approach may be chosen, provided that there are no microbiological contra-indications. The presence of the organism is demonstrated as soon as one positive confirmation is made. A laboratory may, therefore, choose to examine fewer colonies, initially, than when the aim is to estimate the count. However, if the chosen colonies do not confirm, then the sample cannot be assumed to be satisfactory, in terms of presence or absence, since other colonies not chosen for confirmation testing may, if tested, confirm the presence. Hence, the membrane filter should be stored and other colonies tested if the initial colonies selected do not confirm. This sequential testing is acceptable only when refrigerated storage of the membrane is not detrimental to the survival of the relevant organism.

If all presumptive colonies are tested for confirmation, then no further imprecision is introduced into the final result (other than that due to the test method) when the presumptive count is converted into a confirmed count. If a fraction of the colonies is tested for confirmation, then further imprecision is introduced into the estimated confirmed count. For example, if 60 colonies are counted on the filter, and 20 are selected at random for confirmatory testing, and 15 of these colonies are confirmed, then the estimated confirmed count is given by 45, ie 15x60/20.

It is assumed that the n colonies are selected at random, or by some other equivalent procedure which ensures that the chosen colonies represent a typical subsample of the N colonies, and that all N colonies were equally likely to arise from the relevant organism group. The conditional probability that y is the true count, given that x colonies have confirmed, can be calculated from the equation of possible combinations:

$$P(x / y) = {}^{y}C_{x} \,.\, {}^{N-y}C_{n-x} / {}^{N}C_{n}$$

The 95% confidence interval for the confirmed count can be found by examination of the probabilities for all possible values of the true count, y, using the observed value tested, x. The confidence interval will exclude end of range (high and low) values of y, such that their cumulative conditional probabilities sum to less than, or equal to, 0.05. This procedure follows that described elsewhere[19]. For example, if 10 colonies are observed (ie N = 10) and two colonies are tested (ie n = 2) then Table 5.2 shows the complete range of probabilities.

Table 5.2 Range of probability values if 10 colonies are counted, of which two are tested for confirmation

Given that the true count, y, is	and the number of colonies confirmed, x, is		
	x = 0	x = 1	x = 2
	then the probability is		
0	1.000}	–	–
1	0.800}	0.200}	–
2	0.622}	0.356}	0.022
3	0.467}	0.467}	0.067}
4	0.333}	0.533}	0.133}
5	0.222}	0.556}	0.222}
6	0.133}	0.533}	0.333}
7	0.067}	0.467}	0.467}
8	0.022	0.356}	0.622}
9	–	0.200}	0.800}
10	–	–	1.000}

The braces show the range of true counts, y, which should be included in the 95% confidence interval for the true count. Note that if one of the two colonies tested is confirmed (ie x = 1) then all the possible values of y are within the 95% confidence interval because all the probabilities exceed 0.05. See example b in Table 5.3.

In general, if only small numbers of the total colonies are tested for confirmation, then the confidence interval can be very wide. Confidence intervals also tend to be wider if a substantial number of colonies turn out not to confirm. Some examples are shown in Table 5.3

Table 5.3 Variation in the 95% confidence intervals with variation in the proportion of tested colonies confirming

	Colonies observed (presumptive count) ie N	Number tested ie n	Number confirmed ie x	Confirmed count	Confidence interval (95%)
(a)	10	2	0	0	0 - 7
(b)	10	2	1	5	1 - 9
(c)	10	2	2	10	3 - 10
(d)	14	7	5	10	6 - 12
(e)	50	10	5	25	9 - 41

It should be noted that the practice of confirming 10 colonies can still introduce potential imprecision, especially if the presumptive count is large and some colonies fail to confirm. Hence, the higher the percentage of colonies used for confirmation, the more accurate the result.

5.4 Reporting results

The report should be a clear statement of the findings and a further statement on error, to qualify these findings, should not be necessary for routine samples. The sampling strategy should have been established with the aim of acquiring an adequate level of information. The laboratory should have in place a programme of quality assurance to assure adequate performance. If it is necessary that a report for a particular sample warrants a statement on accuracy and precision, then a clear distinction should be made between sampling error due to variability at the water source and possible error introduced by the laboratory methods.

Absence of organisms and unmeasurably high counts should be reported as follows:

(i) *No organisms detected*. - When the sample is examined and no relevant organisms are detected, this should be reported as "none found in the sample examined", ie 0/100 ml. If a dilution of the sample is taken and an aliquot is tested, and no organisms are detected, for example, 0/10 ml, then this should be equated to the reporting volume, for example, less than 10 per 100 ml. However, since there is no corresponding equivalence to the concept of "limit of detection" used with chemical measurements, an expression of "less than 1 per unit volume" is often meaningless. For reporting purposes, it is recognised that computers used to store this information cannot recognise text in a numeric field. In addition, if the growth of target organisms is obscured by non-target organisms, then a note should be made of this effect and consideration should be given to repeating the examination, possibly by a different technique.

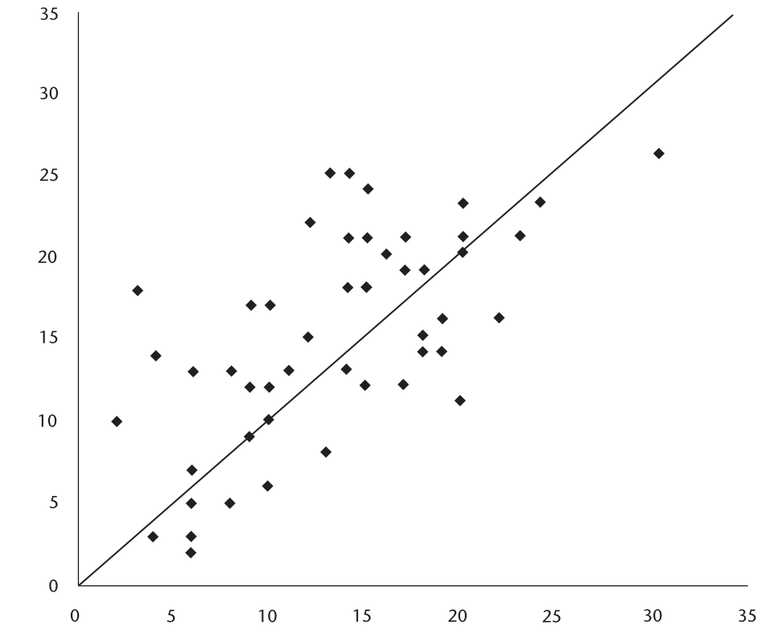
(ii) *Overgrowth of membrane filters, or all tubes exhibiting growth in MPN methods.* – This means that the laboratory has failed to estimate the true count because of insufficient pre-dilution. With the multiple tube method it is customary to report this, in the appropriate units, as "greater than 180" for the 11-tube series or "greater than 1800" for the 15-tube series. In reality, the count could be very much higher. With membrane filtration and similar methods, the report should include a statement of "count too high to be estimated at the dilution used". Again, it is recognised that computers used to store this information cannot recognise text in a numeric field.

5.5 Correlation between parameters

Recreational and environmental waters are often examined for several microbial parameters, and there has been discussion about which parameters do, or do not, correlate well with each other. Examining a sample of water for a second parameter that correlates well with the first parameter will provide little extra information about water quality. Examining the water for a second parameter that does not correlate well with the first parameter may provide extra, different information, which may be of interest. Levels of correlation may be better than at first appears, because of the random variation in the number of organisms occurring between subsamples as the two parameters, by most methods, cannot be measured from the same subsample.

The following example illustrates "best possible" correlation. A series of 50 samples, each of which, after thorough mixing, is divided into two subsamples. These are then examined as duplicates for the same parameter (in this case, total coliform organisms). The pairs of counts are then plotted as shown in Figure 5.1. The counts might be expected to be identical, and lie on a straight line, except for the differences arising from random variation in the number of organisms between the two subsamples. Figure 5.1 shows considerable scatter and the "correlation", as expressed, is $r = 0.63$ ($r^2 = 0.39$), where r is the product-moment correlation coefficient. This statistic, r, assumes Normal distribution of the variables, which is often not applicable with microbiological data. Therefore, a non-parametric measure of correlation, such as Spearman's rho, may be more appropriate. In Figure 5.1, rho is 0.62. These data were taken from a series of natural samples of untreated water examined as part of a quality control scheme[30] and were demonstrated to be "in control" and satisfactory. This example of correlation can be considered as "best possible", and careful consideration should be given to assessing any apparent lack of correlation between different parameters.

Figure 5.1 Pairs of replicate counts of total coliform organisms



Counts are duplicates and in theory should correlate "exactly", ie $r^2 = 1$
Random variation leads to an r vaue of 0.63 (ie $r^2 = 0.39$)
Samples were "natural" and consisted of 100 ml subsample volumes

6. Sampling

6.1 Surface waters

6.1.1 Introduction

The procedures used to obtain samples can significantly affect the quality of environmental data. While much attention has been paid to the accuracy and precision of analytical techniques applied to the sample, less detail has been given to sampling and sample preservation. Any sample should reflect the quality of the bulk of water being sampled.

In addition, there should be an understanding of the purpose for which the sample is being taken and the reason why information is recorded at the time of sampling. Use of appropriate containers and the correct handling and transport of samples are also important if significant changes in the constituents of the sample are not to occur between the time of sampling and its subsequent analysis.

6.1.2 **Regulatory monitoring**

All sites designated under the Bathing Water Directive[2] will have routine monitoring programmes designed to measure water quality during the defined bathing season. The sampling frequency is prescribed, and all samples should be taken from defined locations where the bathing density is recognised as being at its greatest. If the water quality deteriorates, additional samples should be taken.

6.1.3 **Site investigations**

Where any site requires recreational or environmental water quality data to be provided for background information, or needs to be investigated for a source of pollution, a review of all existing knowledge (likely to influence microbiological quality) is an important prerequisite to the design of an appropriate sampling programme. Factors for consideration should include knowledge of polluting, or potentially polluting, sources. Flow and current conditions are important characteristics, whilst geographical features, depth profiles and weather conditions may all influence the distribution of micro-organisms.

6.1.4 **Sampling locations**

Site locations should be such so as to produce adequate data about water quality considering the factors described above. For investigational purposes, a wider range of locations may be required taking into account recreational activity, known sources of pollution, tidal states and weather conditions. A number of locations should be established, within defined geographical limits, sufficient to provide data about water quality. This should take into account user activity and known or perceived sources of pollution. Where recreational activities extend offshore, boats and satellite-tracking systems may be required to pin-point exact sampling positions. Similarly, the monitoring and investigation of offshore discharges may require boats and tracer-type studies. For inland waters, bridges provide ideal sampling positions. Each location should be selected after due consideration has been given to safety and ease of access, particularly in adverse weather conditions.

6.1.5 **Sampling frequency**

The sampling frequencies for designated waters are defined under various directives[2,9–13]. Samples are taken when conditions are appropriate and should reflect all tidal conditions. Additional pre- and post-season sampling may be considered as appropriate. For non-regulatory circumstances, similar frequencies may be adopted for recreational and environmental waters. When sufficient data have been collected and where there are no obvious signs of pollution, it might be considered adequate to sample less frequently, unless significant changes occur. Where fundamental changes are planned or areas receive intense public interest, it may be prudent to increase the sampling frequency.

The sampling frequency for investigational purposes will depend upon the information required. Tidal waters should be sampled at all tidal states and in different weather conditions. Fresh waters should be sampled during dry weather and after heavy rainfall. In addition, they should be sampled either side of known and suspected sources of pollution. Investigational programmes often involve many samples and the accumulation of substantial amounts of data. It is important that such investigations are planned carefully and that data are reviewed frequently in order to modify programmes of work, should circumstances require it.

6.1.6 **Sampling equipment**

The type of sampling equipment used will depend on the individual circumstances encountered at the sampling site. In shallow waters with good access, direct immersion of the appropriate container to a fixed depth may be satisfactory. This may require sampling staff to wade out to a position where the fixed depth can be accommodated. However, care should be taken when wading out to the position that disturbances in the water are not

caused that may result in an unrepresentative sample being taken. It is important that sampling staff have appropriate protective clothing. The use of disposable gloves minimises skin contact with water and reduces contamination. Where access is difficult, or samples are required to be taken some distance from the bank of a river, or reservoir, telescopic poles fitted with clamps may be more appropriate. Sampling from bridges, or boats, can be facilitated with the use of stainless steel buckets, or jugs, that can be suitably disinfected and rinsed before use.

Where samples are required at greater depth, depth-samplers or pumping equipment will be needed. Two types of design are available, air displacement samplers and open-ended samplers. With these types of equipment, sterilization between sampling occasions will be difficult and adequate flushing of the sample may be the most appropriate procedure to use. Air displacement samplers are lowered (on a rope) with the “openings” in a closed position, which are then released at the appropriate depth. Open-ended samplers are “free-flushing” as they are lowered and are then sealed when in the appropriate position. These samplers can be used for sampling from depths, generally, up to 100 m. When sampling takes place in strong currents, or at greater depths, the cable used to support the sampler is unlikely to be vertical. Hence, the location of the sampling equipment in the water may need to be determined. Further guidance, particularly for sediments, can be found elsewhere in this series[31].

6.1.7 **Sample containers**

Sample containers may consist of glass or plastic and should be of sufficient volume to provide a sample suitable for subsequent analysis. Containers should be non-toxic and should not possess bacteriostatic properties or provide nutrients to support the growth of micro-organisms. Chipped, etched or cracked containers should be discarded. Closures should produce a water-tight seal. Prior to use, containers and closures should be thoroughly cleaned and sterilized by autoclaving at 121 ± 1°C for 15 minutes. Alternatively, sterile disposable containers can be used and discarded after use. Each batch of sterile containers should be identifiable, for example, marked with a batch number, and checked for sterility and marked with a “use by” date. Preservatives are not, generally, required and guidance on the amount of sample required for specific examinations is given in Table 6.1. Samples of mud and sediments can be placed into sterile, wide-necked, plastic or glass containers of suitable volume.

Table 6.1 Approximate sample volumes

Organism	Minimum volume (ml)
Faecal indicators	300
Salmonella	1000
Enteroviruses	10000

6.1.8 **Sampling procedures**

Care should be exercised at all times during the sampling procedures so as to avoid contamination. When the cap or seal is removed from the container, the inside of the cap should be protected. For surface samples, the sample container should be held at the base and plunged into the water to an appropriate depth, for example 300 mm is used for sampling bathing waters. The neck of the container should be positioned uppermost, allowing air to escape and the container to fill. In still or static waters, filling of the container can be encouraged by moving the container in a horizontal position with the neck of the container pointing away from sampling staff or boats. In flowing waters, the container should be positioned with the neck of the container pointing up-stream, away from sampling staff or boats. Sterile sample containers should not be rinsed prior to filling and should be filled leaving a small air space. This will enable adequate mixing of the sample to be undertaken in the laboratory prior to analysis. The cap or seal should be replaced immediately after filling, ensuring the inside of the cap has not been contaminated. Where it is appropriate to avoid sampling surface film, the cap should only be removed once the container has been immersed to the correct depth. A similar principle can be used to sample from boats. Samples should be taken from the bow of the boat to minimise contamination from the vessel itself. Mud and sediments can be transferred directly to containers using a sterile

spatula or stainless steel trowel. When sterile sampling jugs or buckets are used, the water can be poured directly into the containers.

6.1.9 **Sample labelling**
The sample should be labelled on-site in a clear and durable manner so as to permit unambiguous identification. Additional relevant details can also be recorded at the same time. These may include date and time of sample, location, analysis required, and environmental observations such as tidal state, weather conditions etc.

6.1.10 **Sample transport**
After collection and labelling, samples should be transferred immediately to light-proof boxes and kept at a temperature of between 2 - 8°C. This temperature range may be maintained using, for example, insulated boxes or refrigerated conditions. The arrangements for transport to the laboratory should ensure that spillage does not occur. Studies[32, 33] to assess microbial levels during transport and storage have yet to provide clear explanations of the effects of transport and storage on resulting analyses. However, the longer the delay between sampling and analysis the more likely the opportunity for change to take place. Samples should therefore, be examined as soon as practicable on the day of collection, and storage, under the conditions stated above, should not exceed 24 hours before commencement of analysis.

6.1.11 **Safety aspects of sampling**
Staff involved in sampling marine or surface waters will frequently encounter a wide range of conditions and be subject to a variety of health and safety issues. This booklet is not intended as a comprehensive guide, and a thorough assessment of safety should be performed before any sampling programme is undertaken.

Sampling from unsafe sites, such as insecure banks of rivers or reservoirs, should be avoided. If necessary, more than one person should be considered for sampling procedures to be undertaken safely. Reasonable access to sampling locations in all weather conditions is important and may be essential for some routine sampling purposes. When samples are to be taken in streams, rivers or estuaries, sampling staff should take into account the possible presence of mud, sand, holes and swift currents. A wading rod, or similar probing instrument, may be essential for safe wading. In addition, traffic can be a hazard when sampling from bridges is being undertaken, or when sampling from bridges over navigable streams. Care should also be taken not to cause injury to others whilst sampling is being carried out.

Suitable safety equipment should be used when working from boats or other vessels, and weather conditions constantly observed. Care should be taken when using electrical equipment near water, and staff should always be aware of the risk of handling toxic or flammable materials, and the risks connected with infections from sewage associated materials. Further discussion of safety aspects can be found elsewhere[34].

6.1.12 **Quality assurance and quality control**
Much attention has already been paid to the quality assurance and quality control practices associated with analytical techniques. Where appropriate, these principles should be extended to sampling. A good quality assurance system should provide an organised, documented and supervised approach to any sampling programme. The documentation should include a clear definition of the programme, its aims and appropriate accuracy and precision requirements. There will need to be selection of sampling methods and containers appropriate to the analytical requirements. Sampling staff should be identified and a documented approach to training used. Sampling sites should also be clearly identified. There should be a system of audit and review of the sampling programme covering all aspects of documentation, and extending to quality control and maintenance of the system of records to support sampling activity. This should include recording information relating to individual samples and related activities, such as, for example, the servicing of sampling equipment.

There are a number of quality control measures that can be adopted for microbiological sampling. These include the use of field blank samples which, as far as possible, are exposed to as many of the sources of contamination as “real” samples. Sterile water can be transported to the appropriate location, placed into containers similar to those used for “real” samples and transported and analysed in the same way as real samples. With standard cultures, it is possible to use field check samples to assess potential loss of determinands

during transport and storage of samples. In addition, routine swabbing of vehicles and insulated boxes, which are used in the sampling process, may help to identify sources of contamination. Where suitable, sampling boxes should be cleaned and disinfected on a regular basis. Further advice on sampling can be found elsewhere[31, 35, 36, 37].

6.2 Swimming pool waters and spa pool waters

6.2.1 **Introduction**
There are many different kinds of pool waters used for swimming, hydrotherapy and other recreational uses. They often operate under different regimes and at different temperatures, and include a range of different water treatments and disinfection. Properly maintained pool waters with the correct treatment regimes, pH and levels of disinfectant are unlikely to be sources of infection for users. Infections are more likely to occur as a result of poor pool water management and hygiene practices. Failure to achieve good pool water maintenance can lead to deterioration of the microbiological quality of the water. Further advice is given elsewhere[14].

6.2.2 **Treatment and disinfection**
Most pools include filtration and disinfection procedures as part of water treatment practices[14]. Filters usually consist of sand filtration and are designed to remove particulate and colloidal material. To be effective, filters should be back-washed at regular intervals.

Chlorine is the most common disinfectant and is used either in the gaseous phase or as hypochlorite solution. Generally, the free chlorine level should be between 1.0 - 2.0 mgl^{-1} and the pH between 7.2 - 7.8 for effective disinfection. Ammonia and organic material can reduce free chlorine levels and produce disinfectant by-products. Ozone is also used as a disinfectant and removes many pollutants. Chloroisocyanurate compounds are also used as disinfectants. These compounds stabilise the free chlorine in ultra-violet light and are useful in outdoor pool waters. However, levels of cyanuric acid, a dissociation product, should be controlled and monitored. Bromine and bromine-based disinfectants utilise hypobromous acid, the properties of which are similar to those of hypochlorous acid. Bromine-based disinfectants and, in particular, bromochlorodimethylhydantoin are commonly used in whirlpool waters and spa pool waters. Other disinfectants include chlorine dioxide, ultra-violet light with chlorine, metallic ions of copper and silver with chlorine and polymeric biguanide compounds.

6.2.3 **Frequency of sampling**
Properly maintained pool waters should be sampled at regular intervals and random samples taken when there is a heavy bathing load to help establish that treatment regimes are working correctly. Samples should be taken just before pool waters are closed to users (for whatever reason) and after they are returned for use. Samples should also be taken when obvious contamination has occurred or when medical or epidemiological evidence suggests sufficient cause.

6.2.4 **Microbiological parameters used for assessing pool water quality**

6.2.4.1 **Colony counts**
The total viable colony count of recreational pool waters gives the best overall assessment of water quality. See Table 3.2. The colony count at 37°C provides information about the possible presence of pathogenic bacteria resulting from human contamination. In general, colony counts greater than 100 colony forming units per ml at 37°C may indicate a water treatment, or disinfection, problem that requires investigation. In addition, a trend with a continuing increase in colony counts may indicate that a water treatment system is beginning to fail and that an investigation should be undertaken.

6.2.4.2 **Coliform organisms and faecal coliform organisms**
The treatment and disinfection regimes operating for pool waters should be sufficient to ensure that the levels of coliform organisms and faecal coliform organisms shown in Table 3.2 are not exceeded. Occasionally, small numbers of coliform organisms may be isolated from a sample. Provided there are less than 10 coliform organisms per 100 ml, with a satisfactory colony count and no faecal coliform organisms, and the pH and disinfectant concentration

are satisfactory, and that no coliform organisms are isolated on a subsequent re-examination, the pool water quality may be considered satisfactory.

6.2.4.3 ***Pseudomonas aeruginosa***
Pseudomonads and in particular *Pseudomonas aeruginosa* are able to grow in moist, warm conditions with low levels of organic nutrients. Many strains are resistant to reasonably high levels of disinfectant, particularly when they are associated with biofilms. Under such circumstances, these organisms may cause problems with pool water quality. Since these organisms are widespread environmental organisms and common skin contaminants, they can be introduced into pool waters directly by users. Failure to maintain disinfectant levels, or to provide adequate treatment, or cleaning regimes, may result in pool waters, or treatment systems, becoming colonised with *Pseudomonas aeruginosa*.

6.2.4.4 ***Staphylococcus aureus***
Staphylococci are common skin organisms and may gain ready access to recreational waters. It is, therefore, likely that they may be found in pool waters, especially where the bathing load is heavy.

6.2.4.5 ***Legionella***
Outbreaks of legionnaires' disease have been documented[38] where *Legionella pneumophila* have been found in whirlpool waters that have not been properly maintained. Where pool water quality is poor, particularly with whirlpool waters, tests for legionella should be considered. Advice on sampling and analysis is given in section 10.

6.2.4.6 **Intestinal parasites**
There have been a number of documented outbreaks of *Cryptosporidium* and *Giardia* associated with swimming pool waters[39]. Advice on sampling and analysis is given in section 9.

6.2.5 **Sample containers**
Glass bottles should not be used in pool waters because of the associated risk of breakage. Plastic, disposable bottles, containing thiosulphate, should be used. The required capacity should not be less than 300 ml. Each batch of sterilized bottles should be tested for sterility and should not be used before this test is satisfactorily completed and results reported. Sterile bottles should be marked with an appropriate "use by" date and stored in suitable locations. Sample bottles should not be used after the "use by" date has expired.

