Monitoring Bathing Waters - A Practical Guide to the Design and Implementation of Assessments and Monitoring Programmes
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Chapter 8*: SANITARY INSPECTION AND MICROBIOLOGICAL WATER QUALITY

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Sanitary inspection, water quality determination and data analysis and interpretation are essential elements in characterising the microbiological safety of water in recreational areas. Sanitary inspection is a necessary adjunct to water microbiological analysis. A well-conducted sanitary inspection can identify sources of microbiological hazards, microbiological water quality data confirm the presence of hazards, and the two together allow an estimation of the risk of illness to bathers and other users. In assessing the microbiological quality of recreational waters, it will normally be necessary to conduct:

- An intensive sanitary inspection (only once as part of an assessment or annually in monitoring programmes).

- Periodic appraisal visits in which water quality analysis and shortened inspections are undertaken.

- Follow-up appraisals to investigate abnormal events, new sources of pollution and extreme values of pollution indicators.

One of the most important aspects of aquatic microbiology is related to several human diseases transmitted via water. The design and development of epidemiological surveillance studies described in Chapter 13 have led to the awareness of the magnitude of human morbidity and mortality associated with waterborne infectious diseases. The most relevant micro-organisms and the associated waterborne infectious diseases are summarised in the *WHO Guidelines for Safe Recreational Water Environments* (WHO, 1998). The derivation of guideline values for microbiological quality are also discussed in the *WHO Guidelines for Safe Recreational Water Environments* (WHO, 1998).

This present chapter deals with sanitary inspection, microbiological analytical methods and data handling and reporting. Strategies to implement sanitary inspections and recommendations for selection of the site and frequency of water sampling are given in Chapter 9. Specific methods for sampling and analysis are detailed in the following sections together with the different statistical procedures to express the overall microbiological water quality at a specific recreational water use area. It should be noted that a single beach or recreational area may vary widely in relation to microbiological measures of health risk within relatively short periods of time and thus the commonly

used methods of defining a recreational water as passing or failing a defined microbiological standard has inherent limitations; these are discussed in this chapter and also in Chapter 9.

8.1 Sanitary inspection and sampling programmes

A sanitary inspection is a search for, and evaluation of, existing and potential microbiological hazards that could affect the safe use of a particular stretch of recreational water or bathing beach. It provides the foundation required to design and implement an effective water quality sampling programme and provides valuable information to assist in the interpretation of water quality data. In particular, it provides public health authorities with information to aid the selection of sampling locations, times and frequencies, in order to estimate more accurately water quality and therefore to allow for sound risk management decisions (see Chapter 9).

A comprehensive sanitary inspection of an existing recreational area should be conducted annually, just prior to the bathing season. The annual inspection should not only look for new sources of microbiological hazards but also review the adequacy of any sampling programme and corrective measures in place to deal with existing hazards. Further inspections should be conducted along with routine sampling during the bathing season, in order to identify recent events and their impact on water quality. During the peak bathing season additional inspections at different days and times of the day may provide a more complete picture of the bathing area.

Comprehensive inspections should be conducted prior to any major new or proposed activity which could significantly alter the microbiological quality of the water in an existing recreational water use area. A sanitary inspection should therefore be carried out as part of, or in response to, any proposal to expand or develop a new recreational bathing area. The findings of the inspection should receive prime consideration in any decision to proceed with development. A comprehensive annual sanitary inspection consists of four steps:

- Pre-inspection preparations.

- An on-site visit.

- The preparation of a preliminary report including recommendations on location of sampling sites and changes to the sampling frequency if necessary.

- The preparation of a final assessment report often in combination with water quality data.

While sampling, important field data can be obtained at each bathing area by inspecting specific sources of pollution. Microbial contamination may be suspected, for example, when inspection reveals abnormal colouration or odour of the water at the bathing site. In the Mediterranean coastal area where the influence of tides is minimal, changes in microbiological quality are mainly due to riverine and direct, especially urban, discharges at the bathing site. The microbiological contamination produced by long sea outfalls, if well designed, is normally diluted and should not influence the microbiological quality of

the bathing area. Land-based sources of contamination are normally associated with smaller discharges or with the likelihood of heavy rain events, characteristic of the Mediterranean climate at the end of the summer period, where a great amount of water falls in a very short period of time. Heavy rain may wash out faeces from pastures or other agricultural land and directly influence microbiological water quality. Studies in other regions also document pulses of poor water quality associated with rainfall events (O'Shea and Field, 1992; Vonstille *et al.,* 1993; Armstrong *et al.,* 1997; Wyer *et al.,* 1994, 1995, 1997). In inland recreational waters the main sources of pollution are water inlets (PHLS, 1995). Therefore, influences from rivers, natural watercourses and, particularly around populated areas, combined sewer overflows, produce important changes in the microbiological quality of bathing waters. Sporadic malfunctioning of sewerage systems can produce similar problems (Davis *et al.,* 1995; Marsalek *et al.,* 1996). These events, if recent, can sometimes be recognised visually at the recreational site by changes in the appearance of the water. In marine recreational waters, a field analysis of the salinity can indicate the discharges of freshwater at the bathing site. Such measurements indicate indirectly, that land-borne discharges are occurring.

8.1.1 Pre-inspection preparations

The collection and review of any existing data or reports on the area, including reports of previous inspections, will allow a thorough and efficient on-site evaluation. Topographical maps and aerial photographs are useful tools for locating activities and features that could affect water quality and for establishing sampling sites. Historical data on tides, currents, prevailing winds, rainfall and discharges of sewage, storm overflows and combined sewer overflows, and urban and agricultural effluents should be collected and reviewed to determine the impact of these events, (either singly or collectively) on water quality. Depending on the availability of water quality data, experts conducting the annual inspection may need to collect samples for microbiological analyses. Therefore, adequate numbers of sterile sample bottles and sampling equipment should be readily available and prior arrangements should be made with the microbiology laboratory to process samples promptly after collection. Arrangements should be made to meet with user groups and with individuals in charge of any facility or activity that affects, or has the potential to affect, water quality in the recreational area. It will be essential to obtain the trust and co-operation of the groups or individuals if the survey is to provide an accurate assessment of water quality and to identify and remedy unacceptable water quality (see Chapter 6).

8.1.2 On-site visit

The purpose of the on-site visit is to identify and evaluate all existing and potential sources of microbiological contamination that could affect the safe use of the area. Attention should be paid to the presence of sewage disposal facilities, including long sea outfalls, industrial outfalls, seabird colonies, sanitary sewers, and rivers, tributaries, streams or ditches receiving sewage, storm water or agricultural runoff. All data recorded should be added to the catalogue of basic characteristics to form a catalogue of inspections that would enable the tracking of trends and influences (see Chapter 2).

Visual faecal pollution (including sanitary plastics), sewage odour and suspicious water colour should also be considered as an immediate indication of unacceptable water quality. Adjacent industries should also be identified and their impact assessed. The

impact of local geography and meteorological conditions on water quality should also be evaluated. In most cases it will be necessary to collect representative water samples to confirm the presence of faecal pollution, to establish its variability and to identify the source. Non-toxic fluorescent tracer dyes, bacteriophages (such as PDR-1) or faecal sterol biomarkers (coprostanol and 24-ethylcoprostanol) may also be helpful to identify sources of contamination.

Epidemiological studies have shown that bathers can be a significant source of pathogenic micro-organisms (Seyfried *et al.,* 1985; Calderon *et al.,* 1991; Cheung *et al.,* 1991). In small bathing areas with a lot of bathers and a low rate of water turnover, the person to person disease transmission has to be considered, even if there is no source of faecal pollution from the outside. The assessment may therefore need to consider measures to control microbiological water pollution by bathers in the area. This is especially important in shallow, enclosed areas used by young children where water circulation and flushing rates are low. Intensive studies to locate sources of pollution and to propose remedial actions have been undertaken successfully (Wyer *et al.,* 1994; Tsanis *et al.,* 1995; Marsalek *et al.,* 1996). A specially designed form can assist in the process of comprehensive sanitary inspection (Box 8.1).

Box 8.1 Sanitary inspection form

Background information

Area name and code number: _____
Location: _____

Type of water: Fresh ☐ Marine ☐ Estuarine ☐
Responsible authority: _____
Address: _____
Tel. _____ Fax. _____ E-mail _____

Laboratory of analysis:
Name: _____
Distance (km) _____ Sample transport time (h) _____
Person responsible for samples during transport: _____

What land or human activity surrounds the bathing area? (check all that apply)

Forest ☐ Fields ☐ Desert ☐ Hills ☐ Swamp ☐ River/stream/ditch ☐

Agriculture (specify) ☐ _____ Urban ☐ Commercial ☐

Residential ☐ Industry (specify) ☐ _____ Hotel ☐

Harbour ☐ Airport ☐ Road/rail ☐ Military ☐ Waste tip ☐ Other ☐
Is the area surrounding the bathing area urban? ____

Additional details (historical information, reason for assessment, other contacts, etc.):
Size of bathing area: Area (m^2) ___ Length (m) ___ Mean width (m) ___
Is there a beach? ___ Average area (m^2) ___ Length (m) ___
Width (m) at high tide ___ Width (m) at low tide ___
Prevailing onshore winds: Direction ____ Typical speed (km/h) ____
Prevailing water currents: Direction ____ Typical speed ($m\ s^{-1}$) ____
Shoreline configuration ____ Presence of sandbars ____

Average wave heights: ____
Rainfall: Total annual ____ Seasonal patterns _____
Temperature:

Water: Average ___ Annual low __ Annual high ___
Air: Average ___ Annual low __ Annual high ___
Public facilities: No. of toilets ___ Showers ___ Drinking water fountains ___
Litter bins ___ (are they animal and/or bird-proof? ___)
Are methods in place to warn the public of danger? ____
Are the above facilities adequate?

Accessibility: Road ❑ Path ❑ No access ❑
Is there an adequate parking area? _____
Additional details _____

Microbiological hazards

a) Sewage and animal wastes. Is the water quality affected, or likely to be affected, by discharges from:

On-site or other private sewage disposal systems ❑ Communal sewage disposal or treatment facilities ❑ Long sea outfalls ❑ Agricultural activities ❑ Aquacultural activities ❑ Unconfined domestic or wild animals and birds ❑ Confined animals or birds (i.e. feedlots) ❑

Are discharges continuous or sporadic? _____

Is wastewater from toilets, showers, etc. likely to contaminate the bathing area? _____

Will typical bather densities impair water quality? _____

b) Storm water runoff. Is the water quality affected or likely to be affected by non-point discharges from:

Municipal storm drains or combined sewer overflows? ❑ Agricultural fields? ❑ Natural drainage? ❑
Are onshore winds likely to carry polluted water into the bathing area? _____
Are currents likely to carry polluted water into the bathing area? _____
Are tides likely to affect water quality in the bathing area? _____
Microbiological water quality data or additional information:_____

Note: Any of the above with a “yes” answer require a detailed investigation and risk analysis. This investigation should include:

- Proximity of potential contamination source to bathing area.

- Background and contamination incident flow rates.

• Effective rainfall which triggers contamination events (and typical duration of contamination).

• For discharges from sewage systems or treatment facilities, include what type of treatment is used, the system capacity, flow rates and variability, and indicator standards.

• For animals/birds, stocking densities and types of animals, indicator data will be necessary to support and supplement this information.

Chemical and other hazards

Water quality

Is the water likely to be affected by: Discharges from industrial sources? ❑Agricultural drainage? ❑Water craft mooring or use? ❑Urban surface runoff? ❑
Are onshore winds likely to carry polluted water into the bathing area? ____
Are currents likely to carry polluted water into the bathing area? ____
Are tides likely to affect water quality in the bathing area? ____

Sand quality

Is the sand likely to be affected by: Discharges from industrial sources? ❑Agricultural drainage? ❑Water craft mooring or use? ❑Urban surface runoff? ❑
Are plastic residues present? ___ Are tar residues present? ___
Are algae present? ___ Are other residues present? ___
Supporting chemical water quality data or additional comments: _____

Note: Any of the above with a “yes” answer require a detailed investigation and risk analysis. This investigation should include:

• Proximity of potential contamination source to bathing area.

• For boats, densities and pumpouts.

• For urban surface runoff, the effective rainfall.

• For discharge from industrial sites, the type of discharge, treatment being used, flow rates and variability, system capacity and chemical/indicator standards.

Please attach a map of the beach area included in this sanitary inspection, with possible contamination sources (rivers, storm drains, outfalls, etc.) marked. If possible, maps of the entire catchment area indicating land-use, topography, and infrastructure networks (i.e. wastewater and storm drain systems, etc.) should also be attached.

