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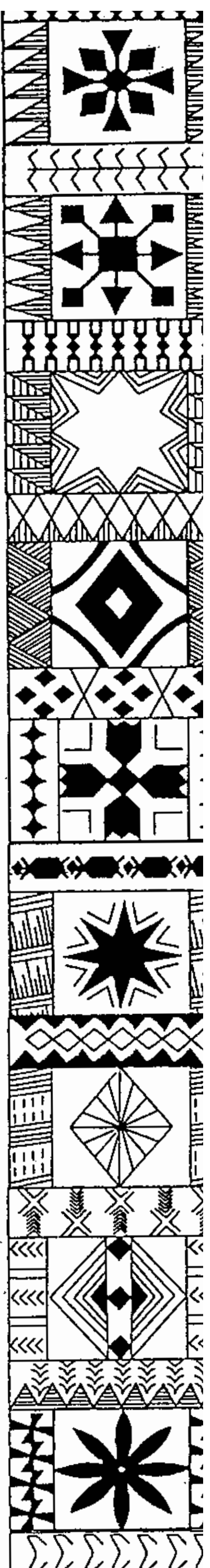

LABORATORY ENVIRONMENTAL
MONITORING WORKSHOP
8th - 12th May, 1995

VOLUME II - MICROBIOLOGY

IAS TECHNICAL REPORT NO 95/02

INSTITUTE OF APPLIED SCIENCES
THE UNIVERSITY OF THE SOUTH PACIFIC

REPORT

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July 1995

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OBSERVERS

1.	Mr Derrick Depledge	-	SOPAC
2.	Mr Leone Limalevu	-	Mineral Resources Department, Fiji

RESOURCE PERSONS

1.	Dr Philomena Gangaiya	-	SPAS (USP)
2.	Ms Dhana Rao	-	SPAS (USP)
3.	Ms Bale Tamata	-	IAS (USP)
4.	Dr Ed Anderson	-	IAS (USP)
5.	Dr Yogendra Singh	-	IAS (USP)
6.	Ms Sereana Kubuabola	-	IAS (USP)
7.	Mrs Arun Lata Pande	-	IAS (USP)

ANNEX 23

OPENING - MONDAY 8TH MAY, 1995

The programme for the opening will be as follows

8.45 am	Introduction Remarks - Mr B.W.M. Fisher
	Opening Speech - Dr S.T. Han, Regional Director (WHO)
	Welcome Address - Ministry of Health, Fiji
9. 15 am	Morning Tea
9.40 am	Administrative Briefing, WHO/USP Payment of per diem to participants
10.00 am	1st lecture

DAY TO DAY SCHEDULE OF THE

LABORATORY ENVIRONMENTAL MONITORING WORKSHOP

GROUP 2 - MICROBIOLOGY

MONDAY, MAY 8 DAY 1

10.00 am - 11.00 am Lecture 1 (combined with Group 1)

Introduction to environmental monitoring including water quality criteria (Dr P. Gangaiya)

11.00 am - 12.00 noon Lecture 1
- General water quality
- Sampling

12.00 noon - 1.00 pm L U N C H

1.00pm - 4.00pm Shellfish demonstration
- preparation
- dilutions
- transfer into broth
- incubation
- autoclaving bottles for Tuesday

TUESDAY, MAY 9 DAY 2

8.00 am - 9.00 am	Lecture 2 Testing for water purity	
9.00 am - 10.00 am	Water sample collection	
10.10 am - 10.40 am	MORNING TEA	
10.45 am - 11.45 am	Demonstration of Membrane Filteration Technique Labelling	
11.45 am - 1.00 pm	L U N C H	
1.00 pm - 3.00 pm	Membrane filteration	- for total coliforms - faecal coliforms - faecal streptococci
3.00 pm - 4.00 pm	Total aerobic count	- dilutions and pour plating

WEDNESDAY, MAY 10 DAY 3

8.00 am - 10.00 am	Checking MPN tubes and sub-culturing into BGBB and tryptone water	
10.10 am - 10.40 am	MORNING TEA	
10.40 am - 11.45 am	Counting coliform colonies and recording results (Membrane Filtration)	
11.45 am - 1.00 pm	L U N C H	
1.00 pm - 4.00 pm	Demonstration	- streaking for pure cultures - pouring agar plates streaking the tubes culture EMB agar

THURSDAY, MAY 11 DAY 4

8.00 am - 10.00 am	Addition of reagent to tryptone water after 24 hours of incubation Checking BGBB tubes after 24 hours incubation
10.10 am - 10.40 am	MORNING TEA
10.40 am - 11.45 am	Counting faecal streptococci plates after 48 hours
11.45 am - 1.00 pm	L U N C H
1.00 pm - 4.00 pm	Gramstain of cultures inoculated Microscopic examination of prepared slides and Gramstain slides Aerobic plate counting

FRIDAY, MAY 12 DAY 5

8.00 am - 10.00 am	Discussion of results
	Maintenance - equipment
	- chemicals
	Quality Control

ANNEX 24

AQUATIC MICROBIOLOGY

Natural waters may serve as habitats for many microorganisms. They may be *freshwater habitats*, such as lakes, ponds, springs, swamps, and rivers; *marine habitats* (the oceans); or *estuarine habitats*, the regions between freshwater sources and the oceans. The kinds of microorganisms found in aquatic environments are, to a large extent, determined by the physical and chemical conditions. These conditions vary from one extreme to another in terms of factors of light, temperature, pH and nutrients.

Temperature

Temperatures of surface waters range from 0°C in polar regions to 40°C in equatorial regions. Beneath the surface more than 90% of the marine environment is below 5°C, a condition favourable for the growth of cold loving microorganisms. Some heat loving bacteria have been isolated from cracks in the ocean floor where temperatures can reach as high as 105°C.

Hydrostatic Pressure

This is the pressure at the bottom of a vertical column of water. It increases with water depth at the rate of 1 atmosphere of pressure for every 10 m. At great depths the hydrostatic pressure is enormous and can cause many changes that affect biological systems, such as changes in the rates of chemical reactions and the solubility of nutrients. Some barophyllic (requiring high hydrostatic pressure) bacteria have been isolated from Pacific Ocean trenches at depths of 1000 to 10,000 m.

Light

Most aquatic life forms depend directly, or indirectly, upon the metabolic products of photosynthetic organisms. The principal photosynthetic organisms in most aquatic habits are algae and cyanobacteria; their growth is restricted to the upper layers of water through which light can penetrate. The depth of the water layer in which photosynthesis occurs is called the photic zone. Generally the photic zone is confined to the upper 50 to 125 m of a body of water.

Salinity

The salinity or sodium chloride conditions, of natural waters ranges from near zero in fresh water to saturation (32% NaCl) in salt lakes. Seawater contains approximately 3.3 to 3.7% of salts. Most marine microorganisms are halophytic, they grow best at NaCl concentrations of 2.5 to 4.0%. Besides NaCl, the principal salts found in water are sulphates and carbonates of sodium, potassium, calcium and magnesium.

Turbidity

There is a marked variation in the clarity of surface waters. The suspended mater responsible for water turbidity includes:

1) particles of mineral material, which comes from coastal erosion

2) detritus, which is particulate organic matter such as fragments of cellulose, hemicellulose and chitin from decomposing plant and animal matter

3) suspended microorganims

The greater the turbidity of the water, the less the penetration of light, and the less the depth of the photic zone.

Particulate matter may also serve as a surface to which microorganisms adhere. Many species of bacteria attach themselves to a solid surface in order to colonise.

Hydrogen Ion Concentration

Aquatic microorganisms usually grow best at pH 6.5 to 8.5. The pH of seawater is 7.5-8.5. Lakes and rivers may show a wider range in pH depending upon local environmental conditions.

Nutrients

The amount and kind of organic and inorganic materials (nutrients) present in an aquatic environment significantly influences microbial growth. Nitrates and phosphates are common inorganic constituents and they promote the growth of algae. The quantity of nutrients in a body of water is referred to as the nutrient load of an environment. Nearshore waters which receive domestic wastewater are subject to variations in their nutrient load, whereas the open sea has a nutrient load that is comparatively low and stable.

Distribution of microorganisms in the aquatic environment

Microorganisms in the aquatic environment may occur at all depths - ranging from the surface to the very bottom of ocean trenches. The upper layers and the bottom sediments contain the larger numbers of microorganisms, particularly in deep waters.

The collection of floating and drifting microbial life in the surface regions of ponds, lakes, and oceans is called plankton. The plankton population may be primarily algae and cyanobacteria (phytoplankton) or it may consist predominantly of protozoa and other microscopic life (zooplankton). Phototropic microorganims are regarded as the most important plankton because they are primary producers of organic matter via photosynthesis. Most phytoplanktonic

organisms can maintain their location in the photosynthetic zone by being motile. Others contain structural features which gives them buoyancy. A multitude of conditions- such as sunlight, winds, tides, currents, nutrients, and ingestion of higher forms, may affect the numbers and types of organisms in the planktonic populations.

Microbial inhabitants at the bottom of a body of water are called benthic organisms. The benthic zone is the richest region of an aquatic system in terms of numbers and kinds of microorganisms.

Water Pollution

The drinking water of most communities and municipalities comes from surface sources - rivers, streams and lakes. Such natural water supplies, particularly streams and rivers are likely to be polluted with domestic, agricultural and industrial wastes. Water can be perfectly clear, odourless, and tasteless and yet be unsafe to drink. Contaminants that pollute water are classified into three categories: chemical, physical and biological. Our discussion will focus on the biological pollutants, namely, microorganisms.

Water can endanger life and health if it contains pathogenic microorganisms. The pathogens that are frequently transmitted through water are those which cause infections of the intestinal tract. Examples are the causative agents of typhoid and paratyphoid fevers, shigellosis, cholera, viral enteritis and amoebiasis. These organisms are present in the faeces or urine of an infected person and when discharged, may enter a body of water that ultimately serves as a source of drinking water. Shellfish that feed by filtering tend to concentrate waterborne viruses and bacteria in their tissues, so shellfish from polluted water may be dangerous to eat. However, not all pathogens must be ingested to cause disease. For example, the helminth disease schistosomiasis is spread among people who swim or wade in waters contaminated by human wastes. The infective pathogens are not usually ingested but are free-swimming cercaria that bore through the skin.

In order to prevent transmission of these pathogens, there must be procedures whereby water can be examined to determine its microbiological quality. The routine microbiological examination of water to determine its portability (free of disease-producing microorganisms and chemical substances harmful to health) is not based on the isolation of pathogenic microorganims. The reasons for this are:

1) Pathogens are likely to enter a water supply sporadically, and since they may not survive for long periods of time, they could be missed in a sample submitted to the laboratory.

2) If they are present in very small numbers, pathogens are likely to escape detection by laboratory procedures.

3) It takes 24 h or longer to obtain results from a routine laboratory examination for pathogenic microorganisms. By the time pathogens are found, many people would have consumed the water and would be exposed to these pathogenic microbes before action could be taken to correct the situation.

For these reasons, microbiologists have developed water testing procedures that do not rely on the isolation and identification of pathogens. Instead, tests are based upon finding a microorganism whose presence indicates the possibility of the presence of pathogenic microorganisms. The indicator organism serves as an "alarm" system.

Indicator Microorganisms

The term indicator microorganism refers to a kind of microorganism whose presence in water is evidence that the water is polluted with faecal material from humans or other warm-blooded animals. This kind of pollution means that any pathogenic microorganisms that occur in the intestinal tract of these animals may also be present. Some of the characteristics of an indicator organism are:

1) It is present in polluted water and absent from unpolluted water.

2) It is present in water when pathogens are present.

3) The quantity of indicator organism correlates with the amount of pollution.

4) It survives better and longer than the pathogens.

5) It has uniform and stable properties.

6) It is generally harmless to humans and other animals.

7) It is present in greater numbers than those of pathogens (making detection relatively easy).

8) It is easily detected by standard laboratory techniques.

Escherichia coli most closely satisfies the requirements of an ideal indicator of pollution and is the organism most widely used. Other bacteria have been suggested and sometimes used as pollution indicators. These include *Streptococcus faecalis* and *Clostridium perfringens*, both are normal inhabitants of the large intestine of humans and other animals. There is also considerable interest in the development of a routine procedure for the detection of virus as an indicator of pollution.

Like the coliform bacteria, enteric viruses can be carried by human wastes into water. Analysis of a water sample for presence of viruses requires

elaborate procedures than those used for isolation of bacteria. Although considerable research is under way, no "standard method" to detect viruses in water has been adopted.

Sampling

Before conducting a survey, specific data quality objectives have to be determined and the information sought has to be defined clearly. For example, the frequency of repetitive sampling may vary from hourly for a detailed study to every third month for a general assessment depending on objectives. The scope of the study must be adjusted to limitations in personnel, time and budget. Before the development of a study plan, it is important to seek advice from an expert and use existing information to assist in planning. This information might include data from other investigations, hydrological data, sources and types of effluents, descriptions of local conditions including unusual events eg. excavations.

The following questions should be answered before preparing the analytical instructions:

1) For what purposes are the measurements required?

2) What are the most important parameters enabling a general assessment to be made?

3) Which sampling points and measurement frequencies are absolutely necessary?

4) How should the sampling and measuring programs be organised with respect to time and place?

5) How should the obtained data be documented?

6) What are the personal requirements and costs?

After gaining a thorough understanding of the factors involved with a particular body of water, specific areas to be sampled can be selected. There is no set number of sampling stations that will be sufficient to monitor all possible waste discharges. The preliminary studies can be used to determine the minimum number of samples, the frequency of sampling, the time of sampling and the sampling sites.

Some basic rules that must be followed when collecting samples:

1) The sample must be collected in a clear sterile bottle with a tight fitting stopper.