It is essential that sample bottles contain the appropriate neutralising agent commensurate with the disinfectant used in pool water treatment. It is equally important that the laboratory is advised of the type of disinfectant used. Chlorine- and bromine-based disinfectants may be neutralised by the addition of a solution of sodium thiosulphate at a concentration of 18 mgl^{-1} added to sample bottles before sterilization. Copper and silver ions may be neutralised[40] by the addition of disodium ethylenediaminotetraacetate solution at a concentration of 50 mgl^{-1}. A solution can be sterilized by filtration and 1 ml added after sterilization of the sample bottles. For polymeric biguanide disinfection compounds, lecithin (3% m/v) with polyoxyethylene sorbitan mono-oleate solution, for example Tween 80 (20% m/v) can be used. Sodium thiosulphate solution should also be incorporated, if a chlorine residual is maintained.

6.2.6 **Sampling procedures**
Remove the stopper or cap from the sample bottle, ensuring that the neck of the bottle and the inside of the cap do not become contaminated. Immerse the bottle to about 150 mm below the surface of the pool water and allow the bottle to fill, leaving a small air space. Remove the bottle from the water and replace the stopper immediately. Store the sample bottle in the dark in an insulated cool box. Transport the sample box to the laboratory as described in section 6.1.10. The disinfectant residual and the pH should be determined on-site at the same time as the sample is taken and the results recorded and sent with the sample to the laboratory.

6.2.7 **Sample location**
The sample site should be chosen where the water velocity is low and situated away from any inlet systems. Depending on the size of the pool, it may be necessary to take additional

samples from other locations within the pool. Many leisure pool waters include features, such as flumes, islands, whirlpools and backwaters with complex systems of water flow. Consequently, several water samples may be necessary in order to obtain an overall assessment of water quality.

7. Bacteriological methods

7.1 General laboratory practice

7.1.1 **Introduction**
The analysis of waters for micro-organisms involves the filtration of measured volumes of water through membrane filters, ie membrane filtration (MF). Alternatively, this may involve the direct inoculation of measured volumes of water into liquid media contained in a series of tubes, or bottles, ie multiple tube method or most probable number (MPN) technique. It is essential that reasonable precautions are taken to prevent contamination of water samples, apparatus or test materials. Such contamination will lead to the production of misleading data and erroneous assumptions about water quality, and may lead to substantial amounts of unnecessary investigative work and expenditure on remedial work that is not required. It is important, therefore, that good laboratory practice and good aseptic techniques are adhered to.

7.1.2 **Laboratory preparation**
All contaminating materials, including samples from previous analyses, should be kept separate from areas where analytical work is to be conducted. All work surfaces should be cleaned with suitable disinfectants at the appropriate concentration. Any commercial "wipe" that is used should be discarded after use. Laboratory coats should be clean and changed regularly or when they become contaminated. The materials and media used in test procedures should be sterilized and checked for sterility prior to use.

7.1.3 **Sample preparation**

7.1.3.1 **Liquid samples**
Samples should be thoroughly mixed, for example inverted rapidly several times, in order to distribute the organisms uniformly throughout the water. The sample bottle should also be thoroughly shaken before any dilutions are prepared. In general, the results from previous routine sampling and testing may provide information on the number of organisms present at particular locations. This may facilitate the preparation of the appropriate number of dilutions that may be required. Where this information is not available, a range of suitable dilutions should be prepared to ensure that at least one of the test dilutions possesses a suitable number of organisms for counting purposes. For MF, this should be between 20-80 colonies, and for MPN, this should be demonstrated by the absence of growth in some of, but not all, the dilutions tested. For practical purposes, sub-samples of 10 or 1 ml may be tested, or suitable dilutions prepared, and appropriate aliquots tested. For example, measure out suitable volumes (90 or 9 ml) of sterile diluent, such as Ringer's solution, into suitable sterile bottles or tubes. Prepare an appropriate range of dilutions by transferring one volume of sample (or previously diluted sample) into 9 volumes of diluent. A clean, fresh, sterile pipette should be used for each occasion. Provided that the dilutions are tested in increasing order of concentration (ie starting with the least concentration first) a single membrane filter apparatus may be used without the need for disinfection between filtrations.

7.1.3.2 **Solid samples**
Where the number of organisms is very high and the dilutions required sufficiently large to minimise the amount of particulate matter, then semi-solid samples may be treated as above for MF. Alternatively, appropriate dilutions should be prepared and inoculated into isolation media as described for the MPN technique.

Solid samples should be thoroughly mixed and known amounts taken, for example 1x10 g and 5x1 g amounts, and treated as described for the MPN technique. The sample can be weighed directly into the isolation media, ensuring there is sufficient media for the sample. For example, 10 g of sample can be added to 90 ml of media. The sample should be dispersed with a sterile spatula. Alternatively, a known amount of the sample can be added to recovery diluent in a sterile "stomacher" bag and homogenised before being dispensed into isolation media.

7.1.4 **Apparatus**

Incubators and water baths should be capable of meeting the temperature tolerances specified in the method. Membrane filtration apparatus and filter units should be able to withstand steam or dry-heat sterilization. Filter units can be disinfected between use, for example by immersion in a boiling water bath for one minute, and then being allowed to cool before use. The base and funnel should be sterilized at the end of each working day.

Membrane filters are often made from cellulose esters, usually cellulose acetate or cellulose nitrate, and are, usually, 47 mm in diameter with a nominal pore size of 0.45 µm. Other sizes, made from different materials, are available and may be equally applicable. Membrane filters should be free of toxic substances, grid-marked (to ease counting) and, when used, should show no signs of growth inhibition or stimulation along the grid lines. Membrane filters should be sterilized before use and only used once.

Absorbent pads should be the same diameter as the membrane filter and be of at least 1 mm thickness. The pads should be manufactured from high quality paper fibres, free of toxic substances and uniformly absorbent. Pads are available pre-sterilized, or, if necessary, can be sterilized by autoclaving at 121 ± 1°C for 15 minutes.

Petri dishes are available pre-sterilized, or aseptically manufactured, in a variety of sizes for membrane filtration, used with either an incubation pad or a pre-poured medium. Petri dishes can also be used with selective and nutrient agars for plating broth cultures to obtain isolated colonies. It is important that Petri dishes used for colony counts are of a common size to ensure that the surface area and depth of the medium remain constant.

Pipettes of 10 or 1 ml volumes are available pre-sterilized as individually wrapped items to be discarded after use and should be the straight-sided, total delivery type. Glass pipettes should be plugged with non-absorbent cotton wool before being packed into suitable containers, or wrapped in brown paper or aluminium foil, and sterilized. Glass pipettes may be sterilized by autoclaving at 121 ± 1°C for 15 minutes or by heating to approximately 160°C for 60 minutes. Plastic disposable pipettes can also be used. Air displacement pipettes, calibrated gravimetrically, may also be used with sterile polypropylene tips. Alternatively, appropriately calibrated automatic pipettes with sterile disposable tips are satisfactory. Where applicable, after use, cotton wool plugs should be removed from pipettes, which should then be placed in jars containing disinfectant solution. Pipettes should be left for at least one hour, or preferably, overnight. Pipettes should then be rinsed with tap water and soaked in a suitable non-toxic detergent solution followed by rinsing with tap water. They should then be rinsed with distilled or de-ionised water and dried. Finally, pipettes should be plugged with non-absorbent cotton wool and stacked into metal containers for sterilization.

Forceps should be flat-ended and sterilized by immersion in alcohol, followed by flaming or heating in a Bunsen burner. A plate drier, if required, should be heated to approximately 45°C. Petri dishes may be dried by inverting, and then leaving for approximately 30 minutes.

The 50 ml volumes of double-strength media required for any multiple tube method should be dispensed into screw-capped bottles with a capacity of at least 125 ml. The 10 ml volumes of double-strength media should be dispensed into test-tubes or containers with a capacity of about 30 ml. Sterile disposable containers may be used for certain tests provided the medium is dispensed aseptically before use. For 10 ml volumes of single-strength medium and other liquid media, tubes or containers with a capacity of about 22 ml should be used. When media have to be stored for up to 1 month, screw-capped containers should be used. Routine media should be prepared and stored in volumes no larger than 500 ml, and bottle capacities should be of a sufficient size to prevent boiling-over during sterilization, and subsequent melting.

Test tubes, bottles and flasks should be autoclaved at 121 ± 1°C for 15 minutes after use and cleaned with a brush, washed in water with a non-toxic detergent solution, rinsed in tap water and finally in distilled or de-ionised water. Appropriate laboratory washing machines may be used. Before sterilization, test-tubes and flasks should be plugged with non-absorbent cotton wool or covered with suitable close-fitting caps. The necks and stoppers of flasks and bottles should be covered with brown paper or aluminium foil to prevent contamination.

Glassware may be sterilized in hot air ovens at approximately 160°C for a minimum of 1 hour. If this temperature is exceeded, the exposure time may be reduced proportionately. A temperature of above 170°C should be avoided since cotton wool is rendered friable. Alternatively, bottles and tubes may be sterilized by autoclaving at 121 ± 1°C for a minimum of 15 minutes. If bottles with ground-glass stoppers are used, a strip of brown paper or aluminium foil, approximately 75 mm x 10 mm, should be inserted between the stopper and the neck of the bottle before sterilization. This helps to prevent jamming of the stopper in the neck of the bottle and subsequent damage on cooling.

7.1.5 **Membrane filtration**

Membrane filters are incubated either on absorbent pads (soaked in liquid medium) or on pre-poured agar Petri dishes. Where pads are used, aseptically place sterile pads into Petri dishes. Add sufficient medium to saturate the pad and then drain off the excess medium.

Place the sterile filtration apparatus in position and connect to a source of vacuum with all the stopcocks turned off. Remove the funnel and place a sterile membrane filter, grid-side upwards on to the porous disc of the filter base. Hold the outer part of the membrane filter with sterile forceps. Replace the sterile funnel securely on the filter base. Pour, or pipette, the required volume of sample, or diluted sample, into the funnel. For volumes of 10 ml or less, at least 20 ml of sterile diluent should first be added to the funnel. Open the stopcock and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered, to prevent excess air being drawn through the membrane filter.

Transfer the membrane filter to the growth medium, ensuring that no air bubbles are trapped between the membrane filter and the medium. Remove the funnel to a boiling water bath. Where different volumes of the same sample are to be filtered, the funnel may be re-used without further disinfection, provided the samples are filtered in sequence, starting with the smallest volume. For different samples, remove a funnel from the boiling water bath and allow it to cool. Repeat the filtration process as before.

Petri dishes containing membranes should be inverted during incubation. Where an incubation pad is used, Petri dishes should be incubated in sealed containers to prevent the pad from drying out.

7.1.6 **Multiple tube method or most probable number technique**

The multiple tube method is most often used where samples are too dirty to permit membrane filtration or where organisms are present in very low numbers and a concentration step is required in order to detect them. Volumes of the sample, or dilutions of the sample, are inoculated into bottles, or tubes, containing liquid culture media. During incubation, each tube that contains one or more organisms should show growth. Provided that some, but not all, of the tubes exhibit no growth of the organism, ie are negative, the most probable number of organisms in the sample can be estimated from tables.

The volume of sample added to the tube should not significantly change the concentration of the medium. Volumes of 1 ml, or less, are usually added to single-strength growth medium and volumes of between 10 and 50 ml are normally added to double-strength medium. For larger volumes, more concentrated medium can be used, and for special purposes, dehydrated medium can be dissolved in the sample. When the test is completed, positive tests, ie tubes that show growth of the particular organism under study, can be subcultured on to agar or inoculated directly into confirmatory media.

Typically, 1x50 ml volume, 5x10 ml volumes and 5x1 ml volumes are aseptically inoculated into tubes containing media. With more polluted samples, additional volumes of 5x0.1 ml may be added to extra tubes in order to produce tubes exhibiting no growth, ie produce negative results. The addition of smaller volumes, to accommodate heavily polluted samples, may be undertaken using appropriately diluted samples. Inoculated tubes are incubated at the appropriate temperature, and the proportion of positive and negative tubes can then be ascertained and the most probable number of organisms in the sample determined from tables. If necessary, positive tubes may be subcultured into enrichment or selective media for confirmation.

Where samples are clear and expected to contain very few organisms, they can be concentrated by MF and then analysed by the MPN technique. For example, 1x500 ml, 5x100 ml and 5x10 ml volumes of sample can be filtered and the respective membrane filters added to tubes containing appropriate medium. The test is then completed in the usual manner.

7.1.7 **Analytical quality control**

All media prepared in the laboratory should be subjected to quality control procedures before being released for laboratory use. This will, usually, include checking the final pH of the medium and ensuring that the medium is suitable for the type of micro-organism being determined, whilst inhibiting or clearly differentiating the growth of unwanted micro-organisms. This type of testing should be carried out on each batch of prepared medium using suspensions of organisms that are traceable to national culture collections. The results of quality control testing should be recorded at the time of sample testing, and positive, negative and blank controls should be included. The positive control may be an environmental sample known to contain the micro-organism being sought, or a suspension from a stock culture as described above. Negative controls should contain non-target micro-organisms which do not grow characteristically on the medium. The blank control may consist of 100 ml of sterile water and is used to ensure sterility of the test media and any apparatus used. Positive, negative and blank controls should be incubated under the same conditions and at the same time as the test samples are analysed and should ensure that the method is satisfactory for the micro-organisms under test. A failure in any of these controls should invalidate the results of the test and consideration should be given to repeating the analysis. However, it is often impracticable to repeat the analysis of a sample as the number of organisms in the original sample may change during storage. Hence, laboratories should undertake thorough audits of all procedures after unexpected results are obtained.

The recovery efficiency of any method can be determined by carrying out the analysis using suspensions of known concentrations of organisms. This may be undertaken by inoculating laboratory cultures into the types of sample normally tested, or by participating in appropriate proficiency testing schemes.

7.2 Detection and enumeration of coliform organisms and faecal coliform organisms by membrane filtration

7.2.1 **Introduction**

Coliform organisms and faecal coliform organisms have become the standard faecal indicators for recreational and environmental water quality. The presence of these organisms is suggestive of faecal contamination and the numbers enumerated give some indication of the degree of contamination. However, the levels of coliform organisms and faecal coliform organisms do not indicate potential pathogen levels, and the absence of these indicator organisms does not indicate the absence of pathogenic organisms in that water. Techniques involving MF are subject to considerable statistical variation (see section 5) and great care should be taken over the interpretation of particular results, especially if aliquots of diluted samples have been prepared and analysed.

7.2.2 **Scope and field of application**

This method is suitable for the isolation of coliform organisms and faecal coliform organisms from all types of waters. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes in order to produce a membrane filter containing a sufficient number of colonies.

7.2.2.1 **Definition and description of the organisms**

The family Enterobacteriaceae is a large and complex family of organisms and is subject to considerable re-classification. A significant number of the genera possess the ability to ferment lactose and a prerequisite of lactose fermentation is possession of the enzyme ß-galactosidase[41]. This enzyme catalyses the hydrolysis of lactose to form glucose and galactose.

Coliform organisms are members of a genus or species within the family Enterobacteriaceae that grow at 37°C and possess ß-galactosidase. Coliform organisms are also oxidase-negative. In the context of the method, organisms that produce acid from lactose and form yellow

colonies on membrane filters after incubation for 4 hours at 30°C and 14 hours at 37°C are regarded as coliform organisms. Some spore-forming bacteria may also produce acid, and subculture should be made to confirm fermentative properties or the possession of ß-galactosidase and the absence of oxidase.

Some lactose-fermenting strains of *Aeromonas* species and staphylococcus and bacillus may produce typical small yellow colonies. Where the presence of *Aeromonas* is suspected, subcultures should be made and the presence of oxidase confirmed[41]. Where staphylococcus and bacillus are suspected, isolates should be Gram stained to confirm Gram-positive cocci or rods respectively.

Faecal coliform organisms are coliform organisms that grow at 44°C and produce acid and, generally, gas from lactose, and indole from tryptophan. This group of organisms may include some genera, for example *Klebsiella* species that may not be faecal in origin. It is assumed that, for most cases, these particular organisms will be in the minority and that the majority of organisms isolated will be *Escherichia coli (E. coli)*. Most strains of *E. coli* possess the enzyme ß-glucuronidase and substrates specific for this enzyme have been used for the detection and confirmation of *E. coli* isolates.

7.2.2.2 **Pathogenicity**

Coliform organisms and faecal coliform organisms are present in large numbers in the intestines of humans and animals, and certain strains cause illness. Enterotoxigenic *E. coli* are probably the commonest causes of travellers' diarrhoea. This may relate to lack of previous exposure to the organism, and predisposition to other illnesses.

7.2.3 **Principle**

A choice of media is available for the isolation and identification of coliform organisms and faecal coliform organisms. Only procedures employing membrane lauryl sulphate broth (MLSB) are described. A number of trials comparing MLSB with other media have been reported[42, 43]. Volumes of sample, or appropriate aliquots of suitable dilutions, are filtered, and the filters incubated on absorbent pads soaked in MLSB. The organisms produce characteristic yellow colonies on MLSB at 37°C and 44°C. Confirmation of isolates is carried out by subculture to nutrient agar for 24 hours at 37°C, together with appropriate testing for the production of acid from lactose at 37°C and 44°C, the production of indole at 44°C and the absence of the enzyme oxidase.

7.2.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms, in particular aeromonads and non-lactose-fermenting bacteria, may inhibit the growth, or detection, of lactose-fermenting organisms. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the identification of colonies under investigation. Alternatively, highly turbid waters can be analysed using the MPN technique. After incubating for 18 hours, membrane filters are examined for characteristic colonies. Confirmation of the organisms isolated may take a further 48 hours.

7.2.5 **Reagents and media**

7.2.5.1 **Membrane lauryl sulphate broth**

Bacteriological peptone	40.0 g
Yeast extract	6.0 g
Lactose	30.0 g
Phenol red (0.4% m/v aqueous solution)	50 ml
Sodium lauryl sulphate	1.0 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 0.2. Dispense the resulting

solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.4 ± 0.2. An agar form of the medium can be prepared by adding 10 - 15 g of agar per litre of solution.

7.2.5.2 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark and used within one month. Most non-selective agars are suitable for producing pure cultures for oxidase testing, provided they do not contain fermentable carbohydrates.

7.2.5.3 **Lactose peptone water**

Peptone	10.0 g
Sodium chloride	5.0 g
Phenol red (0.4% m/v aqueous solution)	2.5 ml
Lactose	10.0 g
Water	1000 ml

Dissolve the ingredients (except phenol red) in the water and adjust the pH to 7.5 ± 0.2. Add the phenol red solution and dispense the resulting solution in appropriate volumes into suitable containers, or test tubes, appropriately capped. Sterilize by autoclaving at 115 ± 1°C for 10 minutes. The medium should be stored at between 2 - 8 °C and used within one month.

7.2.5.4 **Tryptone water**

Tryptone	20.0 g
Sodium chloride	5.0 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.5 ± 0.2. Dispense the resulting solution (in 5 ml volumes) into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes. The sterilized medium should be stored at between 2 - 8°C and used within one month.

7.2.5.5 **Kovács' reagent**

4-dimethylaminobenzaldehyde	5.0 g
Iso-amyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the aldehyde in the alcohol. Cautiously, add the concentrated acid. The reagent should be light yellow to light brown in colour and should be stored at between 2 - 8°C and used within six months.

7.2.5.6 **Oxidase reagent**

Tetramethyl-p-phenylenediamine dihydrochloride	100 mg
Water	10 ml

Weigh out the tetramethyl-p-phenylenediamine dihydrochloride into a suitable container and dissolve in the water. This reagent does not keep and should be prepared immediately before use. "Test sticks" are available commercially and may be stored and used according to the manufacturer's instructions.

7.2.6 **Analytical procedure**

Duplicate volumes of sample, or aliquots of diluted sample, are filtered through membrane filters, which are then placed onto pads soaked in MLSB contained in Petri dishes. The dish is then placed in a container with a tight-fitting lid to prevent pads drying out. Membrane filters used for the isolation of coliform organisms should be incubated for 4 ± 1 hour at 30 ± 1°C followed by 14 hours at 37 ± 1°C. Membrane filters used for the isolation of faecal coliform organisms should be incubated for 4 ± 1 hour at 30 ± 1°C followed by 14 hours at 44.0 ± 0.5°C.

After incubating for 18 hours, count all yellow colonies (irrespective of size) on each membrane filter. Where a range of dilutions has been filtered, it should, ideally, be possible to count from a membrane filter showing between 20 - 80 colonies (section 5.3.1). Where no specific dilution gives a count within this range, it may usually be satisfactory to average the counts for two mutually consistent dilutions with counts falling just outside this range. Depending on the purposes of the sampling, there may be occasions where it is necessary to report a value, even though insufficient dilutions have been examined or were able to satisfy the above criteria. In these cases, the limitations of the reliability of the count should be acknowledged when reporting the value. Where more than one membrane filter has been used for a given volume of sample, determine the average number of colonies per membrane filter.

The yellow colonies incubated at 37°C are regarded as presumptive coliform organisms and those at 44°C are regarded as presumptive faecal coliform organisms. If membrane filters are examined before 18 hours, for example to obtain a provisional assessment, then they should be quickly returned to the incubator so that the full incubation is completed. Membrane filters removed from the incubator should be examined as soon as possible, preferably within 10 minutes.

7.2.7 **Confirmation**

See also section 7.3.7. Where the number of organisms is less than the statutory limit, then consideration should be given as to whether the results need to be confirmed. Where the number of organisms exceeds the statutory limits, then confirmation will often be required; see section 5.3.2.3. Selected colonies are subcultured on nutrient agar. At the same time, suitable control organisms, for example *E. coli* and *Pseudomonas aeruginosa (Ps. aeruginosa)* can be subcultured as part of ongoing quality control. All membrane filters should be stored at between 2 - 8°C, until the confirmatory tests are complete.

Incubate all nutrient and MacConkey agar dishes for a minimum of 18 hours at 37°C and check each culture for purity. Where cultures are pure, the nutrient agar dishes should be tested for the absence of the enzyme oxidase by transferring some of the colonies to a pad soaked in oxidase reagent. The oxidase test should not be carried out using a nichrome wire. If an organism is oxidase-positive, a blue colour develops, normally within approximately 10 seconds. A negative reaction is indicated when no colour change is produced. The control organisms should give satisfactory oxidase reactions, ie negative for *E. coli* and positive for *Ps. aeruginosa*. Where cultures are not pure, an isolated colony of each colony type should be subcultured onto fresh nutrient agar.

Inoculate all oxidase-negative isolates into lactose peptone water and incubate at 37 ± 1°C for 24 hours, and into lactose peptone water and tryptone water and incubate at 44.0 ± 0.5°C for 24 hours. Include the appropriate control cultures.

Examine lactose peptone waters for the production of acid (as demonstrated by the production of a yellow colour). Negative tubes at 37°C should be returned to the incubator for a further 24 hours. Add approximately 0.25 ml of Kovács' reagent to the tryptone water and shake. The appearance of a red colour indicates a positive reaction confirming the presence of indole, while a yellow colour indicates a negative reaction. A range of commercially available systems is available for this test. These should be assessed for suitability of confirmation against reference procedures.

7.2.8 **Reporting results**

For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported; see sections 5.4 and 7.2.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter to be obtained that contains a sufficient number of organisms. The report may also contain details of presumptive organisms, as well as confirmed organisms.

7.3 Detection and enumeration of coliform organisms and faecal coliform organisms by the multiple tube method or most probable number technique

7.3.1 **Introduction**

Coliform organisms and faecal coliform organisms have become the standard faecal indicators for recreational and environmental water quality. The presence of these organisms indicates faecal contamination and the numbers enumerated give some indication of the degree of faecal contamination. However, the levels of coliform organisms and faecal coliform organisms do not indicate potential pathogen levels, and the absence of these indicator organisms does not indicate the absence of pathogenic organisms in that water. The multiple tube method or MPN technique is subject to considerable statistical variation (see section 5) and great care should be taken over the interpretation of particular results, especially if aliquots of diluted samples have been prepared and analysed.