Reporting systems

Are there formal mechanisms for reporting waste discharges, spills, treatment bypasses, etc. to the local health authorities? ___

Is there an illness or injury reporting mechanism in place that would be effective for

epidemiological investigations? ___

Sampling or posting recommendations. This section should describe circumstances which indicate the need to post warning notices or close beaches and provide information such as sampling locations, times and frequencies.

8.1.3 Sampling location, time and frequency

The first step in planning a sampling activity is to define clearly the objective; in most cases the objective will be either exploratory (assessment) or monitoring (surveillance). While the former is designed to provide preliminary or "one-off information about a site, the latter is undertaken for regulatory or non-regulatory purposes" (Keith, 1990). For a recreational water use area, both objectives may initially coincide. Exploratory sampling will be required to define subsequent sampling. Special requirements for epidemiological studies will be necessary as highlighted in Chapter 13.

The selection of sampling sites, time and frequency of sample collection should attempt to capture the overall microbiological quality of the water at the recreational water use area. These choices should be based upon the information gathered during the sanitary inspection. The selection of sampling stations and time of sampling should take into consideration, variables known to affect water quality, such as the length of the bathing area, presence and periodicity of point and non-point sources of faecal contamination, influences of local weather, the physical characteristics of the bathing area and the presence of bathers. For example, at bathing areas with no detectable sources of external faecal contamination, samples should be collected at the places with the greatest bather densities. Bathing areas known to be influenced by direct or indirect faecal contamination will require additional sampling sites to help define the degree and extent of pollution. The time of day can be an important source of variation (Brenniman *et al.,* 1981; Fleisher, 1985; Tillett, 1993; PHLS, 1995) especially at beaches with significant tides (Cheung *et al.,* 1991). Consideration should also be given to collecting samples at times when bather densities are greatest for example, afternoons at weekends (Cheung *et al.,* 1991; APHA, 1995). Chapter 9 gives an example of an approach to a sampling programme.

Sampling frequency can also influence the acquisition of reliable information on microbiological pollution in a bathing area (Fleisher, 1990; Tillett, 1993). For those laboratories with limited economic or human resources it is better to direct efforts towards increasing sampling frequency instead of confirming presumptive results for *Escherichia coli* and faecal streptococci. The sampling frequency adopted in many programmes and assessments is fortnightly during the bathing season. Some authors have advocated more frequent samplings such as weekly or more, especially in the peak season in temperate climates (Figueras *et al.,* 1997) and others maintain lower intensity monitoring (e.g. monthly) outside the bathing season. Evidence suggests that once an understanding of quality behaviour has been developed through relatively intensive monitoring and sanitary surveys, then reduced sampling frequencies may be justifiable and can contribute to reducing the burden of monitoring (Chapter 9). For colder climates where the bathing season is restricted by weather, water sampling should be concentrated in that period where historical data show a higher probability of favourable weather conditions for recreational activities. If abnormal favourable weather conditions

appear, more frequent sampling should be carried out, especially in freshwater resources with poor water circulation that may be overcrowded under those circumstances.

Monitoring a bathing area or a site to reconfirm repeated failure to meet a guideline or poor water quality has little value. Equally, sampling frequency can be reduced when an area is known, through historical microbiological data, to have consistently good microbiological quality and when it is known from the catalogue of basic characteristics that it will not be subjected to pollution influences because potential sources of contamination are absent. In these situations only occasional confirmatory sampling will be required. Such an approach will direct resources to those beaches known to have variable water quality (see Chapter 9).

Resampling and new sanitary inspection, following the detection of unexpected peak values, is essential to establish the cause of the observed peak. An exhaustive investigation, including an inspection of the site and possible collection of additional samples to locate the source or sources of pollution, is also essential where the cause is known not to be due to a sporadic event. The effect of episodic events, such as heavy rainfall, on the water quality of bathing beaches, and the management response to such events, is discussed in Chapter 9.

8.2 Sampling

8.2.1 Sampling procedures

Sampling in chest depth water, typically 1.2-1.5 m depth, represents areas of greatest bather density although sampling at ankle depth may be appropriate to determine risk to young children.

Microbiological counts from surface samples have been shown to have a tendency to be higher than those beneath the surface (PHLS, 1995), but the epidemiological significance of this has yet to be studied. Therefore samples should be collected from beneath the surface. Precise sampling recommendations vary, for example 30 cm below the surface is indicated by the American Public Health Association (APHA) (APHA/AWWA/WPCF, 1992) and the European Community (EC) Directive (EEC, 1976), while the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) (WHO/UNEP, 1994a) have proposed 25 cm. Every sample within a monitoring programme should be taken as near as possible to the defined sampling location.

Care must be taken to avoid external contamination during sample collection. Sterilised sample bottles should be opened with the opening facing downward and should be held by the base and submerged in the water. At the appropriate depth, the bottle should be turned upwards with the mouth facing the current (if any). After retrieving the bottle, some water should be discarded to leave an air space of at least 2.5 cm to allow mixing by shaking before examination (APHA/AWWA/WPCF, 1989; Bartram and Ballance, 1996). The utmost care must be taken at all times not to touch the top of the bottle during removal or replacement of the cap.

The sample volume should be sufficient to carry out all the required tests. In practice, 300-500 ml are adequate. If *Salmonella, Vibrio cholerae* or enteroviruses are to be analysed, as required by some authorities or under certain circumstances, greater volumes of water will be necessary (1.5 litres, 10 litres and 10 litres respectively). Bottles of borosilicate glass or suitable autoclavable plastic (PHLS, 1994; Bartram and Ballance, 1996) are recommended. They should have screw caps that withstand repeated sterilisation at 121°C or 180°C. Quality assurance procedures, as described in Chapter 4, should be followed. All sampling bottles should be correctly labelled with the reference of the sampling point. Additional information of the time of collection, temperature of water and other observations should be recorded on sample record sheets designed for this purpose.

8.2.2 Sample storage

There is little published information available that gives a consensus on the time limit for storage of samples to avoid changes in the concentrations of indicator organisms (Gameson and Munro, 1980; Tillet and Benton, 1993). Storage times should be as short as possible and it is recommended here that samples should be analysed as soon as possible, preferably within 8 hours of collection. If samples cannot be analysed within 24 hours field analysis should be considered. Immediately after collection, the samples should be stored in insulated boxes with cooling packs (prefrozen packs) and/or ice. Samples should be kept in the dark and the temperature of the cooling box maintained below 10°C where possible (APHA/AWWA/WPCF, 1995). This temperature may be difficult to reach and so in practice samples should be kept as cold as possible, but not frozen. In practical terms these storage conditions can have at best only a limited effect on reducing variations in bacterial populations. It is generally accepted that changes in microbial populations in water samples will begin to occur around 2 hours after collection; within 6 hours the samples are likely to have altered significantly particularly if no cooling mechanism was available and the samples were exposed to light. The key factor to consider in storage and transport of samples is time between collection and analysis rather than the time between collection and receipt at the laboratory. Ideally, the temperature of the insulated box should be controlled and recorded, as should the storage time. This information should be considered in the interpretation of results. Storage under these conditions should be as short as possible, and samples should be analysed promptly after collection.

8.3 Index and indicator organisms

Natural waters are subject to important changes in their microbial quality that arise from agricultural use, discharges of sewage or wastewater resulting from human activity or storm water runoff. Sewage effluents contain a wide variety of pathogenic micro-organisms that may pose a health hazard to the human population when the effects are discharged into recreational waters. The density and variety of these pathogens are related to the size of the human population, the seasonal incidence of the illness, and dissemination of pathogens within the community (Pipes, 1982). Appropriate indicators of faecal contamination under various conditions are discussed in Chapter 9 and in the *Guidelines for Safe Recreational Water Environments* (WHO, 1998).

Many waterborne pathogens are difficult to detect and/or quantify and the specific methodology to detect them in environmental water samples has still to be developed

(Borrego, 1994). While faecal streptococci are suggested as the recommended indicator for salt water, either faecal streptococci or *Escherichia coli* can be used for monitoring freshwaters. Additional variables can be investigated if they are considered relevant, such as the spores of *Clostridium perfringens* in tropical waters where the traditional indicators may increase in number in soil and water (Hardina and Fujioka, 1991; Anon, 1996). Staphylococci are generally assumed to serve as indicators of water pollution deriving from bathers themselves (i.e. by shedding from the body surface). The epidemiological significance of the recovery of Staphylococci remains unclear.

8.3.1 Thermotolerant coliforms and *E. coli*

Thermotolerant (faecal) coliforms constitute the subset of total coliforms that possess a more direct and closer relationship with homeothermic faecal pollution (Geldreich, 1967). These bacteria conform to all the criteria used to define total coliforms (all are aerobic and facultatively anaerobic, Gram-negative, non-spore forming rod-shaped bacteria that ferment lactose with gas and acid production in 24-48 hours at 36 ± 1°C), but in addition they grow and ferment lactose with production of gas and acid at 44.5 ± 0.2°C within the first 48 hours of incubation. For this reason, the term “thermotolerant coliforms” rather than “faecal coliforms” is a more accurate name for this group (WHO, 1993). The physiological basis of the elevated temperature phenotype in the thermotolerant coliforms has been described as a thermotolerant adaptation of proteins to, and their stability at, the temperatures found in the enteric tracts of animals (Clark, 1990). Thermotolerant coliforms include strains of the genera *Klebsiella* and *Escherichia* (Dufour, 1977). The thermotolerant coliform definition is not based on strictly taxonomic criteria, but on specific biochemical reactions or on the appearance of characteristic colonies on selective and/or differential culture media. Certain *Enterobacter* and *Citrobacter* strains are also able to grow under the conditions defined for thermotolerant coliforms (Figueras *et al.,* 1994; Gleeson and Gray, 1997). *E. coli* is, however, the only biotype of the family Enterobacteriaceae that is almost always faecal in origin (Bonde, 1977; Hardina and Fujioka, 1991). Therefore, the thermotolerant coliform group when used should ideally be replaced by *E. coli* as an indicator of faecal pollution. For the purpose of water testing, most *E. coli* can be confirmed by a positive indole test and by their inability to use citrate (as the only carbon source) in the culture medium. Alternatively, *E. coli* can be distinguished easily enzymatically by the lack of urease or presence of β-glucuronidase enzymes. The enzymes can be recognised easily using culture media that contain specific substrata (Gauthier *et al.,* 1991; Brenner *et al.,* 1993; Walter *et al.,* 1994).

However, several studies have indicated the limitation of both the thermotolerant coliform group and *E. coli* as ideal faecal indicators or pathogen index organisms. Several thermotolerant *Klebsiella* strains have been isolated from environmental samples with high levels of carbohydrates in the apparent absence of faecal pollution (Dufour and Cabelli, 1976; Knittel *et al.,* 1977; Niemi *et al.,* 1997). Similarly, other members of the thermotolerant coliform group, including *E. coli,* have been detected in some pristine areas (Rivera *et al.,* 1988; Ashbolt *et al.,* 1997) and have been associated with regrowth in drinking water distribution systems (Lechevallier, 1990). The principal disadvantages of this organism as an indicator in water are: (i) its detection in other environments without faecal contamination (Hazen and Toranzos, 1990; Hardina and Fujioka, 1991), and (ii) its low survival capability in aquatic environments when compared with faecal pathogens (Borrego *et al.,* 1983; Cornax *et al.,* 1990).

8.3.2 Faecal streptococci and enterococci

Faecal streptococci have received widespread acceptance as useful indicators of faecal pollution in natural aquatic ecosystems. These organisms show a close relationship with health hazards (mainly for gastrointestinal symptoms) associated with bathing in marine and freshwater environments, (Cabelli *et al.,* 1982, 1983; Dufour, 1984; Kay *et al.,* 1994; WHO, 1998). They are not as ubiquitous as coliforms (Borrego *et al.,* 1982), they are always present in the faeces of warm-blooded animals (Volterra *et al.,* 1986), and it is believed that they do not multiply in sewage-contaminated waters (Slanetz and Bartley, 1965). Enterococci, however, have been shown to grow in freshly stored urine (Höglund *et al.,* 1998). Nonetheless, their die-off rate is slower than the decline in coliforms in seawater (Evison and Tosti, 1980; Borrego *et al.,* 1983) and persistence patterns are similar to those of potential water-borne pathogenic bacteria (Richardson *et al.,* 1991). Reviews of all these aspects have been carried out by Sinton *et al.,* (1993a,b).