2) If chlorinated water samples are to be taken, the sample bottles must contain 0.1 ml of a 3% solution of sodium thiosulphate to neutralise residual chlorine. This would eliminate growth inhibiting substances and make it possible for any live organisms to grow.

3) Before sampling from taps, the tap should be fully opened and closed several times to get rid of dirt particles. The tap exit is then flamed for a sufficiently long time and then the water is allowed to run for 5 minutes before filling the bottle.

4) Contamination of the sample must be avoided during and after sampling. When sampling from a stream, the bottle should be placed below the surface and moved forward while being filled so that water coming into contact with the hands does not enter the bottle.

5) The sample must be representative of the supply from which it is taken. If there are horizontal and vertical variations in water composition, then collect from specific depths at a series of verticals or have representative concentrations for the cross section.

6) The sample should be tested as promptly as possible after collection to ensure meaningful results. Otherwise there will be changes in microbial number during storage.

7) If there is a delay in examination, the sample should be stored at a temperature between 0 and 10°C.

Biochemical Oxygen Demand

The biochemical oxygen demand is the amount of dissolved oxygen required by microorganisms for the aerobic degradation of organic matter present in water. The magnitude of BOD is an indication of the amount of organic matter in the water. The more oxidisable organic material present, the higher the BOD. The "strength" of the water is expressed in terms of the BOD level. The life of any body of water depends to a large extent upon its ability to maintain a certain amount of dissolved oxygen, which is needed to maintain aquatic life.

The classic method of measurement is to use special bottles with airtight stoppers. Each bottle is first filled with the test water. The water is initially aerated to provide a relatively high level of dissolved oxygen and is seeded with bacteria if necessary. The seeded bottles are then incubated in the dark for five days at 20°C. The decrease in dissolved oxygen is determined by a chemical or electronic testing method. The more oxygen that is used up as the bacteria degrade the organic matter in the sample, the greater is the BOD, which is usually expressed in milligrams of oxygen per litre of water. The amount of oxygen that can be normally dissolved in water is only about 10 mg/litre, typical BOD values of waste water may be twenty times this amount.

ANNEX 25

TESTS FOR WATER PURITY

Most of our concern about water purity has been related to transmission of disease. Therefore tests have been developed to determine the safety of water. It is not practical to look only for pathogens in water supplies. For one thing, if we were to find the pathogen causing typhoid or cholera in the water system, the discovery would already be too late to permit an outbreak of the disease. Moreover, such pathogens would probably be present only in small numbers and might not be included in tested samples.

The tests for water safety in use today are aimed instead at detecting particular indicator organisms. There are several criteria for an indicator organism. The most important criterion is that the organism be consistently present in human faeces in substantial numbers so that its detection is a good indication that human wastes are entering the water. The indicator organisms should also survive in the water as well as the pathogenic organisms would. The indicator organisms must also be detected by simple tests.

The usual indicator species are the *coliform* bacteria. They are a group of gram-negative, rod-shaped, lactose fermenting bacteria native to the intestinal tract of mammals. They are members of the family of bacteria called Enterobacteriaceae and like the other members can be cultured from mammalian faeces.

Total coliform refers to species of *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter* and *Edwardsiella.* All these species except *Escherichia* can exist as free living saprophytes as well as being intestinal organisms. Faecal coliforms defines primarily *Escherichia coli* and occasionally *Klebsiella* sp. They are distinguished from the total coliform by having the capability of fermenting lactose at elevated temperatures of 44.5°C. There are specialised tests to distinguish between faecal coliforms and nonfaecal coliforms.

Standard Plate Counts

Counts of viable bacteria are usually calculated from the number of colonies that develop on agar plates which have been inoculated with certain quantities of sample and incubated under prescribed conditions. Such counts are sometimes wrongly called total plate counts when in fact only those bacteria which will grow under the particular conditions selected will grow. A wide variety of conditions can be obtained by changing the composition of the agar medium, the gaseous environment and/or the time and temperature of incubation. The aerobic plate count at a particular temperature comprises all those organisms capable of growth in air on a non-selective agar medium.

Plate counts can be carried out by a number of different methods, two of which include the pour plate and the spread plate methods. The pour plate method is

simple to perform and can accommodate volumes of samples ranging from 0.1 to 2.0 ml. The colonies produced are relatively small and compact, showing less tendency to encroach on each other than those produced by surface growth. On the other hand, submerged colonies are slower growing, are difficult to transfer and significant heat shock can occur from the transient exposure of temperatures upto 45°C.

The spread plate method causes no heat shock and all colonies are on the agar surface where they can be distinguished readily from particles and bubbles. Colonies can be transferred quickly and compared easily to published descriptions. However, this method is limited by the small volume of sample that can be absorbed by the agar (0.1-0.5 ml).

The standard plate count technique provides a measure of the degree of microbiological contamination of the water gives information about sudden bacterial invasions. When using the standard plate count to test water, there are no particular numbers of bacteria that are officially acceptable. This is because water with a few pathogenic bacteria is obviously more dangerous than water containing many nonpathogenic bacteria. Nevertheless, water of good quality has a low total bacterial count, fewer than 100 per ml. A value exceeding 100 is rounded up to the nearest ten; a value exceeding 1000 is rounded up to the nearest hundred etc.

Plate counts are also useful in determining the efficiency of various procedures for removing or destroying organisms (sedimentation, filtration and chlorination). A standard plate count can be made before and after a specific treatment to measure the reduction of the microbial population.

Membrane Filter Technique

The membrane filter technique is a popular lab test in water microbiology, because it is straightforward and can be used in the field. It has a high degree of reproducibility and relatively large volumes of sample can be tested. It yields definite tests more rapidly than the standard tubes test.
The advantages with this test are:

1) Results can be obtained in approximately 24 hours, as compared with the 48-96 hours for the standard fermentation tube method.

2) Much larger, and hence more representative, samples of water can be sampled routinely with membrane filters.

3) Numerical results from membrane filters have much greater precision (reproducibility) than is possible with the fermentation tube method.

4) The equipment and supplies required are not bulky. A great many samples can be examined with minimum requirements for laboratory space, equipment and supplies.

The membrane-filter technique for the bacteriological examination of water consists of the following steps:

1) A sterile filter disk is placed in a filtration unit.

2) A measured volume of water is drawn through the filter disk; the bacteria are retained on the surface of the membrane -filter disk.

3) The filter disk is removed and placed upon an absorbent pad that has previously been saturated with an appropriate culture medium. Special Petri dishes that will hold both the absorbent pad and the filtration disk are used for incubation.

4) During incubation, colonies develop on the filter disk wherever bacteria were entrapped.

5) By counting the colonies the technician may determine the original number of bacteria in the samples. To obtain a count of the coliform bacteria, a selective medium may be used.

However the membrane filter technique has limitations, particularly when testing waters with high turbidity or noncoliform (background bacteria).

Total Coliform

After proper sampling and filtration, the bacterial retentive membrane filter is placed on top of MF-endo media containing lactose, protein digest, vitamins, selective chemicals and Schiff's reagent. As the membranes incubate for 24 hours at 35°C, the media diffuses through the pores in the filter, supplying nutrients to the multiplying bacteria. Many kinds of bacteria can grow and form colonies under these conditions, but only the coliforms will ferment lactose. One by-product of the reaction is an aldehyde that will combine with Schiff's reagent to form an iridescent green coating over the growing colonies.

Faecal Coliforms

The filtration test is similar. The media used for this test is M-FC containing lactose, protein digest, vitamins, selective chemicals and aniline blue dye. The membrane is incubated at 44.5°C, allowing only coliforms of faecal origin to grow into visible colonies. The non-faecal coliforms, due to heat shock, will not grow. As the faecal coliforms grow, they ferment lactose, producing acid which reacts with the aniline dye to produce a blue colour. When viewed under the stereomicroscope at 10-20X magnification, all the colonies exhibiting a blue colour are faecal coliforms.

Coliforms have been very useful as indicator organisms but they have limitations. One problem is that coliform bacteria can grow as biofilms on the inner surface of water pipes. Their presence in tap water has led to a number

of community orders to boil water, even though these coliforms are not necessarily related to faecal contamination and there is no evidence that they have significant effect on public health.

A more serious problem is that some pathogens are even more resistant than coliforms to chemical disinfection. Water samples that are free of coliforms are often contaminated with enteric viruses. The cysts of *Giardia lamblia* and *Cryptosporidium* are very resistant to chlorination. The Legionellaceae, because they are not enteric bear no relationship to faecal bacteria. Nevertheless, the coliform test has been, and continues to be, a useful test for assessing the quality of water.

Faecal Streptococci

Some laboratories also test for faecal streptococci such as *Streptococcus faecalis*. As they are normally present in the intestinal tract of man and animals, these organisms are also indicators of faecal contamination of water. They rarely multiply in water but possess an above average resistance to heat, alkali and salts. Thus they grow at pH 9.6 and at temperatures of 10-45°C in a medium with 6.5% NaCl content and are not inhibited by azide.

These bacteria provide additional evidence of water contamination. The membrane-filter technique may be adapted for faecal streptococci by using a medium that supports these organisms, and further identification can be made by culturing water samples on plates of media selective for streptococci.

Pseudomonas aeruginosa

These bacteria are also faecal indicators. They are facultative human pathogens, often giving rise to eye and ear infections, and are extremely resistant to antibiotics. These organisms have been isolated from waters that have passed the *E. coli*-coliform bacteria test.

Clostridium perfringens

This anaerobic spore forming bacterium is also present in the faeces of warm-blooded animals but at lower concentrations than *E. Coli*. Since clostridial spores can survive for a long time in the environment, their detection in the absence of coliform bacteria points to an older water contamination and is not evidence of a current contamination.

Multiple-tube fermentation technique

When this technique is used, the coliform group is defined as all aerobic and facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C.

Tubes are incubated and coliform organisms are identified by their production of gas from lactose. Referring to a Most Probable Number (MPN) table, a

statistical range of the number of coliform bacteria is determined by observing how many broth tubes showed gas. The MPN test does not detect the total number of bacteria in the water, nor does it pinpoint noncoliform bacteria such as *Salmonella*. However, it does indicate that coliform bacteria are present and it gives an estimation of their number. It is also fairly rapid to perform and easy to interpret.

The precision of the test depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in the majority of the tubes. MPN tables are based on a Poisson distribution (random dispersion). However, if the sample is not adequately shaken before portions are removed or if clumping of bacterial cells occurs, the MPN value will be an underestimate of the actual bacterial density.

The maximum number of allowable coliform organisms is prescribed in terms of standard portion volume (10 ml or 100 ml) and the number of portions examined. The absence of gas in all tubes, when five 10 ml portions are examined generally indicates that the single sample meets the standards. A positive confirmed phase in three or more tubes (10 ml portions) indicates the need for immediate remedial action and additional examinations.

MICROBIOLOGY LABORATORY RULES

The following rules should be regarded as obligatory requirements for the safe handling of food and water samples during microbiological analyses. Laboratory personnel must have training in the procedures conducted in the laboratory.

1. The laboratory should be easy to clean. Bench surfaces should be impervious to water and resistant to acids, alkalis, solvents and disinfectants.

2. If the laboratory is mechanically ventilated, an inward airflow into the laboratory should be maintained by extracting room air to the atmosphere.

3. The laboratory must contain a sink for hands washing only.

4. An autoclave for sterilization of waste materials should be available on site.

5. The laboratory door should be closed when work is in progress.

6. Laboratory coats or gowns should be worn in the laboratory and removed when leaving the laboratory.

7. Eating, drinking, smoking, storing of food and applying cosmetics must not take place in the laboratory.

8. Unhygienic practices such as mouth pipetting must not take place.

9. Hands must be disinfected or washed immediately when contamination is suspected, after handling viable materials, and also before leaving the laboratory.

10. Work may be generally conducted on the open bench, but all procedures must be performed so as to minimise the production of aerosols.

11. Effective disinfectants must be available for immediate use in the events of spillage.

12. Bench tops should be cleaned regularly after use.

13. Used laboratory glassware and other materials awaiting sterilization must be stored in a safe manner. Pipettes if placed in disinfectant, must be totally immersed.

14. All waste material must be rendered non-infective before disposal.

15. Materials for autoclaving must be transported to the autoclave without spillage in leakproof and robust containers.

16. All accidents and incidents must be reported to the Laboratory Manager and the incident recorded in a record book.

17. Opening samples, cultures etc. - All parcels containing microorganisms must be opened in a laboratory by trained personnel and, ideally, in a cabinet that will prevent inhalation of aerosols.

18. The laboratory must be left clean and tidy at the end of the session.

REMEMBER:

The safe disposal of unwanted cultures is as important in the microbiological laboratory as is the safe disposal of radioactive waste material in an isotopes laboratory.

LABORATORY PROCEDURE FOR DISCARDING UNWANTED CULTURES AND OTHER MATERIAL

a. Uncontaminated Waste Paper is to be placed in bins provided.

b. Slides are to be discarded into containers of disinfectant (SAVLON or 4% sodium hypochlorite). Containers should be on each bench.

c. Pasteur Pipettes are to be discarded into the plastic cylinders found on each bench. The tip of the pipette is held below the surface of the disinfectant while removing the rubber teat. The rubber teats are not discarded into the disinfectant.

d. Graduated Pipettes are to be discarded into the measuring cylinder of disinfectant found on each bench.

e. Plastic Petri Plates should be discarded into a leakproof, autoclavable "discard container" provided. Plates will be autoclaved separately to glassware at 121° for 20 minutes.

f. "Tubes and Bottles". Aluminum screw caps must be loosened a little before discarding, standing upright so leakage does not occur. Again place in a leakproof autoclavable container and sterilise at 121° for 20 minutes.

g. Discard procedures for all other contaminated materials should be ascertained from the laboratory manager.

DESCRIPTION OF BACTERIAL COLONIES

The following characteristics of morphology should be noted.