7.3.2 **Scope and field of application**

This method is suitable for the isolation of coliform organisms and faecal coliform organisms from all types of waters, but is particularly suited for testing sediments and turbid waters that cannot be processed by MF. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes, or dilutions, in order to produce a series of tubes in which some of the tubes exhibit no growth, ie negative results are obtained.

7.3.2.1 **Definition and description of the organisms**

The family Enterobacteriaceae is a large and complex family of organisms and is subject to considerable re-classification. A significant number of the genera possess the ability to ferment lactose and a prerequisite of lactose fermentation is possession of the enzyme ß-galactosidase[41]. This enzyme catalyses the hydrolysis of lactose to form glucose and galactose.

Coliform organisms are members of a genus or species within the family Enterobacteriaceae that grow at 37°C and possess ß-galactosidase. Coliform organisms are also oxidase-negative. In the context of the method, organisms that exhibit growth after incubating for 48 hours at 30°C and 14 hours at 37°C are regarded as coliform organisms. Some spore-forming bacteria may also grow, and subculture should be made to confirm fermentative properties or the possession of ß-galactosidase and the absence of oxidase.

Some lactose-fermenting strains of *Aeromonas* species and staphylococcus and bacillus may also demonstrate growth. Where the presence of *Aeromonas* is suspected, subcultures should be made and the presence of oxidase confirmed[41]. Where staphylococcus and bacillus are suspected, isolates should be Gram stained to confirm Gram positive cocci or rods respectively.

Faecal coliform organisms are coliform organisms that will grow at 44°C and produce acid and, generally, gas from lactose, and indole from tryptophan. This group of organisms may include some genera, for example *Klebsiella* species that may not be faecal in origin. It is assumed that, for most cases, these organisms will be in the minority and that the majority of organisms isolated will be *E. coli*. Most strains of *E. coli* possess the enzyme ß-glucuronidase, and substrates specific for this enzyme have been used for the detection and confirmation of *E. coli* isolates.

7.3.2.2 **Pathogenicity**

Coliform organisms and faecal coliform organisms are present in large numbers in the intestines of humans and animals, and certain strains cause illness. Enterotoxigenic *E. coli* are probably the commonest causes of travellers' diarrhoea. This may relate to lack of previous exposure to the organism, and predisposition to other illnesses.

7.3.3 **Principle**

Volumes of sample, or appropriate aliquots of suitable dilutions, are added to a series of tubes, or bottles, containing the isolation medium. Each container that is inoculated with the organism should show growth and acid production after incubation. Provided that some, but not, of all the tubes, exhibit no growth, ie are negative, the most probable number of organisms in the sample can be determined from probability tables. Positive tests, ie those tubes exhibiting growth, are subcultured to confirmatory media to demonstrate the production of acid from lactose at 37°C and 44°C, and the production of indole.

7.3.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volumes of sample that are used. To increase the detection, or volume of sample, MF may be used to concentrate volumes of sample before inoculation into tubes containing media. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. Tubes may be examined after 24 hours but should be incubated for 48 hours before being discarded. Confirmation will take a further 48 hours.

7.3.5 **Reagents and media**

7.3.5.1 **Double-strength minerals modified glutamate 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	20 mg
Thiamin (aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1% m/v ethanolic solution)	2.0 ml
Water to	1000 ml

Dissolve all the ingredients in the water. Dispense the resulting solution (in 5 ml volumes) into suitable containers. Sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. The sterile medium should be stored in the dark at room temperature and used within one month.

The medium is conveniently prepared in quantities of 10 litres and several of the ingredients added as separate solutions.

Solution 1	
L(+) Arginine monohydrochloride	400 mg
L(-) Aspartic acid	480 mg
Water	50 ml

Dissolve the ingredients in the water. This may require gentle heating.

Solution 2	
L(-) Cystine	400 mg
5M sodium hydroxide	10 ml
Water	90 ml

Dissolve the ingredients in the water. This may require gentle heating.

Solution 3	
Nicotinic acid	20 mg
Pantothenic acid	20 mg
Water	5 ml

Dissolve the ingredients in the water.

Solution 4	
Iron(III) citrate	200 mg
Water	10 ml

Dissolve the ingredient in the water. This may require gentle heating.

Solution 5	
Calcium chloride dihydrate	5.0 g
Water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water and sterilize by autoclaving at 121 ± 1°C for 20 minutes. Keep as a stock solution.

Solution 6	
Thiamin	100 mg
Water	100 ml

Dissolve the ingredient in the water and sterilize by filtration through a sterile 0.2 µm pore size membrane filter. This solution should be stored at room temperature and used within six weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L(+) glutamic acid, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate in 9 litres of hot water. Add solutions 1, 2, 3 and 4, and 4.0 ml of solution 5. Adjust the pH to 6.8 or such that after sterilization the final pH is 6.7 ± 0.2. Add 20.0 ml of bromocresol purple solution and make the final volume up to 10 litres. For immediate use, add the appropriate quantity of lactose and the thiamin solution, dispense into tubes, or bottles, and sterilize by autoclaving at 115 ± 1°C for 10 minutes. For storage without thiamin and lactose, dispense in 500 ml volumes in suitable containers and autoclave at 115 ± 1°C for 10 minutes.

Prepare single-strength medium by diluting double-strength medium with an equal volume of water, dispense (in 5 ml volumes) into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes.

A variety of confirmation media is available and should be chosen as applicable. Confirmatory tests that detect the expression of ß-galactosidase and ß-glucuronidase are also available. Confirmation is carried out by subculture to a selective medium that permits the differentiation of lactose-fermenting organisms from non-lactose-fermenting organisms, for example MacConkey agar, followed by inoculation of typical colonies into confirmatory media.

7.3.5.2 **MacConkey agar**

Bile salts	5.0 g
Peptone	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Agar	12.0 g
Neutral red (1% m/v aqueous solution)	5 ml
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored in the dark at room temperature and used within one month.

7.3.5.3 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10-15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark and used within 1 month. Most non-selective agars are suitable for producing pure cultures for oxidase testing, provided they do not contain fermentable carbohydrates.

7.3.5.4 **Lactose peptone water**

Peptone	10.0 g
Sodium chloride	5.0 g
Phenol red (0.4% m/v aqueous solution)	2.5 ml
Lactose	10.0 g
Water	1000 ml

Dissolve the ingredients (except phenol red) in the water and adjust the pH to 7.5 ± 0.2. Add the phenol red solution and dispense the resulting solution in appropriate volumes into suitable containers, or test tubes, appropriately capped. Sterilize by autoclaving at 115 ± 1°C for 10 minutes. The medium should be stored at between 2 - 8°C and used within one month.

7.3.5.5 **Tryptone water**

Tryptone	20.0 g
Sodium chloride	5.0 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.5 ± 0.2. Dispense the resulting solution (in 5 ml volumes) into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes. The sterilized medium should be stored at between 2 - 8°C and used within one month.

7.3.5.6 **Kovács' reagent**

4-dimethylaminobenzaldehyde	5.0 g
Iso-amyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the aldehyde in the alcohol. Cautiously, add the concentrated acid. The reagent should be light yellow to light brown in colour and should be stored at between 2 - 8°C and used within six months.

7.3.5.7 **Oxidase reagent**

Tetramethyl-p-phenylenediamine dihydrochloride	100 mg
Water	10 ml

Weigh out the tetramethyl-p-phenylenediamine dihydrochloride into a suitable container and dissolve in the water. This reagent does not keep and should be prepared immediately before use. "Test sticks" are available commercially and may be stored and used according to the manufacturer's instructions.

7.3.6 **Analytical procedure**

Thoroughly mix the sample and inoculate 1x50 ml and 5x10 ml sample volumes into tubes containing equal volumes of double-strength minerals modified glutamate medium (MMGM). In addition, inoculate 5x1 ml sample volumes into 5 ml of single-strength MMGM. Where polluted samples are to be tested, omit the 50 ml inoculation and include 5x0.1 ml sample volumes (or 5x1 ml volumes of an appropriately diluted sample, ie a 10-fold dilution) into single-strength MMGM. For heavily polluted samples, additional aliquots of 100-fold dilutions, or 1000-fold or higher dilutions, should be included to ensure that some of the tubes exhibit no growth, ie show negative reactions. For solid and semi-solid samples, see section 7.1.3.2.

Inoculated tubes should be incubated at 37 ± 1°C for 18 - 24 hours. The number of tubes, or bottles, showing growth of organisms, as indicated by acid production, is recorded. Cultures from these tubes are subcultured to two separate Petri dishes containing MacConkey agar. One Petri dish is incubated at 37 ± 1°C for 18 - 24 hours, and the other at 44.0 ± 0.5°C for 18 - 24 hours. All tubes showing growth are retained for a period, since they may be required for repeat subcultures. All tubes showing no growth of organisms are incubated for a further 24 hours and then examined again. Any new tubes showing growth of organisms are inoculated onto MacConkey agar.

7.3.7 **Confirmation**

See also section 7.2.7. Colonies of coliform organisms on the MacConkey agar are usually circular in shape, convex or low convex with a smooth surface and entire edge. They are usually red in colour, but the depth of colour may vary and colonial differences cannot be relied upon for differentiation within the group. The colony may be surrounded by a halo due to acid precipitation of the bile salts.

Typical isolated colonies from each Petri dish at 37°C should be inoculated into lactose peptone water and onto nutrient agar for incubation at 37°C. Typical isolated colonies from each Petri dish at 44°C should be inoculated into lactose peptone water and onto nutrient agar for incubation at 37°C, and into lactose peptone water and tryptone water for incubation at 44°C. See also section 5.3.2.3. The MacConkey agar Petri dishes should be retained in case repeat confirmatory tests are required. Appropriate control cultures should also be included. Examine lactose peptone waters for the production of acid (as demonstrated by the production of a yellow colour). Negative tubes at 37°C should be returned to the incubator for a further 24 hours. Add approximately 0.25 ml of Kovács' reagent to the tryptone water and shake. The appearance of a red colour indicates a positive reaction confirming the presence of indole, while a yellow colour indicates a negative reaction. A range of commercially available systems is available for this test. These should be assessed for suitability of confirmation against reference procedures.

Check the nutrient agar cultures for purity. Where cultures are pure, they should be tested for the absence of the enzyme oxidase by transferring some of the colonies to a pad soaked in oxidase reagent. The oxidase test should not be carried out using a nichrome wire. If an organism is oxidase-positive, a blue colour develops, normally within approximately 10 seconds. A negative reaction is indicated when no colour change is produced. The control organisms should give satisfactory oxidase reactions, ie negative for *E. coli* and positive for *Ps. aeruginosa*. Where cultures are not pure, an isolated colony of each colony type should be subcultured onto fresh nutrient agar.

Where there is interest in the different types of coliform organisms present in a sample, a number of morphologically different colonies can be subcultured from the MacConkey agar dishes. These can be confirmed and subjected to further testing with commercially available products.

7.3.8 **Reporting results**

For each sample, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4 and 7.3.4. Every effort should be made to prepare sufficient dilutions to enable a series of tubes to be obtained to show that some tubes exhibit no growth while others exhibit some growth. The report may also contain details of presumptive organisms, as well as confirmed organisms.

7.4 Detection and enumeration of enterococci

7.4.1 **Introduction**

Enterococci include a number of different species that occur in the faeces of humans and warm-blooded animals and can therefore provide an indication of such pollution. In human faeces, the organisms rarely exceed one million per gram, while in animal faeces enterococci are often more numerous than *E. coli*. Enterococci of faecal origin do not multiply in water and are more resistant to environmental stress than *E. coli*. They can be found in food products and are often unrelated to direct faecal contamination. A related group of bacteria, Aerococcus species, is often found in water and on vegetation.

The species of enterococci in faeces, and therefore those most likely to be found in polluted waters can be found in two main groups. The first group includes *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans*, which are normally present in humans and various animals. The second group comprises *Streptococcus bovis*, *Streptococcus equinus* and *Enterococcus avium*, which are not usually found in humans. Hence, a count of the actual enterococcal species present in water may facilitate the tracing of sources of pollution. The number of *E. coli* organisms in water, compared with enterococci, is sometimes used to assess the nature of recent faecal contamination. A ratio greater than four is regarded as an indication of human pollution, and a ratio of less than one is regarded as an animal source of pollution. This kind of approach is, however, limited owing to such variables as time, temperature and pH. Differential survival rates of the organisms may also affect the validity of the results. While enterococci are regarded as secondary indicator organisms of faecal pollution, they are considered to be better indicators of past, or less recent, contamination, and may be of more value in assessing health risks associated with recreational and environmental waters.

7.4.2 **Scope and field of application**

The MF method[44] is suitable for the isolation of enterococci from all types of waters and the MPN technique is suitable for sediments and sludges and waters containing high levels of particulate matter. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes, or dilutions, in order to produce a membrane filter containing sufficient colonies, or a series of tubes, some of which exhibit no growth.

7.4.2.1 **Definition and description of the organisms**

Enterococci are Gram-positive cocci that tend to form pairs and chains. They are non-sporing, oxidase-negative and catalase-negative, possess Lancefield's Group D antigen and can hydrolyse aesculin. They can grow aerobically and anaerobically in the presence of bile salts, and in concentrations of sodium azide which are inhibitory to coliform organisms and most Gram-negative bacteria. *Enterococcus faecalis* and some related species can reduce 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble red dye, formazan.

7.4.2.2 **Pathogenicity**

Enterococci often occur in urinary tract infections and in sub-acute bacterial endocarditis.

7.4.3 **Principle**

Membrane filters from filtered samples are incubated on Slanetz and Bartley agar and the number of characteristic colonies, after 48 hours incubation, is recorded. Confirmation is based on the ability of isolated colonies to hydrolyse aesculin.

For the MPN technique, a series of tubes containing glucose azide broth can be incubated and examined for growth and reduction of TTC or production of acid respectively.

Presumptive positive tubes may be confirmed by sub-culture to the confirmatory media and incubation at 44°C for 24 hours.

7.4.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered or series of tubes tested. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth, or detection, of enterococci. Some species of spore-bearing bacillus may produce small red colonies on the medium and confirmation by aesculin hydrolysis is important. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the identification of colonies under investigation. After incubating for 48 hours, membrane filters should be examined for colonies. Confirmation takes a further 2 hours.

7.4.5 **Reagents and media**

Sodium azide is toxic. Solutions containing sodium azide should not be discharged through metal pipework or drains, as explosive compounds may be formed. Sodium azide can be decomposed by addition of excess nitrite solution.

7.4.5.1 **Slanetz and Bartley agar**

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dipotassium hydrogen phosphate	4.0 g
Sodium azide	400 mg
2,3,5-triphenyltetrazolium chloride (TTC)	100 mg
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Excessive heating should be avoided to minimise the reduction of TTC. Cool quickly to approximately 50°C. The pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Pour appropriate aliquots of the resulting solution into sterile Petri dishes. Allow the agar to solidify, store at between 2 - 8°C, and use within one month.

7.4.5.2 **Bile aesculin agar**

Peptone	8.0 g
Bile salts	20.0 g
Aesculin	1.0 g
Ammonium iron(III) citrate	500 mg
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.1 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes. Allow the agar to solidify, store at room temperature in the dark and use within one month.

7.4.5.3 **Kanamycin aesculin azide agar**

Tryptone	20.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Sodium citrate	1.0 g
Aesculin	1.0 g
Ammonium iron(III) citrate	500 mg
Sodium azide	150 mg
Kanamycin sulphate	20 mg
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes. Allow the agar to solidify, store between 2 - 8°C and use within one month.

7.4.5.4 **Glucose azide broth**

	Single-strength	Double-strength
Peptone	10.0 g	20.0 g
Yeast extract	3.0 g	6.0 g
Sodium chloride	5.0 g	10.0 g
Dipotassium hydrogen phosphate	5.0 g	10.0 g
Potassium dihydrogen phosphate	2.0 g	4.0 g
Glucose	5.0 g	10.0 g
Sodium azide	250 mg	500 mg
Bromocresol purple (1.6% m/v ethanolic solution)	2.0 ml	4.0 ml
Water	1000 ml	1000 ml

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. Store at between 2 - 8°C and use within one month.

7.4.6 **Analytical procedure**

A volume of sample, or an aliquot of a diluted sample, is filtered through a membrane filter. The filter is then placed onto Slanetz and Bartley agar and incubated at 37 ± 1°C for 4 hours followed by 44.0 ± 0.5°C for 44 hours. After incubation, examine the membrane filters for typical colonies. Enterococci form red or maroon coloured colonies, 1 - 2 mm in diameter, that may, or may not, have a whitish border. Other organisms, in particular spore-bearing bacilli, may produce pin-pointed red colonies. Record all colonies as presumptive enterococci.

Alternatively, measured volumes of sample, or dilutions of sample, are added to a series of tubes, or bottles, containing the isolation broth medium. Each container that is inoculated with the organism should show growth after incubation. Provided that some of the tubes exhibit no growth, ie are negative, the most probable number of organisms in the sample can be determined from probability tables. Positive tests are subcultured to confirmatory media.

7.4.7 **Confirmation**

Presumptive colonies should be confirmed (see section 5.3.2.3) by transferring the membrane filter to bile aesculin azide agar, or kanamycin aesculin azide agar, and incubating at 44.0 ± 0.5°C for 2 hours. Development of a black or brown colour in and around the colony confirms presumptive colonies as enterococci. This is due to the hydrolysis of aesculin.

7.4.8 **Reporting results**

For each sample, record the number of colonies per volume tested. Alternatively, record the number of tubes that confirm as positive, ie demonstrate growth of organisms, and from

tables determine the number of organisms. Convert this number to the volume to be reported; see sections 5.4 and 7.4.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter to be obtained that contains sufficient organisms, or a series of tubes to be obtained that shows that some tubes exhibit no growth while others exhibit some growth. The report may also contain details of presumptive organisms.

Table 7.1 Biochemical differentiation of enterococci

Table 7.1 summarises different reactions for a number of organisms.

	S. bovis	*S. equinus*	*Ent. durans*	*Ent. faecalis*	*Ent. Faecium*
Antigen group	D	D*	D	D	D
Growth at 45°C	++	+	d	+	+
Survives 60°C - 30 minutes	-	-	++	+	+
Growth in 6.5% sodium chloride	-	-	++	+	+
Growth at pH 9.6	-	-	d	+	+
Growth on 40% bile	++	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+
Arginine hydrolysis	-	-	+	+	+
Arabinose (acid)	d	-	-	-	+
Raffinose (acid)	+	-	-	-	-
Glycerol (acid)	-	-	+	+	+
Sorbitol (acid)	-	-	-	+	-
Haemolysis	∝/-	∝	ß/-	ß/-	∝/ß

* In the precipitin test, extracts of some strains do not react with antisera

7.5 Detection and enumeration of spore-forming sulphite-reducing clostridia and *Clostridium perfringens*

7.5.1 Introduction

Clostridium species are environmental bacteria. *Clostridium perfringens (Cl. perfringens)* is an important species of the group of anaerobic sulphite-reducing clostridia and is commonly found in human and animal faeces. Most species are saprophytic, normally inhabiting soil, water and decomposing plant and animal material. Since these organisms are usually present in much lower numbers than *E. coli* and enterococci, they are less sensitive as direct indicator organisms of faecal contamination. *Cl. perfringens* can form resistant spores that survive in water and in the environment for much longer periods than *E. coli* and other faecal indicator organisms.

7.5.2 Scope and field of application

Several methods[41, 45] are suitable for the isolation of *Cl. perfringens* from waters including river waters, estuarine and sea waters, sewage and sewage effluents, and spa pool waters used for recreational or therapeutic purposes. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes in order to produce a membrane filter containing sufficient colonies, or a series of tubes, some of which exhibit no growth.

7.5.2.1 Definition and description of the organisms

Sulphite-reducing clostridia are Gram-positive, spore-forming anaerobic rods. The rods are typically large, straight or slightly curved with slightly rounded ends. Most members of the genus are motile, but *Cl. perfringens* is an important exception. Clostridia are biochemically active, frequently possessing both saccharolytic and proteolytic enzymes.

7.5.2.2 Pathogenicity

The presence of such organisms in recreational and environmental water can be regarded as being a result of faecal contamination. The genus, whilst consisting mainly of saprophytes, contains some species which are, generally, regarded as opportunistic pathogenic organisms. For example, *Cl. perfringens* is responsible for gas gangrene and also a substantial number of cases of food poisoning.

7.5.3 Principle

The methods describe the isolation of sulphite-reducing clostridia by membrane filtration. After preliminary heat treatment, to destroy vegetative bacteria, test volumes of sample, or diluted sample, are filtered. The membrane filters are incubated under anaerobic conditions at 37°C for 48 hours, and the number of typical black colonies that develop is recorded. Typical colonies may be confirmed as *Cl. perfringens* by subculture to Crossley's milk. The production of acid and the formation of the characteristic "stormy clot" by coagulation of casein constitute confirmation.

7.5.4 Performance characteristics

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth, or detection, of the organism. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the identification of colonies under investigation. After incubating for 18 hours, membrane filters may be examined but should be incubated for up to 48 hours before a final record is made. Confirmation will take a further 24 hours.

7.5.5 Reagents and media

7.5.5.1 Tryptose sulphite cycloserine (TSC) agar[46]

Tryptose	15.0 g
Yeast extract	5.0 g
Soya peptone	5.0 g
Ammonium iron(III) citrate	1.0 g
Sodium metabisulphite	1.0 g
D-cycloserine	400 mg
Agar	15 g
Water	1000 ml

Dissolve the ingredients (except D-cycloserine) in the water. To achieve this, it will be necessary to heat to boiling. Adjust the pH to 7.6 ± 0.2 and dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.6 ± 0.2. Add the appropriate amount of D-cycloserine from a suitable stock solution. This solution should be sterilized by membrane filtration through a 0.2 µm pore size filter. Alternatively, the partial medium may be cooled to approximately 50°C and the appropriate amount of D-cycloserine solution added. The complete medium is poured into sterile Petri dishes, allowed to solidify, stored at between 2 - 8°C and used within seven days. Any medium that is removed from the refrigerator and remains unused should be discarded. It has been shown that warming the medium to room temperature with subsequent cooling for storage reduces the effectiveness of the medium.

7.5.5.2 Perfringens (OPSP) agar[47]

Tryptone	15.0 g
Yeast extract	5.0 g
Soya peptone	5.0 g
Ammonium iron(III) citrate	1.0 g
Sodium metabisulphite	1.0 g
Sodium sulphadiazine	100 mg
Oleandomycin phosphate	500 µg
Polymixin B sulphate	10000 iu
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients (except the antibiotics, sodium sulphadiazine, oleandomycin phosphate and polymixin B sulphate) in the water. To achieve this, it will be necessary to heat to boiling. Adjust the pH to 7.6 ± 0.2 and dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.6 ± 0.2. Add the appropriate amounts of sodium sulphadiazine, oleandomycin phosphate and polymixin B sulphate from suitable stock solutions. These solutions should be sterilized by membrane filtration through 0.2 µm pore size filters. Alternatively, the partial medium may be cooled to approximately 50°C and the appropriate amounts of sterile antibiotic solutions added. The complete medium should be thoroughly mixed and poured into sterile Petri dishes, allowed to solidify, stored at between 2 - 8°C and used within seven days. Any medium that is removed from the refrigerator and remains unused should be discarded. It has been shown that warming the medium to room temperature with subsequent cooling for storage reduces the effectiveness of the medium.

7.5.5.3 **Crossley's milk medium**

Skimmed-milk powder	100.0 g
Peptone	10.0 g
Bromocresol purple	100 mg
Water	1000 ml

Mix the skimmed-milk powder with the water, adding the water gradually. Add the peptone and indicator solution and mix well. Distribute in 10 ml quantities into suitable screw-capped containers and autoclave at 121 ± 1°C for 5 minutes. Remove containers from the autoclave whilst still hot, and tighten the caps to prevent excess air entering the bottles. The pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. The complete medium should be stored at between 2 - 8°C and used within one month.