The group called faecal streptococci includes species of different sanitary significance and survival characteristics (Gauci, 1991; Sinton and Donnison, 1994). In addition, the proportion of the species of this group is not the same in animal and human faeces (Rutkowski and Sjogren, 1987; Poucher *et al.,* 1991). The taxonomy of this group, comprising species of two genera *Enterococcus* and *Streptococcus* (Holt *et al.,* 1993), has been subject to extensive revision in recent years (Ruoff, 1990; Devriese *et al.,* 1993; Janda, 1994; Leclerc *et al.,* 1996). Although several species of both genera are included under the term enterococci (Leclerc *et al.,* 1996), the species most predominant in polluted aquatic environments are *Enterococcus faecalis, E. faecium* and *E. durans* (Volterra *et al.,* 1986; Sinton and Donnison, 1994; Audicana *et al.,* 1995).

Enterococci, a term commonly used in the USA, includes all the species described as members of the genus *Enterococcus* that fulfil the following criteria: growth at 10°C and 45°C, resistance to 60°C for 30 minutes, growth at pH 9.6 and at 6.5 per cent NaCl, and the ability to reduce 0.1 per cent methylene blue. The most common environmental species fulfil these criteria and thus in practice the terms faecal streptococci, enterococci, intestinal enterococci and *Enterococcus* group can be considered synonymous.

8.3.3 Alternative faecal indicators

The lack of a strong relationship between faecal indicators and health outcomes in a number of epidemiological studies in warm tropical waters may, in part, relate to the inappropriate nature of *E. coli* or faecal streptococci as indices of waterborne pathogens in these recreational waters. In this context an alternative index group, sulphite-reducing clostridia or spores of *Clostridium perfringens,* have been proposed and are used in Hawaii (Anon, 1996).

Spores of *C. perfringens* are largely faecal in origin (Sorensen *et al.,* 1989), they are always present in sewage (about 10^4-10^5 colony forming units (cfu) per 100 ml), they are highly resistant in the environment and appear not to reproduce in aquatic sediments (which appears to be the case with thermotolerant coliforms) (Davies *et al.,* 1995). It is interesting to note, however, that dog faeces may have some 9×10^8 cfu *C. perfringens* per gram dry weight (dw), whereas pig faeces are similar to humans (4.8×10^5 cfu *C. perfringens* per gram dw). *C. perfringens* is generally less common or absent in other warm blooded animals. Hence, although dogs have a similar number of thermotolerant

coliforms and faecal streptococci to that found in humans, the relatively higher ratio of *C. perfringens* spores found in dog faeces may be a useful indicator when fresh faecal contamination is being investigated (Leeming *et al.,* 1998).

It is important to note that spores of *C. perfringens* do not act as an indicator for non-sewage or animal faecal contamination in general, and therefore they are only suitable as indicator organisms for parasitic protozoa and viruses from sewage-impacted waters (Payment and Franco, 1993; Ferguson *et al.,* 1996). Their resistance to disinfectants may also be an advantage for indexing disinfectant-resistant pathogens. Simple anaerobic culture is possible for *C. perfringens* spores after a short heat treatment to remove vegetative cells. Confirmation of their presence may be assisted by the addition of a methylumbelliferyl phosphate substrate to the growth medium (Davies *et al.,* 1995).

Other indicator organisms for sewage, but also specific for human sewage are the bacteriophages to *Bacteroides fragilis* HSP40. These *B. fragilis* phages appear to survive in a manner that is similar to the hardier human enteric viruses under a range of conditions (Jofre *et al.,* 1995; Lucena *et al.,* 1996). Their numbers in sewage such as the F-specific RNA bacteriophages may be an order of magnitude lower than various coliphages. Furthermore, only 1-5 per cent of humans may excrete these phages (Leeming *et al.,* 1998), and thus they may be unsuitable pathogen indicator organisms for small communities. The International Office for Standardization (ISO) standard methods for these phages are under final review (ISO, 1999c).

The ratio between thermotolerant coliforms and faecal streptococci has been proposed by Geldreich (1976) as a means of distinguishing between human and animal-derived faecal matter. However, this method is no longer recommended (Howell *et al.,* 1995) and none of the currently-used bacterial indicators distinguish different sources of faecal matter confidently when used alone (Cabelli *et al.,* 1983), although genetic typing of *E. coli* shows some potential (Muhldorfer *et al.,* 1996). Identification of human enteric viruses can identify specifically the presence of human faecal material although the necessary procedures are difficult and expensive, and not readily quantifiable. Other microbiological options include specific identification of phenotypes of *Bifidobacterium* spp. (Gavini *et al.,* 1991), *Bacteroides* spp. (Kreader, 1995), serotypes of F-specific RNA bacteriophages (Osawa *et al.,* 1981) or, as previously discussed, the bacteriophages to *Bacteroides fragilis* (Puig *et al.,* 1997). However none of these organisms are suitable for quantifying the proportion of human faecal contamination. Moreover, no one indicator or single approach is likely to represent all the facets and issues associated with faecal contamination of waters.

Recently, Leeming *et al.* (1994, 1996) demonstrated the ability to distinguish human from herbivore-derived faecal matter using a range of faecal sterol biomarkers (Table 8.1). The distribution of sterols found in faeces, and hence their source-specificity, is caused by a combination of diet, the animal's ability to synthesise its own sterols and the intestinal microbiota in the digestive tract. The combination of these factors determines “the sterol fingerprint”. The principal human faecal sterol is coprostanol (5β(H)-cholestan-3β-ol), which constitutes about 60 per cent of the total sterols found in human faeces. The C29 homologue of coprostanol is 24-ethylcoprostanol (24-ethyl-5β(H)-cholestan-3β-ol). In large quantities (relative to coprostanol), this faecal sterol is indicative of faecal contamination from herbivores. It is possible to determine the contribution of faecal matter from these two sources relative to each other by calculating

the ratio of coprostanol to 24-ethylcoprostanol in human and herbivore (sheep and cow) faeces (Leeming *et al.,* 1996) and comparing these to ratios obtained for water samples (Leeming *et al.,* 1998). Other animals that are ubiquitous in urban areas such as dogs and birds, either do not have coprostanol in their faeces or have it in trace amounts only (Leeming *et al.,* 1994).

Table 8.1 Examples of faecal sterol biomarkers

Systematic name	Common name	Comments
C_{27} sterols		
5β-cholestan-3α-ol	Coprostanol	Human faecal biomarker; high relative amounts indicate fresh human faecal contamination
5β-cholestan-3α-ol	Epi-coprostanol	Present in sewage sludges; high relative amounts suggest older faecal contamination
cholest-5-en-3β-ol	Cholesterol	C_{27} precursor to 5α- and 5β-stanols
5α-cholestan-3β-ol	Cholestanol	The thermodynamically most stable isomer is ubiquitous; if the ratio of coprostanol to cholestanol is < 0.5, origin of 5β-stanols may not be faecal
C_{29} sterols		
24-ethyl-5β-cholestan-3β-ol	24-ethylcoprostanol	Herbivore faecal biomarker; high relative amounts indicate herbivore faecal contamination
24-ethyl-5β-cholestan-3α-ol	24-ethyl-*epi*-coprostanol	Present in some herbivore faeces
24-ethylcholest-5-en-3β-ol	24-ethylcholesterol	C_{27} precursor to 5α- and 5β-stanols
24-ethyl-5α-cholestan-3β-ol	24-ethylcholestanol	The thermodynamically most stable isomer is ubiquitous

Faecal sterols generally associate with particulate matter, and can be concentrated from 1-10 litres of water by simply filtering the water through a glass fibre filter (such as type OFF, Whatman). The lipids are extracted by acetone, concentrated, derivatised and quantified by gas chromatography. Thus the method requires a suitable chemistry laboratory and may cost ten times more than that for the analysis of *E. coli* and enterococci. Nonetheless, it is an appropriate method for specific studies investigating the proportion of human and animal faecal contamination.

8.4 Analytical methods

8.4.1 Most Probable Number

Most Probable Number (MPN) analysis is a statistical method based on the random dispersion (Poisson) of micro-organisms per volume in a given sample. Classically, this assay has been performed as a multiple-tube fermentation test. Although the technique

is rather time consuming (taking between five and seven days), several laboratories prefer it to other methods of water analysis because it is applicable to all sample types.

The MPN technique is generally conducted in three sequential phases (presumptive, confirmatory, and complete), each phase requiring 1 to 2 days of incubation. In the initial or presumptive phase, three volumes of samples (usually 10, 1, and 0.1 ml) (Table 8.2 and Figures 8.1 and 8.2) are inoculated into 3, 5, or 10 tubes containing the appropriate medium to allow the target bacteria to grow (Figure 8.2; Tables 8.3 and 8.4). In this test, it is assumed that any single viable target organism in the sample will result in growth or a positive reaction in the medium.

Table 8.2 Recommended serial dilutions for water samples in relation to the degree of microbiological contamination and type of indicator

Type of water	Serial dilutions for thermotolerant coliforms[1]					Serial dilutions for faecal streptococci				
Sewage	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Secondary effluent	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	1	10^{-1}	10^{-2}	10^{-3}	
Contaminated bathing water	10	1	10^{-1}	10^{-2}	10^{-3}	10	1	10^{-1}		
Clean water	10	1	10^{-1}			100	10	1		

[1] E. Coli
Source: Anon, 1983

Figure 8.1 Preparation of a series of dilutions

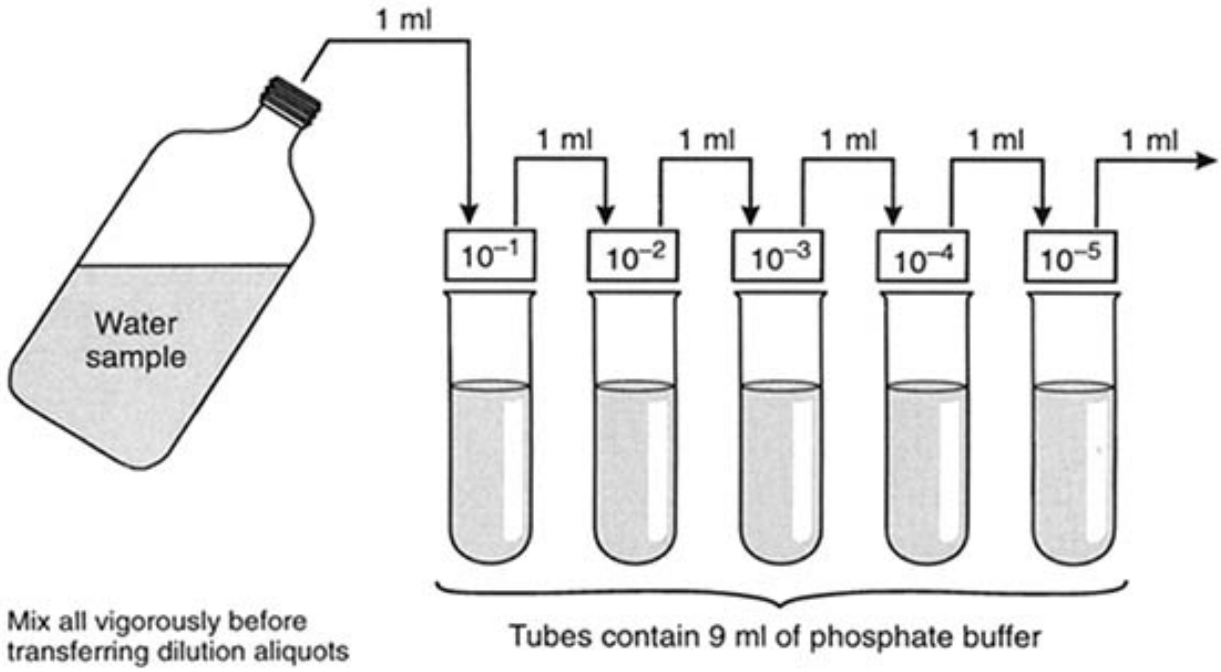
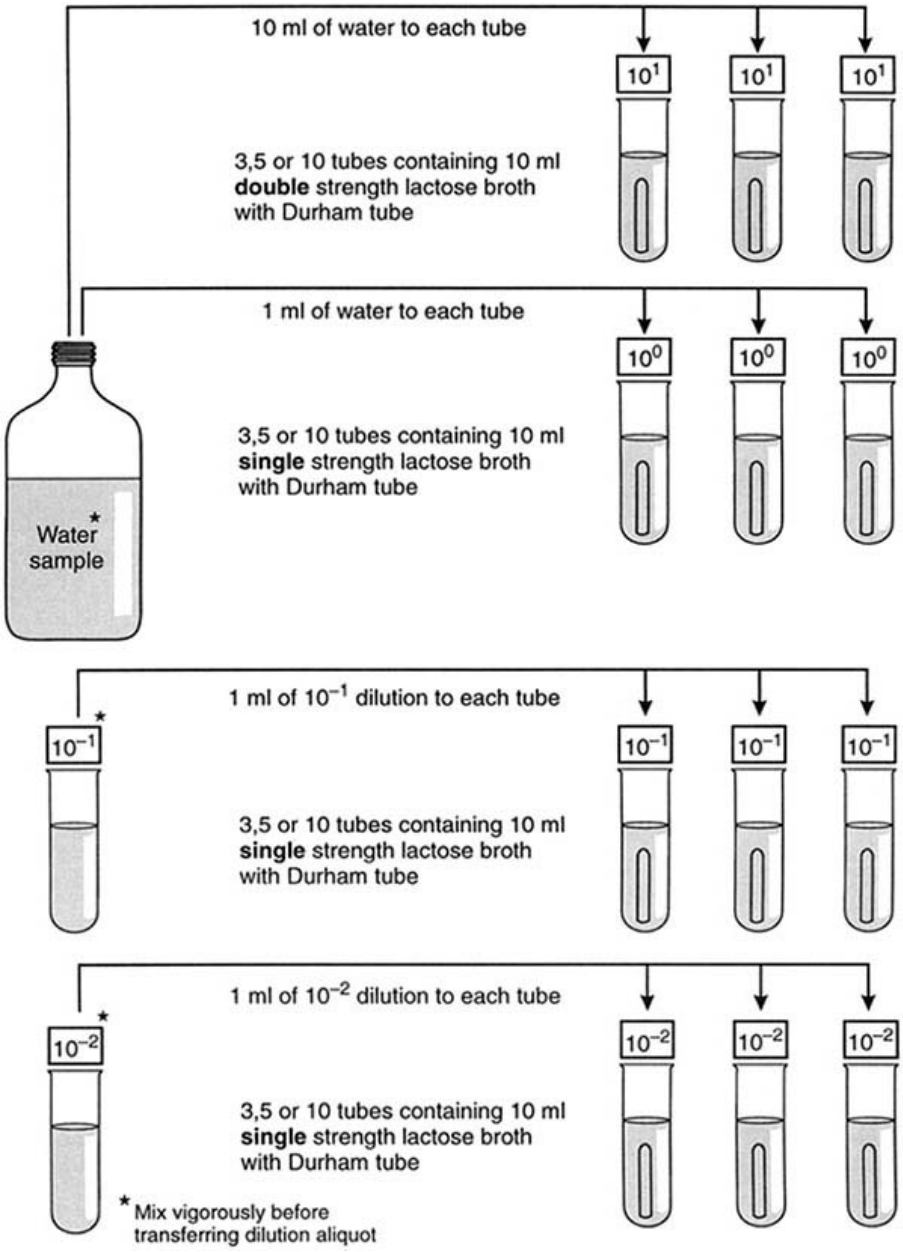