1. Colony Surface:

   Smooth, rough, wrinkled (rugose), contoured, granular, glistening.

2. Elevation:

   Flat, raised, convex, umbonate, pulvinate.

3. Form:

   Circular, irregular, filamentous, rhizoid, punctiliform.

4. Optical Characteristics:

   Opaque - light will not pass through
   Translucent - light will pass through
   Opalescent - (colour of opals; perhaps soapy)
   Iridescent in reflected light

5. Consistency:

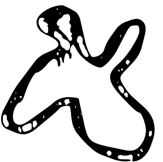
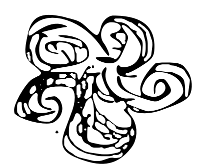
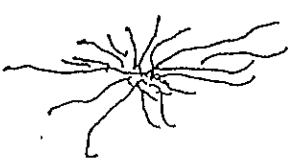
   (when tested with a sterile loop) viscid; butyrous, fria
   (brittle), membranous.

6. Colour and/or Pigmentation:

   white, buff, straw, etc.
   Is pigment associated with colony?
   Does pigment diffuse into agar?

7. Margin or Edge:

   entire, undulate, lobate, curled, filamentous.

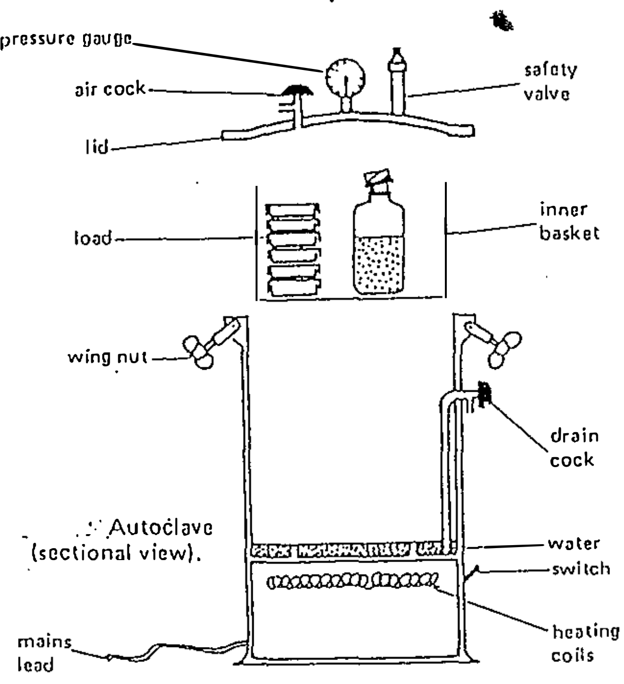
Autoclaving:

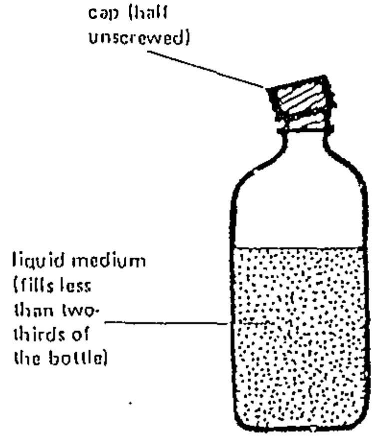
Bottles of liquid simulating old media, unwanted cultures and discarded samples will be placed in the autoclave and the operation of the autoclave demonstrated.

Sterilisation using the autoclave:

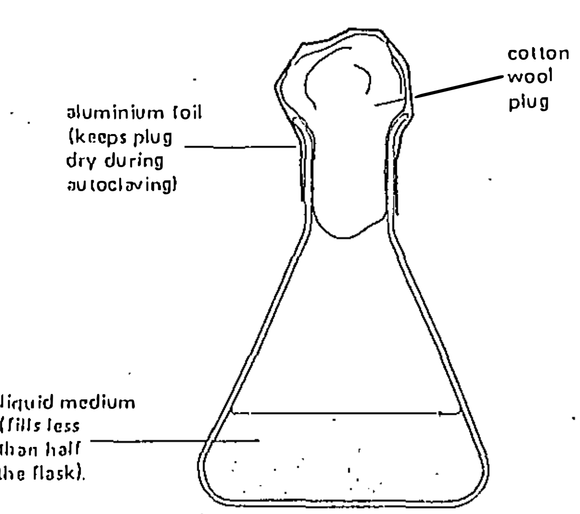
An autoclave should be used whenever the medium or equipment to be sterilized is *not* heat-labile. Heat-labile ingredients include vitamins, growth factors, sugars and antibiotics. Heat-labile equipment includes most plastics and low-quality glass.

The autoclave sterilizes by raising the temperature inside the autoclave to 121 °C using steam under pressure (about 15 psi above atmospheric pressure). The moist heat denatures proteins and other cellular constituents resulting in cell death. Since proteins are affected, viruses are also destroyed in this process. To ensure complete sterility, the material must be kept at 121 °C for 15 minutes. Since the thermometer of the autoclave measures the temperature in the *air-space* in the autoclave, the required time in the autoclave may exceed 15 minutes after the thermometer has reached 121°, to compensate for the time it takes for the *material* inside to reach 121°. Typically, 15-20 minutes of autoclaving is sufficient for small loads in an autoclave, and more time is needed if either the total amount of material in the autoclave or the volume of any container (for example, a 6-litre container) is large.





Bottle of medium ready for autoclaving.



Flask of medium ready for autoclaving.

The autoclave

This is simply a laboratory version of the humble domestic pressure cooker; it is a pressure steamer and its function is the efficient killing of micro-organisms, i.e. sterilisation of articles. Boiling alone is quite useless for killing endospores of certain bacteria; more drastic methods are necessary. The following procedure applies only to the autoclave illustrated (fig. 10.1, p. 26), but the general principles apply to nearly all autoclaves.

PROCEDURE (about 1 hour)

(*a*) Remove the basket from the chamber and pour about 1200 cm^3 water into the autoclave (the bottom should be covered to a depth of 2 cm); replace the basket.

(*b*) Pack the basket with the items to be sterilised (see notes on packing on p. 30).

(*c*) Place the lid (fig. 10.7) in position and fasten down the wing nuts; they need not be screwed down very hard but should be turned lightly until they touch the lugs on the lid; when they are all touching the lid, give a final tightening up to *hand* tightness only by screwing down each *pair* of nuts in turn, i.e. 1 and 5, 2 and 6, 3 and 7, 4 and 8; this ensures even pressure on the lid.

(*d*) Set the adjustable safety valve (fig. 10.8) to 103 kPa pressure i.e. the knurled thumb nut at the top should be in the depressed position; it can also be adjusted to the 69 kPa position.

(*e*) Close the drain cock and open fully the air cock.

(*f*) Switch on the electric current to 'high' position, i.e. both switches should be down.

(*g*) Wait for the water to boil (about 10 minutes); allow steam issue from the air cock for fully 5 minutes and then close tl air cock; it is essential to remove as much air as possible, b from the chamber and from the load, because the presence air considerably lowers the temperature at a given pressure.

(*h*) Wait for the pressure to reach 103 kPa (about 5 minutes), when steam will begin escaping from the safety valve; immediately switch the current to *medium* — when the medium switch *only* is operating the pressure will be kept 103 kPa; leave for 15 to 30 minutes according to the natu of the items being sterilised (see notes on sterilising times p. 30).

(*i*) At the end of the sterilisation time switch off the curren completely; do not open either drain cock or air cock.

(*j*) Leave until pressure falls to zero (about 15 minutes – longer, a negative pressure may develop); open the air co unfasten the wing nuts and remove the lid; the sterilised items may then be removed.

PACKING

Glass petri dishes should be sterilised in a hot air oven.

Plastic petri dishes cannot be sterilised without melting; t melting point of the plastic is somewhat below 121 °C. Old cultures in plastic plates can be conveniently disposed autoclave: 20 minutes at 121 °C. Fig. 10.10 serves to the futility of any attempt to use a disposable petri dish m than once.

Bottles of media should never be more than two-thirds allow for vigorous boiling; if screw-cap bottles are used, must be loosened so that steam may penetrate the interio bottle (fig. 10.11); sterilisation time is 15 minutes at 121 ° longer time might alter the chemical composition of the m

Flasks of media should be plugged with dry non-absor o cotton wool, or be covered with aluminium foil; if cotton used, it should be covered with aluminium foil to prevent sive wetting (fig. 10.12); total sterilisation time is 15 minu 121 °C.

Old cultures should be sterilised for 20 minutes at 121

Steps for autoclaving are outlined below:

1. Place material to be sterilized in the autoclave. Be sure all containers are heat- and pressure-resistant and that all closures are self-venting. Containers with liquids should not be more than 80% full. To allow maximum heat exchange, allow enough space between each container for steam to contact all sides.

2. Close the autoclave door firmly. If the door is closed properly, no steam or liquid should escape during the actual autoclaving.

3. Read and follow the directions carefully for use of your particular autoclave. If the autoclave has automatic controls, set the timer for the length of time appropriate for the volume of material to be autoclaved (but *not* for less than 15 minutes) and set the exhaust routine for "slow exhaust" for loads containing liquids or "fast exhaust" for dry loads such as empty tubes. Slow exhaust is required for liquids because during sterilization the liquid is at 121°, (and thus 21° above its boiling point at atmospheric pressure). Slow exhaust allows the liquid to cool below its boiling point as the pressure descends. Using fast exhaust with liquids will result in loss of the liquid from the container as the pressure descends and the super-heated liquid boils over.

4. Turn on the steam (or if automatic, set to start). Begin timing when the temperature reaches 121°.

5. For non-automatic autoclaves: after the appropriate period of time, turn off the steam. For slow exhaust, do not open the exhaust vent; for fast exhaust, open it.

6. Allow the autoclave to exhaust properly (indicated by a return to 0 on the pressure gauge on non-automatic autoclaves or by a signal on automatic ones). Open the autoclave *carefully*, keeping the arms and face away from the opening to avoid being burned by any steam that may leave the autoclave as the door is opened. Wear safety gloves, apron, visor.

7. *Using properly insulated gloves*, remove the material from the autoclave. *Do not* remove any containers while the liquid is still boiling or bumping, as the liquid in these containers may

boil out of the container and cause a burn. Place materials in an appropriate area for cooling. Any materials which do not need to be treated further prior to use should be placed in an area away from normal use to avoid possible burns to personnel. Tubes which contain media for agar slants should be tilted in baskets or racks for cooling. Flasks containing media for plates should be placed in a 50-55° water bath until cool enough for pouring.

Preparation of culture media

Utensils

Copper or zinc containers must not be used. Small amounts of these metals will dissolve in the culture media and are bactericidal. Large amounts of media can be made in the stainless steel buckets that are used in the dairy and food trades. Smaller amounts can be made in resistance-glass laboratory flasks. Large stainless steel funnels can be used for filtration, with folded filter-papers of the heavy Chardin type. Filtration and adjustment of pH should be completed before agar is added. The present-day agars give a clear gel but if it is necessary to filter agar medium, cut a sheet of heavy filter-paper to fit a large Büchner funnel and place wet filter-paper pulp on it to a depth of 5 mm. Place the funnel on a filter flask and suck the pulp dry. Heat the whole apparatus in a steamer in order to prevent gelling of the medium in the filter.

Inhibitory substances

Metals such as zinc and copper (*see above*) and other inhibitory substances such as fatty acids may be present in peptones, agar, carbohydrates and other chemicals which are not specifically made for culture media. It is inadvisable, for reasons of cost and convenience, to use any material that is not specified as being of 'bacteriological quality' and purchasing officers should be severely discouraged from seeking, for reasons of 'economy', suppliers of media ingredients, including chemicals and dyes, who are not known to bacteriologists.

Distilled water from commercial bulk supplies, 'factory' or 'battery' quality may contain substances that inhibit bacterial growth, e.g. oily materials from stripper stills attached to steam lines. Manesty-type distilled water, glass-distilled water and deionized water should be used, although it must be realized that some trace elements necessary for bacterial growth are in fact supplied by the distilled water, by glassware, or by recognized impurities in analytical quality reagents.

Glucose, when autoclaved with salts such as phosphate, may yield inhibitory substances. It is best to add carbohydrates as sterile solutions after the medium has been sterilized.

Some cotton wools, when sterilized in a hot-air oven, yield fatty materials.

Sometimes these materials can be seen as condensates on glass tubes. Again, cotton wool from a reliable source should be used.

Excessive heating and re-melting may destroy growth factors and gelling capacity and cause darkening or pH drift of culture media.

Adjustment of pH

The pH of reconstituted and laboratory-prepared media should be checked with the meter, but for practical purposes devices such as the BDH Lovibond Comparator is good enough.

Pipette 10 ml of the medium into each of two 152 × 16 mm test-tubes. To one add 0.5 ml of 0.04% phenol red solution. Place the tubes in a Lovibond Comparator with the blank tube (without indicator) behind the phenol red colour disc, which must be used with the appropriate screen. Rotate the disc until the colours seen through the apertures match. The pH of the medium can be read in the scale aperture. Rotate the disc until the required pH figure is seen, add 0.05 N sodium hydroxide solution or hydrochloric acid to the medium plus indicator tube and mix until the required pH is obtained.

From the amount added, calculate the volume of 1 or 5 N alkali or acid that must be added to the bulk of the medium. After adding, check the pH again.

Example 10 ml of medium at pH 6.4 requires 0.6 ml of 0.05 N sodium hydroxide solution to give the required pH of 7.2.

Then the bulk medium will require

$$\frac{0.6 \times 1000}{20 \times 10} \text{ ml of 1 N NaOH/litre} = 3.0 \text{ ml}$$

Discs of other pH ranges are available.

All final readings of pH must be made with the medium at room temperature because hot medium will give a false reaction with some indicators.