7.5.6 **Analytical procedure**

For liquid samples, filter a volume of sample, or appropriate dilution of the sample, through a membrane filter and place the filter onto an incubation pad containing sterile quarter-strength Ringer's solution. Raise the temperature of the solution to 80°C and then incubate at 80 ± 1°C for 10 minutes. Allow the membrane filter to cool to room temperature. Transfer the membrane filter to the TSC or OPSP agar. Invert the Petri dish and incubate in an anaerobic jar at 37 ± 1°C for 48 hours. Examine the membrane filter for typical black colonies (2 - 4 mm in diameter). For selective enumeration of *Cl. perfringens*, the Petri dishes can be incubated at 44 ± 0.5°C. Alternatively, heat the volume of sample, or appropriate dilution of the sample, to 80°C and then incubate at 80 ± 1°C for 10 minutes. Cool to room temperature and filter. Transfer the membrane filter to the TSC or OPSP agar. Invert the Petri dish and incubate in an anaerobic jar at 37 ± 1°C for 48 hours. Commercial systems are available for the generation of the correct anaerobic conditions within jars. Indicator strips are also available and should be added to each jar to demonstrate complete anaerobiosis.

For semi-solid or solid samples, aseptically, weigh out 10 g of sample into a sterile "stomacher" bag. Add 90 ml of sterile Ringer's solution and homogenise for 30 seconds. Place the homogenised suspension into a sterile bottle and incubate at 80 ± 1°C for 30 minutes. Allow the bottle to cool to room temperature and pipette suitable aliquots, or aliquots of suitable dilutions, into sterile Petri dishes. Add 15 - 25 ml of sterile molten agar, which has been cooled to approximately 50°C, and carefully mix thoroughly. Allow the agar to solidify, and incubate under anaerobic conditions at 37 ± 1°C for 48 hours.

7.5.7 **Confirmation**

Where membrane filters or Petri dishes contain typical black colonies, subculture the colonies into Crossley's milk medium; see section 5.3.2.3. At the same time, subculture a suitable control culture of *Cl. perfringens*. Incubate the milk medium at 37 ± 1°C for 24 hours and examine for the production of acid, as demonstrated by the change in colour of the medium, and coagulation of the casein to produce a typical clot. A number of other species of clostridium, for example *Cl. chavoei*, may also produce the "stormy clot" reaction. Confirmation may, therefore, need to be based upon motility, nitrate reduction, lactose fermentation and/or gelatin liquefaction[48].

7.5.8 **Reporting results**

For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported. See sections 5.4 and 7.5.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter or Petri dish to be obtained that contains sufficient organisms. The final report may also contain details of presumptive as well as confirmed clostridia.

7.6 Determination of viable colony count using the pour plate method

7.6.1 **Introduction**

The determination of the viable colony count is usually of little interest for environmental waters. Such waters, generally, possess high bacterial numbers due to the organisms normally present. The determination is, however, of importance in recreational waters where treatment and disinfection are routinely practised and where such procedures are monitored. Such pools operate at elevated water temperatures and, often, under heavy bathing loads, which encourage the growth of undesirable organisms. The organisms that grow in water at 37°C, generally, only survive with difficulty and are more likely to reflect poor hygiene conditions. Micro-organisms which survive in water at 22°C usually reflect the normal microbial population. Hence, colony counts form an important part in the assessment of the hygienic quality of such waters.

7.6.2 **Scope and field of application**

The pour plate method is suitable for the determination of the viable colony count of waters such as swimming pool waters and spa pool waters. The method detects all bacteria, yeasts and moulds capable of growing in or on the medium specified under the test conditions. Many bacteria, once in water, become stressed, or injured, and find it difficult to recover on high-nutrient media. Other bacteria become adapted to a low-nutrient environment and find conventional culture media too nutrient-rich. Hence, a low-nutrient medium[49] for the enumeration of colony counts may also be used. The use of this medium involves longer incubation periods, and, generally, results in more bacteria being recovered than with the conventional colony count medium. This medium may be particularly useful for the isolation of specific organisms, for example *Flavobacterium*. In addition, a method for determining the total viable and non-viable colony count can be carried out using acridine orange[41].

7.6.3 **Principle**

The normal sample volume is 1 ml, but appropriate dilutions of the sample may need to be used in order to obtain a sufficient number of colonies, normally between 30 - 300. The sample is mixed with yeast extract agar and incubated at 22°C for 72 hours and at 37°C for 24 hours.

7.6.4 **Performance characteristics**

When low numbers of bacteria are present, their detection is dependent only on the volume of sample that can be incorporated into the pour plate procedure and still enable the agar to solidify. When high numbers of bacteria are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one colony in the largest volume of sample that can be processed. High numbers of competing organisms may inhibit the growth, or detection, of the bacteria.

7.6.5 **Reagents and media**

7.6.5.1 **Yeast extract agar**

Yeast extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. Allow the medium to solidify, store at room temperature in the dark and use within one month. For use, heat the medium to

approximately 100°C, cool to approximately 50°C and maintain at this temperature until ready for use.

7.6.5.2 **R2A agar**[49]

Yeast extract	500 mg
Protease peptone number 3 or polypeptone	500 mg
Casamino acids	500 mg
Glucose	500 mg
Soluble starch	500 mg
Dipotassium hydrogen phosphate	300 mg
Magnesium sulphate heptahydrate	50 mg
Sodium pyruvate	300 mg
Agar	12.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Allow the medium to solidify, store at room temperature in the dark and use within one month. For use, heat the medium to approximately 100°C, cool to approximately 50°C and maintain at this temperature until ready for use.

7.6.6 **Analytical procedure**

For each incubation temperature, pipette 1 ml of the sample, or suitable dilution of the sample, into a 90 mm Petri dish. Add approximately 20 ml of molten medium and mix immediately using rapid, but gentle, backwards, forwards and rotational movements, taking care that the medium does not spill over the side of the dish or adhere to the lid. The time between pipetting the sample and the addition of the medium should not exceed 20 minutes. Allow the medium to solidify, invert the dish and incubate at the appropriate temperature. For the yeast extract agar procedure, incubate one Petri dish at 37 ± 1°C for 24 ± 3 hours and another at 22 ± 1°C for 72 ± 3 hours. For the R2A agar procedure, incubate one Petri dish at 20 ± 1°C for seven days. After incubation, remove the dish from the incubator and record the number of colonies. The volume of sample, or dilution of sample, should have been prepared and used which enables a colony count of between 30 - 300 to be recorded. If the number of colonies on the agar exceeds 300, count the dish prepared with the appropriately diluted sample (which should contain between 30 - 300 colonies). In cases where all prepared dishes result in counts in excess of 300 colonies, then consideration should be given to repeating the determination using a freshly submitted sample, or recording the number of colonies in a randomly chosen segment of the dish. See section 5.3.2.2. If the colonies are unevenly distributed, a number of opposing segments of the dish can be used to provide a better estimate of the count. Spreading colonies may interfere with counts or make counting difficult. Where a chain of colonies appears to be caused by the disintegration of a clump of organisms, or the growth appears as a film around the edge or surface or at the base of the medium, then these colonies should be counted as 1 colony.

As an alternative technique, surface spreading can be useful where it is intended to isolate and identify the organisms grown under the test conditions. The molten medium may be poured into the dish and allowed to solidify. A volume of sample, or dilution of the sample, normally 0.1 ml, is then pipetted onto the surface of the agar. The sample is evenly distributed onto the surface of the agar until all the liquid has been absorbed into the agar. Invert the dish and incubate at the appropriate temperature.

7.6.7 **Reporting results**

For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported. See sections 5.4 and 7.6.4. Every effort should be made to prepare sufficient dilutions to enable a Petri dish to be obtained that contains sufficient colonies. It may not be satisfactory to provide an estimate of the result that is reported as, for example, greater than 300, since the correct value might be much higher.

7.7 Detection and enumeration of *Salmonella* species

7.7.1 **Introduction**

Many different serotypes of *Salmonellae* are present to varying extents in humans, animals and birds. All members of the genus are potentially pathogenic. The low numbers of *Salmonellae* found in waters mainly originate from sewage and sewage effluents. The numbers of salmonellas present in water are, generally, much lower than those of other micro-organisms.

7.7.2 **Scope and field of application**

The MPN technique is suitable for the isolation of *Salmonella* species (other than *Salmonella typhi*) from surface water, sewage and related materials. The sample volume, or diluted sample, to be tested depends upon the degree of faecal contamination but is usually between 100 - 1000 ml.

7.7.2.1 **Definition and description of the organisms**

Salmonellas normally conform to the general definition of the family Enterobacteriacae[50] and can be further differentiated, biochemically, into 4 subgroups, subgenus I to IV. Those of subgenus I, the largest group, are considered pathogenic towards humans and are ß-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

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

7.7.2.2 **Pathogenicity**

Salmonella infections give rise to symptoms of diarrhoea and vomiting. The incubation period ranges from 12 - 72 hours and symptoms persist for 2 - 3 days. Most cases occur from the consumption of raw, or undercooked, food, in particular poultry, and food containing eggs.

7.7.3 **Principle**

Isolation is based on concentration from water by membrane filtration, or the use of a filter aid, followed by pre-enrichment (to recover environmentally stressed organisms) involving incubation in a non-selective medium, for example, buffered peptone water at 37°C for 24 hours. Selective enrichment is then carried out using Rappaport Vassiliadis enrichment broth at 41.5°C, subculturing to selective agar for 24 and 48 hours.

7.7.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volumes of sample that are used. To increase the detection, or volume of sample, MF may be used to concentrate volumes of sample before inoculation into tubes containing media. When membrane filtration is impracticable, a suitable filter-aid, for example diatomaceous earth, may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. High numbers of competing organisms, in particular *Ps. aeruginosa*, *Proteus* species and coliform organisms may inhibit the growth, or detection, of the organisms, and *Citrobacter* species may produce colonies that closely resemble salmonellas. Initial isolation takes 3 days but tests may be incubated for up to 4 days. Subculture and confirmation take a further 48 hours.

7.7.5 **Reagents and media**

7.7.5.1 **Buffered peptone water**[51]

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1000 ml

Dissolve the ingredients in the water. Dispense the resulting solution in appropriate volumes into suitable screw-capped bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature and used within one month.

7.7.5.2 **Rappaport Vassiliadis enrichment broth**[52, 53]

Solution A	
Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	126 mg
Dipotassium hydrogen phosphate	180 mg
Water	800 ml
Solution B	
Magnesium chloride anhydrous	13.6 g
Water	100 ml
Solution C	
Malachite green	40 mg
Water	100 ml

Dissolve the ingredients of solution A in water. To achieve this, it may be necessary to heat to boiling. Prepare this solution on the day of use. To 1000 ml of solution A, add 100 ml of solution B and 10 ml of solution C and mix well. Dispense the resulting solution in 10 ml volumes into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 5.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature and used within one month.

7.7.5.3 **Brilliant green agar**[54]

Yeast extract	3.0 g
Proteose peptone or polypeptone	10.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red (0.2% m/v aqueous solution)	40 ml
Brilliant green (0.5% m/v aqueous solution)	2.5 ml
Agar	20.0 g
Water	960 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.9 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8°C and use within one month. Alternatively, the bottled medium may be stored in the dark at room temperature and used within one month.

The medium may be more selective by the addition of sulphonamide, for example, sulphapyridine at 1000 mgl^{-1}, or sulphadiazine at 800 mgl^{-1}, or sulphamandelate supplement[55] (comprising an aqueous filter-sterilized solution of sodium sulphacetamide at 1000 mgl^{-1} and sodium mandelate at 250 mgl^{-1}). Sodium desoxycholate at 2500 mgl^{-1} has also been used to prevent swarming of *Proteus* species.

7.7.5.4 **Xylose lysine desoxycholate agar**[56]

Basal medium	
Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5.0 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red (0.4% m/v aqueous solution)	20 ml
Agar	12.0 g
Water	1000 ml
Solution A	
Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Water	100 ml
Solution B	
Sodium desoxycholate	10.0 g
Water	100 ml

Dissolve the ingredients of the basal medium in the water. This will require gentle heating. Dispense the resulting solution in appropriate volumes into suitable screw-capped bottles and sterilize by autoclaving at 115 ± 1°C for 10 minutes. The basal medium may be stored in the dark at room temperature and used within one month. Dissolve the ingredients of solution A and solution B in the water and separately pasteurise the individual solutions by heating at approximately 60°C for 1 hour. To prepare the complete medium, melt the basal medium and cool to approximately 50°C. Aseptically, add 2.0 ml of solution A and 2.5 ml of solution B to 100 ml of basal medium and mix well. The pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Pour the medium into sterile Petri dishes and allow it to solidify. Solutions A and B and the prepared dishes may be stored in the dark at between 2 - 8°C and used within one month.

7.7.5.5 **Lysine iron agar**[57]

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
L (-) Lysine	10.0 g
Ammonium iron(III) citrate	500 mg
Sodium thiosulphate pentahydrate	40 mg
Bromocresol purple (1% m/v ethanolic solution)	2 ml
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solutions in small volumes, ie 5 - 10 ml, into suitable containers. Sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.7 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8°C and used within one month.

7.7.5.6 **Triple sugar iron agar**[58]

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4% m/v aqueous solution)	6 ml
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients (except phenol red) in the water. To achieve this, it will be necessary to heat to boiling. Add the indicator solution and mix well. Dispense the resulting solution in small volumes, ie 5 - 10 ml, into suitable containers and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a

pH of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8°C, and used within one month.

7.7.5.7 **Urea broth**

Broth base	
Peptone	1.0 g
Glucose	1.0 g
Disodium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5.0 g
Phenol red (0.4% m/v aqueous solution)	1.0 ml
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution in 95 ml volumes into suitable screw-capped bottles and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. (Prepared medium may be stored in the dark at room temperature and used within one month.) Add 5 ml of an aqueous 40% m/v filter-sterilized solution of urea to 95 ml of broth base and aseptically dispense in 2 - 3 ml volumes in sterile containers.

7.7.5.8 **Filter-aid**[59]

Diatomaceous earth	1 g (approximately)
Water	20 ml

Weigh out appropriate amounts of filter-aid into suitable bottles and add the water. Sterilize by autoclaving at 121 ± 1°C for 15 minutes. Store in the dark at room temperature and use within 12 months.

7.7.5.9 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes. Allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. The above medium may be dispensed into sterile containers and allowed to solidify in a sloping position. For serological confirmation, slopes should be stored at between 2 - 8°C and used within one month. The same medium, without the addition of agar, may be used to moisten the slopes for flagellar agglutination. Alternatively, the bottled medium may be stored at room temperature in the dark and used within one month.

7.7.6 **Analytical procedure**

7.7.6.1 **Membrane filtration**

For qualitative (presence or absence) determinations, filter an appropriate volume of sample, or diluted sample, for example 100 - 1000 ml. If the sample is turbid, several membranes may be required. After filtration, place the membrane filters into buffered peptone water (BPW) and incubate at 37 ± 1°C for 24 hours. For quantitative determinations using an MPN technique, 1x500 ml, 5x100 ml and 5x10 ml volumes of sample can be filtered and the filters added to approximately 20 ml of BPW in suitable containers. If required, the 11-tube series can be extended by the addition of 1 ml volumes of sample to 9 ml volumes of BPW. Turbid waters and solid samples can be similarly treated.

7.7.6.2 **Filter-aid**

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. One bottle of filter-aid is filtered to form an initial layer on the absorbent pad. The contents of a second bottle are mixed with the sample, which is then filtered. For very dirty waters, additional bottles of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to BPW. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml. Mix well. For qualitative determinations, the contents of the vessel are incubated at 37 ± 1°C for 24 hours. For quantitative determinations, 5x10 ml volumes of sample are pipetted into sterile containers. The remaining 50 ml volume is kept as a separate culture. If high counts are suspected, from the 50 ml volume, pipette 5x1 ml and 5x0.1 ml volumes into separate containers each containing 10 ml of BPW. Incubate BPW at 37 ± 1°C for 24 hours.

Subculture 0.1 ml of the incubated BPW solutions into 10 ml of Rappaport Vassiliadis enrichment broth (RVB) and incubate at 41.5 ± 1°C. After incubating for 24 and 48 hours, inoculate the RVB onto xylose lysine desoxycholate agar (XLDA) and brilliant green agar (BGA). Incubate the selective agars at 37 ± 1°C for 24 hours.

Salmonellas produce smooth red colonies 2 - 3 mm in diameter on XLDA, typically with a black centre. Xylose-fermenting coliform organisms produce yellow colonies. *Pseudomonas* species produce red or yellow colonies with grey-black centres. Shigellas produce small pink-red colonies. *Proteus* species produce red colonies that are irregular and may have small black centres. *Salmonella* produce smooth red colonies, approximately 2 mm in diameter on BGA. Lactose- and sucrose-fermenting coliform organisms produce yellow/green colonies. *Proteus* species produce small red colonies, and *Pseudomonas* species produce small crenated colonies. Where isolates are overgrown, subculture to fresh XLDA and BGA. This facilitates the production of pure cultures and enables typical colonial morphology to be observed.

7.7.7 **Confirmation**

7.7.7.1 **Biochemical confirmation**

Subculture characteristic colonies from each Petri dish, using a straight wire, to lysine iron agar (LIA), triple sugar iron agar (TSI), urea broth (UB) and nutrient agar (NA) as a check for purity. The wire should be stabbed into the butt and streaked along the slant as it is withdrawn. Avoid stabbing through the butt to the bottom of the tube; the end of the wire should remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be blown out of the tube. Incubate at 37 ± 1°C for 18 - 24 hours. Regard cultures that give characteristic reactions in these confirmatory media as presumptive salmonellas (see Tables 7.2 and 7.3). Alternatively, one of the commercially available identification systems may be used.

Table 7.2 Reaction 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 is acid (yellow). Alk is alkaline (purple). NC is no change.
+ is blackening. - is no blackening.

Table 7.3 Reaction in triple sugar iron agar and urea broth

Genus	Slope	Butt	H_2S	Urea broth
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 is acid (yellow). AG is acid (yellow) and gas formation.
NC is no change. Alk is alkaline (red).
For H_2S, + is blackening and - is no change.
For urea broth, + is alkaline (red) and - is no change.

7.7.7.2 **Serological confirmation**
Subculture characteristic colonies onto moist NA slopes. For optimum flagellar formation, it is essential that fluid is present in the tube and sterile broth should be added if required. Incubate overnight at 37 ± 1°C. Carry out a slide agglutination test. For example, using a wire loop or pipette, place 3 separate drops (0.02 ml) of saline solution onto a clean microscope slide. Emulsify growth from the moist butt of the slope in each separate drop to produce homogeneous suspensions. Mix a loopful of *Salmonella* polyvalent 'O' (PSO) antiserum with the first drop of suspension and a loopful of *Salmonella* polyvalent 'H' (PSH) serum with the second drop. Gently rock the slide back and forth and examine for agglutination against a black background. The third drop containing no antiserum indicates whether or not the culture auto-agglutinates. Auto-agglutination strains should be re-plated on XLDA or BGA and dry smooth colonies dealt with as previously described.

Organisms which agglutinate with PSO and PSH antisera or strains which agglutinate with PSH serum only can be regarded as presumptive members of the *Salmonella* group. It is advisable to check colonial characteristic PSO-negative, PSH-positive and PSH-negative strains for agglutination with *Salmonella* Vi antiserum in order to exclude *S. typhi*, as the Vi antigen may mask the 'O' and 'H' antigens.

7.7.8 **Reporting results**
For qualitative determinations, report the presence or absence of salmonellas in the volume of sample examined. For quantitative determinations, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4, 7.7.4 and 7.7.6. Every effort should be made to prepare sufficient dilutions to enable a series of tubes, or containers, to be obtained to show that some tubes exhibit no growth while others exhibit some growth. The report may also contain details of presumptive organisms, as well as confirmed salmonellas.

7.8 Detection and enumeration of thermophilic campylobacter species

7.8.1 **Introduction**
Thermophilic campylobacters are not thought to be free-living but are obligate parasites of humans, animals and birds. They are found in the gastrointestinal system, and, being excreted in faeces, can be isolated from sewage and surface waters.

7.8.2 **Scope and field of application**
The MPN technique using enrichment and selective agars is suitable for the isolation of thermophilic campylobacters from surface waters, sewage and sewage effluents. The sample volume, or diluted sample, depends upon the degree of faecal contamination but is usually between 100 - 1000 ml.

7.8.2.1 **Definition and description of the organisms**
Bacteria in the genus campylobacter are slender, spirally curved, Gram-negative rods, 0.2 - 0.8 µm diameter and 0.5 - 8 µm in length. Rods may have one or more spirals and may be S-shaped or appear typically as "gull's wings". In older cultures, coccoid forms may be present and these may be degenerative non-viable forms, or viable forms which are not readily cultured. They do not form spores and are motile by means of a single polar flagellum at one or both ends of the cell, giving a characteristic darting or corkscrew motility. They are micro-aerophilic, requiring oxygen concentration between 3 - 15%, but some will grow anaerobically. The thermo-tolerant group comprising *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari (Campylobacter laridis)* grow well at 43°C, but fail to grow at 25°C.

7.8.2.2 **Pathogenicity**
The thermophilic campylobacter group is recognised as a common cause of enteritis in humans. Campylobacter infections give rise to a flu-like illness with malaise, fever and myalgia followed by diarrhoea. The incubation period ranges from 1 - 7 days with an average of 3 days. Most cases occur from the consumption of contaminated raw, or improperly cooked, foods.

7.8.3 **Principle**
A variety of enrichment and plating media is available for the isolation of campylobacters. Membrane filtration should be used for the concentration of campylobacters from water, followed by enrichment and culture on selective agar. Filtration aids are, generally, not as efficient at removing all the organisms from the sample. A choice of nutrient media is available, including nutrient broth number 2, brain heart infusion broth, blood agar base number 2, columbia agar base and brucella medium base, or the Preston enrichment broth and blood-free agar base[60, 61].

7.8.4 **Performance characteristics**
When low numbers of organisms are present, their detection is dependent only on the volumes of sample that are filtered. To increase the detection, or volume of sample, MF may be used to concentrate volumes of sample before inoculation into tubes containing media. Several filters may be used to increase this limit. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. High numbers of competing organisms, in particular *Ps. aeruginosa* and *Proteus* species may inhibit the growth or detection of the organisms. Tubes, or containers, should be incubated for 48 hours but tests may involve incubation for up to 4 days. Subculture and confirmation take a further 48 hours.

7.8.5 **Reagents and media**
For enrichment, an aero-tolerant supplement may be added. Lysed horse-blood may be obtained commercially, but defibrinated horse-blood can also be used. Defibrinated horse-blood may be lysed by dispensing suitable volumes of horse-blood into sterile containers which are then frozen. Blood, lysed in this way, can be kept frozen until required.

The aero-tolerance supplement should be added to enrichment broth and bottles that have been filled after inoculation and the caps screwed down tightly. Alternatively, the caps on inoculated bottles can be left loose and the bottles incubated in micro-aerobic conditions. Selective agar Petri dishes should be incubated in an anaerobic jar containing the appropriate atmosphere. This may be achieved by commercially available gas generating kits. It is important that the kit used is appropriate to the volume of the jar. In addition, since hydrogen is produced, jars should not be opened near a source of ignition.

7.8.5.1 **Enrichment broth number 1[41]**

Nutrient broth number 2	25.0 g
Iron(II) sulphate heptahydrate	250 mg
Sodium metabisulphate	250 mg
Sodium pyruvate	250 mg
Water	950 ml

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Autoclaved base may be stored in the dark at room temperature and used within one month.

To improve recovery of the organisms, activated charcoal and lysed horse-blood are added to give final concentrations of 2% and 5% respectively, and the following filter-sterilized solutions added:

(i) Trimethoprim solution - dissolve 500 mg of trimethoprim lactate in 100 ml of water and add 2 ml of this solution to the medium to give a final concentration of 10 mgl^{-1}.