Figure 8.2 Inoculation scheme for the multiple test tube method



After the incubation period, all the inoculated presumptive positive tubes must be inoculated into a more selective medium to confirm the presence of the target bacteria (confirmatory phase). The confirmation test is reliable evidence, but not proof, that the target bacteria have been detected. Therefore, subsamples of the confirmed positive reactions should be inoculated onto a selective agar medium and several verification tests (Gram stain, and biochemical, serological or enzymatic tests) should be carried out (Tables 8.3 and 8.4). This completed test is generally conducted on 10 per cent of the positive tubes as a quality control measure. For practical purposes, the number of

positive and negative tubes in the confirmatory phase of the technique is generally used to determine the MPN of the target bacteria by using tables of positive and negative tube reactions (WHO/UNEP, 1994a; APHA/AWWA/WPCF, 1995).

The major advantages of the MPN technique are (Fujioka, 1997):

- It will accept both clear and turbid samples.

- It inherently allows the resuscitation and growth of injured bacteria.

- The results may be recorded by personnel with minimal skill.

- Minimal preparation time and effort are required to start the test, and therefore processing of samples can be initiated at any time of the day.

By contrast the MPN technique may also have several disadvantages, such as:

- The total time, labour, material and costs required to analyse one sample.

- The substantial increase in reagents, tubes, incubation space and cleanup requirements when multiple samples need to be analysed or when the sample volume must be increased to 100 ml.

- The multiphase nature of the technique, each phase requiring a 24 hour or 48 hour incubation period.

- The fact that MPN is a simple estimated number, while the true number (95 per cent confidence limit) may show extreme variation from the MPN.

The choice of precision level of the technique (using 3, 5 or 10 tubes of each dilution) depends on the required detection sensitivity, because the total volumes analysed by each are 33.3, 55.5, and 111 ml, respectively. Miniaturised MPN methods with 96 incubation wells (e.g. ISO 1996a,b) are more precise than traditional five-tube tests with three descending decimal dilutions and equivalent to membrane filtration (Hernandez *et al.,* 1991, 1995). The existing standardised procedures for the MPN technique are given in Tables 8.3 and 8.4.

Table 8.3 Standard methods for the determination of thermotolerant coliforms (*E. coli* presumptive) - MPN methods

ISO 9308-2 (ISO, 1990c)[1]	ISO 9308-3 (96 wells)[2] (ISO, 1998)	APHA
Isolation media		
a) Lactose broth b) MacConkey broth c) Lauryl tryptose (lactose) broth d) Formate lactose glutamate medium	Tryptone salicine triton MUG broth (MU/EC)	a) EC medium, or b) A-1 medium
Incubation conditions	*Incubation conditions*	*Incubation conditions*
24-48 h at 35 ± 1 °C or 37 ± 1 °C	36-72 h at 44 ± 0.5 °C	a) 24 ± 2 h at 44.5 ± 0.2 °C b) 3 h at 35 ± 0.5 °C followed by 21 ± 2 hat 44.5 ± 0.2 °C
Reaction	*Reaction*	*Reaction*
Turbidity = (+)	Blue fluorescence = (+) for *E. coli*	a) and b) Gas production = (+) for thermotolerant coliforms
Confirmatory media tests		
Two confirmatory methods can be used:	Confirmatory tests are not required	If using EC medium, verify with the following test:
A. With two steps		
1. a) EC medium b) Brilliant green lactose (bile) broth		Brilliant green lactose (bile) broth
Incubation conditions		*Incubation conditions*
24 h at 44 ± 0.25°C or 44.5 ± 0.25°C		24 ± 2 h at 44.5 ± 0.2 °C
Reaction		*Reaction*
Gas production = (+) for thermotolerant coliforms		Gas production = (+) for thermotolerant coliforms
2. Tryptone water		If using A-1 medium, a confirmatory test is not required
Incubation conditions		
24 h at 44 ± 0.25°C or 44.5 ± 0.25°C		
Reaction		
Indol production with indol reagent Kovacs = (+) for *E. coli*		
B. With one step		
Lauryl tryptose mannitol broth with tryptophan		
Incubation conditions		
24 h at 44 ± 0.25 °C or 44.5 ± 0.25 °C		
Reactions		
Gas production = (+) and indol = (+/-) for thermotolerant coliforms; gas production = (+) and indol = (+) for *E. coli*		

MPN Most probable number

APHA American Public Health Association

MUG 4-methylumbelliferyl-β-D-glucoside

[1] ISO 9308-2 is at an early stage of revision by an ISO working group

[2] Not suitable for drinking water - lower limit of detection is 15 counts per 100 ml

Table 8.4 Standard methods for the determination of faecal streptococci (enterococci) - MPN methods

ISO 7899-1 (ISO, 1984a)[1]	**APHA**
Isolation media	
Azide dextrose broth	Azide dextrose broth
Incubation conditions	*Incubation conditions*
22 ± 2 h at 35 ± 1 °C or 37 ± 1 °C; negative tubes may be re-incubated for 22 ± 2 h	24 ± 2 h at 35 ± 0.5 °C; negative tubes may be re-incubated until 48 ± 3 h
Reaction	*Reaction*
Turbidity = (+)	Turbidity = (+)
Confirmatory media tests	
Two tests are recommended:	Two tests are recommended:
1. BEAA	1. PSE agar
Incubation conditions	*Incubation conditions*
44 ± 4 h at 44 ± 0.5 °C	24 ± 2 h at 35 ± 0.5 °C
	Reaction
	Brownish-black colonies with brown halos (+) for faecal streptococci
2. Catalase	2. BHIB containing 6.5% NaCl
	Incubation conditions
	24 h at 45°C
Reaction	*Reaction*
Dark brown to black colonies surrounded by black halos are (+) BEAA, with a (-) catalase test = faecal streptococci	Turbidity = (+) A (+) PSE with a (+) BHIB (6.5% NaCl) = enterococcus group

MPN Most probable number

APHA American Public Health Association

BEAA Bile esculin azide agar

PSE Pfizer selective enterococcus

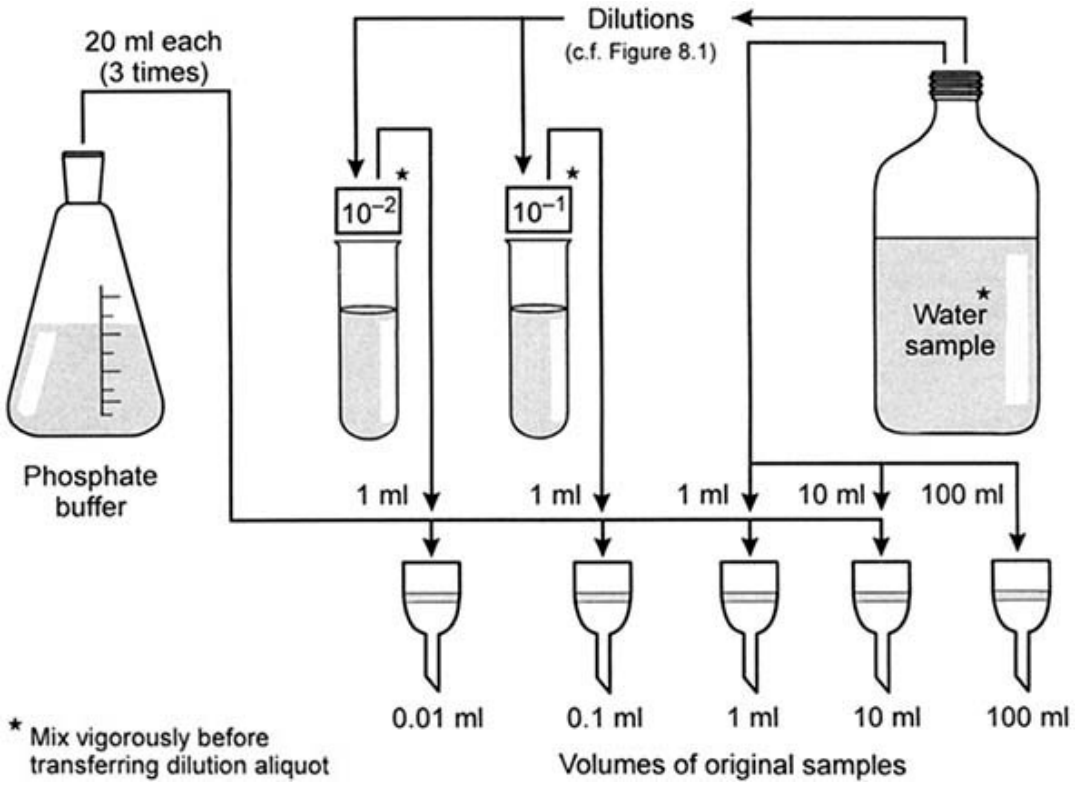
BHIB Brain heart infusion broth

[1] ISO 7899-1 has been replaced recently with a new methodology proposed under the same ISO reference as in Table 8.7

8.4.2 Membrane filtration

The membrane filtration (MF) technique is based on the entrapment of the bacterial cells by a membrane filter (pore size of 0.45 µm) (Figure 8.3). After the water is filtered, the membrane is placed on an appropriate medium and incubated (Tables 8.5, 8.6 and 8.7). Discrete colonies with typical appearance are counted after 24-48 hours, and the population density of the target bacteria, usually described as cfu per 100 ml in the original sample, can be calculated from the filtered volumes and dilutions used. This technique is more precise than the MPN technique, but the MF test can only be used for low-turbidity waters with low concentrations of background micro-organisms.