Identification and storage

Most laboratories employ a colour code to identify media that look alike. Coloured beads may be placed in bottles of bulk medium. Coloured cotton wool or coloured aluminium or polypropylene caps can be used for tubed media, and the caps of bottled media can be dabbed with coloured enamel paint. This is particularly useful for carbohydrate fermentation test media.

The labelling machines used to place prices on goods in supermarkets are very useful for labelling and dating tubes, bottles and petri dishes of culture media.

Bulk bottled media in screw-capped bottles can be stored at room temperature.

Filling, tubing and pouring culture media

Liquid media can be tubed with the aid of a large funnel held in a retort stand and fitted with a short piece of rubber tubing and a spring clip. The semi-automatic commercial fillers are more satisfactory.

To make agar slopes, tube the medium in 3–7-ml amounts according to the size of the tube required. After sterilization and before the medium sets, slope the tubes individually on the bench by leaning them against a length of glass or metal rod 6 or 7 mm in diameter. When making large batches, slope the tubes or bottles on wire racks, e.g. old refrigerator shelves, sloped at a convenient angle.

When pouring plates, raise the lid only far enough to permit the mouth of the tube or bottle to enter. Pour about 12–15 ml in each plate. Dry the plates slightly open in an incubator, and store them medium side-up in a refrigerator. An automatic plate pouring machine is useful in laboratories where many plates are used. This reduces the number of plates contaminated with airborne organisms to almost nil.

Contamination of sterile media during tubing and pouring may be a serious problem in some buildings. It can be minimized by using clean air cabinets of the simple, horizontal outflow variety.

Quality control and performance tests

Manufacturers of certain media and their ingredients impose stringent controls on the quality and performance of their products. Reconstitution and handling of these media in the laboratory (e.g. in overheating) may reduce its efficiency. Laboratory-prepared media are rarely tested.

Some kind of quality control should therefore be imposed on all materials used. The national system of quality control (in the UK) and performance tests (in the USA) operate satisfactorily only in clinical laboratories and problems arise mainly in the smaller industrial and food control establishments. Two simple systems are described here, one for isolation and selective media, the other for identification media.

Isolation and selective media: efficiency of plating technique (EOP)

New batches of culture media may vary considerably and should be tested in the following way.

Prepare serial tenfold dilutions, for example, 10^{-2} to 10^{-7} of cultures of various organisms which will grow on or be inhibited by the medium. For example, when testing DCA use *S. sonnei*, *S. typhi*, *S. typhimurium*, several other salmonellas and *E. coli*. Use at least four well dried plates of the test medium for each organism and at least two plates each of a known satisfactory control medium, and of a general medium, e.g. nutrient or MacConkey agar. Do Miles and Misra drop counts with the serial dilutions of the organisms on these plates so that each plate is used for several dilutions.

Count the colonies, tabulate the results and compare the performances of the various media. These may suggest that in a new batch of a special medium it is necessary to alter the proportion of certain ingredients.

Stock cultures, however, will frequently grow on media which will not

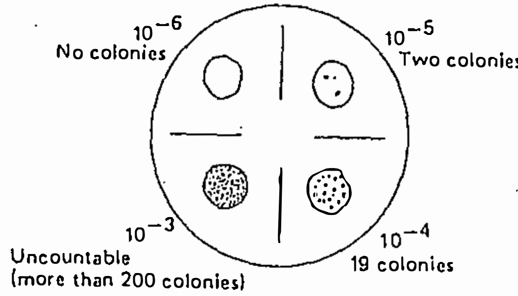


Figure 4.1 Miles and Misra count

support the growth of 'damaged' organisms from natural materials. It is best, therefore, to use dilutions or suspensions of such material.

With some culture media, these drop counts do not give satisfactory results because of reduced surface tension. An alternative procedure is recommended.

Make cardboard masks to fit over the tops of petri dishes and squares 25 × 25 mm in the centre of each. Place a mask over a test plate and drop one drop of the suspension through the square on the medium. Spread the drop over the area limited by the mask. This method has the additional advantage of making colony counts easier to perform but more plates must, of course, be tested.

In addition to these EOP tests, ordinary plating methods should be used to compare colony size and appearance.

Identification media

Maintain stock cultures of organisms known to give positive or negative reactions with the identification tests in routine use. The same organism may often be used for a number of tests. When a new batch of any identification medium is anticipated, subculture the appropriate stock strain so that a young, active culture is available. Test the new medium with this culture.

ANNEX 27

SECTION A: MICROBIOLOGICAL EXAMINATION OF WATER

Ref:Collins, C H & Lyne, P M (1984).
5th edition ch 25. "Water", pp 253-260. Microbiological Methods,
MUL 576.028 Col. Butterworths, London.

Natural waters and stored treated waters contain a wide variety of bacteria (pseudomonads, flavobacteria, micrococci, aerobic spore formers, enterobacteria and streptomycetes). Piped water may also contain iron bacteria while in river waters many kinds are found including both iron bacteria and sulphate reducing bacteria. Consequently, because of this great diversity, a single medium will detect only a fraction of the total microbial population present. Not all microbial groups attract universal interest except those organisms important to public health. Government and municipal authorities regularly perform bacteriological tests on water to ensure its safety for drinking, food and beverage manufacture, recreation and industrial uses. Such examination is intended to identify waters that have been contaminated with potential disease causing bacteria, viruses and protozoans. Such contamination may arise directly from human or animal sources, often faeces, or perhaps through breakdowns in sewage treatment systems or sewerage. As it is impossible to test for all pathogens, alternative procedures that test for are used.

Coliform tests are the most important as the presence of coliforms, particularly *E. coli* indicates if not actual pollution then a less than satisfactory supply. Other useful indicator tests depend on the detection of enteric streptococci and *Clostridium perfringens*.

In this laboratory session an examination of tap water, river water and pond water will be made and will involve: total heterotrophic plate counts, coliforms, *E. coli*,

Sampling

Samples will be provided for analysis. However, if students wish, they may bring in water samples from wells, ponds, rivers or lagoons for assay.

Normally about 100ml sample is taken in a clear sterile 120ml glass bottle (available from the laboratory). If chlorinated water samples are to be taken, the sample bottles must contain 0.1ml of a 3% solution of sodium thiosulphate to neutralize residual chlorine. These sample bottles should be prepared freshly.

Samples should be assayed within <u>six hours</u> of collection to ensure meaningful results.

What is a Coliform?

The coliforms are a group of gram-negative, rod-shaped, lactose fermenting bacteria native to the intestinal tract of mammals. They are members of the family of bacteria called Enterobacteriaceae and, like the other members, can be cultured from mammalian feces.

Total coliform refers to species of *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Edwardsiella*. All of these species, except *Escherichia*, can exist as free-living saprophytes as well as being intestinal organisms (ref. 1).

Fecal coliform defines primarily *Escherichia coli*, and occasionally *Klebsiella* sp. They are distinguished from the total coliform by having the capability of fermenting lactose at elevated temperatures as well as at 35°C, the optimum for coliform. The elevated temperature of 44.5°C ±0.2 has been shown to be the best temperature to select for coliforms specifically of fecal origin. Any total coliform count may include fecal coliform organisms.

Why Test for Coliform?

Epidemics such as typhoid fever, dysentery and cholera are caused by pathogenic bacteria transmitted via polluted drinking water. The organisms are difficult to culture *in vitro*. Waterborne intestinal parasites and viruses are an even greater challenge to laboratory analysis. Yet, it is extremely important to be able to detect their presence in order to determine whether or not the water is safe to drink.

Coliform organisms, while relatively harmless themselves, are almost always present in water that contains enteric pathogens. Thus, because they are relatively easy to isolate and because they normally survive longer than the disease-producing organisms, coliforms are a useful indicator of the possible presence of enteric pathogenic bacteria and viruses. In most cases, water that is free of total coliforms is considered free of disease-producing bacteria.

Fecal coliform analysis is a more definitive test for recent fecal pollution than the total coliform test, and fecal coliform is the standard test organism used in many laboratories testing treated sewage, untreated public water supplies, and such primary contact waters as swimming areas.

MEMBRANE-FILTER TECHNIQUE

Ref 1: Fresenius, W; Quentin, K.E. and Scheider, W. (1988). Water Analysis, A Practical Guide to Physico-Chemical, Chemical and Microbiological Water Examination and Quality Assurance, p640. Springer Verlag, Berlin. M.U.L. No. 628 161 Wat.

Ref 2: Standard Methods for the Examination of Water and Wastewater, 16th edition; A.P.H.A., A.W.W.A., W.P.C.F., N.Y., 1985 - Section 909 and 914 C p887 and p978).

Unlike the multiple-tube fermentation technic, the membrane-filter procedure permits a direct count of colonies.. The widespread use of the technic has confirmed its value, especially its high degree of reproducibility, the possibility of testing relatively larger volumes of sample, and its ability to yield definite results more rapidly than the standard tube procedure.

The wide acceptance of the Membrane Filtration (MF) technique is documented in the following quotation from Standard Methods for the Examination of Water and Wastewater (ref. 3, page 928).

"Since publication of the 11th Edition, widespread use of the (membrane filtration) technique has confirmed its value, especially its high degree of reproducibility, the possibility of testing relatively larger volumes of sample, and its ability to yield definite results more rapidly than the standard tube procedure . . ."

Public Health Service Drinking Water Standards (revised 1962) recommend the membrane filter method for analysis of interstate waters. The following advantages are cited by the National Training Center of the U. S. Environmental Protection Agency (EPA).

1. Results are obtained in approximately 24 hours, as compared with 48-96 hours for the standard fermentation tube method.

2. Much larger, and hence more representative, samples of water can be sampled routinely with membrane filters.

3. Numerical results from membrane filters have much greater precision (reproducibility) than is expected with the fermentation tube method.

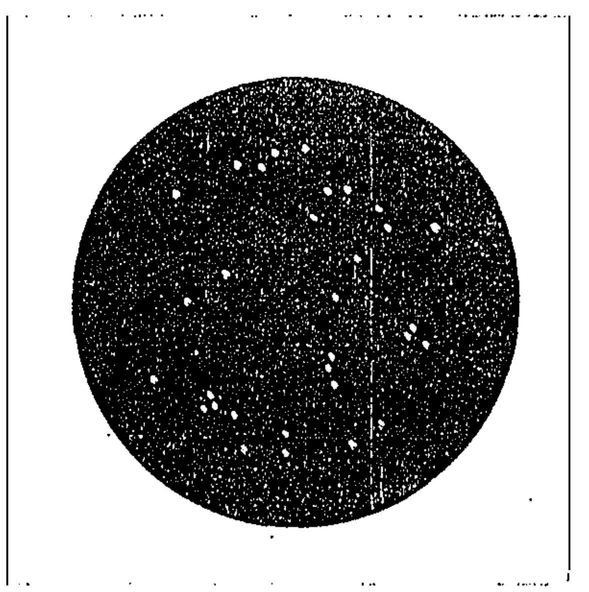
4. The equipment and supplies required are not bulky. A great many samples can be examined with minimum requirements for laboratory space, equipment, and supplies.

In the recent "Manual for the Interim Certification of Laboratories Involved in Analyzing Public Drinking Water Supplies", the EPA states that the MF method is preferred because it permits analysis of large sample volumes in reduced analysis time (ref.10).

In addition, the MF technique costs about half as much as the MPN method per test. This means considerable savings to any laboratory routinely performing coliform analyses.

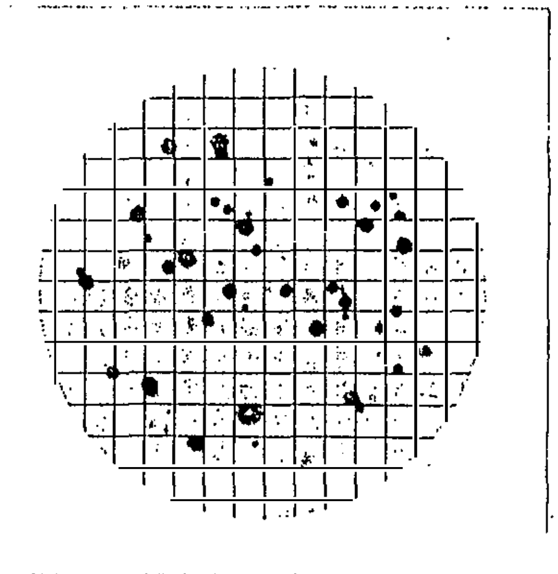
The results of bacteriological water testing can often be invalidated by delays in processing. For this reason, Standard Methods (ref. 3, page 907) recommends field testing when there would otherwise be more than a six-hour delay between sampling and processing. The MF method is the *only* approved method that lends itself to field testing.

How Do Coliforms Look When Cultured?



Total Coliform

This is how a total coliform culture might appear. After proper sampling and filtration, the bacterial retentive membrane filter is placed on top of MF-Endo media containing lactose, protein digest, vitamins, selective chemicals, and Schiff's Reagent. As the membrane incubates for 24 hours at 35°C ± 0.5, the media diffuses through the pores in the filter, supplying nutrients to the multiplying bacteria. Many kinds of bacteria from the water sample can grow and form colonies under these conditions, but only the coliforms will ferment lactose. One by-product of this reaction is an acid aldehyde complex that will combine with the Schiff's Reagent to form an iridescent green coating over the growing colonies. Thus, the coliforms can be identified as dark red colonies with a greenish-gold "sheen," when seen with 10-20X magnification and fluorescent illumination.