(ii) Polymixin solution - dissolve 500000 iu of polymixin B sulphate in 40 ml of water and add 0.4 ml of this solution to the medium to give a final concentration of 5000 iul^{-1}.

(iii) Rifampicin solution - dissolve 200 mg in 20 ml of methanol and add 1 ml of this solution to the medium to give a final concentration of 10 mgl^{-1}.

(iv) Actidione solution - dissolve 2000 mg in 10 ml of methanol and add 1 ml of this solution to the medium to give a final concentration of 100 mgl^{-1}.

7.8.5.2 **Enrichment broth number 2**[62]

Brain heart infusion	37.0 g
Iron(II) sulphate heptahydrate	250 mg
Sodium metabisulphate	250 mg
Sodium pyruvate	250 mg
Water	950 ml

Dissolve the ingredients in the water and adjust the pH to 7.5 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. Autoclaved medium may be stored at room temperature and used within one month.

Activated charcoal and lysed horse-blood are added to give final concentrations of 2% and 5% respectively, and the following filter-sterilized solution added:

(i) Antibiotic supplement solution (Butzler supplement) consisting of bacitracin (33,000 iul^{-1}) cycloheximide (66 mgl^{-1}) colistin sulphate (20 mgl^{-1}) and novobiocin (6.6 mgl^{-1}). The supplement should be added at a concentration of 0.8 ml of supplement per 100 ml of medium. This concentration provides better inhibition of competing organisms, in particular *Pseudomonas*.

7.8.5.3 **Selective agar number 1**[63, 64]

Nutrient broth	25.0 g
Bacteriological charcoal	4.0 g
Casein hydrolysate	3.0 g
Sodium desoxycholate	1.0 g
Iron(II) sulphate	250 mg
Sodium pyruvate	250 mg
Agar	12.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. Cool the medium to approximately 50°C and add 1 ml quantities of each of filter-sterilized aqueous solutions of cefoperazone (containing 32,000 mgl^{-1}) to give a final concentration of 32 mgl^{-1}, and amphotericin (containing 10000 mgl^{-1}) to give a final concentration of 10 mgl^{-1}. Alternatively, the antibiotic supplement described in section 7.8.5.1 can be used. Pour into sterile Petri dishes, allow the agar to solidify, store between 2 - 8°C and use within one month. Alternatively, the bottled medium (without antibiotic) can be stored in the dark at room temperature and used within one month.

7.8.5.4 **Selective agar number 2**[62]

Columbia agar base	39.0 g
Iron(II) sulphate heptahydrate	250 mg
Sodium metabisulphate	250 mg
Sodium pyruvate	250 mg
Water	950 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. Cool the medium to approximately 50°C, add lysed horse-blood to give a final concentration of 5% and 1 ml of a filter-sterilized aqueous solution of cefoperazone (containing 32,000 mgl^{-1}) to give a final concentration of 32 mgl^{-1}. Alternatively, the supplement described in section 7.8.5.1 can be used. Pour the resulting medium into sterile Petri dishes and allow the agar to solidify. Agar Petri dishes should be prepared fresh but may be stored between 2 - 8°C and used within 7 days. Alternatively, the base medium can be stored at room temperature and used within one month.

7.8.6 **Analytical procedure**

For quantitative counts using an MPN technique, organisms may be concentrated from large volumes of water by membrane filtration, through 0.2 µm pore size membrane filters. Smaller volumes (for example, 10 ml) may be inoculated directly into double-strength medium or 1 ml into single-strength medium. For enrichment medium number 1, screw-capped containers should then be filled with medium and the caps screwed down tightly. For enrichment medium number 2, containers are incubated with the appropriate gas mixture (5% oxygen, 10% carbon dioxide and 85% nitrogen) in an anaerobic jar. Enrichment broths are incubated at 42 ± 1°C for 48 hours, subculturing onto selective agar at 24 and 48 hours.

Agar Petri dishes are incubated in anaerobic jars containing the appropriate gas mixture. After incubating for 24 hours, the dishes should be examined and those exhibiting no growth should be incubated for a further 24 hours and re-examined.

Preliminary identification may be made on the basis of colonial morphology and Gram stain. Colonies may vary in size from pin-point to 2 - 4 mm in diameter. The size will depend to some extent on the number of competing organisms growing on the agar dish. Large numbers of *Proteus* or *Pseudomonas* will restrict the growth, and under such circumstances careful examination of each dish, for example with a hand lens or plate microscope, is important. Colonies are typically small, flat and transparent. They may be circular or ovoid and may extend along the line of inoculation. They resemble small flat droplets of water. Where competing organisms are minimal or absent, colonies are slightly convex, with an entire edge, and, again, may extend along the line of inoculation. They are grey or buff in colour and may also exhibit swarming. Occasionally two colony types occur in one culture. One is, typically, slightly convex as above, and the other is smaller and more dome-shaped

7.8.7 **Confirmation**

Carry out an oxidase test on suspect colonies, subculturing onto a blood-free medium and incubate at 42 ± 1°C for 48 hours. Gram-stain any oxidase-positive cultures using 1:20 carbol fuchsin or 0.5% m/v safranin solutions. Campylobacters are Gram-negative curved rods with typical "gull's wings", S-shapes or short spirals. For most practical purposes, colonial and cellular morphology, together with the oxidase test, are adequate for identification. If required, species and sub-species identification may be carried out by using biotyping schemes[61, 65, 66].

7.8.8 **Reporting results**

For each sample, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4 and 7.8.4. Every effort should be made to prepare sufficient dilutions to enable a series of tubes to be obtained to show that some tubes exhibit no growth while others exhibit some growth. The report may also contain details of presumptive organisms, as well as confirmed campylobacters.

7.9 Detection and enumeration of *Staphylococcus aureus*

7.9.1 Introduction

Staphylococci have been advocated as indicator organisms of water quality in recreational and environmental waters, and, where appropriate, provide a measure of effective water treatment and disinfection.

7.9.2 Scope and field of application

The MF method is suitable for the isolation of staphylococci, and, in particular, *Staphylococcus aureus (S. aureus)* from surface water, sewage and related samples. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes in order to produce a membrane filter containing a sufficient number of colonies.

7.9.2.1 Definition and description of the organisms

Bacteria in the genus staphylococcus are Gram-positive cocci, approximately 1 µm in diameter occurring singly, in pairs or in clumps. They are facultatively anaerobic, non-motile and do not form spores, and are catalase-positive and sensitive to lysostaphin. Staphylococci ferment mannitol at 37°C in the presence of 7.5% m/v sodium chloride and 0.005% m/v sodium azide. *S. aureus* also produces coagulase.

7.9.2.2 Pathogenicity

Staphylococci are mainly associated with the skin, respiratory tract and gastrointestinal tract of humans and warm-blooded animals. They are also part of the normal flora of the nose. They are readily shed into water when a body is immersed. *S. aureus* is a pathogenic organism causing wound and localised skin infections. It may also cause urinary tract infections. Some strains produce a heat-stable enterotoxin in food products.

7.9.3 Principle

Isolation of staphylococci, including *S. aureus*, is carried out by membrane filtration followed by growth on solid selective media. Baird-Parker agar may be used, which contains sodium pyruvate (to enhance damaged cell recovery) glycine, lithium and tellurite (as selective agents) and egg yolk (to demonstrate lipolytic and proteolytic activity). Confirmation of presumptive staphylococci is determined by sensitivity to lysostaphin, and for *S. aureus*, by coagulase reaction.

7.9.4 Performance characteristics

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, inoculum detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth or detection, of staphylococci. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the recognition of typical colonies. After incubating for 48 hours, membrane filters may be examined and confirmation takes a further 24 hours.

7.9.5 Reagents and media

7.9.5.1 Baird-Parker agar (BPA)[67]

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Sodium pyruvate	10.0 g
Glycine	12.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Adjust the pH to 6.8 ± 0.2 and dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. Cool the molten medium to approximately 50°C and add sterile egg yolk emulsion containing 0.2% m/v potassium tellurite to give a final concentration of 5% egg yolk and 0.1% potassium tellurite. Mix thoroughly, pour into sterile Petri dishes and allow the medium to solidify. Agar dishes may be stored at between 2 - 8°C and used within one month. Alternatively, the medium without the addition of egg yolk emulsion can be stored at room temperature in the dark and used within one month.

7.9.5.2 Nutrient agar

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark for up to one month.

7.9.6 Analytical procedure

A volume of sample, or an aliquot of a diluted sample, is filtered and the membrane filter placed onto the appropriate agar medium. Incubate at 37 ± 1°C for 48 hours. After incubation, examine the filters for typical colonies. On BPA, typical colonies are up to 3 mm diameter, convex, shiny and pale black in colour. Count all typical colonies and record as presumptive staphylococci.

7.9.7 Confirmation

The presence of *S. aureus* may be confirmed by testing for the ability of staphylococci to produce coagulase. Commercial coagulase test kits are available. Select individual colonies from each membrane filter, see section 5.3.2.3, and suspend in 0.5 ml of sterile water. Saturate the surface of a nutrient agar Petri dish with the suspension and dry at 37 ± 1°C for 10 to 20 minutes. Place one drop of aqueous lysostaphin solution (200 $mgml^{-1}$) on one side of the agar dish and one drop of aqueous lysozyme solution (400 $mgml^{-1}$) on the opposing side. Incubate plates at 37 ± 1°C for 18 hours. Staphylococci are confirmed by the total, or partial, lysis of the bacterial culture in proximity to the drop of lysostaphin. No lysis should occur in proximity to the drop of lysozyme.

7.9.8 Reporting results

For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported. See sections 5.4 and 7.9.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter to be obtained that contains a sufficient number of organisms. The final report may also contain details of presumptive as well as confirmed staphylococci and *S. aureus.*

7.10 Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration

7.10.1 Introduction

Pseudomonas aeruginosa is an environmental bacterium commonly found in soil and on plants. It is able to grow in waters containing very low levels of nutrients, and is frequently present, in small numbers, in the normal intestinal flora of humans and animals.

7.10.2 Scope and field of application

The MF method is suitable for the isolation of *Ps. aeruginosa* from surface waters and sewage and related samples[41, 68]. The volume of sample, or diluted sample, to be tested depends

upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes in order to produce a membrane filter containing a sufficient number of colonies.

7.10.2.1 **Definition and description of the organisms**
Ps. aeruginosa is a Gram-negative, non-sporing bacillus approximately 0.5 x 1.5 µm in length. It is oxidase-positive and catalase-positive, and is essentially aerobic, with a temperature range for growth of between 6 - 43°C and an optimum temperature of 37°C. Glucose is oxidised, nitrates are reduced to nitrogen, and casein is hydrolysed. The organisms produce characteristic colonies on Pseudomonas agar, recognised by the production of characteristic green colonies that fluoresce under ultra-violet light. Greenish-blue pigments (fluorescein and pyocyanin) are produced by most isolates.

7.10.2.2 **Pathogenicity**
Ps. aeruginosa is an opportunistic pathogen, particularly in humans who are immuno-compromised. The principal infections include septicaemia, skin and burn infections, and respiratory, urinary tract and ear infections. Large numbers growing in polluted waters, swimming pool waters or spa pool waters may produce ear infections or a follicular dermatitis following immersion. The organism is important because of its antibiotic resistance.

7.10.3 **Principle**
This method describes the isolation of *Ps. aeruginosa* by membrane filtration. The organisms can be isolated on Pseudomonas selective medium, the base of which is a modification of King's A medium containing magnesium chloride and potassium sulphate to enhance pigment production. The medium is made selective by the addition of cetrimide and nalidixic acid. The organisms produce characteristic blue-green or brown coloured colonies. Confirmation of isolates is by subculture to milk agar to demonstrate hydrolysis of casein.

7.10.4 **Performance characteristics**
When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth, or detection, of the organism. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the recognition of typical colonies. After incubating for 48 hours, membrane filters should be examined and confirmation takes a further 24 hours.

7.10.5 **Reagents and media**

7.10.5.1 **Pseudomonas agar base medium**

Gelatin peptone	16.0 g
Casein hydrolysate	10.0 g
Potassium sulphate	10.0 g
Magnesium chloride	1.4 g
Agar	11.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the base medium should be checked to confirm a pH of 7.1 ± 0.2. Cool the molten base medium to approximately 50°C and add cetyl trimethylammonium bromide (cetrimide) and nalidixic acid sodium salt as aqueous sterile solutions to give final concentrations of 200 mgl^{-1} and 15 mgl^{-1} respectively. Mix thoroughly, and dispense into sterile Petri dishes. Allow the complete medium to solidify, store at between 2 - 8°C and use within one month. Alternatively, the base medium can be stored in the dark at room temperature and used within one month.

7.10.5.2 **Yeast extract broth**

Bacteriological peptone	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Water	1000 ml

Dissolve the ingredients in the water. Adjust the pH to 7.3 ± 0.2 and sterilize by autoclaving at 121 ± 1°C for 15 minutes.

7.10.5.3 **Milk agar with cetrimide**

Skimmed-milk powder	100.0 g
Yeast extract broth (see section 7.10.5.2)	250.0 ml
Agar	15.0 g
Cetrimide	300 mg
Water to	750 ml

Mix the sterile yeast extract broth with the cetrimide and agar, and steam to dissolve. Thoroughly mix the skimmed-milk powder with water. Autoclave the individual solutions separately at 121 ± 1°C for 5 minutes. Cool to approximately 50 - 55°C, add the skimmed-milk powder solution to the agar solution, mix thoroughly and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8°C and use within one month.

7.10.6 **Analytical procedure**
Filter a volume of sample, or appropriate dilution of the sample, and place the filter onto the Pseudomonas agar. Incubate at 37 ± 1°C for 48 hours. Examine the membrane filter for colonies that are blue-green or greenish brown in colour. These colonies should be considered as presumptive *Ps. aeruginosa*. Membrane filters may also be examined for fluorescence under ultra-violet light at a wavelength of 360 ± 20 nm.

Other members of the Pseudomonas group, in particular the fluorescent Pseudomonads, may be isolated on Pseudomonas agar base supplemented with the following aqueous filter-sterilized solutions of cetrimide, fucidin and cephaloridine to give final concentrations of 10 mgl^{-1}, 10 mgl^{-1} and 50 mgl^{-1} respectively. Incubation should be carried out at 30 ± 1oC for 48 hours. This medium allows the growth of all non-pigmented and pigmented psychrophilic Pseudomonads as well as *Ps. aeruginosa*.

7.10.7 **Confirmation**
Colonies should be inoculated onto milk agar and incubated at 37 ± 1°C for 24 hours for confirmation. See section 5.3.2.3. Colonies are 2 - 4 mm in diameter and should show typical pigment production and possess a "halo of clearing" around the colony where the casein has been hydrolysed. Typical colonies are recorded as confirmed *Ps. aeruginosa*. Positive and negative control samples should be included with each batch of confirmatory tests.

Some strains of *Ps. aeruginosa* fail to produce pigment on the membrane filter. In such cases, the milk agar dishes subcultured for confirmation should be exposed to daylight at room temperature before they are examined. Where pigment is still not produced, a colony should be subcultured onto a fresh milk agar Petri dish and incubated at 37 ± 1°C for 24 hours in order to provide a pure culture, which can then be confirmed using a commercially available test kit.

7.10.8 **Reporting results**
For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported. See sections 5.4 and 7.10.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter or Petri dish to be obtained that contains a sufficient number of organisms. The final report may also contain details of presumptive as well as confirmed *Ps. aeruginosa*.

7.11 Detection and enumeration of *Aeromonas* species

7.11.1 **Introduction**
Bacteria of the genus *Aeromonas* are commonly found in fresh and estuarine waters and

sewage. At present, all the aeromonads may be considered equally significant, and for practical purposes it is not necessary to identify aeromonads beyond the level of genus.

7.11.2 Scope and field of application

A variety of media has been developed for the isolation of aeromonads, including ampicillin-dextrin agar[69, 70, 71, 72]. The MF method using selective agar is suitable for the isolation of *Aeromonas* species from surface waters, sewage, pool waters and other related materials. The sample volume to be tested depends on the degree of faecal contamination but should be between 1 - 100 ml. Samples of sewage may require dilution before testing. The medium used to grow *Aeromonas* also encourages the growth of certain *Vibrio* species. If samples are likely to contain strains of *Vibrio*, as might be expected for estuarine waters, then the medium can be made more selective by the addition of 2,4-diamino-6,7-diisopropyl pteridine phosphate (O/129 phosphate) to produce a final concentration of 50 mgl^{-1}.

7.11.2.1 Definition and description of the organisms

The taxonomy of the genus *Aeromonas* is subject to considerable debate. Most isolates can be divided into three groups, corresponding broadly to *A. hydrophila*, *A. caviae* and *A. veronii* biotype *sobria*. The genus *Aeromonas* comprises non-sporing, Gram-negative rods approximately 1.0 - 4.4 µm x 0.4 - 1.0 µm in length. Members are motile with predominant polar flagellation, and are facultative anaerobes, catalase-positive and oxidase-positive. They ferment glucose and other carbohydrates with the production of acid, or acid and gas. The optimum growth temperature is about 30°C, with a maximum temperature of 38 - 40°C. *A. salmonicida* grows poorly, if at all, at 37°C, and the minimum growth temperature is about 10°C.

Strains are regarded as *Aeromonas* if they are oxidase-positive, ferment dextrin and mannitol, are fermentative in the test of Hugh and Leifson[73], are able to grow in 1% tryptone water in the absence of sodium chloride but not in 1% tryptone water containing 6% sodium chloride, are resistant to 50 mgl^{-1} of O/129 phosphate, and hydrolyse arginine in the test of Thornley[74].

7.11.2.2 Pathogenicity

Some species, notably *A. salmonicida*, are pathogenic towards fish and reptiles. Other species have been incriminated as pathogenic towards humans. They may colonise, or infect, wounds contaminated with water, and can cause septicaemia in immuno-compromised individuals. They have been incriminated as a cause of diarrhoea. Wound infections related to contact with soil and water have also been reported. Aeromonads may also cause post-operative wound infections, urinary tract infections and rare cases of peritonitis, otitis and endocarditis.

There is some evidence to suggest that environmental strains are non-pathogenic and that pathogenic strains produce cytotoxins. This can be demonstrated using classical cytotoxicity tests, or ß-haemolysis of horse-blood on horse-blood agar, or other biochemical tests.

7.11.3 Principle

The isolation of *Aeromonas* species is undertaken by membrane filtration, followed by growth on solid selective media. Membrane filters are incubated on ampicillin-dextrin agar containing ampicillin, (as the selective agent) dextrin, (as the fermentable carbohydrate) and bromothymol blue, (as the indicator). Typical yellow colonies are subcultured for confirmation by the oxidase test and fermentative reaction in the Hugh and Leifson O/F test.

7.11.4 Performance characteristics

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth, or detection, of the organisms. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the recognition of typical colonies. After incubating for 20 - 24 hours, membrane filters may be examined, and confirmation takes a further 48 hours.

7.11.5 Reagents and media

Samples from estuarine or marine environments may contain *Vibrio* species, which, under these conditions, can produce colonies similar to *Aeromonas* species. For these samples, the isolation medium, before being autoclaved, should be supplemented with 0.05 g of 2,4-diamino-6,7-diisopropyl pteridine phosphate (O/129 phosphate) to each litre of medium.

7.11.5.1 Ampicillin-dextrin agar

Tryptose	5.0 g
Dextrin	10.0 g
Yeast extract	2.0 g
Sodium chloride	3.0 g
Potassium chloride	2.0 g
Magnesium sulphate heptahydrate	200 mg
Iron(III) chloride	100 mg
Bromothymol blue (1% m/v aqueous solution)	8 ml
Agar	15.0 g
Sodium desoxycholate	100 mg
Ampicillin	10 mg
Water	1000 ml

Dissolve all the ingredients except the agar, ampicillin and desoxycholate in the water. Adjust the pH to 8.0 ± 0.2. Add the agar and dissolve. This will require heating the solution to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 8.0 ± 0.2. Cool the molten medium to approximately 50°C and add 10 ml of a freshly prepared aqueous filter-sterilized solution of ampicillin (containing 1 $mgml^{-1}$) and 10 ml of an aqueous filter-sterilized solution of desoxycholate (containing 10 $mgml^{-1}$) per litre of medium. Mix thoroughly, pour into sterile Petri dishes and allow the medium to solidify. The agar plates should be stored at between 2 - 8°C and used within seven days. Alternatively, the medium without ampicillin and desoxycholate can be stored at room temperature and used within one month.

7.11.5.2 Nutrient agar

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10-15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to ensure that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark and used within one month. Most non-selective agars are suitable for producing pure cultures and oxidase testing providing they do not contain fermentable carbohydrates.

7.11.5.3 Oxidase reagent

Tetramethyl-p-phenylenediamine dihydrochloride	100 mg
Water	10 ml

Weigh out the tetramethyl-p-phenylenediamine dihydrochloride into a suitable container and dissolve in the water. This reagent does not keep and should be prepared immediately before use. “Test sticks” are available commercially and may be stored and used according to the manufacturer’s instructions.

7.11.5.4 **Hugh and Leifson's base medium**

Peptone	2.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	300 mg
Bromothymol blue (1% m/v aqueous solution)	3.0 ml
Agar	3.0 g
Water	900 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Adjust the pH to 7.1 ± 0.2 and dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the base medium should be checked to confirm a pH of 7.1 ± 0.2. Cool the molten base medium to approximately 50°C and add an appropriate volume of an aqueous filter-sterilized solution of dextrin (containing 10% m/v) to give a final concentration of 1%. Dispense the medium into sterile test tubes, or containers, to a depth of about 40 mm, store at between 2 - 8°C and use within one month. Alternatively, the base medium without the dextrin can be stored in the dark at room temperature and used within one month.

7.11.6 **Analytical procedure**

A volume of sample, or an aliquot of diluted sample, is filtered and the membrane filter placed onto ampicillin-dextrin agar. Incubate at 30 ± 1°C for 20 - 24 hours. After incubation, examine the filters for typical colonies that are 2 - 3 mm in diameter, smooth with an entire edge, and yellow or yellow with a greenish-yellow periphery. Colonies that are completely blue or white, and translucent in appearance should not be counted.

7.11.7 **Confirmation**

With a straight wire, transfer representative colonies to a nutrient agar Petri dish and then to the bottom of two Hugh and Leifson O/F tubes. See also section 5.3.2.3. With a looped-wire, distribute the colonies on the surface of the agar and incubate the dish at 30°C for 20 - 24 hours. Cover the medium in one of the O/F tubes with a small amount of sterile mineral oil and incubate both tubes at 30°C for 20 - 24 hours. Alternatively, incubate the tube at 30°C for 20 - 24 hours, without mineral oil, in an anaerobe jar.

Examine the agar dishes for growth and test each isolate for cytochrome oxidase production. Remove some of the colonies from the agar and transfer to pads soaked in oxidase reagent. If an organism is oxidase-positive, a blue colour develops within approximately 10 seconds. Where no colour change is observed, this should be regarded as a negative reaction. Control organisms should be tested at the same time. Negative reactions are given by *E. coli* and positive reactions are given by *A. hydrophila*. Examine the O/F tubes for growth and oxidative or fermentative reactions. An oxidative reaction should show acid production in the tube open to the atmosphere. There should be little or no acid production in the tube containing the mineral oil or the tube incubated anaerobically. A fermentative reaction will show acid production in both tubes. Presumptive *Aeromonas* species will ferment dextrin, give a fermentative reaction in the Hugh and Leifson test and be oxidase-positive. Record these numbers for each sample. Confirmation of isolates may also be carried out using commercially available biochemical tests[75, 76].