Figure 8.3 Preparation of dilution series and procedure for the membrane filtration method



The advantages of the MF technique include (Fujioka, 1997):

- Savings in terms of time, labour, and cost compared with the MPN technique.

- Direct determination of the concentrations of bacteria with high precision and accuracy.

- The formation of the target bacteria as colonies which can be purified for further identification and characterisation.

- The ability to process large volumes of water samples to increase greatly the sensitivity of this method.

Several disadvantages of the MF technique have also been reported:
• Inapplicability of the method to turbid samples which can clog the membrane or prevent the growth of the target bacteria on the filter.

• False negative results due to the inability of viable but non-culturable bacteria present in environmental waters to grow with standard MF methods.

• False positive results when non-target bacteria form colonies similar to the target colonies (Figueras *et al.,* 1994. 1996; Hernandez-Lopez and Vargas-Albores, 1994).

Table 8.5 Standard methods for the determination of thermotolerant coliforms (*E. coli* presumptive) - MF methods

ISO 9308-1 (ISO, 1990b)[1]	**APHA**
Isolation media	
a) TTC agar with Tergitol-7 or Teepol b) Lactose agar with Tergitol-7 or Teepol c) Membrane enrichment Teepol broth d) m-FC medium with 1% rosolic acid in 0.2N NaOH added e) Laurylsulphate broth	m-FC medium with 1% rosolic acid in 0.2N NaOH added (if there is interference with background growth)
Incubation conditions	*Incubation conditions*
18-24 h at 44 ± 0.25°C or 44.5 ± 0.25°C; a pre-incubation of 4 h at 30°C is recommended	24 ± 2 h at 44.5 ± 0.2°C
Reaction	*Reaction*
Depends on the media selected	Blue colonies = (+)
Confirmatory media tests	
Verify a representative number of colonies. Two confirmatory methods can be used:	Verify by picking at least 10 typical colonies; by two tests:
A. With two steps	
1. Lactose peptone water	1. Lauryl tryptose broth
Incubation conditions	*Incubation conditions*
24 h at 44 ± 0.25°C or 44.5 ± 0.25°C	24-48 h at 35 ± 0.5°C
Reaction	*Reaction*
Gas production = (+) for thermotolerant coliforms	Gas production = (+)
2. Tryptone water	2. EC broth
Incubation conditions	*Incubation conditions*
24 h at 44 ± 0.25°C or 44.5 ± 0.25°C	24 h at 44.5 ± 0.2°C
Reaction	*Reaction*
Indol production with indol reagent Kovacs = (+) for *E. coli*	Growth (+) and gas production = (+) for thermotolerant coliforms
B. With one step	
Lauryl tryptose mannitol broth with tryptophan	
Incubation conditions	
24 h at 44 ± 0.25°C or 44.5 ± 0.25°C	
Reaction	
Gas production = (+) and indol = (+/-) for thermotolerant coliforms; gas production = (+) and indol = (+) for *E. coli*	

MF Membrane filtration

APHA American Public Health Association

TTC Triphenyl-tetrazolium chloride

[1] ISO 9308-1 is under revision. Only one culture medium (a) has been chosen and is proposed under the same ISO reference in Table 8.7

Table 8.6 Standard methods for the determination of faecal streptococci (enterococci) - MF methods

ISO 7899-2 (ISO, 1984b)[1]	**APHA**
Isolation media	
a) KF streptococcus agar with 1% sterile solution of TTC added to cooled basal medium b) Slanetz-Bartley agar[2] with 1% sterile solution of TTC added to cooled basal medium	a) m-E agar for enterococci, or b) m-Enterococcus agar[2] for faecal streptococci
Incubation conditions	*Incubation conditions*
44 ± 4 h at 35 ± 1 °C or 37 ± 1 °C; however, if other types of micro-organisms are expected use 5 ± 1 h at 37 ± 1 °C followed by 44 ± 0.5°C until 48 h	a) 48 h at 41 ± 0.5 °C; transfer membrane fil- to esculin iron agar for 20 min ± 2 h at 41 ± 0.5 °C b) 48 h at 35 ± 0.5 °C
Reaction	*Reaction*
a) and b) Colonies: red, brown or pink (+)	a) and b) Colonies: pink to red (+)
Confirmatory media tests	
Verify a representative number of colonies by two tests:	Verify at least 10 well-isolated typical colonies by sub-culturing on:
1. BEAA	BHIA
Incubation conditions	*Incubation conditions*
48 h at 44 ± 0.5 °C	24-48 h at 35 ± 0.5 °C
2. Catalase	Transfer a loop-full of growth to:
Reaction	BHIB
Dark brown to black colonies surrounded by black halos are (+) BEAA, with a (-) catalase test = faecal streptococci	*Incubation conditions*
	24 h at 35 ± 0.5 °C
	A series of five tests are recommended for confirmation:
	1. Catalase
	2. Gram
	3. BEA
	Incubation conditions
	48 h at 35 ± 0.5 °C
	Reaction
	Growth = (+)

4. BHIB

Incubation conditions

48 h at 45 ± 0.5 °C

Reaction

Growth = (+)

5. BHIB containing 6.5% NaCl

Incubation conditions

48 h at 35 ± 0.5 °C

Reaction

Turbidity = (+)

Final reaction

A (+) BEA with a (+) BHIB 45 °C (test number 4) = faecal streptococci; a (+) BEA with (+) BHIB 45 °C (test number 4) and (+) BHIB 6.5% NCI (test number 5) = enterococci

MF Membrane filtration
KF KF streptococcus agar
BEAA Bile esculin azide agar
BHIB Brain heart infusion broth
APHA American Public Health Association
TTC Triphenyl-tetrazolium chloride
BHIA Brain heart infusion agar
BEA Bile esculin agar

[1] ISO 7899-2 is currently under revision; the new proposed version under the same ISO reference is given in Table 8.7

[2] Slanetz-Bartley has the same formulation as m-Enterococcus but the latter already includes TTC

Table 8.7 Recently proposed modifications to ISO standard methods

MF methods for conforms and *E. Coli* ISO DIS 9308-1 (ISO, 1997)[1]	**MF methods for intestinal enterococci ISO DIS 7899-2 (ISO, 1999b)[2]**	**MPN methods for intestinal enterococci ISO DIS 7899-1 (96 wells) (ISO, 1999a)[3]**
Isolation media	***Isolation media***	***Isolation media***
For a standard test use a lactose TTC agar with Tergitol-7, incubate for 21 ± 3 h at 36 ± 2°C[4]; typical colonies will turn the medium yellow For a rapid test use tryptone soya agar and incubate for 4-5 h at 36 ± 2°C[4]	Use a m-Enterococcus agar (Slanetz-Bartley) with a 1% sterile solution of TTC added to the cooled basal medium, incubate for 44 ± 4 h at 36 ± 2°C[4]; typical colonies are light and dark red	Use a medium with tryptose, nalidixic acid, TTC thallium acetate and MUD (MUD/SF medium); incubate for 36-72 h at 44 ± 0.5°C; fluorescence indicates intestinal enterococci

Confirmatory media tests	***Confirmatory media tests***	***Confirmatory media tests***
In the case of the standard test, verify all or a representative number of typical colonies (at least 10), using the following series of tests: 1. Non selective agar (i.e. tryptone soya agar); incubate for 21 ± 3 h at 36 ± 2°C[4] 2. Oxidase test; the non-appearance of a dark purple colour within 5-10 s indicates a negative result; a (-) oxidase = coliform bacteria 3. Tryptophane broth, incubate for 21 ± 3 h at 44 ± 0.5°C[4] Add indol reagent; indol production (i.e. a red ring) indicates a positive result A (-) oxidase and (+) indol = *E. coli* For a rapid test, transfer the membrane filter to tryptone bile agar, incubate for 19-20 h at 44 ± 0.5°C[4]; place the membrane filter on a filter paper saturated with indol reagent; the appearance of red colonies = *E. coli*	Transfer the membrane filter to a bile esculin azide agar, preheated at 44°C; incubate at 44 ± 0.5 °C for 1 h The appearance of dark brown to black colonies surrounded by black halos = intestinal enterococci	Tests are not required

MF Membrane filtration

MPN Most probable number

TTC Triphyl-tetrazolium chloride

MUD 4-methylumbelliferyl-β-D-glucoside

[1] Suitable for drinking water with low background growth

[2] Suitable for drinking water, swimming pools and other water with low intestinal enterococci

[3] Not suitable for drinking water; lower limit of detection is 15 counts per 100 ml

[4] These conditions substitute and standardise those from the previous ISO 9308-1 and 7899-2

E. coli has been demonstrated to be a more specific indicator for the presence of faecal contamination than the thermotolerant coliform group (Dufour, 1977). Improvements in both MPN and MF techniques have been carried out for the rapid and selective enumeration of *E. coli.* Barnes *et al.* (1989) designed a rapid seven hour membrane filter test for quantification of thermotolerant coliforms from drinking water samples and other freshwaters and salt waters, although it is not suitable for salt water due to the high proportion of false positives obtained. Fluorogenic and chromogenic tests using 4-methylumbelliferyl-β-D-glucuronide (MUG) have been applied in MPN and MF techniques, for the detection of β-glucuronidase that is specific to *E. coli* (Manafi and Kneifel, 1989; Balebona *et al.,* 1990; Gauthier *et al.,* 1991; Rice *et al.,* 1991). A miniaturised MPN method with a 96-well microplate has been developed for *E. coli* (Hernandez *et al.,* 1991; ISO, 1996b) (Table 8.3). Based on this principle a number of different media have been developed for the use in MF and MPN techniques (Frampton *et al.,* 1988; McCarty *et al.,* 1992). Commercially available media include Colisure (formerly Millipore, now IDEXX) (McFeters *et al.,* 1995), Colilert (IDEXX) (Edberg *et al.,* 1988; Palmer *et al.,* 1993), m-ColiBlue (Hach), ColiComplete (BioControl), Chromocult (Merck) and MicroSure (Gelman). Similar media for the detection of *E. coli* in water have also been described (Sartory and Howard, 1992; Brenner *et al.,* 1993; Walter *et al.,* 1994). Molecular methods have also been designed to detect specifically *E. coli* from water samples, such as PCR-gene probes for the *uid* gene (Bej *et al.,* 1991a,b; Tsai *et al.,* 1993; McDaniels *et al.,* 1996). In addition, other alternative techniques, i.e. enzyme capture (Kaspar *et al.,* 1987) and radioisotopes (Reasoner and Geldreich, 1989) have been proposed.

8.5 Laboratory procedures

8.5.1 Faecal streptococci and enterococci

Early attempts to quantify faecal streptococci relied on enrichment tube procedures and the MPN technique; Rothe Azide Dextrose broth followed by a confirmation in Ethyl Violet Azide (Litsky) broth being the procedure most widely accepted by researchers. A rapid system for enumeration of faecal streptococci or enterococci in water samples using a miniaturised fluorogenic assay based on a 96-well microplate MPN system has been described by several workers (Hernandez *et al.,* 1991; Poucher *et al.,* 1991; Budnicki *et al.,* 1996) and the technique has recently been proposed as an ISO method (Table 8.7). In addition, Enterolert (IDEXX) is available for the MPN technique with up to 100 ml of sample, and has been shown to be reliable (Fricker and Fricker, 1996).

The enumeration of faecal streptococci by a MF procedure using a selective medium was first reported by Slanetz and Bartley (1957). Since then, several media have been proposed, including Thallous Acetate agar (Barnes, 1959), KF agar (Kenner *et al.,* 1961), PSE agar (Isenberg *et al.,* 1970), Kanamycin Aesculin Azide (KEA) agar (Mossel *et al.,* 1973), mSD agar (Levin *et al.,* 1975), and mE agar (APHA/AWWA/WPCF, 1989). The accepted standardised procedures for the MF method are given in Table 8.6. Other media formulations and incubation procedures for faecal streptococci have been proposed for specific situations (Lin, 1974), such as increasing the membrane incubation period from 48 hours to 72 hours to recover stressed faecal streptococci. Rutkowski and Sjogren (1987) developed a medium, designated M2, to distinguish between human and animal pollution sources.