Fecal Coliform

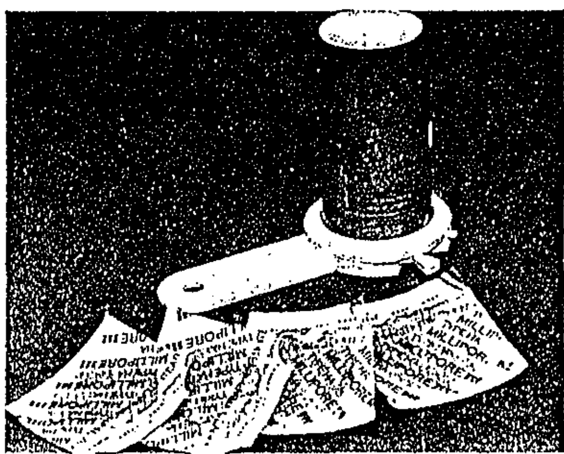
This is how a fecal coliform culture might appear. The filtration step is similar to that for total coliform. The media used for this test is M-FC, containing lactose, protein digest, vitamins, selective chemicals, and aniline blue dye. The membrane is incubated for 24 hours at 44.5°C ± 0.2, allowing only coliforms of fecal origin to grow into visible colonies. The non-fecal coliforms, due to heat shock, will not grow. As the fecal coliforms grow, they ferment lactose, producing acid which reacts with the aniline dye to produce a blue color. When viewed with a stereomicroscope at 10-20X magnification, all colonies exhibiting a blue color are fecal coliforms.

Expendables

Membrane Filters

The .45μm pore size membrane filter described below is recommended by Standard Methods for total and fecal coliform analysis. To help your laboratory comply with the legal requirements of the Safe Drinking Water Act of 1974, Millipore has introduced a comprehensive Certification Program that:

- Ensures greater precision, with fewer false positives and negatives.
- Minimizes quality control expenses.
- Complies with the legal requirements of the Safe Drinking Water Act.
- Improves test reliability.
- Ensures that you're using the most well-defined test materials that are available.

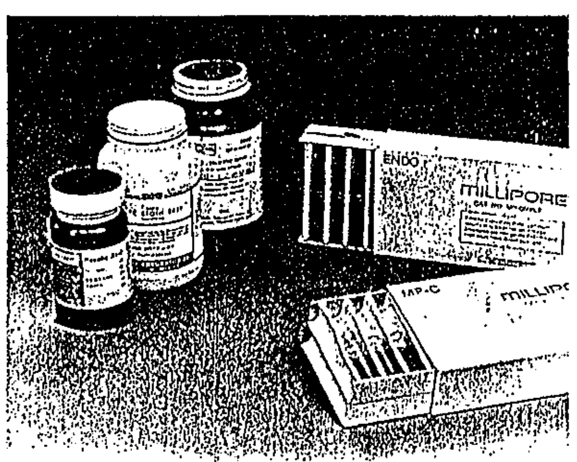


The membrane filter used for total and fecal coliform analysis is a 47 mm, white grid-marked filter with a 0.45μm pore size. White absorbent pads are used as a support for nutrient media. The *pre-sterilized* filters and pads are available in packages of different size and configuration to meet the needs of any laboratory.

Cat. No.	Description
HAWG 047 S0	100 HA Filters and 100 Pads 10 pkg. of 10/pkg.
HAWG 047 S1	1,000 HA Filters individually sealed in peel-back envelopes, 5 boxes of 200 envelopes each
HAWG 047 S2	1,000 HA Filters individually sealed and 1,000 Pads packed in 10 dispenser tubes, plus 2 plastic pad dispensers
HCWG 047 S1*	1,000 HC Filters individually sealed in peel-back envelopes, 5 boxes of 200 envelopes each
HCWG047 S2*	1,000 HC Filters individually sealed and 1,000 Pads packed in 10 dispenser tubes, plus 2 plastic pad dispensers.
HCWG 047 S3*	200 HC Filters individually sealed in peel-back envelopes.
HCWG 047 S4*	200 HC Filters individually sealed and 200 pads, plus 1 plastic pad dispenser.

Media

Growth media may be prepared from a dehydrated powder or used directly from premeasured, ready-to-use ampoules. The ampouled MF-Endo media is tested and certified by Millipore to comply with the Safe Drinking Water Act of 1974.

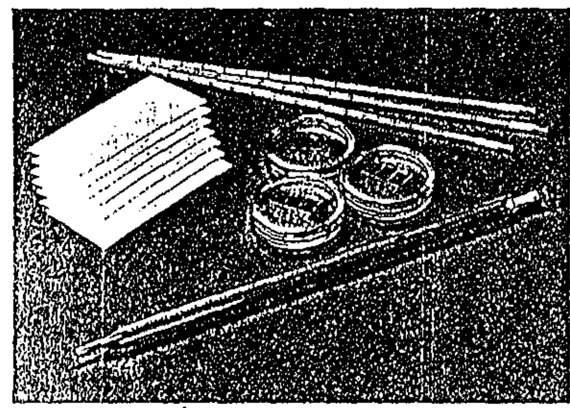


Cat. No.	Description
M000 000 2E	MF Endo Broth (Total Coliform) 2 ml ampoules, 24/pkg.
MB00 0000E	MF Endo Broth (Total Coliform) dehydrated, ¼ lb
M000 000 2F	M-FC Broth (Fecal Coliform) 2 ml ampoules, 24/pkg.
MB00 000 0F	M-FC Broth (Fecal Coliform) dehydrated, ¼ lb
MB00 000 0R	Rosalic Acid, 25 gm (used with dehydrated M-FC Broth)

Ampouled media for total count, yeast and mold, and other bacteria are also available.

* The HC filter (.7μm pore size) is designed for optimal recovery of stressed fecal coliforms from chlorinated effluents.

Additional Expendables



Cat. No.	Description
PD10 047 00	1. Sterile Plastic Petri Dishes, 100/pkg.
XX10 047 09	2. Graduated Pipette, 10 ml
XX63 001 35	3. Sterile Graduated Pipettes, plastic, 1 ml, 25/pkg.
EDWP 031 00	4. Blotter Pads, 2" × 4". 100 pkg.

Media Preparation

Both the MF-Endo media and the M-FC media can be purchased in ready-to-use 2 ml ampoules. These ampoules are easy to use and are warranted for one year. Small laboratories (doing less than 20 tests per day) should use ampouled media, as it is easier and less expensive than preparing broth media. If broth media are used, prepare according to the following instructions and be sure to use reagent grade water (ASTM Type I)* suitable for bacterial growth.

MF-Endo Broth (Total Coliform)

1. Weigh out 4.8 gm of dehydrate into a weighing dish.
2. Add 2 ml of 95% ethyl alcohol to 100 ml reagent grade water in a graduated cylinder.
3. Decant approximately 20 ml of solution from the graduated cylinder to a 250 ml screw-cap Erlenmeyer flask.
4. Empty the contents of the weighing dish into the Erlenmeyer flask.
5. Add the remaining contents from the graduated cylinder to the flask.
6. Place the flask, loosely covered, in a boiling water bath (or in makeshift beaker and hot-plate water bath).
7. Heat the medium for 3-5 minutes. Bring to the point of boiling, but do not allow to boil.
8. Remove and cool to 45°C. Adjust the pH to between 7.1 and 7.3 with 1N NaOH.
9. Left-over medium may be refrigerated at 2-10°C for 96 hours maximum and then discarded. It is best, however, to prepare fresh medium each day.

M-FC Broth (Fecal Coliform)

1. Add 100 ml of reagent grade water to 3.7 gm of dehydrated M-FC medium in a 250 ml screw-cap Erlenmeyer flask.
2. In a separate flask, add 100 ml of 0.2 N NaOH solution to 1 gm of rosalic acid dehydrate to produce a 1% rosalic acid solution.
3. Pipette 1 ml of 1% rosalic acid solution into the rehydrated M-FC broth.
4. Heat medium to the boiling point in a loosely covered flask; then promptly remove it and cool it to below 45°C. Do not autoclave.
5. Final pH should be 7.4.
6. Dispense at room temperature. Store the unused portion at 2-10°C and discard after 96 hours.

Phosphate Buffer

The Phosphate Buffer is used for diluting and rinsing samples. This solution must be sterile, since any organisms present in the buffer may interfere with coliform counts by overcrowding the membrane, producing toxic substances, or competing for nutrients. To prepare the buffer:

Stock Solution I — Dissolve 34.0 gm of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of reagent grade water. Adjust the pH to 7.2 with 1N NaOH (available commercially). Then dilute to 1,000 ml with reagent grade water to produce 1 liter of stock buffer solution.

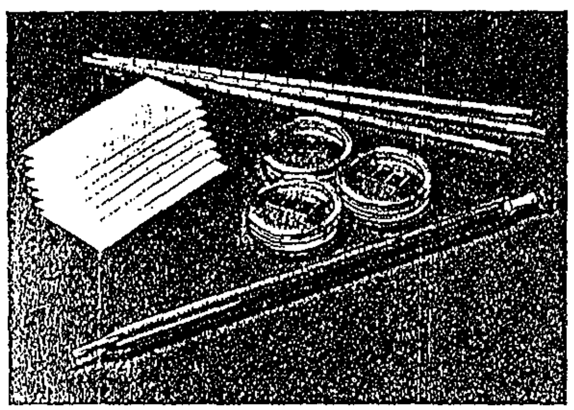
Stock Solution II — Dissolve 50 gm of magnesium sulfate ($MgSO_4 7H_2O$) in 1 liter of reagent grade water.**

Working Solution — Add together 1.25 ml of Stock Solution I and 5.0 ml of Stock Solution II and dilute to 1 liter with reagent grade water.

* Reagent Grade Water (ASTM Type I) is purified by reverse osmosis (RO) followed by deionization and membrane filtration.

** Magnesium sulphate is added to the buffer to increase the recovery of organisms with metabolic injury due to high-quality water or to waters containing concentrations of heavy metal ions (ref. 4, p.).

Additional Expendables



Cat. No.	Description
PD10047 00	1. Sterile Plastic Petri Dishes, 100/pkg.
XX10 047 09	2. Graduated Pipette, 10 ml
XX63 001 35	3. Sterile Graduated Pipettes, plastic, 1 ml; 25/pkg.
EDWP 031 00	4. Blotter Pads, 2" × 4", 100 pkg.

Media Preparation

Both the MF-Endo media and the M-FC media can be purchased in ready-to-use 2 ml ampoules. These ampoules are easy to use and are warranted for one year. Small laboratories (doing less than 20 tests per day) should use ampouled media, as it is easier and less expensive than preparing broth media. If broth media are used, prepare according to the following instructions and be sure to use reagent grade water (ASTM Type I)* suitable for bacterial growth.

MF-Endo Broth (Total Coliform)

1. Weigh out 4.8 gm of dehydrate into a weighing dish.

2. Add 2 ml of 95% ethyl alcohol to 100 ml reagent grade water in a graduated cylinder.

3. Decant approximately 20 ml of solution from the graduated cylinder to a 250 ml screw-cap Erlenmeyer flask.

4. Empty the contents of the weighing dish into the Erlenmeyer flask.

5. Add the remaining contents from the graduated cylinder to the flask.

6. Place the flask, loosely covered, in a boiling water bath (or in makeshift beaker and hot-plate water bath).

7. Heat the medium for 3-5 minutes. Bring to the point of boiling, but do not allow to boil.

8. Remove and cool to 45°C. Adjust the pH to between 7.1 and 7.3 with 1N NaOH.

9. Left-over medium may be refrigerated at 2 - 10°C for 96 hours maximum and then discarded. It is best, however, to prepare fresh medium each day.

M-FC Broth (Fecal Coliform)

1. Add 100 ml of reagent grade water to 3.7 gm of dehydrated M-FC medium in a 250 ml screw-cap Erlenmeyer flask.

2. In a separate flask, add 100 ml of 0.2 N NaOH solution to 1 gm of rosalic acid dehydrate to produce a 1% rosalic acid solution.

3. Pipette 1 ml of 1% rosalic acid solution into the rehydrated M-FC broth.

4. Heat medium to the boiling point in a loosely covered flask; then promptly remove it and cool it to below 45°C. Do not autoclave.

5. Final pH should be 7.4.

6. Dispense at room temperature. Store the unused portion at 2-10°C and discard after 96 hours.

Phosphate Buffer

The Phosphate Buffer is used for diluting and rinsing samples. This solution must be sterile, since any organisms present in the buffer may interfere with coliform counts by overcrowding the membrane, producing toxic substances, or competing for nutrients. To prepare the buffer:

Stock Solution I — Dissolve 34.0 gm of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of reagent grade water. Adjust the pH to 7.2 with 1N NaOH (available commercially). Then dilute to 1,000 ml with reagent grade water to produce 1 liter of stock buffer solution.

Stock Solution II — Dissolve 50 gm of magnesium sulfate ($MgSO_4 7H_2O$) in 1 liter of reagent grade water.**

Working Solution — Add together 1.25 ml of Stock Solution I and 5.0 ml of Stock Solution II and dilute to 1 liter with reagent grade water.

* Reagent Grade Water (ASTM Type I) is purified by reverse osmosis (RO) followed by deionization and membrane filtration.

** Magnesium sulphate is added to the buffer to increase the recovery of organisms with metabolic injury due to high-quality water or to waters containing significant concentrations of heavy metal ions (ref. 4, p. 71).

Sterilization Procedures*

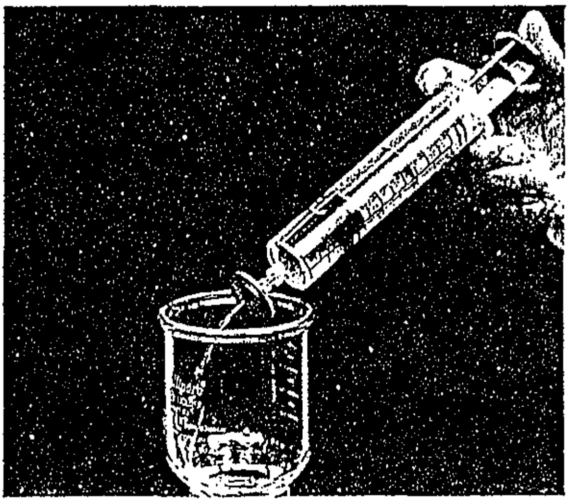
Sterilize the phosphate buffer via autoclave or membrane filtration.