Isolates may be speciated using the following biochemical testing procedure:

	A. hydrophila	*A. sobria*	*A. caviae*
Gas from glucose	+	+	-
Aesculin hydrolysis	+	-	+

7.11.8 **Reporting results**

For each sample, record the number of colonies per volume tested. Convert this number to the volume to be reported. See sections 5.4 and 7.11.4. Every effort should be made to prepare sufficient dilutions to enable a membrane filter to be obtained that contains a sufficient number of organisms. The final report may also contain details of presumptive as well as confirmed *Aeromonas* species.

7.12 Detection and enumeration of *Vibrio cholerae* and other *Vibrio* species

7.12.1 **Introduction**

Vibrio species occur naturally in brackish and saline waters, but only a few species are capable of surviving in fresh water ecosystems. Most species, including the pathogenic species, are not, normally, able to grow except under highly eutrophic conditions. *Vibrio* species have been reported in tropical waters where the temperature remains reasonably constant at about 25°C.

7.12.2 **Scope and field of application**

The MF method is suitable for the isolation of *Vibrio* species from surface waters, sewage, pool waters, sediments and other related materials. The sample volume to be tested depends upon the nature of the sample and the degree of faecal contamination but is, usually, between 1 - 100 ml. Samples of sewage and brackish waters may require dilution before testing.

7.12.2.1 **Definition and description of the organisms**

Vibrio species are Gram-negative, curved or "comma-shaped" rods that are oxidase-positive. They are motile by means of a single sheathed polar flagellum. Growth is stimulated by sodium chloride at an optimum concentration of between 1 - 3%.

Isolates are identified as *Vibrio* species by these procedures if they grow at pH 8.6, are oxidase-positive, Gram-negative and grow within 24 hours at 37°C in the presence of bile salts, 1% sodium thiosulphate and 1% sodium citrate without the production of sulphide. Usually, they can also ferment glucose without the production of gas, and are sensitive to 2,4-diamino-6,7-diisopropyl pteridine phosphate (O/129 phosphate). *Vibrio metschnikovii (V. metschnikovii)* grows well on thiosulphate citrate bile salt sucrose agar[77] but is oxidase-negative and is not an enteropathogen.

7.12.2.2 **Pathogenicity**

The species *V. cholerae* can be divided into approximately 140 O-serovars. The organisms that usually produce outbreaks of epidemic cholera are toxin-producing strains of the O1 serovar, including a more recently reported serovar, O139. Non-O1 *V. cholerae* can also cause gastroenteritis. *V. parahaemolyticus* also causes diarrhoea often through the consumption of raw, contaminated seafood. *V. fluvialis* and *V. mimicus* also cause diarrhoea. Outbreaks of cholera have been reported following consumption of crops irrigated with sewage-contaminated water.

7.12.3 **Principle**

Isolation of *Vibrio* species is carried out by membrane filtration followed by growth on selective media such as alkaline peptone water and thiosulphate citrate bile salt sucrose agar (TCBSA). A modification to this method has been described[78]. Turbid samples and sediments can be inoculated directly into double-strength and single-strength enrichment media. Typical colonies may be subcultured for confirmation by oxidase and biochemical or serological testing.

7.12.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. Samples from estuarine or marine environments may contain large numbers of *Vibrio* species and may well require dilution prior to testing. High numbers of competing organisms may inhibit the growth, or detection, of the organisms. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the recognition of typical colonies. Under such circumstances, alkaline peptone water may be inoculated directly using the appropriate volumes of double-strength and single-strength media and a multiple tube method used, ensuring an adequate number of dilutions are prepared. Enrichment takes 16-24 hours and membrane filters can be examined after 20 - 24 hours. Confirmation takes a further 48 hours. It is not advisable to

place membrane filters directly onto TCBSA because of the risk of overgrowth of competing organisms and failure to recognise typical *Vibrio* species.

7.12.5 **Reagents and media**

7.12.5.1 **Alkaline peptone water (single-strength)**

Peptone	10.0 g
Sodium chloride	5.0 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 8.6 ± 0.2. Sterilize the resulting solution by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 8.6 ± 0.2. The medium should be stored at room temperature and used within one month.

7.12.5.2 **Thiosulphate citrate bile salt sucrose agar**

Yeast extract	5.0 g
Peptone	10.0 g
Sodium thiosulphate pentahydrate	10.0 g
Sodium citrate	10.0 g
Ox bile	8.0 g
Sucrose	20.0 g
Sodium chloride	10.0 g
Iron(III) citrate	1.0 g
Bromothymol blue (1% m/v aqueous solution)	4 ml
Thymol blue (1% m/v aqueous solution)	4 ml
Agar	14.0 g
Water	1000 ml

Dissolve the ingredients in the water. This will require heating the solution to boiling. Do not autoclave. After boiling, cool the medium to approximately 50°C and check the pH of the medium to confirm a pH of 8.6 ± 0.2. Pour the medium into sterile Petri dishes and allow it to solidify, store at between 2 - 8°C and use within one month.

7.12.5.3 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium can be stored at room temperature in the dark and used within one month. Most non-selective agars are suitable for producing pure cultures and oxidase testing providing they do not contain fermentable carbohydrates.

7.12.5.4 **Oxidase reagent**

Tetramethyl-p-phenylenediamine dihydrochloride	100 mg
Water	10 ml

Weigh out the tetramethyl-p-phenylenediamine dihydrochloride into a suitable container and dissolve in the water. This reagent does not keep and should be prepared immediately before use. "Test sticks" are available commercially and may be stored and used according to the manufacturer's instructions.

7.12.6 **Analytical procedure**

For quantitative determinations using the MPN technique, organisms may be concentrated from large volumes of sample by membrane filtration through 0.2 µm membrane filters. This technique can be used for 500 ml and 100 ml sample volumes. The membrane filters used to filter these aliquots are inoculated directly into single-strength alkaline peptone water. Smaller volumes, for example 10 ml, can be inoculated directly into 10 ml of double-strength medium, and 1 ml volumes into 10 ml of single-strength medium. Where turbidity makes membrane filtration impracticable, larger volumes should be inoculated directly into double-strength medium. Incubate the enrichment alkaline peptone waters at 25 ± 1°C for 2 hours followed by 37 ± 1°C for 12 - 16 hours.

Subculture the enrichment alkaline peptone waters (from the top of the broth) to Petri dishes containing TCBSA and incubate at 37 ± 1°C for 16 - 24 hours. See section 5.3.2.3. Examine the dishes for colonies of Vibrio species, which are usually 1 - 3 mm in diameter, and either yellow (sucrose-fermenting) or blue-green (non-sucrose-fermenting). *V. cholerae* and *V. fluvialis* are seen as yellow colonies and *V. mimicus* and *V. parahaemolyticus* as blue-green colonies. Subculture each colony type to Petri dishes containing nutrient agar and incubate at 37 ± 1°C for 24 hours. Check the cultures for purity and carry out an oxidase test. Remove some of the colonies from the agar and transfer them to pads soaked in oxidase reagent. If an organism is oxidase-positive, a blue colour develops within approximately 10 - 20 seconds. Where no colour change is observed, this should be regarded as a negative reaction. Control organisms should be tested at the same time. Negative reactions are produced by *E. coli*, and positive reactions are given by Vibrio species.

7.12.7 **Confirmation**

Strains that are oxidase-positive require further identification. If epidemic strains of *V. cholerae* O1 are being determined, then colonies that are both sucrose-fermenting (yellow colonies on TCBSA) and oxidase-positive can be checked for agglutination with *V. cholerae* O1 antiserum. An isolate that agglutinates can be regarded as presumptive *V. cholerae* O1. Commercially available kits can be used to identify oxidase-positive, sucrose-fermenting and non-sucrose-fermenting isolates.

7.12.8 **Reporting results**

For each sample, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4 and 7.12.4. Every effort should be made to prepare sufficient dilutions to enable a series of tubes to be obtained to show that some tubes exhibit no growth while others exhibit some growth. The final report may also contain details of presumptive as well as confirmed Vibrio species.

7.13 Detection and enumeration of *Escherichia coli* O157:H7

7.13.1 **Introduction**

The recovery of *Escherichia coli* O157:H7 from environmental samples is often difficult because of the altered physiological state that bacteria sometimes develop in order to survive hostile environments. Infections involving *E. coli* O157:H7 have occasionally been implicated with contaminated water, but food-borne infections are more common.

7.13.2 **Scope and field of application**

The MF method is suitable for the isolation of *E. coli* O157:H7 from all types of water including river waters, estuarine and sea waters, sewage and sewage effluents, and spa pool waters. The volume of sample, or diluted sample, to be tested depends upon the degree of faecal contamination. It may, therefore, be advisable to test a range of volumes in order to produce a membrane filter containing a sufficient number of colonies.

7.13.2.1 **Definition and description of the organism**

E. coli O157:H7 is a Gram-negative, oxidase-negative, non-sporing rod bacillus. Most strains of *E. coli* are thermo-tolerant and grow at 44°C, but *E. coli* O157:H7 grows only poorly at this temperature and does not ferment sorbitol at 37°C. Sorbitol MacConkey agar can, therefore, be used to differentiate *E. coli* O157:H7 from other *E. coli* strains. The organism can be further identified by lack of the enzyme ß-glucuronidase and by agglutination with appropriate antisera or latex/antibody reagents.

7.13.2.2 **Pathogenicity**

E. coli O157:H7 is a recognised cause of haemorrhagic colitis, an illness characterised by bloody diarrhoea and severe abdominal pain but little or no fever. It is also one of the causes of haemolytic uraemic syndrome. Outbreaks have been associated with the consumption of food and contaminated water, and person-to-person contact also occurs. Symptoms can persist for up to 7 days. Strains of *E. coli* O157:H7 produce a toxin similar to that produced by *S. dysenteric* Type 1, which is cytotoxic to Vero cells in cell culture.

E. coli O157:H7 strains which produce verocytotoxin have been re-classified from "Hazard Group 2" to "Hazard Group 3"[79]. However, where samples are expected to contain no *E. coli* O157:H7, the routine examination of water for this organism may be undertaken in "Hazard Group 2" containment facilities. Where substantial subculture work is required, this should be undertaken in "Hazard Group 3" containment facilities. In addition, positive control strains should not produce verocytotoxin. Suitable strains are available commercially. Great care should be exercised in the disposal of contaminated materials, especially those containing *E. coli* O157:H7.

7.13.3 **Principle**

Isolation is based on membrane filtration and enrichment followed by the use of immunomagnetic separation (IMS) and inoculation onto modified sorbitol MacConkey agar. Typical non-sorbitol-fermenting colonies are selected for identification by biochemical and serological tests. Two enrichment broths have been reported[80, 81], namely modified buffered peptone water (MBPW) and modified tryptone soya broth (MTSB).

7.13.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volume of sample that is filtered. To increase the detection, or volume of sample, several membrane filters may be used. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. The limit of detection will, therefore, be one organism in the largest volume of sample that can be filtered. High numbers of competing organisms may inhibit the growth, or detection, of the organisms. The detection of organisms in waters possessing high turbidities depends on the volume of sample that can be filtered. In addition, with these waters, residues may be left on the surface of the membrane filters. These residues may interfere with the recognition of typical colonies. After incubating for 18 hours, membrane filters may be examined, and confirmation takes a further 24 hours.

7.13.5 **Reagents and media**

Immunomagnetic beads[82] are available commercially and should be used according to manufacturer's instructions.

7.13.5.1 **Modified buffered peptone water**

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1000 ml

Dissolve the ingredients in the water and dispense into suitable volumes in screw-capped containers. Sterilize the resulting solution by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. The medium may be stored in the dark at room temperature and used within one month. The following antibiotic solutions should be dissolved, where appropriate, in water and filter-sterilized.

(i) Dissolve 80 mg of vancomycin hydrochloride in 10 ml of water. The filter-sterilized solution should be stored at approximately - 20°C and used within one month. Add 1 ml of this solution to the medium to give a final concentration of 8 mgl^{-1}.

(ii) Dissolve 500 mg of cefixime in 100 ml of ethanol. Store at between 2 - 8°C and use within one month. Add 1 ml of this solution to 100 ml of ethanol and add 1 ml of the resulting solution to the medium to give a final concentration of 50 μgl^{-1}.

(iii) Dissolve 100 mg of cefsulodin sodium salt in 10 ml of water. The filter-sterilized solution should be stored at approximately - 20°C and used within one month. Add 1 ml of this solution to the medium to give a final concentration of 10 mgl^{-1}.

The complete medium should be prepared on the day of use and dispensed in 20 ml volumes into sterile containers.

7.13.5.2 **Modified tryptone soya broth**

Tryptone soya broth	30.0 g
Bile salts number 3	1.5 g
Dipotassium hydrogen phosphate	2.5 g
Novobiocin	20 mg
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.3 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. The sterilized medium may be stored at room temperature in the dark and used within one month.

7.13.5.3 **Modified sorbitol MacConkey agar[83]**

Peptone	20.0 g
Sorbitol	10.0 g
Bile salts number 3	1.5 g
Neutral red	30.0 mg
Crystal violet	10 mg
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense in appropriate volumes into suitable screw-capped bottles and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.1 ± 0.2. Allow the medium to cool, store in the dark at room temperature and use within one month. Cool the molten medium to approximately 50°C and add the following selective supplements, which should be dissolved, where appropriate, in water and filter-sterilized:

(i) Dissolve 500 mg of cefixime in 100 ml of ethanol. Store at between 2 - 8°C and use within one month. Add 1 ml of this solution to 100 ml of ethanol and add 1 ml of the resulting solution to the medium to give a final concentration of 50 μgl^{-1}.

(ii) Dissolve 25 mg of in potassium tellurite 10 ml of water. The filter-sterilized solution should be stored at approximately - 20°C and used within one month. Add 1 ml of this solution to the medium to give a final concentration of 2.5 mgl^{-1}.

Mix the complete medium thoroughly and pour into sterile Petri dishes and allow the agar to solidify. Petri dishes may be stored at between 2 - 8°C and used within one month. Dishes should be dried in a suitable oven at 45 - 50°C for 30 minutes before use.

7.13.5.4 **Phosphate buffered saline solution**

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate	11.5 g
Potassium dihydrogen phosphate	2 g
Polyoxyethylene-sorbitan monolaurate (for example Tween 20)	0.5 ml
Water	1000 ml

Dissolve the ingredients in the water and check that the pH is 7.3 ± 0.2. Sterilize the resulting solution by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the solution should be checked to confirm a pH of 7.3 ± 0.2. Allow the solution to cool, store in the dark at room temperature and use within one month.

7.13.5.5 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to ensure that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark and used within one month. Most non-selective agars are suitable for producing pure cultures for oxidase testing, provided they do not contain fermentable carbohydrates.

7.13.6 **Analytical procedure**

Dispense the modified buffered peptone water into suitable sterile containers. Filter 5x100 ml and 5x10 ml volumes of sample, or diluted sample. Additional 5x1 ml volumes may, if necessary, be inoculated directly into enrichment media. For clear waters containing low numbers of organisms, filter 1x500 ml and 5x100 ml volumes. If a presence or absence determination is to be undertaken, filter 1000 ml of sample. Several membrane filters may need to be used, especially if waters possessing high turbidities are to be analysed. Place the membrane filters in the media and incubate at 37 ± 1°C for 24 hours. Enrichment broths should be subjected to immunomagnetic separation at 6 - 7 hours and after 24 hours.

Thoroughly mix the antibody-coated paramagnetic beads and transfer 20 µl of the suspension to a 1.5 ml Eppendorf tube. Add 1 ml of the well-mixed enrichment broth to the tube and mix thoroughly, but gently, by inversion. Place the Eppendorf tube onto a rotating mixer and gently mix for approximately 30 minutes. Ensure that no air bubbles are trapped at the bottom of the tube. Place the tube into the magnetic particle separator (MPC) with the associated magnetic strip in position. To concentrate the beads into a small pellet onto the side of the tube, gently invert the MPC repeatedly for about 1 minute. With the magnetic strip in position, carefully open the Eppendorf tube and aspirate the liquid from the tube and any remaining liquid that might be inside the cap. Remove the magnetic strip from the MPC and add 1 ml of phosphate buffered saline solution to the Eppendorf tube. Close the cap and gently invert to resuspend the beads. Re-position the magnetic strip in the MPC and concentrate the beads into a small pellet as before. Repeat the rinsing step with more phosphate buffered saline solution. Re-suspend the beads in 50 µl of phosphate buffered saline solution and inoculate the beads onto modified sorbitol MacConkey agar and incubate at 37 ± 1°C for 24 hours. After incubation, examine the dishes for typical non-sorbitol-fermenting colonies that are smooth and circular, 1 - 3 mm in diameter and pale orange in colour. Some strains of *E. coli*, which ferment sorbitol, are pink in colour.

7.13.7 **Confirmation**

Typical colonies should be inoculated onto nutrient agar and incubated at 37 ± 1°C for 24 hours. Isolates can then be subjected to serological identification using commercially available latex suspension kits. Examine the slides for evidence of agglutination and carry out the tests with appropriate positive and negative controls. Some isolates may require further identification by biochemical testing, as non-sorbitol-fermenting coliforms can cross-react in the latex agglutination test. While chromogenic media can be used to demonstrate the lack of ß-glucuronidase, some strains of *E. coli* O157:H7 may produce atypical biochemical profiles, and results should be interpreted with caution.

7.13.8 **Reporting results**

For each sample, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4 and 7.13.4. Every effort should be made to prepare sufficient solutions to enable a series of tubes to be obtained to show that some tubes exhibit no growth while others exhibit some growth.

7.14 Detection and enumeration of *Shigella* species

7.14.1 **Introduction**

Members of the genus *Shigella* normally inhabit the intestinal tract of humans but do not infect animals. Their presence in water is, therefore, an indication of human faecal contamination. Infection is commonly by person-to-person contact, or by the consumption of contaminated food or water.

7.14.2 **Scope and field of application**

The multiple tube method using enrichment and selective agars is suitable for the detection and isolation of *Shigella* from surface waters, including river waters, estuarine and sea waters, sewage effluents and spa pool waters. The sample volume depends upon the degree of faecal contamination but is usually between 100 - 1000 ml.

7.14.2.1 **Definition and description of the organism**

Bacteria in the genus *Shigella* are facultative anaerobes, Gram-negative, non-motile rods approximately 1 µm x 2-5 µm in length. Four species are commonly found, namely, *S. dysenteriae*, *S. sonnei*, *S. flexneri* and *S. boydii*. The organisms are oxidase-negative and catalase-positive (with the exception of *S. dysenteriae* type, which is catalase-negative). Citrate cannot be used as a sole source of carbon, and, with few exceptions, carbohydrates are fermented without gas production.

7.14.2.2 **Pathogenicity**

Gastro-intestinal disease is commonly a symptom of infection of which dysentery is the most severe. The disease is typical of conditions of poor hygiene and sanitation. In the UK, *S. sonnei* is commonly isolated, although the most severe disease is caused by *S. dysenteriae*, type 1, which produces a potent exotoxin (Shiga toxin).

7.14.3 **Principle**

Organisms are isolated by membrane filtration followed by enrichment in modified Hajnia GN broth and subculture to modified desoxycholate citrate agar (MDCA) and modified Hektoen agar (HA)[84], and examination for typical colonies. Filtration aids, whilst, generally, not as efficient at removing all the organisms, may be of value in processing very turbid waters. Characteristic colonies are confirmed by slide agglutination and may be subcultured for further biochemical testing.

7.14.4 **Performance characteristics**

When low numbers of organisms are present, their detection is dependent only on the volumes of sample that are tested. To increase the detection, or volume of sample, MF may be used to concentrate volumes of sample before inoculation into tubes containing media. Several filters may be used to increase this limit. When high numbers of organisms are present, their detection is unlimited, provided that appropriate dilutions can be prepared and analysed. High numbers of competing organisms may inhibit the growth or detection of the organisms. Tubes, or containers, should be incubated for 48 hours but tests may involve incubation for up to 4 days. Subculture and confirmation take a further 48 hours.

7.14.5 **Reagents and media**

7.14.5.1 **Modified Hajnia GN broth**

Tryptone	20.0 g
Glucose	1.0 g
Mannitol	2.0 g
Sodium citrate	5.0 g
Sodium desoxycholate	500 mg
Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
(DL) Serine	1.0 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.2 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by steaming at

100 ± 1°C for 30 minutes. After steaming, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. The medium should be stored at between 2 - 8°C and used within one month.

7.14.5.2 **Modified desoxycholate citrate agar.**

Tryptone	20.0 g
Lactose	10.0 g
Sodium thiosulphate pentahydrate	6.8 g
Ammonium iron(III) citrate	800 mg
Neutral red (1% m/v aqueous solution)	3 ml
Sodium desoxycholate	500 mg
(DL) Serine	1.0 g
Tetracycline hydrochloride	32 mg
Agar	14.0 g
Water	1000 ml

Dissolve the ingredients (except the tetracycline hydrochloride) in the water. To achieve this, it will be necessary to heat to boiling. Cool the resulting solution to approximately 50°C and add the tetracycline as an aqueous filter-sterilized solution to give a final concentration of 32 mgl^{-1}. Thoroughly mix the complete medium and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared dishes should be dried before use.

7.14.5.3 **Modified Hektoen agar**

Yeast extract	3.0 g
Proteose peptone	12.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
Ammonium iron(III) citrate	1.5 g
Acid fuchsin	100 mg
Bromothymol blue (1% m/v aqueous solution)	6.5 ml
Bile salts number 3	9.0 g
Sodium chloride	5.0 g
Sodium thiosulphate pentahydrate	5.0 g
Agar	14.0 g
Novobiocin	15.0 mg
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Cool the resulting solution to approximately 50°C and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8°C and use within one month. Prepared dishes should be dried before use.

7.14.5.4 **Nutrient agar**

Beef extract powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	10 - 15 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm that the pH is 7.2 ± 0.2. Cool the molten medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium may be stored at room temperature in the dark and used within one month. Most non-selective agars are suitable for producing pure cultures for oxidase testing, provided they do not contain fermentable carbohydrates.

7.14.6 **Analytical procedure**

Samples may be concentrated by membrane filtration. The analysis may be carried out either as a presence-absence test or using an MPN technique. After filtration, membrane filters are placed into 20 ml volumes of Hajnia broth in sterile containers and incubated at 37 ± 1°C for 6 - 8 hours. Enrichment broths are gently shaken and inoculated onto MDCA and modified Hektoen agar. Inoculated dishes are incubated at 37 ± 1°C for 18 - 24 hours and examined for typical colonies, see Tables 7.4 and 7.5. Typical colonies should be subcultured onto a non-selective medium, for example nutrient agar and after incubation, subjected to further biochemical serological testing.

Table 7.4 Colonial appearance of *Shigella* and other bacteria on MDCA

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

Table 7.5 Colonial appearance of *Shigella* and other bacteria on Hektoen agar

Organism	Characteristic appearance
Shigella	Moist green coloured colonies 2-4 mm in diameter. *S sonnei* may produce larger irregular colonies.
Pseudomonas	Large rough textured green coloured colonies.
Coliforms	Yellow coloured colonies. The medium around the colonies often turns salmon pink.
Proteus	Pale green or ochre yellow coloured colonies.

7.14.7 **Confirmation**

Typical colonies should be subcultured onto a non-selective medium, for example nutrient agar, and incubated at 37 ± 1°C for 24 hours. Isolates should then be identified using commercially available biochemical test kits and by slide agglutination using prepared antisera.

7.14.8 **Reporting results**

For each sample, record the number of tubes which confirm as positive, ie demonstrate growth of organisms, and from tables determine the number of organisms per volume to be reported; see sections 5.4 and 7.14.4. Every effort should be made to prepare sufficient solutions to enable a series of tubes to be obtained to show that some tubes exhibit no growth while others exhibit some growth. The final report may also contain details of presumptive as well as confirmed Shigella species.