The methods for enumeration of faecal streptococci from natural waters have been compared by different authors (Volterra *et al.,* 1986; Yoshpe-Purer, 1989). Dionisio and Borrego (1995) compared eight methods for the specific recovery of faecal streptococci from natural freshwater and marine waters on the basis of the following characteristics: accuracy, specificity, selectivity, precision and relative recovery efficiency. The results obtained indicated that none of the tested methods showed perfect selectivity. The methods that showed the best performance characteristics were the MPN technique (with Rothe and Litsky media) and the m-Enterococcus agar in conjunction with the MF technique. The latter is the only technique recommended in the “Standard Methods” for faecal streptococci in conjunction with membrane filtration (APHA/AWWA/WPCF, 1995). A rapid confirmation technique, based on the transplantation of the membrane from m-Enterococcus agar after incubation for 48 hours at 36 ± 1°C to Bilis-Esculin-Agar (BEA) for 4 hours additional incubation, improves the low specificity of the m-Enterococcus agar, enabling the confirmation of 100 per cent of the colonies (Figueras *et al.,* 1996). A similar procedure has been proposed by ISO (Table 8.7).

Recently, Audicana *et al.* (1995) designed and tested a modification of the KEA agar, named Oxolinic acid-Aesculin-Azide (OAA) agar, to improve the selectivity in the enumeration of faecal streptococci from water samples by the MF technique. The OAA agar showed higher specificity, selectivity and relative recovery efficiencies than those obtained when using m-Enterococcus and KF agars. In addition, no confirmation of typical colonies was needed when OAA agar was used, which shortens the time taken significantly and increases the accuracy of the method. The excellent performance of this culture medium was recently reconfirmed in a routine monitoring programme for bathing waters (Figueras *et al.,* 1998). A Europe-wide standardisation trial demonstrated that the m-Enterococcus agar with total confirmation of the colonies (Figueras *et al.,* 1996), the OAA medium (Audicana *et al.,* 1995), and the miniaturised MPN method (Hernandez *et al.,* 1991) produced the best results (Hernandez *et al.,* 1995).

8.5.2 Thermotolerant coliforms and *E. coli*

The presumptive detection of thermotolerant coliforms can be considered sufficient to give an estimation for the presence *of E. coli.* The EC and A-1 media are the most widely recommended for the presumptive detection of thermotolerant coliforms with the MPN technique (APHA/AWWA/WPCF, 1992). The differences between the two approaches are based on the incubation periods: 44.5 ± 0.2 °C for 24 hours for the EC medium, and 36 ± 1 °C for 3 hours and transfer to 44.5 ± 0.2 °C for 21 hours for the A-1 medium. The tubes containing gas and acid in EC medium are confirmed in the same medium by subsequent incubation at 44.5 ± 0.2 °C for 24 hours. The A-1 medium does not require a confirmation test. Table 8.3 details accepted media. Thermotolerant coliform density and the 95 per cent confidence limits can be estimated with the use of MPN tables (APHA/AWWA/WPCF 1995; Bartram and Ballance, 1996).

The mFC agar is the most frequent medium used to quantify thermotolerant coliforms in water samples when the MF technique is used. Petri dishes containing filters are incubated at 44.5 ± 0.2 °C for 24 hours. Typical thermotolerant coliform colonies appear various shades of blue, atypical *E. coli* may be pale yellow, and non-thermotolerant coliform colonies are grey to cream in colour. Table 8.5 details accepted standardised media.

The existing ISO methods are now under revision. Table 8.7 shows the proposed modifications. The unification of temperature precision has been introduced by ISO as 36 ± 2 °C and 44 ± 0.5 °C.

8.6 Field analyses

Field analysis embraces all the tests that can be performed completely or partially at the site of sampling. Several field analysis techniques have been developed for drinking waters, where the principal requirement is the absence of indicator organisms (Manja *et al.,* 1982; Bernard *et al.,* 1987; Dange *et al.,* 1988; Dutka and El-Shaarawi, 1990; Smoker, 1991; Ramteke, 1995; Grant and Ziel, 1996). Quantitative on-site analysis using the MF technique is also possible (Bartram and Ballance, 1996).

The microbiological quality of bathing waters is presently assessed by the techniques described previously for indicator organisms. Field analyses may be preferred when the time between the collection of the sample and its examination will be long. Field laboratory equipment with filtration and incubation devices are being marketed. The time taken to obtain presumptive results will be the same as at a standard laboratory.

On-site filtration with a delayed incubation is another possibility when conventional procedures are impractical, i.e. when it is not possible to maintain the desired temperature during transport, and when the time between sample collection and analysis will exceed the optimum time limit. With this procedure, filters are placed in water tight plastic Petri dishes with a transport medium, and in conditions that maintain viability but will not allow visible growth. The test is completed at the laboratory by transferring the membranes to appropriate selective media and incubating them for the period of time required. It has to be recognised that growth will start if high temperatures are encountered during transport. Delayed incubation has been found to produce results consistent with those from immediate standard tests (Chen and Hickey, 1983, 1986; APHA/AWWA/WPCF, 1995; Brodsky *et al.,* 1995).

The continuous demands for more rapid techniques that can be performed on site and provide direct results have yet to be satisfied, despite the advances in analytical methods, particularly those based on DNA chips or arrays (Eggers *et al.,* 1997). By contrast, a one-hour assay for thermotolerant coli-forms has been demonstrated for marine bathing beaches, based on MUG detection of β-glucuronidase activity with a portable fluorometer (Davies and Apte, 1996, 1999).

8.7 Data recording, interpretation and reporting

Analysis may be performed as part of a regulatory monitoring programme, as part of a survey of an area used for water recreation, or as part of an epidemiological study in which water quality is related to risks to health from infectious diseases. Each approach has its own requirements which are specified at the outset by the regulatory authority or the study director. One of the most important functions of the analyst is to provide reliable and accurate results in a form that can be recorded for statistical interpretation and reporting, as described in Chapter 3. The following guidance will help the microbiological analyst achieve this aim.

8.7.1 Forms and records

An individual record must be produced for each site inspection or sample and should include the location and reference of the sampling site (that should ideally be equal to the code number of the sample), the date, the time, weather conditions, tide, water temperature, results of visual inspection for abnormal conditions, sources of contamination and the name of the inspector or sampler. Sampling records should also list the laboratory procedures and results (method of analysis, dilutions or volumes analysed, time of analysis, results for each step, any anomalies in the analysis of results and the name of the analyst). Ideally, record forms should occupy a single page. Forms should be conveniently archived, because they will be used later by the data handlers for transcription to the database and they will be analysed for the purposes of preparing the report of the monitoring or survey programme. Great care should be taken in preparing the report and its contents and format should be agreed by those responsible for analysis, data handling and for reporting results and, if necessary, by those responsible for co-ordinating results of regional, national and international programmes. For quality assurance, it should be possible to conduct an “audit trail” through the whole process of visiting the site, analysing the sample and filing the results on the database (see Chapter 4). An example of a record form for site inspections is given in Box 8.1.

8.7.2 Recording results of microbiological analyses

The results of microbiological analyses of water quality must always be regarded as an estimate of the water quality at the time and site of sampling, rather than as an absolute determination (PHLS 1994, APHA/AWWA/WPCF, 1995). All enumeration methods depend on the assumption that bacteria and other micro-organisms are randomly distributed in water samples and that the samples conform to the Poisson distribution (see Chapter 3). In reality, the clumping of bacteria and their aggregation on particles cause samples to depart from the Poisson distribution, thereby introducing additional error. There is little that can be done to reduce this error, apart from taking representative samples (free of sediment and other solid matter) and mixing the contents of the sampling bottles vigorously before taking sub-samples for analysis. It has been shown that two halves of the same sample can vary widely in the counts observed (PHLS, 1994).

A historical record of water quality in a bathing area, in normal and extreme situations, enables the selection of the most appropriate dilutions and facilitates the correct enumeration of the final density of indicator organisms that has to be reported as the total number of cfu per 100 ml.

Great care should go into the counting and recording of analytical results in order to avoid recording results wrongly, leading to errors that can result in statistical misinterpretations of water quality at the recreational area. Typical sources of error in the laboratory are caused by operator fatigue and mistakes, such as mislabelling of bottles, Petri dishes and tubes, and errors in preparing and transferring volumes and dilutions of samples. Because labels attached to the lids of Petri dishes, tubes and bottles can be transposed, the labels should be placed on the dish, tube or bottle itself, because these contain the culture medium. Although it may not be obvious, operators vary in the accuracy with which they count colonies and, in addition, unsuspected partial colour blindness can interfere with the interpretation of biochemical reactions of target colonies

or in tubes of diagnostic media. Mistakes and errors can be minimised by proper training and supervision of samplers and analysts in their duties. In addition, laboratory quality controls and the careful application of standard procedures for analysis are essential. Standard procedures should be written correctly and copies should always be available for reference in the laboratory.

The multiple tube method is very sensitive for the detection of a small number of indicator organisms, but the MPN is not a precise value. Confidence intervals, i.e. most probable range (MPR), are often published with the MPN and are meant to indicate the imprecision of the method (Tillett, 1995). However, it should be stated clearly that the range applies to the sample and not to the water source (PHLS, 1994).

For thermotolerant coliforms and faecal streptococci membrane filters with 20-60 typical colonies are recommended for counting, with the provision that filters with no more than 200 colonies of all types should be considered; if the counts of colonies on the membranes are all below the minimum recommended, they should be totalled (APHA/AWWA/WPCF, 1995). Counting colonies on all filters has been shown to improve precision (Gameson, 1983) and is the only method specified in other standard procedures (ISO, 1988, 1990b). The count, in cfu per 100 ml, becomes the sum of all colonies counted multiplied by 100 ml and divided by the total volume (in ml) of water filtered.

If confirmation tests have been applied to a number of typical and atypical colonies, the initial count should be adjusted by multiplying it by the percentage of verified colonies. This percentage will be calculated by dividing the number of verified colonies by the total number of colonies subject to verification and then by multiplying the result by 100 (PHLS, 1994; WHO/UNEP, 1994a; APHA/AWWA/WPCF, 1995). This procedure has a considerable effect in reducing the precision of the count, depending on the total number of presumptive colonies selected and the fraction confirming (PHLS, 1994). Nevertheless, all colonies should be selected if there are ten or fewer colonies on a membrane. New proposed ISO methods try to overcome the imprecision by proposing verification methods for all the colonies grown on the filter (Table 8.7).

If the total number of bacteria colonies, including the specific target colonies, exceed 200 per membrane or are not distinctive enough to enable counting, results should be reported as “too numerous to count” (APHA/AWWA/WPCF, 1995) or “count too high to be estimated at the dilution employed” (PHLS, 1994). A new sample should be requested immediately if possible and more appropriate volumes should be selected for filtration (Table 8.2). The resultant data however, represent the results from a different sample; nevertheless the data may help to investigate the event. If this approach is not possible, it is preferable to try to count a sector of the original filter, before the information is lost thereby estimating the total number of colonies (even though these counts may lack precision). This technique is facilitated by the grid printed on the membranes. The details of the estimate should be recorded, for example “Count in n squares = x; diameter of filtration circle = d squares; estimated count = $\pi d^2 x/4n$ colonies”. If 135 colonies were counted in 10 squares and the diameter of the filtration area was 11.5 squares, the count would be estimated to two significant figures as $3.142 \times 11.5^2 \times 135/40 = 1{,}400$ colonies.

Although the statistical reliability of membrane filter results is higher than that of the MPN procedure, membrane counts are not absolute numbers; 95 per cent confidence limits can be calculated using a normal distribution equation (Fleisher and McFadden, 1980; PHLS, 1994; APHA/AWWA/WPCF, 1995).

8.7.3 Statistical procedures

A single water sample from a recreational area gives very little useful information. However, when individual results are accumulated and analysed statistically, then the trend in water quality will become apparent. Statistical analysis also enables evaluation of the improvement of water quality after remedial actions have been applied (e.g. to sewage contamination sources) and enables achievement of comparability between different regions within the same country or across countries. To establish comparability for the concentrations of thermotolerant coliforms from different regions, it is essential to agree what will be analysed (i.e. all the thermotolerant coliforms or only *E. coli*) otherwise comparison is impossible (Figueras *et al.,* 1994, 1997). The type of data analysis needed depends on the nature of the study (see Chapter 3).

For regulatory monitoring programmes, the objective of data analysis is to demonstrate compliance with a standard. The definition of the standard specifies the type of statistical analysis required. Most recreational water quality standards derive from those of the US EPA (Dufour and Ballentine, 1986), UNEP/WHO (1985) or the European Bathing Water Directive (EEC, 1976). More recently, WHO has published the *Guidelines for Safe Recreational Water Environments* (WHO, 1998). Microbiological standards typically specify the frequency of analysis and the number or proportion of samples that must not exceed given limiting values of the target organism. The rules for interpreting compliance differ with each standard. For microbiological surveys or epidemiological studies, the type of data analysis is decided at the planning stage. The procedures and statistics that are most often used to assess compliance during or after a bathing season are described below, together with worked examples from two sets of data from the two different bathing areas shown in Table 8.8, with the calculation of their basic statistics in Table 8.9.