1. *Autoclave technique*: For rinse buffer, either keep the solution in a liter bottle or dispense it into 100 ml bottles. For dilution flasks, dispense in amounts that will provide 99 ± 2.0 ml or 9.0 ± 0.2 ml after autoclaving. Loosely cap or cover and autoclave at 121°C, 15 psi for at least 20 minutes. Use the slow exhaust cycle.

2a. *Membrane filter technique*: If an autoclave is not available, but an oven is, sterilize a one liter empty flask for 1 hour at 170°C. Install a sterile filter holder and sterile HA filter, and process the liter or buffer via vacuum filtration. It may be necessary to use a prefilter (AP20) to prevent membrane clogging. The buffer may be either dispensed from the side-arm or transferred to another sterile bottle. Do not store this buffer for long periods of time.

2b. *Membrane filter technique*: If an autoclave and oven are not available, process the buffer as in 2a to remove particles. Then dispense the buffer directly into the filter funnel or dilution flask as needed with a plastic syringe and attached sterile Millex® filter unit (Cat. No. SLHA 025 0S - see picture below).

Membrane filtration of buffer is actually better than autoclaving because dead bacteria and all particles, as well as live bacteria, are removed.



Proper sterilization of equipment is of utmost importance when performing microbiological analysis. These procedures are necessary to insure that interfering organisms are kept to a minimum. The following section outlines the sterilization procedures that must be performed in order to obtain valid results.

Sampling Bottles And Pipettes

Use sampling bottles made of borosilicate glass or heat resistant polypropylene having a minimum capacity of 120 ml. If the water to be sampled is chlorinated, add 0.1 ml of a 10% sodium thiosulfate solution, a dechlorinating agent, to the bottle prior to sterilization. Wrap the bottle in Kraft paper and autoclave at 121°C and 15 psi for 15 minutes, or, if glass, dry heat sterilize at 170°C for 1 hour. If glass pipettes are used, autoclave them at 121°C and 15 psi for 30 minutes, or dry heat sterilize at 170°C for 1 hour.

Filter Holders

The filter holders can be sterilized by any of the following methods.

1. *Ultraviolet Light*. This method is recommended for all Millipore filter holders because of its speed, safety, and efficient kill (99.9%). The EPA recommends a two-minute exposure period for clean filter holders (ref. 4).

2. *Steam Autoclaving*. Wrap the funnel and base in Kraft paper and autoclave at 121°C and 15 psi for 15-20 minutes. Units remain sterile until opened.

3. *Dry Heat*. This is only recommended for the pyrex holder. Remove stopper, wrap in aluminum foil, and heat 1 hour at 170°C. Units remain sterile until opened.

The filter holders can be sanitized by any one of the following methods:

1. *Boiling*. Any holder can be sanitized, just prior to use by immersion in boiling water for 3-5 minutes. Use promptly upon cooling.

2. *70% Alcohol Immersion and Drying*. Use this procedure only when other methods are not available. Immerse for a few minutes, allow to air dry and use promptly. This works with any holder.

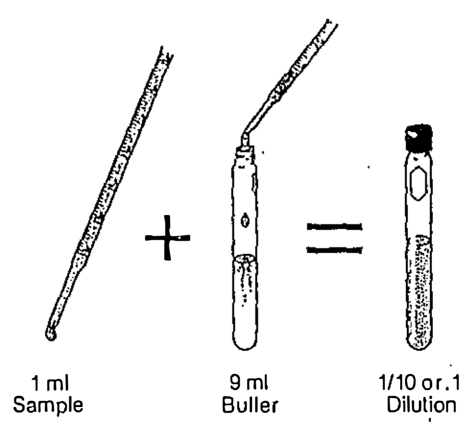
* These sterilization procedures are acceptable for coliform analysis only.

Sampling

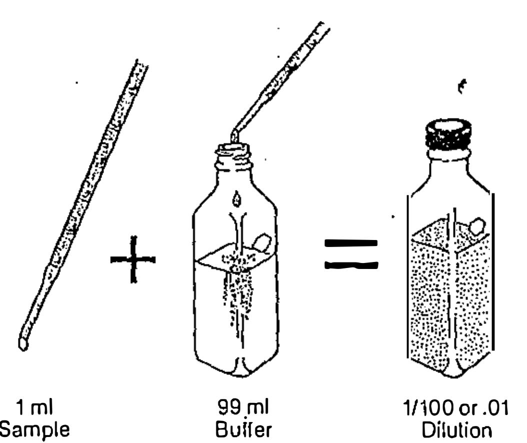
The technologist should ascertain appropriate sample sizes from history of the water source and its expected pollution level. Federal law requires that a drinking water sample be assayed for 100 ml. This can be done with a single sample or two 50 ml samples. Wastewater may contain thousands of coliform per 100 ml, and the sample size should be smaller, perhaps 1 ml or fractions of 1 ml. The following chart recommends trial sampling sizes.

Trial Sample Sizes for Coliform Level	
Drinking Water	50 ml, 100 ml
Well Water	10, 50, 100 ml
Unpolluted Surface Waters	1, 5, 10, 50 ml
Polluted Surface Waters	.05, 0.1, 0.5, 1.0 ml
Treated Sewage	0.1, 0.5, 1.0, 5.0 10.0 ml
Raw Sewage	0.0001, .0005, .001, .005, .01, .05, 0.1 ml

Making Dilutions



1 ml Sample   9 ml Buffer   1/10 or .1 Dilution



1 ml Sample   99 ml Buffer   1/100 or .01 Dilution

Making Dilutions

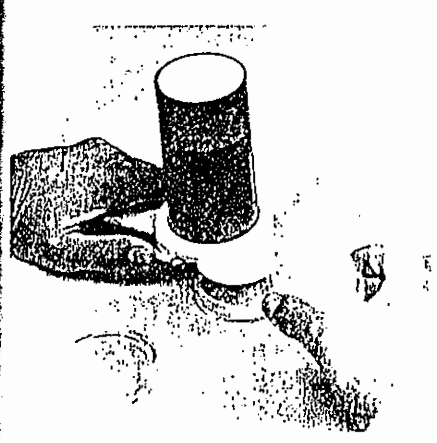
Accurate measurement of sample size below 1.0 ml requires dilutions in sterile buffered water. The most convenient way is to sterilize 9.0 ml or 99.0 ml in dilution bottles or test tubes. To make a 1:10 dilution, aseptically pipette 1.0 ml of sample into 9.0 ml sterile buffer and mix thoroughly. If you remove 1.0 ml from this for filtration, you have a 0.1 ml sample. If you remove 0.5 ml from this, you have a 0.05 ml sample. To make a 1:100 dilution either pipette 1.0 ml from a 1:10 dilution into another 9.0 ml of sterile buffer and mix, removing 1.0 ml for filtration, or pipette 1.0 ml of undiluted sample into 99 ml and remove 1.0 ml for filtration. With this basic technique, any dilution is possible.

Diluted samples should indicate fewer organisms on the membrane. For instance, if a 10 ml sample shows 20 fecal coliform, a 1.0 ml sample should show about 2. Colony counts that are greater than 200 total colonies (both coliform and non-coliform) may give erroneous statistical data due to overcrowding. What may appear as a single colony may actually have originated as many bacteria under crowded conditions. "Mixed colonies" may also interfere with sheen development in total coliform analysis or development of blue color in fecal coliform. If the most dilute sample results in coliform growth that is too numerous to count, we recommend resampling the water supply and using a more diluted sample size.

Laboratory Procedure

Preparation of Petri Dishes

The first step in the laboratory procedure is to prepare the nutrient on which the microorganisms will be cultured. A 47 mm petri dish is prepared by aseptically pouring liquid Endo Agar into the bottom of the dish and allowing it to gel, or by using a sterile absorbent pad saturated with liquid broth from either a stock solution or from premeasured ampoules. The following procedure describes the latter approach:

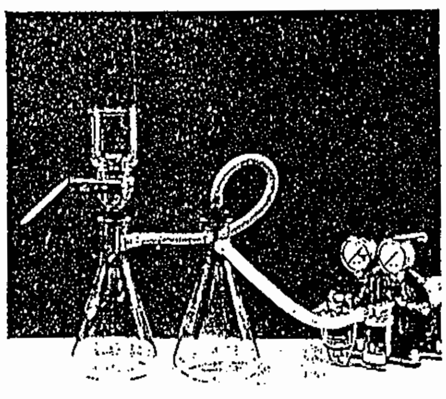


1. Open a petri dish using the handle of the forceps. Place a sterile absorbent pad in the petri dish using the dispenser.

2. Break open an ampoule of appropriate media and aseptically pour the contents evenly over the absorbent pad. If stock media has been prepared pipette 2 ml of this solution onto the absorbent pad. Close the petri dish, mark it appropriately for sample identification and set it aside.

Filtration

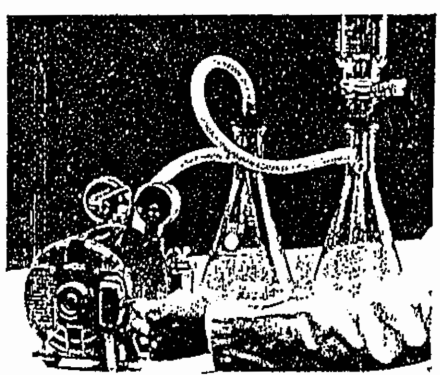
The procedures described below illustrate the use of a single Pyrex Filter Holder; however, the test may be performed with any of several 47 mm Millipore filter holders, either singly or in a manifold.



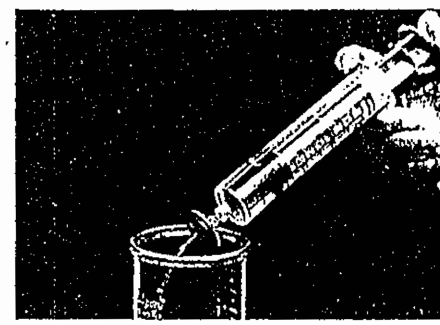
3. Set up the filtration apparatus as shown. Be sure to use a water trap between the pump and the filter holder.

4. Load a sterile membrane, grid side up, in the sterilized filter holder. When handling the filter be sure to use smooth-tipped forceps that have been dipped in alcohol, then quickly flamed to sterilize the tips and allowed to cool for a few seconds.

5. Mix the water sample by vigorously shaking for several seconds. For samples larger than 10 ml, pour the sample into the funnel, wetting the filter. For smaller sample sizes, first pour about 20 ml of sterile buffer into the funnel and then pipette the sample onto the buffer.

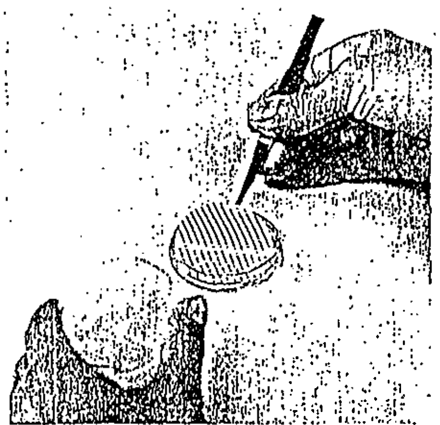


6. Apply the vacuum to filter the sample.



7. Rinse the funnel walls with at least 30 ml sterile buffer. Draw rinse buffer through. Repeat this rinse twice, for a total of three rinses. If autoclaved buffer is not available, a syringe and Millex® filter can be used to deliver sterile buffer.

Incubation

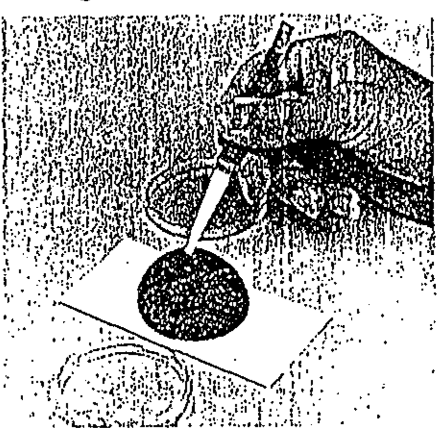


8. Turn off the vacuum and lift off the filter holder funnel. Remove the membrane filter using flame-sterilized forceps and transfer it immediately to a previously prepared petri dish. With a slight rolling motion, center the filter, grid side up, on the absorbent pad. Be careful not to trap air under the filter as this will prevent nutrient media from reaching parts of the membrane surface.

9. Replace the cover tightly. Incubate the petri dish upside down for 24 hours at the appropriate temperature (total coliform at 35.0 ± 0.5°C, fecal coliform at 44.5 ± 0.2°C). The use of a heatsink type incubator, such as the Aluminum Block Incubator, is recommended by the EPA (ref. 4) and Standard Methods (ref. 3). If using a waterbath, be sure to seal the petri dishes with water-proof tape or place them in sealable plastic bags (e.g. Whirl-Pak® bags).

10. Resanitize the filter holder prior to insertion of a new membrane for the next sample by placing the holder into the Ultraviolet Light Sterilizer for a period of 3 minutes. When filtering a series of dilutions from a single water sample, such as 0.1, 1.0 and 10.0 ml, the rinse buffer is appropriate sanitization if the most dilute samples are filtered first.

Identification and Counting



11. There are two procedures for counting coliform colonies on the membrane; either may be used.

Wet Method: After the required incubation period, turn the dish right side up and remove the lid of the petri dish. Place the bottom of the petri dish containing the membrane filter in the microscope field.

or

Dry Method: After removing the lid of the petri dish, gently remove the membrane filter using smooth tipped forceps and place it grid side up on a clean blotter. After the filter has air dried, place it in the microscope field. Air drying will enhance the sheen of coliform colonies.