7.15 Detection and enumeration of F-specific RNA bacteriophages

7.15.1 **Introduction**

Bacteriophages are widely distributed in the environment and may be found in water, soil, sewage and sediments. They occur infrequently in the faeces of humans or animals, but are commonly found in waste waters. The presence of F-specific bacteriophages in waters, generally, indicates contamination by human or animal faeces. They will only replicate in bacteria and, for many, in specific strains of bacteria. Bacteriophages are readily adsorbed to particulate material and are inactivated by sunlight. Their survival in the environment, removal by water and waste water treatment processes and their accumulation in shellfish closely resembles that of food- and water-borne enteric viruses.

7.15.2 **Scope and field of application**

The method involving incubation of the sample with a susceptible host strain is suitable for

the detection and enumeration of F-specific RNA bacteriophages from surface waters, sewage and associated samples, including sediments and sludges, and extracts from shellfish. The volume of sample depends upon the degree of contamination but is usually 1 - 5 ml. Where high numbers of bacteriophages are suspected, dilution of the sample may be required, and where low numbers are present, a pre-concentration step may be used.

7.15.2.1 **Definition and description of the organisms**

F-specific RNA bacteriophages are bacterial viruses that consist of simple capsids of cubic symmetry, 21 - 30 nm in diameter, containing single stranded RNA as the genome. They are infectious for bacteria that possess the F- or sex plasmid, originally detected in *Escherichia coli* K12, and absorb to the F- or sex pili coded by this plasmid. The F-plasmid is transferable to a wide range of Gram-negative bacteria. The infectious process is inhibited by the presence of RNase in the growth medium. This can be used to distinguish between the F-specific RNA bacteriophages and the rod-shaped F-specific DNA bacteriophages of the Inoviridae family. Infection of the host produces visible plaques (clearance zones or zones of inhibition) in a confluent growth obtained under appropriate culture conditions. The infectious process is inhibited in the presence of RNase in the overlay.

7.15.2.2 **Pathogenicity**

Bacteriophages are highly specific to their bacterial hosts. There is no evidence, at present, to suggest that they pose a threat to humans or animals. However, good laboratory practice should be followed at all times during the test procedures, especially as *Salmonella typhimurium* is used as a host for the virus. F-specific RNA bacteriophages are very resistant to drying. Appropriate precautions should, therefore, be taken to prevent cross-contamination of materials, particularly when examining or handling cultures, or when inoculating cultures of the host strain.

7.15.3 **Principle**

The detection and enumeration of F-specific RNA bacteriophages is carried out by direct plating using a semi-solid agar overlay technique[85]. Replication usually leads to lysis of the cell and, in the agar overlay, the production of clear areas of lysis called plaques.

The host bacterium used for the assay is *S. typhimurium* WG 49. Bacteriophage MS2 can be used as a control and *E. coli* K12 can be used to produce the bacteriophage. Samples may need to be de-contaminated using chloroform. F-specific DNA bacteriophages may also produce plaques using these procedures, and incorporation of RNase into the overlay agar may be necessary to confirm this. It is important that during the assay the host bacteria grow in an exponential phase as they express the F-pilus. The concentration of host bacteria for the assay should be approximately 10^8 colony forming units per millilitre ($cfuml^{-1}$). Growth of the host should, therefore, be calibrated by establishing the relationship between absorbance readings (as a measure of turbidity) and colony counts.

7.15.4 **Performance characteristics**

When low numbers of bacteriophages are present, their detection is dependent only on the volumes of sample that can be plated (usually a maximum of 1 ml). To increase the detection, or volume of sample, concentration techniques are used. When high numbers of bacteriophages are present, their detection is unlimited, provided that appropriate dilutions can be prepared. Their detection will, therefore, depend on the largest volume of sample that can be concentrated and plated. A replicate number of plates may be used to increase this detection. High numbers of competing organisms may overgrow the host and mask plaque production. After incubation, agar overlay tests are examined after 18 hours.

7.15.5 **Reagents and media**

Not every nutrient or selective agar is suitable for agar overlay assays and agars should be checked for their suitability before use. Calcium and magnesium ions are important factors for absorption of phages to host bacteria, and highly purified agars are not recommended.

7.15.5.1 **Peptone saline solution**

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1000 ml

Dissolve the ingredients in the water and dispense the resulting solution in appropriate volumes into suitable screw-capped bottles. Sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.1. Store in the dark at room temperature and use within one month.

7.15.5.2 **Tryptone yeast extract glucose broth (TYGB)**

Trypticase peptone (tryptone)	10.0 g
Yeast extract	1.0 g
Sodium chloride	8.0 g
Water	1000 ml

Dissolve the ingredients in the water. Dispense the resulting solution in 200 ml volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.1. Store at between 2 - 8°C and use within one month.

7.15.5.3 **Calcium glucose solution**

Calcium chloride dihydrate	3.0 g
Glucose	10.0 g
Water	100 ml

Dissolve the ingredients in the water. Filter-sterilize the solution through a 0.22 µm pore size membrane filter, store in sterile screw-capped bottles at between 2 - 8°C and use within one month.

7.15.5.4 **Complete medium**

TYGB (see 7.15.5.2)	200 ml
Calcium glucose solution (see 7.15.5.3)	2 ml

Aseptically add the calcium glucose solution to the TYGB solution and mix well. If not used immediately, store at between 2 - 8°C and use within five days.

7.15.5.5 **Tryptone yeast extract glucose agar (TYGA)**

Trypticase peptone (tryptone)	10.0 g
Yeast extract	1.0 g
Sodium chloride	8.0 g
Agar	12 - 20 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in 200 ml volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.1. Cool the molten medium to approximately 50°C and add 2 ml of calcium glucose solution, mix well and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Alternatively, the medium without the calcium glucose solution can be stored at between 2 - 8°C and used within one month.

7.15.5.6 **Semi-solid tryptone yeast extract glucose agar (ssTYGA)**

Prepare the basal medium as described in section 7.15.5.5 using half the amount of agar. The gel strength is critical in order to obtain good results, and different concentrations should be tested. Dispense the resulting solution in 50 ml volumes, sterilize and store as described in section 7.15.5.5.

7.15.5.7 **RNase solution**

RNase	100 mg
Water	100 ml

Dissolve the RNase in the water whilst heating at 100°C for 10 minutes. Dispense the resulting solution in 0.5 ml volumes into sterile vials, store at approximately -15°C and use within 12 months. Thaw at room temperature before use.

7.15.5.8 **Glycerol solution**

Glycerol (87% m/v)	100 ml

Prepare a solution of glycerol in water and dispense in 20 ml volumes into suitable bottles. Sterilize by autoclaving at 121 ± 1°C for 15 minutes. Store in the dark at room temperature and use within 12 months.

7.15.5.9 **MacConkey agar**

Bile salts	5.0 g
Peptone	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Agar	12.0 g
Neutral red (1% m/v aqueous solution)	5 ml
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable bottles and sterilize by autoclaving at 115 ± 1°C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.5 ± 0.2. Cool the medium to approximately 50°C and pour into sterile Petri dishes, allow the agar to solidify, store at between 2 - 8°C and use within one month. Prepared Petri dishes should be dried before use. Alternatively, the bottled medium can be stored in the dark at room temperature and used within one month.

7.15.6 **Analytical procedure**

7.15.6.1 Prepare stock cultures of the host bacterium by re-hydrating the contents of a lyophilised ampoule of *S. typhimurium* WG 49 into a small volume of TYGB solution. Transfer the suspension to 25 ml of TYGB in a 300 ml conical flask and incubate at 37 ± 1°C for 18 ± 2 hours whilst shaking at 100 ± 10 rpm. Add 10 ml of glycerol solution, mix well, dispense 1.2 ml volumes into sterile cryovials and store at - 70 ± 10°C.

7.15.6.2 Prepare working cultures of the host bacterium by thawing a vial of stock culture at room temperature. Inoculate onto MacConkey agar and incubate at 37 ± 1°C for 18 ± 2 hours. Add 50 ml of TYGB to a 300 ml sterile conical flask and warm to room temperature. Select a small number (for example 3 - 5) of lactose-positive colonies from the MacConkey agar and inoculate the flask of TYGB. Incubate for 5 ± 1 hour whilst shaking at 100 ± 10 rpm. Add 10 ml of glycerol solution and mix well. Dispense the resulting suspension in 1.2 ml volumes into sterile cryovials, store at - 70 ± 10°C and use within 2 years.

7.15.6.3 Thaw a vial of the working culture of the host bacterium at room temperature. Add 50 ml of TYGB to a 300 ml conical flask and transfer a sufficient amount to a 10 mm cuvette. Place the cuvette in a spectrophotometer with a filter in the range 500 - 650 nm and band-width of 10 nm, and adjust the reading to zero. Add 0.5 ml of the working culture to the flask containing TYGB and incubate at 37 ± 1°C for up to 3 hours whilst shaking at 100 ± 10 rpm. After 30 minutes, transfer a sufficient amount of the TYGB suspension to the same cuvette used to adjust the spectrophotometer reading to zero and measure the absorbance. At the same time, prepare a sufficient number of serial 10-fold dilutions of the TYGB suspension. These dilutions are used to determine colony counts (of each dilution in duplicate) on TYGA. Incubate the TYGA Petri dishes at 37 ± 1°C for 18 hours and, from those dishes containing 30 - 300 colonies, record the number of colonies for the appropriate dilution, and calculate the number of colony forming units per ml of the TYGB suspension. After each 30 minute period, repeat the process, ie absorbance reading and colony count determinations, using the incubating TYGB suspension. Establish the relationship between absorbance and colony count. From this relationship, determine the absorbance reading corresponding to a colony count of approximately 10^8 cfuml^{-1}. Once established, further work may be based on absorbance readings only.

7.15.6.4 Inoculate 25 ml of TYGB with *E. coli* K12 and incubate at 37 ± 1°C for 18 ± 2 hours whilst shaking at 100 ± 10 rpm. Inoculate 0.25 ml of this culture into 25 ml of TYGB and incubate at 37 ± 1°C for 90 ± 10 minutes whilst shaking at 100 ± 10 rpm. Add bacteriophage MS2 to give a final concentration of approximately 10^7 plaque forming units per ml (pfuml^{-1}).

Incubate at 37 ± 1°C for 4 - 5 hours. To the suspension, add 2.5 ml of chloroform, mix well and allow to stand overnight between 2 - 8°C. After standing overnight, decant the aqueous phase into centrifuge tubes and centrifuge, for example at 3000 g for 20 minutes. Prepare a sufficient number of 10-fold serial dilutions and plate out using *E. coli* K 12. Count the number of plaques from the dilution series and prepare 100 - 1000 ml of a suspension of MS2 in peptone saline solution containing approximately 100 pfuml^{-1}. Add 5 ml of glycerol solution, mix well, dispense the resulting suspension in 1.2 ml volumes into sterile cryovials and store at less than - 15°C.

7.15.6.5 The following tests can be carried out as quality control checks to assess the sensitivity of the host strain to bacteriophages. At the same time, ie at time = 0 hours and time = 3 hours, as the calibration tests are undertaken (see section 7.15.6.3) and using the serial dilutions for the colony counts, plate the broth culture onto MacConkey agar and incubate at 37 ± 1°C for 18 ± 2 hours. From those Petri dishes containing 30 - 300 colonies, record the number of lactose-positive and lactose-negative colonies, and calculate the number of lactose-negative colonies relative to the total number of colonies. This should be less than 5% of the total population.

In addition, for the appropriate dilutions, the colony count on TYGA at 0 hours should be approximately 1 - 3 x 10^7 cfuml^{-1} and at 3 hours should be approximately 7 - 40 x 10^7 cfuml^{-1}. *S. typhimurium* WG 49 should be resistant to nalidixic acid and kanamycin. At the same time, ie at time = 0 hours and time = 3 hours, as the calibration tests are undertaken (see section 7.15.6.3) and using a 100-fold dilution of the growth culture, inoculate a MacConkey agar Petri dish. Place two small discs, for example 9 mm in diameter, one containing 130 µg of nalidixic acid and the other disc containing 100 µg of kanamycin, onto the agar. Incubate at 37 ± 1°C for 18 ± 2 hours. There should be no zone of inhibition surrounding the disc with the nalidixic acid and the zone of inhibition surrounding the disc containing kanamycin should be less than 15 mm.

As a final check, thaw 4 vials of bacteriophage MS2 to room temperature, combine the contents into one tube and plate out 1 ml volumes, in duplicate, on *E. coli* K12 and *S typhimurium* WG 49 as described in section 7.15.6.6. Count the plaques on each plate and compare the recovery using *S. typhimurium* WG 49 with that of *E. coli* K12. The *S. typhimurium* WG 49 strain can be considered acceptable if the recovery is better than 80% of the *E. coli* K12 strain.

7.15.6.6 Prepare an inoculum culture of the host bacterium by thawing a vial of the working culture to room temperature. Add 50 ml of TYGB to a conical flask and warm to approximately 37°C. Add 0.5 ml of the working culture to the flask and incubate at 37 ± 1°C for up to 3 hours whilst shaking at 100 ± 10 rpm. Measure the absorbance at 30 minute-intervals until it corresponds to a cell density of approximately 10^8 cfuml^{-1} (as established in section 7.15.6.3). Place the flask into a water-ice mixture and use within 2 hours.

Melt ssTYGA medium, cool to approximately 50°C, aseptically add calcium glucose solution and dispense in 2.5 ml volumes into suitable tubes or containers. Maintain the temperature at 45 ± 1°C. To each tube, add 1 ml of inoculum culture and 1 ml of sample, or diluted sample and mix carefully. For each tube, pour the suspension over the surface of a TYGA Petri dish. Disperse evenly and allow the suspension to solidify. Invert the Petri dish and incubate at 37 ± 1°C for 18 ± 2 hours. After incubation, record the number of plaques on each dish within 4 hours. Increasing the number of replicate determinations will enhance the detection of bacteriophages. With each series of tests, a blank (consisting of sterile diluent) and a positive control (consisting of a standard preparation of bacteriophage MS2) should be included.

Where samples are likely to be contaminated with high bacterial numbers, for example sewage, the addition of nalidixic acid to a final concentration of 100 µgml^{-1} may be necessary to the TYGA or the ssTYGA (before autoclaving) or to the ssTYGA (after melting). Where samples are likely to contain low numbers of bacteriophage, TYGA Petri dishes, overlaid with 10 ml of ssTYGA containing 1 ml of inoculum culture and 5 ml of sample, or concentrated sample, may be used.

7.15.7 **Confirmation**

The host strain, *S. typhimurium* WG 49, is susceptible to infection with somatic bacteriophages. F-specific RNA bacteriophage infection is inhibited by the addition of RNase at 100 mgml^{-1} to tubes of ssTYGA and analysis of samples in parallel to the standard test. Confirmation is particularly important when large, circular, clear plaques with smooth edges are observed, as these are more likely to reflect somatic bacteriophages.

7.15.8 **Reporting results**

For each sample, record the number of plaques per volume tested. Convert this number to the volume to be reported; see sections 5.4 and 7.15.4. Every effort should be made to obtain a sufficient number of solutions to be tested and plaques to be counted. The final report may also contain details of presumptive as well as confirmed RNA bacteriophages.

7.16 Detection and enumeration of microbial tracers

7.16.1 **Introduction**

Microbial tracers have been used to study the movement of water, retention times and dispersion characteristics in rivers and marine environments, and can provide information on the investigation of sources of pollution. A number of tracers have been developed for environmental use and include spores of *Bacillus globigii* and bacteriophages specific for *Serratia marcescens*, *Escherichia coli* and *Enterobacter cloacae*.

B. globigii spores are relatively resistant in the environment[86] and can be useful in the marine environment where tidal cycles may be studied. Bacteriophages are found naturally in the environment and are useful as tracers. They are easily detected and may be useful in rivers, ground waters and for investigating coastal discharges. Water can, therefore, be spiked with large numbers of phage particles to compensate for absorption and inactivation. Unlike *B. globigii*, bacteriophages are readily removed by water treatment and disinfection, and are considered as having no significant health risk for the environment.

7.16.2 **Scope and field of application**

The methods are suitable for the detection of *B. globigii* and bacteriophages for all types of water including river waters, estuarine and sea waters, sewage and sewage effluents, and spa pool waters. The volume of sample, or diluted sample, depends upon the concentration of tracer organism in the sample but, generally, 100 ml of sample is used for *B. globigii* determinations and 1 ml of sample for bacteriophages. Where the concentration of tracers is known to be high, dilution will be necessary in order to obtain sufficient numbers of tracers.

7.16.2.1 **Definition and description of the organism**

The *Bacillus* species contain a wide range of aerobic, spore-bearing, Gram-positive bacteria. *B. globigii* are heat-resistant and produce characteristic orange colonies on culture. There are a number of bacteriophages available for microbial tracing[87]. The coliphage MS2 is particularly suitable for waters with little pollution. A phage isolated from sewage replicates in a strain of *Enterobacter cloacae*.

7.16.2.2 **Pathogenicity**

Some members of the *Bacillus* species are known to be pathogenic. *B. cereus*, *B. subtilis* and *B. licheniformis* can cause gastro-enteritis. They may also cause wound infections and septicaemia. The spores can pass through water treatment and are resistant to chlorination. The use of *B. globigii* as a tracer in rivers where water is abstracted for drinking or ground water tracing is, therefore, not to be recommended. It is, however, unlikely to germinate in the environment, and for marine tracing is unlikely to represent a risk to public health.

Bacteriophages are removed by water treatment and inactivated by disinfection with chlorine. As they are host-specific, they are unlikely to infect plants, animals or humans and are not considered a risk to public health. The host bacterium should not be a recognised pathogenic organism, for example *Salmonella* species and every effort should be made to ensure that suspensions for use in the environment are host-free.

7.16.3 **Principle**

B. globigii can be isolated from samples by membrane filtration and cultured on tryptone water containing glucose and mannitol. Colonies are easily recognised by the production of a bright orange pigment and can be easily counted after incubation at 30°C for 48 hours. Samples are pasteurised before filtration to reduce background contamination and enhance spore germination[88]. Bacteriophages are detected by plating with a susceptible host using the agar overlay technique (see section 7.15) on selective or non-selective media. For phages that are not susceptible, samples may be decontaminated by the addition of a small amount of chloroform. Bacteriophages produce characteristic plaques in the overlay that are easily counted after overnight incubation. With both types of tracers it is unusual to observe background counts except with coliphages and sewage or heavily polluted surface waters.

7.16.4 **Performance characteristics**

With *B. globigii*, the detection of low levels is dependent on the volume of sample that can be filtered. This will normally be 100 ml. When low numbers of bacteriophages are present, their detection is dependent only on the volumes of sample that can be plated (usually a maximum of 1 ml). To increase the detection, or volume of sample, concentration techniques are used. Membrane filtration has been used as a means of increasing volumes up to 100 ml. Alternatively, samples may be enriched in a broth with the host strain using an MPN technique. When high numbers of bacteriophages are present, their detection is unlimited, provided that appropriate dilutions can be prepared. Their detection will, therefore, depend on the largest volume of sample that can be concentrated and plated. A replicate number of plates may be used to increase this detection. High numbers of competing organisms may overgrow the host and mask plaque production. After incubating for 18 hours, agar overlay tests are examined for plaque formation.

7.16.5 **Reagents and media**

Not every nutrient or selective agar is suitable for agar overlay assays, and agars should be checked for their suitability before use. Calcium and magnesium ions are important factors for absorption of phages to host bacteria, and highly purified agars are not recommended.

7.16.5.1 **Tryptone glucose mannitol broth**

Tryptone	20.0 g
Sodium chloride	5.0 g
Glucose mannitol solution	100 ml
Water	900 ml

Dissolve the ingredients (except the glucose mannitol solution) in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution in volumes of 90 ml, or multiples of 90 ml, into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the partial medium should be checked to confirm a pH of 6.8 ± 0.2. Prepare a solution of glucose and mannitol, each 10% m/v, and filter-sterilize using a 0.2 µm pore size membrane filter. Aseptically dispense the solution into sterile containers in 10 ml volumes, store at between 2 - 8°C and use within one month. To prepare the complete medium, add 10 ml of glucose mannitol solution to 90 ml of partial medium and dispense onto sterile 47 mm incubation pads contained in sterile Petri dishes. Allow the pads to soak for a minimum of 15 minutes and drain off the excess medium. Drained pads should be used within 1 hour. Alternatively, the partial medium can be stored at in the dark at room temperature and used within one month.

7.16.5.2 **Growth medium for bacteriophage hosts**

Brain heart infusion	20.0 g
Casein hydrolysate	20.0 g
Yeast extract	1.0 g
Potassium dihydrogen phosphate	5.0 g
Magnesium sulphate heptahydrate	1.0 g
Glycerol	20 ml
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in suitable volumes (10 ml) into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The prepared medium can be kept in the dark at room temperature and used within one month.

7.16.5.3 **Agar base for agar overlay assay (blood agar base)**

Beef extract powder	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. The pH of the medium should be 7.3 ± 0.2. Dispense the resulting solution in suitable volumes in screw-capped containers and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. Cool the molten medium to approximately 50°C and pour 15 ml into sterile Petri dishes, which should be stored at between 2 - 8°C and used within one month. Petri dishes should be brought to room temperature, but need not be dried, before use. Alternatively, the medium can be stored in the dark at room temperature and used within one month.

7.16.5.4 **Agar overlay**

The concentration of agar given will produce a suitable gel strength and plaque size with 0.1 - 1.0 ml of sample.

Nutrient broth number 2	11.2 g
Sodium chloride	7.0 g
Agar number 1	5.5 g
Water	1000 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. The pH of the medium should be 7.3 ± 0.2. Dispense the resulting solution in volumes of 4.0 ml into suitable containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. The medium should be stored in the dark at room temperature and used within two weeks. Any bottles showing signs of desiccation should be discarded. For use, melt the overlay agar and maintain at a temperature of approximately 50°C.

7.16.6 **Analytical procedure**

7.16.6.1 ***B. globigii* spores**

Heat the sample to approximately 63°C and maintain at this temperature for 30 minutes. After pasteurisation, cool the sample to room temperature and filter 100 ml, or a suitable dilution. Any remaining sample should be stored at temperatures between 2 - 8°C until the analysis is completed. Place the membrane filter onto the isolation medium (section 7.16.5.1). Incubate the filters in sealed containers at 30°C for 48 hours. Examine the filters at 24 hours and, if necessary, prepare suitable dilutions (for re-testing) of any sample where the corresponding filter shows a large number of colonies that would make subsequent counting difficult to undertake. Typical colonies are 0.5 - 2 mm in diameter, rough in appearance and orange in colour. Large numbers of colonies on membrane filters may restrict the size of individual colonies and a hand lens may be required.

7.16.6.2 **Bacteriophages**

Inoculate the host strain into growth medium and incubate overnight at 37°C. Add 0.1 ml of host culture to the molten overlay agar medium. Add 0.1 - 1.0 ml of the sample, mix carefully avoiding formation of air bubbles and pour the resulting suspension onto the agar base, ensuring the base is covered. Allow the overlay agar to solidify, invert the Petri dish and incubate at 37°C for 12 - 24 hours. After incubation, record the number of plaques (areas of clearing) for each sample. Plaques may be confirmed by picking into fresh overlay agar medium with host strain and replating when, after incubation, confluent lysis should be observed.

7.16.7 **Reporting results**

For each sample tested, record the number of colonies or plaque forming units per volume tested. Convert this number to the volume to be reported; see sections 5.4 and 7.16.4. Every effort should be made to obtain a sufficient number of colonies or plaques to be counted.

8 Virological methods

8.1 Introduction

Enteric viruses may be found in both fresh and saline waters where there has been faecal contamination. The majority of enteroviruses detectable in water are the vaccine strains of poliovirus and coxsackievirus B. Enteroviruses are transmitted by person-to-person contact and many infections occur with no associated symptoms. None of the enteroviruses cause gastroenteritis except as part of a generalised febrile illness. Other symptoms may include flu-like illness, meningitis or myalgia. Whilst rotavirus is a major cause of diarrhoea in children and elderly adults, it has not been linked to infections via recreational water use.