Table 8.8 Comparability of methods for assessing compliance with microbiological quality criteria in two bathing areas

Method of assessing compliance	**Interim criteria of quality[1] = 100 cfu per 100 ml 50%**	
	Bathing area A	**Bathing area B**
Percentage compliance	Non-compliance (only 1 result complies)	Compliance (10 results comply)
Ranking method	590 non-compliance	8 compliance
Geometric mean[2] (95% confidence intervals)	597 non-compliance (216, 1,646)	16 compliance (4, 66)
Log normal distribution method[3]	680 non-compliance	13 compliance
50 percentile point 90 percentile point	597 non-compliance	15 compliance
50th percentile 90th percentile	595 non-compliance	20 compliance
Method of assessing compliance	**Interim criteria of quality[1] = 1,000 cfu per 100 ml 90%**	
	Bathing area A	**Bathing area B**
Percentage compliance	Non-compliance (only 8 results comply)	Compliance (11 results comply)
Ranking method	3,390 non-compliance	140 compliance
Geometric mean[2] (95% confidence intervals)	597 non-compliance (216, 1,646)	16 compliance (4, 66)
Log normal distribution method[3]	4,700 non-compliance	530 compliance
50 percentile point 90 percentile point	4,618 non-compliance	288 compliance
50th percentile 90th percentile	5,707 non-compliance	1,162 non-compliance

The sets of data for faecal coliforms obtained consecutively from the two bathing areas are as follows (in units of cfu per 100 ml):
A (16; 170; 3,390; 450; 450; 590; 740; 190; 1,180; 6,700; 2,800; 600) and

B (92; 1,600; 36; 0; 140; 4; 0; 36; 4; 8; 0; 32)

[1] According to the UNEP/WHO (1985) Interim Criteria for Recreational Waters, the concentrations of thermotolerant coliforms in at least 10 water samples should not exceed 100 cfu per 100 ml in 50% of the samples and 1,000 cfu per 100 ml in 90% of the samples

[2] The regulation that applies the geometric mean has only one standard in the interim criteria and not two as in the example and the geometric mean is calculated from at least 5 samples equally spaced over a 30 day period (running geometric mean) (US EPA, 1986)

[3] Data extracted from WHO/UNEP (1994b) and Anon (1983)

Percentage compliance

To assess percentage compliance (EEC, 1976) the regulatory percentage of the total number of data “n” obtained from a sampling station has to be calculated. The individual results of the set of data that comply with the established standards have to be counted in order to see if they are higher or lower than the compliance level. This is the approach used in the European Union. For thermotolerant coliforms the guideline standard is 100 cfu per 100 ml in 80 per cent of the samples and the mandatory standard is 2,000 cfu per 100 ml in 95 per cent of the samples (EEC, 1976). This approach is very easy to calculate but does not take into account the absolute values of all the microbiological counts and does not produce any average numerical value of the concentration of micro-organisms in the bathing area. For the worked example of Table 8.8, 50 per cent and 90 per cent of 12 samples is 6 and 10.8 (=11) respectively, which signifies the number of samples that must have counts under or equal to the standards associated to those percentages. In the case of bathing area A, only one result complies with the 50 per cent standard instead of the six needed, and only eight results comply with the 90 per cent standard when there should be 11. This bathing area is therefore failing the compliance assessment. Bathing area B complies with both the 50 and 90 per cent standards (Table 8.8).

Table 8.9 Basic statistics of worked examples from two sets of data obtained for thermotolerant coliforms from two bathing areas

Sample	Bathing area A			Bathing area B		
	Count[1]	Rank[2]	Log count	Count	Rank	Log (count + 1)[3]
1	16	1	1.20	92	10	1.97
2	170	2	2.23	1,600	12	3.20
3	3,390	11	3.53	36	8	1.56
4	450	4	2.65	< 1	1	0.00
5	450	5	2.65	140	11	2.15
6	590	6	2.77	4	4	0.70
7	740	8	2.87	< 1	2	0.00
8	190	3	2.28	36	9	1.57
9	1,180	9	3.07	4	5	0.70
10	6,700	12	3.83	8	6	0.95
11	2,800	10	3.45	< 1	3	0.00
12	600	7	2.78	32	7	1.52
Total	17,276	78	33.31	1,952	78	14.32
Average	1,440	-	2.7758	162.7	-	1.1933
SD[4]	1,965	-	0.6934	455	-	0.9883

SD Standard deviation

[1] cfu per 100 ml

[2] Ranks are given in ascending order

[3] This transformation has been used to enable the geometric mean and the log standard deviation to be calculated, given that three of the values are below the limit of detection, i.e. < 1 cfu per 100 ml

[4] Calculated as $s = \sqrt{\{[\Sigma x^2 - (\Sigma x)^2/n]/(n - 1)\}}$

Ranking method

The ranking method (WHO/UNEP, 1994b) is a very simple method because it involves ordering and multiplication operations, making the use of any complex formulae or laborious graphical analysis unnecessary. The interim UNEP/WHO Mediterranean criteria for recreational waters specify that thermotolerant coliform counts in at least ten samples taken during the bathing season must not exceed 100 cfu per 100 ml in 50 per cent of samples and 1,000 cfu per 100 ml in 90 per cent of samples (UNEP/WHO, 1985). The "*n*" values obtained are first ranked in ascending order of concentration (by definition, the order number, "*i*", takes values of 1 to *n*) (Table 8.9). Then the appropriate order numbers for a given percentage, P (i.e. 50 and 90 per cent) are calculated as $i = nx\text{P}/100$. If ten samples have been taken, then the 50 per cent is measured directly against the fifth value of cfu per 100 ml in the ranking and the 90 per cent against the ninth value. If the number of samples taken does not give a whole number value, the result should be rounded to the nearest whole number to obtain the order. This concentration has to be lower than or equal to the specified standards to comply with the interim criteria. The order point in the rank for the 50 per cent criterion of 12 samples (examples of Table 8.8 and 8.9) is 50 × 12/100 = 600/100 = 6. The order point for the 90 per cent criterion is 90 × 12/100 = 1,080/100 = 10.8 = 11th position. Thus for bathing area A, the sixth point in rank order corresponds to a concentration of 590 cfu per 100 ml while the eleventh corresponds to a concentration of 3,390 cfu per 100 ml. For bathing area B, the corresponding values are 8 and 140 respectively. Whereas A fails both standards, B complies with both (Table 8.8).

Geometric mean

The other systems of interpreting water quality, i.e. the geometric mean with confidence intervals (US EPA, 1986) and the log-normal distribution method (WHO/UNEP, 1994b), are based on the fact that sets of microbiological data from sampling a recreational area are found to conform to a skewed positive distribution, because normally there are many low values and only a few high values.

The transformation of the microbiological counts obtained into decimal logarithms often produces a more symmetrical distribution. The proper descriptive statistic for central tendency is the geometric mean (equal to the median in the case of a normal distribution) with two associated measures of dispersion: the standard deviation of the logarithms of the values (the log standard deviation) and the 95 per cent confidence interval of the geometric mean.

The geometric mean is equal to the antilogarithm of the arithmetic mean of the logarithms of individual concentrations. In the USA it is considered to be the best estimate of the central tendency and the preferred statistic for summarising microbiological results (APHA/AWWA/WPCF, 1995). If there are values less than 1 cfu per 100 ml (i.e. 0 cfu per 100 ml) in the data set it will be impossible to calculate the

geometric mean, because the logarithm of zero does not exist. In this instance one has to be added to all the results (+1) before their logarithmic transformation and then after the average of the logarithms is calculated and the antilog has been taken, the added value has to be subtracted. The calculation of the measures of dispersion are given in Box 8.2.

Box 8.2 Calculation of the measures of dispersion

The logarithmic standard deviation, s_l is calculated by entering the log values, x, into a scientific calculator, programmed to calculate the sample standard deviation. This can also calculated manually as $s_l = \sqrt{\{[\Sigma x^2 - (\Sigma x)^2/n]/(n - 1)\}}$. The 95 per cent confidence intervals of the geometric mean are calculated in two stages by the method below:

- The standard error (se) of the logarithmic mean "*m*", is calculated as $s_l/\sqrt{n}$.

- The 95 per cent confidence intervals are defined as $m \pm t_{(0.025)} \times$ se, where $t_{(0.025)}$ is the value of Student's t for $\alpha = 0.025$ and for $n - 1$ degrees of freedom (from statistical tables).

- For bathing area A the antilog of the log mean is antilog 2.7758 = 597 cfu per 100 ml (Tables 8.8 and 8.9), the standard error (se) of the logarithmic mean 2.7758 is $0.6934/\sqrt{12} = 0.2001$ and the 95 per cent confidence intervals of the log mean where $t_{(0.025)}$ is the value of Student's t for $\alpha = 0.025$ and for $n - 1$ degrees of freedom (from statistical tables; for 11 degrees of freedom = 2.201) is $2.7758 \pm 2.201 \times 0.2001$, giving 2.7758 - 0.4405 = 2.3353 and 2.7758 + 0.4405 = 3.2163 respectively. The antilogs of these values are 216 and 1,646 respectively.

- For bathing area B three counts are below the limit of detection, so the transformation count +1 had been applied to all counts before taking logarithms. The antilog of the log average 1.1933 is 15.6; and the estimated geometric mean is obtained by subtracting 1 from this, giving 14.6, rounded-off to 15. The standard error is $0.9883/\sqrt{12} = 0.2853$ and the 95 per cent confidence intervals of the log mean are thus $1.1933 \pm 2.201 \times 0.2853$, giving results of 1.1933 - 0.6279 = 0.5654 and 1.1933 + 0.6279 = 1.8212. The antilogs of these are 4 and 66 respectively.

Log-normal distribution method

The log-normal distribution method involves the ranking of results and the transformation of data into logarithms to determine the log-normal distribution that fits most closely the experimental results. This can be done by hand fitting the data directly onto log-normal probability paper together with their corresponding cumulative frequencies. The concentration of micro-organisms corresponding to certain specified percentile points on the frequency distribution cumulative frequencies (50 per cent or 90 per cent) can be deduced by the graphic representation (WHO/UNEP, 1994b). This approach is quite similar to the calculation of percentile points on a continuous distribution of an infinite number of samples.

Percentile points, "*p*", on the distribution of the *n* data values from the mean "*m*", (or log mean) and the standard deviation "*s*" (or log standard deviation), as $p = m + zs$, where *z* is the standard normal variable for the desired percentile, obtained from tables of the quantiles (percentage points) of the standard normal distribution. The values of *z* for the 80-, 90- and 95-percentage points are 0.8416, 1.2816 and 1.6449 respectively. The

value for the 50-percentage point will be equal to the mean "*m*". For completeness, it can be noted that the standard normal distribution is symmetrical, so that the values of z for the 20, 10 and 5 percentile points are respectively -0.8416, -1.2816 and -1.6449. This approach can only be applied when the data follow a normal distribution, whereas calculation of classical percentiles does not require normality. For bathing area A, in the example, the 50 percentile point corresponds to the value of "*m*" (597), while the 90 percentile point is estimated by the log-normal distribution method from the log mean 2.7758 and the log standard deviation 0.6934 (Table 8.9). Hence, the log 90 percentile point is 2.7758 + 1.2816 × 0.6934 = 3.6645 and the antilog is 4,618 cfu per 100 ml. This value is quite similar to that obtained by plotting the data on the log-normal-normal distribution probability paper (4,700 cfu per 100 ml). For bathing area B, the 50 percentile point corresponds to a value of *m* = 15 cfu per 100 ml while the 90 percentile point is 1.1933 + 1.2816 × 0.9883 = 1.1933 + 1.2666 = 2.4599 and its antilog is 288 cfu per 100 ml.

The classical statistical calculation of percentiles estimates the variability of the distribution of a set of results (after ordering them in ascending order) independently of whether they are normally distributed and indicates the concentration of micro-organisms that embraces a specific percentile. For example, 80 per cent and 95 per cent of the data will be below the value (cfu per 100 ml) of the 80th and 95th percentiles, respectively, and 20 per cent and 5 per cent of the data will be above those concentrations, respectively. Some computer programs will not calculate the 95th percentile unless 20 records are available, although they will do so if the individual records are specifically considered the midpoint of an interval. An example of the calculation by hand is given in Box 8.3.