12. Use a low-power (10 - 20X) binocular microscope with a day-light white fluorescent light source held perpendicular to the plane of filter. If determining total coliform, examine the surface of the filter fo colonies having a shiny greenish s face. The sheen may cover the en colony or it may appear only in the central area or on the periphery. Count the total number of these "green sheen" colonies.*

If determining fecal coliform, co all the colonies exhibiting a *blue* color. These are the fecal coliform There may also be some colonies that are cream or gray colored. The are not fecal coliform, but probab some thermophilic organisms tha were in the water sample. In order avoid errors caused by subtle co changes, it is important to examin the filters for fecal coliform within 3 minutes after the samples are re-moved from the incubator.

*In some cases (state-certified public hea labs) verification of colonies for lactose fe mentation in lauryl triptose broth and selec broths, such as brilliant green bile or EC br may be required. Refer to *Standard Metho* for these procedures.

Calculations

13. The accepted way of expressing coliform levels in water is in terms of the number per 100 ml. Bear in mind that each colony observed developed from a single bacterial cell in the original sample. If a 100 ml sample is used (as is most likely the case in potable water analysis), then the number of coliform counted is the same as the number reported. However, for waters where less than 100 ml sample is used, apply the following formula:

$$\frac{\text{No. of coliforms counted} \times 100}{\text{Sample size (ml)}} = \text{No. of coliform per 100 ml of sample}$$

Total Coliform Example:

A 100 ml sample was split into two 50 ml samples and filtered through separate filters. After incubation, 60 "sheen" colonies were counted on one filter and 49 "sheen" colonies counted on the other.

Therefore,

$$\frac{(60+49) \times 100}{50+50} \doteq 109 \text{ coliform/100ml}$$

Fecal Coliform Example:

A 1 ml sample was added to the filter funnel which contained approximately 20 ml of sterile buffer. After filtration and incubation, 36 blue colonies were counted.

Therefore,

$(36 \times 100)/1 = 3{,}600$ fecal coliform/100ml

References

The following is a list of references which may be useful for background information on water microbiology and for problem solving your laboratory procedures.

1. Davis, B. D., et al, *Microbiology*, 2nd Edition, Harper, Row, Publishers, Inc., Hagerstown, Maryland, 1973.

2. Geldreich, E. E., Clark, H. F., Huff, C. B. and Best, L. C., "Fecal-coliform-organism medium for the membrane filter technique." JAWWA, 57, 208-214, 1965.

3. Standard Methods for the Examination of Water and Wastewater, Fourteenth Edition, American Public Health Assoc., American Water Works Assoc., Water Pollution Control Federation.

4. Geldreich, E. E., Handbook for Evaluating Water Bacteriological Laboratories, 2nd Edition, U.S. Environmental Protection Agency, Cincinnati, 1975.

5. Microbiological Methods for Monitoring the Environment: I. Water and Wastes. Prepared by Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268, (In preparation, 1977).

6. ASTM Standard D 3508-76T, "Tentative Method for Evaluating Water Testing Membrane Filters for Fecal Coliform Recovery."

7. Public Law 93-523, Dec. 16, 1974, "Safe Drinking Water Act."

8. Peterson, J., "Comparison of MF Technique and MPN Technique for the Estimation of Coliforms in Water." *Public Health Laboratory*, 32, 182-193, 1974.

9. Pelzcar, M. J., Chan and Reid, R. D., *Microbiology*, McGraw Hill, New York, 1977.

10. Manual for the Interim Certification of Laboratories Involved in Analyzing Public Drinking Water Supplies: Criteria and Procedures. Prepared by The Water Supply Quality Assurance Work Group, The U.S. Environmental Protection Agency, Washington, D.C. 20460 (In preparation, 1977)

MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP

Principles:

The membrane filter (MF) technique is highly reproducible, can be used to test relatively large volumes of sample, and yields numerical results more rapidly than the multiple-tube procedure. The membrane filter technique is extremely useful in monitoring drinking water emergencies and for the examination of a variety of natural waters.

Interferences:

Turbidity caused by the presence of algae or other interfering materials may not permit testing of a sample volume sufficient to yield significant results. Low coliform estimates may be caused by the presence of high numbers of non-coliforms or of toxic substances. The MF technique is applicable to the examination of saline water but not wastewater that have received only primary treatment followed by chlorination because of turbidity in high volume sample or wastewater containing toxic metals or toxic organics such as phenol. For the detection of stress total coliforms in treated drinking water and chlorinated secondary or tertiary wastewater effluents used a method designed for stressed organism recovery (see Section 9212B.1).

1. Sample Collection/Preservation:

Collect samples for microbiological examination in bottles that have been cleaned and rinsed carefully, given a final rinse with distilled water and sterilised as directed in Sections 9030 and 9040. For some applications samples may be collected in presterilized plastic bags.

2. Dechlorination:

Add a reducing agent to containers intended for the collection of water having residual chlorine or other halogen. Sodium thiosulphate ($Na_2S_2O_3$) is a satisfactory dechlorinating agent that neutralises any residual halogen and prevents continuation of bactericidal action during sample transit. The examination then will indicate more accurately the true microbial content of the water at the time of sampling.

For sampling chlorinated wastewater effluents add sufficient $Na_2S_2O_3$ to a clean sample bottle to give a concentration of 100 mg/l in the sample. In a 120 ml bottle 0.1 ml of a 10% solution of $Na_2S_2O_3$ will neutralise a sample containing

about 15 mg/l residual chlorine. For drinking water samples, the concentrations of dechlorinating agent may be reduced to 0.1 ml of a 3% solution of $Na_2S_2O_3$ in a 120 ml bottle will a give a final concentration of 18 am/l in the sample and will neutralise up to 5 mg/l residual chlorine. Cap bottle and sterilise by moist heat at 121°C for 15 minutes.

Collect water samples high in copper or zinc and wastewater samples high in heavy metals in sample bottles containing a chelating agent that will reduce metals toxicity. This is particularly significant when such samples are in transit for 4 hours or more. Use 372 mg/l of the disodium salt of ethylenediaminetetraacetic acid (EDTA). Adjust EDTA solution to pH 6.5 before use. Add EDTA separately to sample bottle before bottle sterilisation (0.3 ml 15% solution in a 120 ml bottle) or combine it with the $Na_2S_2O_3$ solution before addition.

3. Sampling Procedure:

When the sample is collected, leave sample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, before examination. Collect samples that are representative of the water being tested, flush or disinfect sample posts and use aseptic techniques to avoid sample contamination.

Keep sampling bottle closed until it is to be filled. Remove stopper and cap as a unit; do not contaminate inner surface of stopper or cap and neck of bottle. Fill container without rinsing, replace stopper or cap immediately, and if used, secure hood around neck of bottle.

a) Portable Water:

If the water sample is to be taken from a distribution system tap without attachments, select a tap that is supplying water from a service pipe directly connected with the main and is not for example served from cistern or storage tank. Open tap fully and let water run to waste for 2 or 3 mins or for a time sufficient to permit clearing the service line. Reduce water flow to permit filling bottle without splashing. If tap cleanliness is questionable, apply a solution of sodium hypochlorite (100 mg NaOCl/l) to faucet before sampling, let water run for additional 2 to 3 mins after treatment. Do not sample from leaking taps that allow water to flow over the outside of the tap.

b) Raw Water Supply:

In collecting samples directly from a river, stream, lake, reservoir, spring, or shallow well, obtain samples representative of the water that is the source of supply to consumer.

c) Surface Water:

Samples may be collected from a boat or from bridges near critical study point.

d) Bathing Beaches:

Sampling locations for recreational areas should reflect water quality within the entire recreational zone. Include sites from upstream peripheral areas and locations adjacent to drains or natural contours that would discharge stormwater collections or septic wastes. Collect samples in the swimming area from a uniform depth of approximately 1m.

e) Manual Sampling:

Take samples from a river, stream, lake or reservoir by holding the bottle near its base in the hand and plunging it, neck downward, below the surface. Turn the bottle until the neck points slightly upward and mouth is directed toward the current. If there is no current, as in the case of a reservoir, create a current artificially by pushing bottle forward horizontally in a direction away from the hand.

When sampling from a boat, obtain samples from upstream side of boat. If it is not possible to collect samples from these situations in this way, attach a weight to base of bottle and lower it into the water. In any case, take care to avoid contact with bank or stream bed, water fouling may occur.

Size of Sample:

The volume of sample should be sufficient to carry out all test required, preferably not less than 100 ml.

Preservation and Storage:

1. Holding time and temperature: Start microbiological examination of a water sample promptly after collection to avoid unpredictable changes. If samples cannot be processed within 1 hour after collection use an iced cooler for storage during transport to the laboratory. If it is known that the results will be used in legal action, employ a special messenger to delivery samples to the laboratory within 6 hours and maintain chain of custody.

   Hold temperature of all stream pollution, drinking and wastewater samples below 10°C during a maximum transport time of 6 hours.

Laboratory Apparatus:

Sample bottles
Dilution bottles

Pipettes and graduated cylinder
Disposable sterile petri dishes with pad

Filtration units: The filter holder assembly consists of a seamless funnel fastened to a base by a locking device or held in place by magnetic force. The design should permit the membrane filter to be held securely on the porous plate of the receptacle without mechanical damage and allow all fluid to pass through the membrane during filtration.

Separately wrap the two posts of the assembly in heavy wrapping paper, sterilise by autoclaving and store until use.

For filtration, mount receptacle of filter holding assembly in a manifold to hold 3 filter assemblies such that a pressure differential can be exerted on the filter membrane.

Membrane filter: Use membrane filter 0.45 μm.

Forceps: Smooth-tipped, without corrugations on the inner sides of the tips. Sterilise before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.

Incubators: Use incubators to provide a temperature of 35° ± 0.5°C and to maintain a high level of humidity.

Colony counter

Quality Control:

Test each new medium lot for satisfactory productivity by preparing dilutions of a culture of Enterobacter Aerogenes and filtering approximate volumes to give 20-80 colonies per filter. With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies, obtained from natural samples, to establish the differential accuracy of the medium lot.

General Quality Control Procedures:

1. For membrane filter tests, check sterility of media, membrane filter, dilution and rinse with water glassware and equipment as a minimum at the end of each series of samples, using sterile water as the sample.

2. For each lot of medium check analytical procedures by testing with known positive and negative control cultures for the organisms under test.

3. Perform duplicate analyses on 5% of samples and on at least one sample per test run.

Quality Control on Membrane Filter Procedures:

1. Colony verification: For each type of test conducted, verify colonies monthly from a known positive sample.

Materials and Culture Media:

The need for uniformity dictates the use of dehydrated media. Never prepare media for basic ingredients when suitable dehydrated media are available. Follow manufacturers directions for rehydration and sterilisation.

M-Endo broth - Weigh 48g. 1 litre distilled water containing 20 ml 95% ethanol. Heat to near boiling, cool to below 50°C. Do not sterilise by autoclaving. Final pH should be between 7.1 and 7.3. Store finished medium in the dark at 2-10° and discard any unused medium after 96 hours.

Procedure:

a) Selection of sample size.
Size of sample will be governed by expected bacterial density which in drinking water samples will be limited only by the degree of turbidity or by the non-coliform growth on the medium.

An ideal sample volume will yield about 50 coliform colonies and not more than 200 colonies of all types. Analyse drinking waters by filtering 100 to 500 ml or more, or by filtering replicate smaller sample volumes such as duplicate 50 ml or four replicates of 25 ml portions. Analyse other water by filtering three different volumes (diluted or undiluted) depending on the expected bacterial density, which in drinking water samples will be limited only by the degree of turbidity or by non-coliform growth on the medium. An ideal sample volume will yield about 50 coliform colonies and not more than 200 colonies of all types.

Analyse drinking water by filtering 100 to 500 ml or more, or by filtering replicate smaller sample volumes such as duplicate 50 ml or four replicates of 25 ml portions. Analyse other water by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. When less than 20 ml of sample (diluted or undiluted) is to be filtered, add approximately 10 ml sterile dilution water to the funnel before filtration. This increase in water volume in acids is uniform dispersion of the bacterial suspension over the entire effective filtering surface.

Suggested Sample Volumes for Membrane Filter
(Total Coliform Test)
Volume to be filtered

	ml							
Water Source	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking water	x							
Swimming pool	x							
Wells, springs	x	x	x					
Lakes, reservoirs	x	x	x					
Water supply intake			x	x	x			
Bathing beaches			x	x	x			
River water				x	x	x	x	
Chlorinated sewage			x	x	x	x		
Raw sewage					x	x	x	x

b) Filtration of sample.

Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of receptacle. Carefully place matched funnel unit over receptacle and lock it in place. Filter sample under partial vacuum. With filter still in place, rinse funnel by filtering three 20 to 30 ml portions of sterile dilution water. Upon completion of final rinse and the filtration process disengage vacuum, unlock and remove funnel, immediately remove membrane filter with sterile forceps and place it on selected medium with a rolling motion to avoid entrapment of air. Insert a sterile rinse water sample (100 ml) after filtration of a series of 10 samples to check for possible cross-contamination or contaminated rinse water. Incubate the control membrane culture under the same conditions as the sample.

Use sterile filter units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilise all membrane filter holder in use. Decontaminate this equipment between successive filtrations by using an ultraviolet (UV) steriliser, flowing steam or boiling water. In the UV sterilisation procedure, a 2 min exposure to UV radiation is sufficient. Do not expose membrane filter culture preparations to random UV radiation leaks that might emanate from the sterilisation cabinet. Eye protection is recommended, safety glasses, clean UV tube regularly and check it periodically for effectiveness to ensure that it will produce a 99.9% bacterial kill in a 2 min exposure.