Enteroviruses should not be regarded as indicators of the presence of other viruses as the epidemiology of the different groups varies considerably. They are, however, indicators of faecal pollution although not necessarily recent pollution. A direct relationship between enteroviruses and bacterial indicator organisms has not been demonstrated, but viruses have been found when other indicator organisms have not been. Enteroviruses are human parasites and can not replicate in the environment. They may, however, persist for several weeks in cool, dark places, for example on sediments. Routine monitoring may be worth while as part of a wider microbiological profile when particular circumstances arise, for example an outbreak of disease is investigated.

8.2 Methodology

Several techniques are available[89] for the determination of enteric viruses. For example, ten litres of river water or seawater are filtered. A solution of high protein content at high pH is then passed through the filter. Virus particles dissociate from the filter into the solution which is then collected. The pH of the resulting solution is adjusted so that all the protein matter, including viruses, flocculate out of solution. The suspension is centrifuged and any particulate material is re-suspended in 10 ml of phosphate buffer.

At the present time enterovirus and rotavirus can be detected by cell culture-based systems. The suspended buffalo green monkey kidney (BGMK) cell plaque assay is sensitive for poliovirus and coxsackievirus B. Microscopy is used for the examination of rotavirus, which is based on a cell culture technique followed by immuno-chemical stain.

9 Parasitological methods

Giardia duodenalis and *Cryptosporidium parvum* are intestinal parasites which commonly infect humans and warm-blooded animals. They may be found in sewage, surface waters and waste waters. Whilst *Giardia* cysts tend to lose viability quite soon after discharge from the host, *Cryptosporidium* cysts can survive for long periods in surface waters. Details of detection and enumeration are described elsewhere[90]. Methods usually involve concentration followed by clean-up and microscopic examination. Similar methods may also be used for the isolation and identification of free-living amoebae, and the intestinal helminths *Ascaris*, *Trichuris* and *Ankylostoma*, which involve recognition of characteristic ova morphology.

10 Legionella

Most species of *Legionellaceae* have been isolated from the environment and can be found in natural waters, marine waters, cooling towers and spa pool waters. All *Legionella* species are potentially pathogenic towards humans and legionellosis is transmitted via aerosols. Culture isolation of *Legionellae* from recreational and environmental waters is generally the method of choice, although some isolates grow only slowly and can be inhibited by the growth of other competing organisms. Details of sampling, detection and enumeration are described elsewhere[91]. Methods usually involve concentration by membrane filtration, followed by acid and heat pre-treatment and culture on selective media.

Appendix A

Tables of most probable numbers and most probable ranges

Tables A1 - A3 indicate the estimated number of bacteria in 100 ml of sample from the positive and negative reactions for the different volumes examined. It is important to realise that the MPN is only an estimate, based on statistical probability and that the true value may lie within a range of values (see section 5.3.2.1). Approximate 95% confidence intervals, which demonstrate a range of possible counts which could yield the tabulated number of tubes, have been published[25]. A computing procedure for estimating these approximate intervals for other dilution series has also been published[92]. These intervals can be examined more accurately[28] but are seldom of practical use when reporting results, because they apply to the accuracy of the method and not the likely range of organisms at the sampling source. The MPR illustrates situations where the method becomes imprecise, particularly when nearly

all the tubes show growth and, in practice, a further dilution should have been made to give a clearer estimate of the MPN.

Tables A1 and A2 give the MPN (and where applicable the MPR) for a 6-tube series containing 1x50 ml and 5x10 ml volumes: and an 11-tube series with 1x50 ml, 5x10 ml, and 5x1 ml volumes respectively. Table A3 relates to a 15-tube series with 5x10 ml, 5x1 ml and 5x0.1 ml volumes, but only the likely combinations of positive and negative reactions are listed. For example, multiple positive reactions in the 0.1 ml tubes would not be expected if all the 10 ml and 1 ml tubes are negative, and so combinations of 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice, with a frequency of greater than about 1 in 100 tests, it is an indication that the statistical assumptions underlying the MPN estimation are not being fulfilled[25, 26, 27]. For example, the organisms may not have been evenly distributed throughout the sample, or toxic or inhibitory substances may have been present in some of the tubes.

A1 Calculation of MPN values

Record the number of positive reactions, ie the number of tubes that show growth of the particular organism, for each set of tubes and, from the relevant table, read the MPN of organisms present in 100 ml of the sample.

Where a series of dilutions of the sample has been used, apply the following rules:

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

(ii) Select, wherever possible, three consecutive sets of dilutions, where the results are neither all positive, nor all negative. The most efficient statistical estimate will be obtained when about half the tubes are positive. (See examples a, b and c in Table A4).

(iii) If less than three sets of dilutions give positive results, start with the set containing the largest volume of the sample. (See example d in Table A4).

(iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower. (See example e in Table A4).

Where there is obvious evidence on inhibition, start with the highest result and use the next two. (See example f in Table A4).

Table A1 MPN and MPR per 100 ml of sample for a 6-tube series containing 1x50 ml and 5x10 ml volumes

Number of tubes giving a positive reaction		MPN per 100 ml	MPR* per 100 ml
1x50 ml	5x10 ml		
0	0	none found	
0	1	1	
0	2	2	
0	3	3	
0	4	4	4 – 5
0	5	6	
1	0	1	
1	1	2	
1	2	5	4 – 5
1	3	9	8 – 10
1	4	15	13 – 18
1	5	>18**	–

* MPR gives the range of counts that are as correct (at least 95% probability) as the MPN (see section 5.3.2.1).

** There is no discrimination when all tubes show growth; the theoretical MPN is infinity. The true count is likely to exceed 18.

Table A2 MPN and MRN per 100 ml of sample for an 11-tube series containing 1x50 ml, 5x10 ml and 5x1 ml volumes

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
1x50 ml	5x10 ml	5x1 ml		
0	0	0	none found	
0	0	1	1	
0	1	0	1	
0	1	1	2	
0	2	0	2	
0	2	1	3	
0	3	0	3	
1	0	0	1	
1	0	1	2	
1	1	0	2	
1	1	1	4	
1	1	2	6	
1	2	0	4	4 – 5
1	2	1	7	6 – 7
1	2	2	9	9 – 10
1	3	0	8	7 – 9
1	3	1	10	10 – 11
1	3	2	13	12 – 15
1	3	3	17	15 – 18
1	4	0	12	11 – 14
1	4	1	16	15 – 19
1	4	2	21	19 – 24
1	4	3	27	24 – 30
1	4	4	33	30 – 38
1	5	0	23	20 – 27
1	5	1	33	29 – 40
1	5	2	53	44 – 65
1	5	3	91	75 – 110
1	5	4	160	134 – 190
1	5	5	>180**	

* MPR gives counts which are at least 95% as probable as the MPN in being the correct number (see section 5).

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

Table A3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5x10 ml, 5x1 ml and 5x0.1 ml volumes.

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
5x10 ml	5x1 ml	5x0.1 ml		
0	0	0	none found	
0	0	1	2	
0	1	0	2	
1	0	0	2	
1	0	1	4	
1	1	0	4	
1	2	0	5	
2	0	0	4	
2	0	1	5	
2	1	0	5	

Table A3 (contd.)

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
5x10 ml	5x1 ml	5x0.1 ml		
2	1	1	7	
2	2	0	7	7 – 9
2	3	0	11	
3	0	0	7	
3	0	1	9	
3	1	0	9	
3	1	1	13	
3	2	0	13	
3	2	1	16	14 – 16
4	0	0	11	14 – 16
4	0	1	14	11 – 13
4	1	0	16	14 – 16
4	1	1	20	18 – 20
4	2	0	20	18 – 22
4	2	1	25	23 – 27
4	3	0	25	23 – 27
4	3	1	31	29 – 34
4	4	0	32	29 – 34
4	4	1	38	34 – 41
5	0	0	22	20 – 23
5	0	1	29	25 – 34
5	0	2	41	36 – 50
5	1	0	31	27 – 36
5	1	1	43	36 – 50
5	1	2	60	50 – 70
5	1	3	85	70 – 95
5	2	0	50	40 – 55
5	2	1	70	60 – 80
5	2	2	95	80 – 110
5	2	3	120	105 – 135
5	3	0	75	65 – 90
5	3	1	110	90 – 135
5	3	2	140	120 – 160
5	3	3	175	150 – 200
5	3	4	210	185 – 240
5	4	0	130	110 – 150
5	4	1	170	150 – 200
5	4	2	220	190 – 250
5	4	3	280	240 – 320
5	4	4	345	300 – 390
5	5	0	240	200 – 280
5	5	1	350	290 – 420
5	5	2	540	450 – 600
5	5	3	910	750 – 1100
5	5	4	1600	1350 – 1900
5	5	5	>1800**	

* MPR gives the range of counts that are as correct (at least 95% probability) as the MPN (see section 5).

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

Table A4 **Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions***

Example in text	Volume of sample (ml)					MPN per 100 ml
	10	1	0.1	0.01	0.001	
a	***5***	***3***	***2***	0		140
b	5	***5***	***3***	***2***	0	1400
c	5	***5***	***2***	***0***	0	500
d	***3***	***1***	***0***	0		9
e	***0***	***1***	***0***	0		2
f	0	2	***5***	***3***	***2***	14000

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

Appendix B

Additional media and reagents

B1 Choice of constituents

See also section 4.4.4.

B1.1 **Peptone**

In the preparation of media, the choice of peptone can be critical. It should form a clear solution at the concentration specified and should not form a precipitate when alkali is added, for example to adjust the pH.

B1.2 **Agar**

The gel strength of agar depends upon its purity, and the appropriate concentration should be determined. A satisfactory gel strength, at the concentration of agar specified, is assumed. It also assumes that the agar forms a clear solution that remains sufficiently clear for filtration to be unnecessary.

B1.3 **Bile salts**

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

B1.4 **Sterilization of media**

A time-temperature combination of 121 ± 1°C for 15 minutes is usually specified for many microbiological purposes. However, a temperature of 115 ± 1°C for a minimum of 10 minutes is often sufficient for certain media used in water examination.

B1.5 **Storage of media**

Generally, after sterilization, most media in sealed containers may be stored at room temperature in the dark. Media should be used as soon as possible after preparation and completion of satisfactory quality control tests, which are crucial factors in the performance of microbiological methods. Before use, media should be examined for any untoward signs such as contamination or excessive evaporation.

B1.6 **pH adjustment**

The correct pH of media is of vital importance. The measurement and, where necessary, adjustment of pH is part of the preparation of most media. All batches of medium should be checked after sterilization to ensure that the sterile medium is of the correct pH.

B2 Additional reagents and media

B2.1 **Diluents**

A wide range of diluents is available for the preparation of dilutions and for use in the preparation of suspensions for staining or immunological techniques. Whichever diluent is used, the appropriate dilutions should be prepared and inoculated into media, usually, within 5 minutes.

B2.1.1 **Saline solution**

Saline solution may be used for the preparation of slides for staining, for preparing dilutions or for slide and tube agglutination. Isotonic saline solution may be prepared by dissolving 8.5 g of sodium chloride in 1000 ml of water. The solution may be dispensed into screw-capped containers and sterilized by autoclaving at 121 ± 1°C for 15 minutes. Sterile saline solution may be stored in the dark at room temperature and should be used within 12 months.

B2.1.2 **Phosphate buffered saline solution**

Phosphate buffered saline solution (PBS) may be used for many applications including those listed for saline solution.

Sodium chloride	80 g
Potassium chloride	2 g
Disodium hydrogen phosphate	11.5 g
Potassium dihydrogen phosphate	2 g
Water	1000 ml

Dissolve the ingredients in the water and dispense in appropriate volumes into suitable screw-capped bottles. Adjust the pH to 7.3 ± 0.2 and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.3 ± 0.2. Phosphate buffered saline solution may be stored in the dark at room temperature and used within three months.

B2.1.3 **Quarter-strength Ringer's solution**

Quarter-strength Ringer's solution is normally used for the preparation of dilutions.

Sodium chloride	2.25 g
Potassium chloride	105 mg
Calcium chloride hexahydrate	120 mg
Sodium dihydrogen carbonate	50 mg
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The sterilized medium may be stored at room temperature in the dark and used within three months.

B2.1.4 **Maximum recovery diluent**

Maximum recovery diluent is a combination of saline solution with a low concentration of peptone designed to give better recovery than that obtained using water or saline solution alone. Because of the low level of peptone, dilutions should be plated as soon as practicable after preparation.

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH to 7.0 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable screw-capped containers and sterilize by autoclaving at 121 ± 1°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The sterilized medium should be stored at room temperature in the dark and used within one month.

B3 Stains

B3.1 **Gram stain**

The Gram stain is an important test in the correct differentiation of Enterobacteriaceae from other bacteria. The principle of the method involves "fixing" methyl violet or crystal violet in cells. Subsequent washing with acetone or ethanol removes the indicator from certain bacteria. These are termed "Gram-negative". Other organisms retain the stain and are termed "Gram-positive". The original method (described by Gram in 1884) has been modified and improved. Gram-positive and Gram-negative controls should be included with each batch of tests. *Staphylococcus aureus* is a suitable Gram-positive control, and *Escherichia coli* is a suitable Gram-negative control.

B3.1.1 **Methyl violet or crystal violet stain**

Methyl violet or crystal violet	10.0 g
Water	1000 ml

Add the indicator to the water and mix thoroughly to dissolve. Filter the solution and store in stoppered bottles in the dark at room temperature. The solution should remain stable indefinitely but may precipitate on keeping.

B3.1.2 **Gram's iodine**

Iodine	1.0 g
Potassium iodide	2.0 g
Water	300 ml

Dissolve the potassium iodide in 50 ml of water and then dissolve the iodine in the resulting solution. Add the remaining water and store the solution in stoppered bottles. This reagent should be stored at room temperature and should be stable for up to 12 months.

B3.1.3 **Counterstain**

Safranin	5.0 g
Water	1000 ml

Dissolve the safranin in the water and store at room temperature. This stain should be stable indefinitely.

B3.1.4 **Procedure**

Prepare a culture smear on a microscope slide in saline solution. "Thin" smears should be prepared, as "thick" smears tend to make examination of cellular morphology difficult to observe. In addition, some cells may retain the indicator stain and, hence, appear as Gram-positive. Allow the smears to dry and "fix", for example by repeatedly passing the microscope slide quickly through a small Bunsen flame. Saturate the slide with indicator solution and stain for approximately 2 minutes. Remove the excess stain with Gram's iodine solution and saturate the slide with fresh iodine solution. Allow the slide to stain for approximately 2 minutes. Remove excess iodine solution with tap water and allow the slide to drain.

De-colourize the slide with acetone or ethanol. De-colourization is, usually, rapid and the slide should be rapidly moved from side to side for 2 - 3 seconds whilst the stain is washed out. Rinse the slide with tap water. Counterstain the slide with the safranin solution for 2 - 3 minutes, wash with tap water, and air-dry. Examine the slide under oil immersion. Gram-positive bacteria appear as a purple colour and Gram-negative bacteria appear pink. Certain species of organisms, notably *Legionella* and *Campylobacter* species stain poorly with counterstain and extended staining for 5 minutes may be required.

B3.2 **Spore stain**[93]

Generally, spores may be observed in a Gram-stain. The following simple method may be used to confirm the presence of spores. It involves staining with hot aqueous malachite green solution followed by washing to de-colourize vegetative cells, and counter staining with safranin.

B3.2.1 **Malachite green solution**

Malachite green	5.0 g
Water	100 ml

Dissolve the stain in the water and filter. Store the solution in screw-capped bottles at room temperature. This stain should remain stable indefinitely but may precipitate on storage.

B3.2.2 **Counterstain – safranin**

Safranin	5.0 g
Distilled water	1000 ml

Dissolve the safranin in the water and store at room temperature. This stain should be stable indefinitely.

B3.2.3 **Procedure**

Prepare slides as described above, dry and "fix". Place the slide over a beaker of boiling water and saturate with malachite green solution. Alternatively, place the slide on a staining rack, saturate with malachite green solution and heat the slide gently until steam rises from the stain, continue to stain for 1 minute. Wash the slide in cold tap water. Counterstain the slide with the safranin solution for approximately 30 seconds. Remove excess stain with tap water, dry and examine under oil immersion. Spores are stained green and vegetative cells are stained red. Lipid granules remain unstained.

Appendix C

Report on recovery media trial for faecal coliform organisms

C1 Introduction

The need for a recovery phase, as part of the isolation procedure, for the isolation of coliform organisms and faecal coliform organisms from recreational waters was investigated. In some circumstances, direct isolation methods yielded lower recoveries than methods using a non-selective pre-enrichment step. An interlaboratory trial, to compare a membrane filtration method, a multiple tube method, and a method involving membrane filtration with a short period of incubation on a non-selective medium, was, therefore, undertaken[94].

C2 Procedure

Eight laboratories participated in the trial, namely: the Ministry of Agriculture, Fisheries and Food Laboratories, Weymouth; Clyde River Purification Board; Guildford Public Health Laboratory; Hull Public Health Laboratory; Lothian Regional Council; Manchester Public Health Laboratory; Yorkshire Water Authority; and Welsh Water Authority. The study was undertaken between June 1987 and February 1988. Media and materials were purchased centrally from single batches in order to minimise differences due to batch variation. The statistical analysis was carried out at the Communicable Disease Surveillance Centre, Central Public Health Laboratory, Colindale, London.

Samples of water, collected aseptically from recreational areas, were examined, quantitatively, for coliform organisms and faecal coliform organisms by membrane filtration and the multiple tube method using membrane lauryl sulphate broth and minerals modified glutamate medium respectively. Each sample was examined using membrane filtration with and without a recovery phase. The recovery phase comprised incubation on tryptone soy agar containing 0.1% m/v yeast extract at 30°C for 4 hours before transfer of the membrane filter to membrane lauryl sulphate broth and incubation at 44°C. Confirmation of faecal coliform organisms was established by subculture to nutrient agar, followed by oxidase testing and acid and gas production at 37°C and 44°C, and indole production at 44°C.

C3 Results

A total of 408 samples were examined, 212 samples from fresh water sites and 196 samples from sea water sites. Figure C1 shows the distribution of confirmed faecal coliform counts for the three methods, namely, membrane filtration with recovery phase, membrane filtration without recovery phase, and the multiple tube method. As indicated, there is no significant difference in the median count values observed between the three methods.

Table C1 shows the confirmed faecal coliform organism counts by membrane filtration with a recovery phase compared with the count from the same sample obtained by membrane filtration without the recovery phase. Generally, there was no consensus that the methods differed, except that laboratory G reported significantly different results, where 61 out of 84 samples gave higher counts with the recovery phase. The bottom row of Table C1 shows the results without the inclusion of those obtained from laboratory G.

Table C2 shows the same comparison between counts from each sample, with and without the recovery phase. The results from laboratory G are separately identified because the results differed from those results obtained by the other laboratories. With fresh waters, there was no significant difference between the methods, except for laboratory G. With sea waters, there were 81 higher counts obtained with the recovery phase compared with 68 obtained without the recovery phase. This difference is not significant (McNemar's test, p = 0.3). Again the results from laboratory G were significantly different (p = 0.0001).

C4 Observations

Except for laboratory G, no method, with or without a recovery phase, gave significantly different results. The proportion of isolates at 44°C confirming as faecal coliform organisms was approximately 95%.

Table C1 Confirmed faecal coliform counts by membrane filtration with and without recovery phase

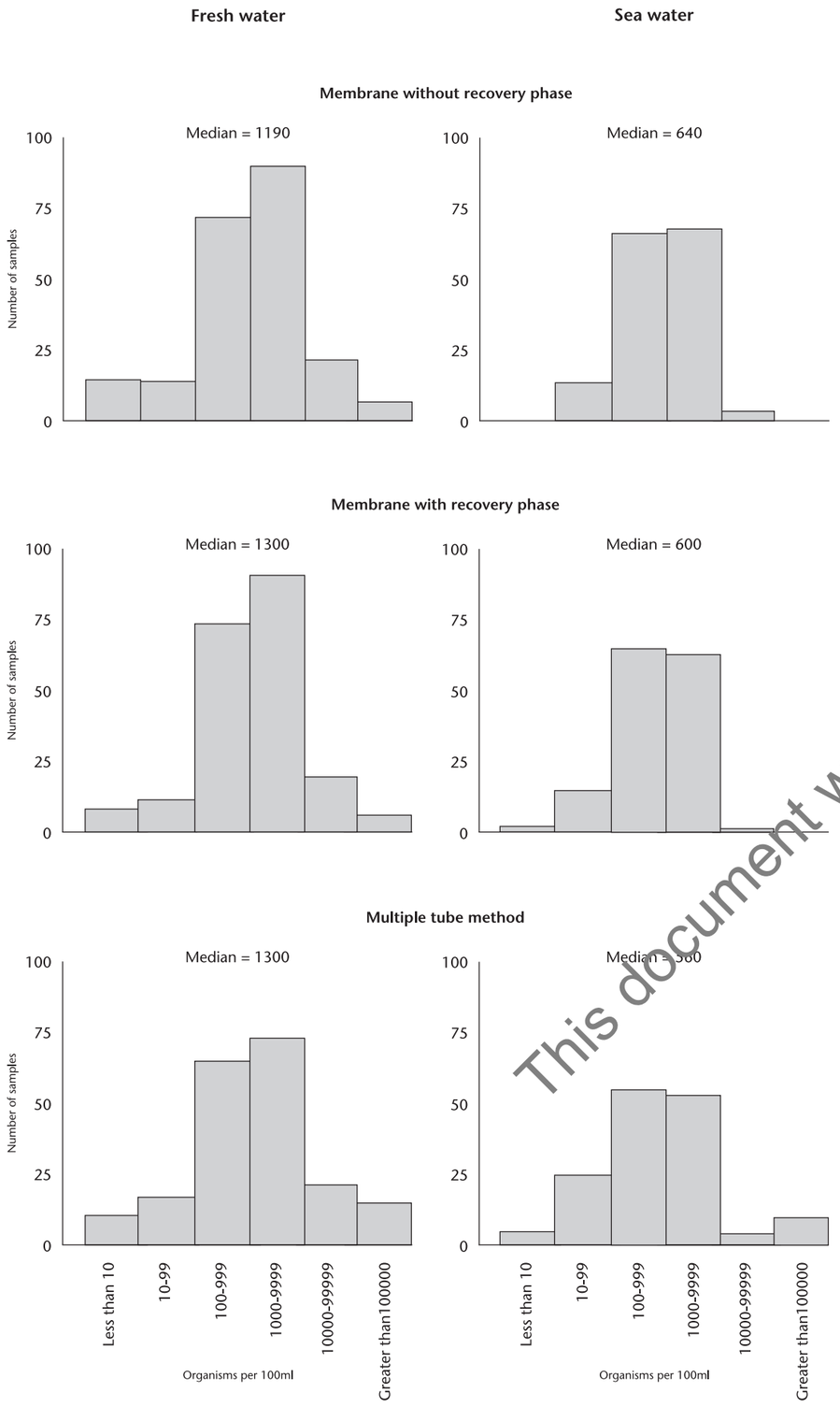
Laboratory	Higher count with recovery phase	Equal	Higher count without recovery phase	Total samples
A	19	2	23	44
B	8	2	15	25
C	54	4	35	93
D	5	4	3	12
E	6	3	6	15
F	42	6	38	86
G	61	12	11	84
H	26	1	22	49
Total	221	34	153	408
Total - G	160	22	142	324

For an additional 47 samples, the confirmed counts were not reported, but the unconfirmed counts showed no significant difference between the methods.

Table C2 Comparison of results with and without the recovery phase

	Higher count with recovery phase	Equal	Higher count without recovery phase	Total Samples
Fresh water				
Laboratory G	30	8	6	44
All other laboratories	79	15	74	168
Sea water				
Laboratory G	31	4	5	40
All other laboratories	81	7	68	156

Figure C1 Confirmed thermo-tolerant coliform organism counts



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

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