Box 8.3 Calculation of percentiles

$Pr = x_i + (j - i)\ (x_{i+1} - x_i)$

where:

Pr is the percentile required (i.e. P_{50}, P_{80}, P_{90} or P_{95})
x_i is the concentration that corresponds with an i position in the ranking corresponding to that Pr
j is the next position in the ranking (calculated as j = r (n + 1)/100)
x_{i+1} is the next concentration in the ranking

- For bathing areas A and B of Table 8.8 and 8.9, the calculation of j is:

50 (12 + 1)/100 = 650/100 = 6.5 = j for P_{50}, and
90 (12 + 1) = 1,170/100 = 11.7 = j for P_{90}

- For bathing area A:

x_i = 590 is the concentration at position 6 while 600 is the concentration at position x_{i+1} (= 7)

Then

P_{50} = 590 + (6.5 - 6) (600 - 590) = 590 + 5 = 595 cfu per 100 ml, and
P_{90} = 3,390 + (11.7 - 11) × (6,700 - 3,390) = 3,390 + 2,317 = 5,707 cfu per 100 ml

- For bathing area B, applying the same criteria:

P_{50} = 8 + (6.5 - 6) × (32 - 8) = 8 + 12 = 20 cfu per 100 ml, and
P_{90} = 140 + (11.7 - 11) × (1,600 - 140) = 140 + 1,022 = 1,162 cfu per 100 ml

8.7.4 Interpretation and reporting

Interpretation of results and reporting do not normally involve the analyst. Nevertheless, strict adherence to analytical control procedures make it possible for queries about unusual or anomalous results to be referred back to the analyst and sampler, through an audit trail.

Although the absolute values may differ with different approaches described, there is a high level of agreement on the water microbiological quality qualification of the beach in relation to compliance. Notice from Table 8.8 how, in bathing area A, only the geometric mean allows compliance in relation to a 1,000 cfu per 100 ml standard. However, in the regulations that govern the use of the geometric mean there is only one standard and the mean is calculated from five consecutive results taken over a period of one month (i.e. the running geometric mean). In this approach there is a strong influence from the most frequent values (8 of the 12 samples rank from 16 to 740 cfu per 100 ml). In the second example, bathing area B, the 90th percentile is the most restrictive, because this approach is highly influenced by the single high value obtained.

One of the features of microbiological studies of water quality at recreational areas is the wide variations in results, temporally and spatially (Fleisher and McFadden, 1980; Gameson, 1982; Tillett, 1993; PHLS, 1994) that are much greater than those caused by laboratory procedures. With effective quality control in the laboratory, variability caused by procedures, such as sub-sampling from the same bottle or by the enumeration procedures themselves, are little greater than expected from the assumption of random distribution of bacteria in the sample and of the Poisson theory, particularly when the mean number is low.

A single limit standard leads to water of borderline quality and with low variability, consistently passing, whereas water which is usually of high quality, but is occasionally affected by intermittent pollution, would fail, even though the former arguably poses a greater risk to health. More detailed study of the results in the latter case might identify the causes of failure and enable advice to be given to the public not to use the water when poor conditions are expected, or enable remedial action to be taken. Bathing area A shows consistently bad quality, with a higher geometric mean (597) and a lower log standard deviation (0.69) than bathing area B (15 and 0.98 respectively). The failure of bathing area B is caused by a single, unusually high count (1,600 cfu per 100 ml) that may be due to a rain effect on the second sampling date.

8.7.5 Control charts

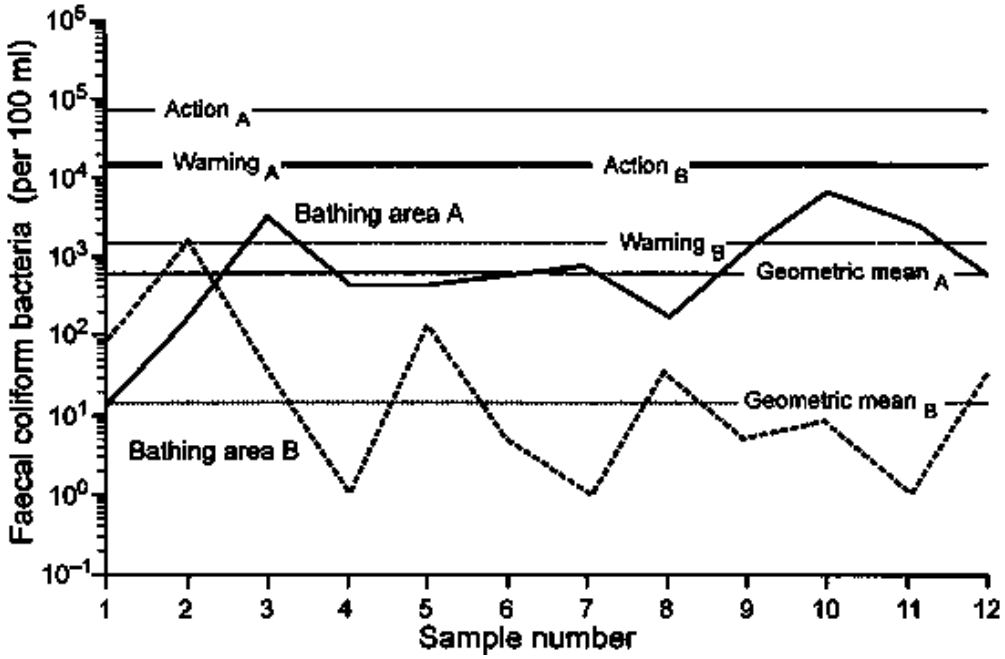
One way of identifying systematic changes in water quality, or of pinpointing sudden deterioration of water quality at a recreational area, is to create control charts (see Chapter 4). Water quality data points are plotted sequentially, as they are obtained, against time on a chart. Any existing historical data from specific bathing areas can be used to create control charts and helps to identify patterns of behaviour. The occurrence of high values is used to initiate investigation. Such values may be set to coincide to a guideline standard. More conventionally, two upper limit values are set on a control chart, at the mean plus twice and three-times the sample standard deviation, i.e. at $m + 2s$ and $m + 3s$ respectively. These represent values that would be expected to be exceeded

only once in 20 or 100 samples respectively, and that would indicate a warning (for example, the need for checking the results and/or for resampling) and then the need for taking remedial action (such as closing the beach until conditions improve and identifying and removing the source of pollution). The bacteriological data in Table 8.8 are presented as two control charts in Figure 8.4, showing the generally poor quality at bathing area A and the greater variability at B, which fails the 90 percentile criterion solely through the high count at the second sampling, even though it is otherwise of good quality.

8.7.6 Technical assessment report

Once the investigation (generally of sanitary conditions and water quality) or monitoring programme is completed, the information is assembled into a comprehensive report. The main body of the report should state the objectives; the manner in which the programme was conducted, with a full description of the recreational area; a historical account of problems and developments; the strategy and reports of inspection, sampling and analysis; significant results obtained; a discussion of the results; and conclusions and recommendations for action. Many of the readers will not have a technical background and therefore an easily readable and accurate “executive summary” should be provided at the front of the report. This gives such readers the main points of the report and invites them to follow-up areas of interest. The report itself should enable the technical reader to understand fully the way in which the study was carried out. Larger bodies of data should be placed in an appendix, so as not to interrupt the flow of the report. A typical report of a sanitary inspection and microbiological analysis includes the following: a description of the survey area(s) and sampling stations, and of any identified hazard(s) and source(s) of pollution (photographs and maps would be useful); the results of the study, including those of the sanitary inspections and microbiological analysis; an in-depth assessment of the risks associated with identified hazards and/or poor water quality; recommendations about the suitability of the area for recreational water use; description and evaluation of various options for improving conditions and thereby for reducing aesthetic and health hazards to users; and recommendations for action, including modifications if necessary, to the monitoring programmes.

Figure 8.4 Counts of faecal coliform bacteria per 100 ml at bathing areas A and B (see Table 8.8) displayed as control charts, with warning and action limits at two and three logarithmic standard deviations respectively above geometric mean counts



The results and recommendations should be discussed with any interested parties before the report is released formally. A contingency plan should also be developed, with the assistance of any interested parties, to investigate and respond to cases of illness or to any unforeseen event or condition that could lead to a deterioration in water quality and possibly increase the risk of illness or danger to bathers. Consideration should also be given to preparing a nontechnical report for the general public.

8.8 Quality control

All laboratories should guarantee that the results of the microbiological analysis of a water sample actually originated from the sample and were not introduced accidentally during sampling or analysis. To support this guarantee, internal and external quality controls should be implemented (Tillett and Lightfoot, 1995). Quality control is described in detail in Chapter 4. Internal quality control includes constant monitoring of equipment (pH meters, balances, pipettes, sterilising equipment, incubators, etc.) and reagents (membrane filters, culture media, buffer solutions, etc.) using controls and reference materials (PHLS, 1994; APHA/AWWA/WPCF, 1995; Janning *et al.,* 1995). Working practice should also be included in this quality control, as well as the precision of the techniques (MPN and MF techniques). In addition, controls have to be made at regular intervals (PHLS, 1994; WHO/UNEP, 1994c). External quality controls are meant to establish good performance by comparing laboratory results with those of other laboratories testing the same artificially prepared sample (Tillet *et al.,* 1993; PHLS, 1994).

8.9 Presenting information to the public

Aspects of public information are considered in Chapter 6. It is sufficient to note here that the quality of recreational water is of great public concern and is often used in publicity to attract visitors to recreational areas. Several countries have developed regular information services, using television, teletext, newspapers and radio (EEC, 1996) to supplement bulletins in municipal buildings and on public notice boards at the recreational areas. The implementation of a monitoring programme with these characteristics is described by Figueras *et al.* (1997). Generally, the public simply want to know if it is safe to use the water and most people have little understanding of the meaning of bacterial counts, let alone their variability. The information presented to the public, therefore, should be direct and unequivocal, up-to-date and not open to misinterpretation.

8.10 Elements of good practice

- Sanitary inspection should be undertaken as a necessary adjunct to microbiological analysis of waters to identify all real and potential sources of microbiological contamination. It should assess the impact of any microbiological contamination present on the quality of the recreational water and on the health of bathers. During the inspection, the temporal and spatial influences of pollution on water quality should receive full consideration.

- An exhaustive sanitary inspection should be carried out immediately prior to the main bathing season. Inspections of specific conditions should be conducted in conjunction with routine sampling during the bathing season. Pertinent information should be recorded on standardised checklists and used to update the catalogue of basic characteristics. If a problem is identified, it may be necessary to collect supplementary samples or information to characterise the problem.

- Visual faecal pollution or sewage odour should be considered a definite sign of elevated microbiological pollution and the necessary steps should be taken to prevent health risks to bathers.

- Standard operating procedures for sanitary inspections, water sampling (including depth) and analyses should be well described to ensure uniform assessments.

- Sample point location and the distance between each location should reflect local conditions (overall water quality, bather usage, predicted sources of faecal pollution, temporal and spatial variations due to tidal cycles, rainfall, currents, onshore winds and point or non-point discharges) and may vary widely between sites.

- Sterile sample containers should be used for microbiological samples. Scrupulous care should be taken to avoid accidental contamination during handling and during sample collection. Every sample should be identified clearly with the time of collection, date and location.

- The most appropriate depth for sampling should be selected and adhered to consistently.

• The sample should be kept in the dark and maintained as cool as possible within a chilled insulated container and returned to the laboratory promptly after collection. Samples should be analysed as soon as possible and preferably within 8 hours of collection. It is recommended that samples should not be stored for more than 24 hours at 5°C.

• Additional information should be collected at the time of sampling, including: water temperature, weather conditions, water transparency, presence of faecal material, abnormal colouration of the water, floating debris, cyanobacterial or algal blooms, flocks of sea birds and any other unusual factors. All information should be recorded on standardised checklists.

• The minimum microbiological variables that should be investigated are faecal streptococci or enterococci and thermotolerant coliforms or *E. coli.* While the former is a recommended indicator for salt water both can be used for freshwater. Additional variables should be investigated if considered relevant and if resources allow.

• The influence of specific events, such as the influence of rain on the recreational water use areas, should be established particularly in relation to the duration of the peak contamination period.

• Extreme events, such as epidemics and natural disasters, may require additional measures to ensure there is no additional risk associated with recreational water use areas.

• The procedures to be used for transformation of raw data, to meet the statistical requirements, should be agreed with the statistical expert prior to analysis. It is usually necessary to transform bacterial counts to logarithms and to convert their approximately log-normal frequency distribution to normality.

• When unexpectedly high microbiological results are obtained, resampling should be carried out to determine whether the unexpected results were due to sporadic events or persistent contamination. In the latter case, the source of pollution should be established and appropriate action taken.

8.11 References

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