Enrichment Technique

Place a sterile absorbent pad in the upper half of a sterile culture dish and pipette enough enrichment medium 1.8-2.0 ml M-Endo broth to saturate pad. Aseptically place filter through while the sample has been passed on pad. Transfer filter carefully onto pads. Incorrect filter placement is at once obvious because patches of unstained membrane indicate entrapment of air. Where such patches occur, carefully reseat filter on to pad. Invert dish and incubate for 20 to 22 hours at 35 $\pm$ 0.5°C.

Counting : The typical coliform colony has a pink to dark red colour with a metallic surface sheen. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Count sheen colonies with the aid of a low-power 10x binocular identified dissecting microscope or other optical device. Colonies that lack sheen may be pink noncoliforms. The total count of colonies (coliform or noncoliform) on Endotype medium has no relation to the total number of bacteria present in the original sample. However, a high count of noncoliform colonies may interfere with the maximum development of coliforms. Anaerobic incubation suppress noncoliform colonies but must be carefully evaluated to ensure no loss of coliform recovery. Samples of disinfected water or wastewater effluent may

include stressed organisms that grow relatively slowly and produce maximum sheen in 22 to 24 hours. Organisms from disinfected sources may produce sheen at 16 to 18 hours, and the sheen subsequently may fade after 24 to 30 hours.

Coliform verification : Typical sheen colonies may be produced occasionally by noncoliform organisms. Verify by a test for lactose fermentation or by using alternative procedures involving either a rapid (4 hour) test or two key biochemical reactions or a API.

Calculation of Coliform Density : Report coliform density as (total) coliforms/100 ml. Compute the count using membrane filters with 20 to 80 coliform colonies and not more than 200 colonies of all types per membrane by the following equation:

(Total) coliform colonies/100 ml

$$= \frac{\text{Coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

For verified counts, adjust the initial count based upon the positive verification percentage and report as "verified coliform count per 100 ml".

Percentage verified coliforms

$$= \frac{\text{Number of verified colonies}}{\text{total number of sheen colonies}} \times 100$$

Notes

a) Quality of Drinking Water : With water of good quality, the occurrence of coliforms generally will be minimal. Therefore, count all coliform colonies and use the formula given above to obtain coliform density. If the confluent growth occurs, that is, growth covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with (or without) coliforms" and request a new sample from the same location. On re-examination, divide 100 ml sample into duplicate 50 ml test portions to reduce interference from overcrowding of the MF surface. If the total number of bacterial colonies, coliforms plus non-coliforms exceed 200 per membrane, or if the colonies are not distinct enough for accurate counting, report results as "too numerous to count" TNTC. The presence of coliforms in such cultures showing no sheen may be indicated by placing the entire membrane filter culture into a sterile tube of brilliant green lactose bile broth. As an alternative, brush the entire filter surface with a sterile loop or applicator stick and inoculate this growth to the tube of brilliant green lactose bile broth. If gas if produced from this culture within 48 hours at 35 $\pm$ 0.5°C, conclude that coliforms are present.

FAECAL COLIFORM MEMBRANE FILTER PROCEDURE

Principles:

Faecal coliform bacterial densities may be determined either by the multiple-tube procedure or by a membrane filter (MF) technique. The MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2°C for selectivity and gives 93% accuracy in differentiating between coliforms found in the faeces of warm-blooded animals and those from other environmental sources. Because incubation temperature is critical, submerge waterproof (plastic bag enclosures) MF cultures in a water bath for incubation at an elevated temperature or use an appropriate, accurate solid heat sink incubator.

Interference:

For most samples M-FC medium may be used without the 1% rosalic acid addition, provided there is no interference with background growth. Such interference may be expected in stormwater samples collected during the first runoff after a long dry period.

Sample Collection/Preservation:

As for total coliform precision bias and detection limit.

Quality Control:

See section 9020 of APHA Method pages 9-4-9.

Materials and Culture Medium:

a) M-FC medium: The need for uniformity dictates the use of dehydrated media.

   Directions: Suspend 3.7g of the powder in 100 ml of purified water, containing 1 ml of rosalic acid, 1% in 0.2N (NaOH) sodium hydroxide. Heat with agitation until the broth boils. Cool and use. Do not autoclave. Final pH 7.4 ± 0.2. Test samples of the finished product for performance using stable, typical control cultures, Escherichia Coli.

b) Culture petri dish: Use tight-fitting plastic dishes because the MF cultures are submerged in a waterbath during incubation. Enclose groups of faecal coliform cultures in plastic bags or seal individual dishes with waterproof tape to prevent leakage during submission.

c) Incubator: The specificity of the faecal coliform test is related directly to the incubation temperature. Static air incubation is undesirable because of potential heat laying within the chamber and the slow recovery of temperature each time the incubator is opened during daily operations. To meet the need for greater temperature control use a waterbath. A temperature tolerance of 44.5 ±0.2°C can be obtained with most types of waterbaths that also are equipped with a tap for the reduction of water and heat losses. A circulating waterbath is excellent but may not be essential to this test if the maximum permissible variation of 0.2°C in temperature can be maintained with other equipment.

Procedure:

a) Selection of sample size: Select volume of water sample to be examined from the table below.

Sample Volumes for Membrane Filter Faecal Coliform Test
Volume (x) to be Filtered

Water Source	ml						
	100	50	10	1	0.1	0.01	0.001
Lakes, reservoirs	x	x					
Wells, spring	x	x	x				
Water supply intake		x	x	x			
Natural bathing water		x	x	x			
Sewage treatment plant			x	x	x		
Secondary effluent			x	x	x		
Farm ponds, rivers				x	x	x	
Stormwater runoff				x	x	x	
Raw municipal sewage					x	x	x
Feedlot runoff					x	x	x

Use sample volumes that will yield counts between 20 and 60 faecal coliform colonies per membrane. When the bacterial density of the sample is unknown, filter several decimal volumes to establish faecal coliform density. Estimate volume expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively.

Filtration of sample : Follow the same procedure and precaution as for total coliforms.

Preparation of culture dish : Pipette approximately 2 ml MFC medium, prepared as directed above to saturate pad. Carefully remove any excess liquid from culture dish. Place prepared filter on medium impregnated pad as above.

Incubation : Place prepared cultures in waterproof plastic bags or seal petri dishes in waterbath and incubate for 24 $\pm$ 2 hours at 44.5 $\pm$ 0.2°C. Anchor dishes below water surface to maintain critical temperature requirements. Place all prepared cultures in the waterbath within 30 mins after filtration.

Counting : Colonies produced by faecal coliform bacteria on MFC medium are various shades of blue. Pale yellow colonies may be a typical E. coli. Verify for gas production in mannitol at 44.5°C. Non-faecal coliform colonies are grey to cream-coloured. Normally, few non-faecal coliform colonies will be observed on MFC medium because of selective action of the elevated temperature and addition of rosalic acid salt reagent. Count colonies with a low-power binocular, widefield dissecting microscope or other optical device.

Calculation of faecal coliform density : Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 faecal colonies. This colony density range is more restrictive than the 20 to 80 total coliform range because the large colony on MFC medium. Calculate faecal coliform density as for total coliforms. Record densities as faecal coliforms per 100 ml.

ANNEX 28

EXERCISE 1 : AEROBIC PLATE COUNTS

INTRODUCTION

Counts of viable bacteria are usually calculated from the number of colonies that develop on nutrient agar plates which have been inoculated with known amounts of food homogenate and incubated under prescribed conditions. Such counts are sometimes erroneously called total plate counts when in fact only those bacteria which will grow under the particular conditions selected will be counted. A wide variety of conditions can be obtained by changing the composition of the agar medium, the gaseous environment and/or the time and temperature of incubation.

The aerobic mesophilic plate count (incubation at 35-37°C) is often used when examining foods. The presence of numerous mesophilic bacteria, which grow readily at or near body temperature, means that conditions may have existed which would favour the multiplication of pathogens. However the mesophile count is of little value in predicting the shelf life of a food in chill storage, as mesophilic bacteria will not grow below 5°C while the psychrotrophic organisms which cause spoilage do not grow above 30°C.

Plate counts serve as a useful guide to conditions of sanitation, holding temperatures, and of time elapsed during food production and transportation. For example, a high count of mesophilic bacteria on carcasses after dressing is evidence of poor dressing hygiene, and points to an increased likelihood of contamination with pathogens. Similarly, the presence of large numbers of psychrotrophic organisms on chilled meat suggests that there has been opportunity for microbial growth during storage, which although not necessarily a health hazard reduces product shelf life. However, there are limitations to the value of any counts, particularly in fermented foods where a large multiplication of bacteria is required for fermentation or ripening. In these products, a high count alone has no hygienic significance unless objectionable organisms can be differentiated from desirable members of the microflora.

DEFINITION

The aerobic plate count at a particular temperature comprises all those organisms capable of growth in air on a non-selective agar medium.

METHOD RATIONALE

Standard Methods Agar or Plate Count Agar contain simple carbon and nitrogen sources, glucose as an extra carbon source and agar to solidify the media. The absence of selective agents allows growth of a wide range of organisms. The pour plate technique is suitable for examination of most samples. It is, however, unsatisfactory for (a) samples from frozen products where cells may be damaged by heat shock from the molten agar, and (b) where colony identification is desired, as colonial morphology and characteristics are not recognizable within the agar. The spread plate technique should be used in such cases.

As some microorganisms are capable of growth over a wide range of temperatures, no single incubation temperature will completely exclude all organisms from another group. The choice of incubation temperature depends on the purpose of the examination. A count at 25°C would provide the most information about general plant sanitation and give an indication of probable shelf life at chill temperatures, whereas a count at 37°C would reflect the degree of contamination of mesophiles. Where specific counts are required, appropriate temperatures should be selected, e.g. for psychrotrophic and thermoduric organisms, 0-5°C and 50-55°C respectively.

TOTAL VIABLE COUNTS FOR MESOPHILIC AEROBIC BACTERIA

Ref: Standard Methods for the Examination of Water and Waste Water, 16th ed. 1985 pages 860-870.

1. DEFINITION (MIRINZ):

The aerobic plate count at a particular temperature comprises all of those microorganisms capable of growth in air on a non-selective agar medium.

There exist several methods for determining the number of aerobic viable cells in a sample. All methods depend upon:

(1) dispersing bacterial clumps to single cells

(2) diluting this suspension usually in 10 fold steps

(3) "plating" samples of some dilutions <u>onto</u> or <u>into</u> a suitable growth medium so that each single cell gives rise to a colony

(4) counting colonies after a suitable incubation period.

The colony counting technique is employed as a means of estimating the number of viable cells in a pure or mixed population. It is based on the knowledge that a single colony may arise from a single cell.

In each of the three established techniques (the "pour-plate", the "spread-plate" and the "drop-plate") accuracy in counting depends on the number of colonies developing in or on medium being between the limits 30 and 300. This usually requires dilution of the samples and selection of the most suitable dilutions for performance of the count.

2. NOTES ON TECHNIQUE:

2.1 <u>Selection of Dilution Range</u>

The number of dilution tubes selected for plating should permit the count per ml to be reduced to 30-300 in the second highest dilution. Preliminary assessment is not ordinarily possible in opaque liquid (e.g. sewage or blood) or solids (minces), unless microscopic examination of a known sample can fir be made (e.g. Breed smear for liquids), and in such cases <u>all dilutions</u> are frequently plated. Microscopic examination does have severe limitations e.g. a density of 500,000 per ml will reveal onl 1 cell per field under oil immersion in a Breed smear.

The ultimate practical limit for dilution is 10^{-12}. A population of 10^{12} cells per ml of a 2µm x 1µm would form a solid mass. Normally counts in liquids never exceed 10^{11} cells per ml under the most favourable conditions. For most practical purposes a dilution limit of 10^{-8} and 10^{-9} is quite adequa for sewage samples or for broth cultures. For meat surfaces, river water or beach waters, counts ar usually in the range of 10^3 to 10^4 colony forming units per unit of measurement. Consequently a dilution range of 10^{-2} to 10^{-5} is adequate.

2.2 <u>Selection of Dilutions to be "Plated Out"</u>

For pour plates, a 1 ml sample is usually mixed with 10 to 15 ml of melted and cooled agar. If the approximate count is known, three dilutions (the decimal dilution calculated to contain between 30 to 300 colonies, one dilution lower and one dilution higher) are selected for plating. This allows sufficient margin for error in judgement and provides at least two successive dilutions for comparison. Unless something unforeseen has occurred, their counts on successive plates should be in the ratio of about 10:1.

2.3 Preparation and Layout of Materials

2.3.1 Disinfectant

Safety is essential. Counts are being made on viable cells, sometimes these are pathogens. It is essential that a vessel containing sufficient disinfectant to assure total immersion of any contaminated apparatus is available and that it be located in such a position that it can be reached from the sitting position without the necessity to reach over other apparatus to gain access to it. A wide-mouthed cylinder e.g. 1 litre is suitable.

2.3.2 Diluents

Diluents are best dispensed in 100 ml quantities in McCartney bottles. From this bottle, accurate 9 ml aliquots can be aseptically dispensed.

The selection of a suitable diluent is important. To be suitable a diluent must:

(i) maintain the viability of the cells suspended in it.
(ii) achieve a uniform degree of dispersion of the sample diluted in it.

A number of diluents are now listed:-

2.3.2.1 Distilled water

- in general should be avoided, especially from copper stills. It is hypotonic to all cellular protoplasm and may cause cells to burst. Ionic copper is lethal.

2.3.2.2 Sterile tap water

- is frequently used but because of its variable composition and pH, even traces of chlorine, it is not to be recommended.

2.3.2.3 Sterile physiological saline

- (i.e. 0.85% NaCl) has found wide acceptance as a diluting fluid and is suitable for most aerobic bacteria. It is NOT suitable for the truely HALOPHILIC bacteria that may occur in bacon brines. It is not recommended for use with ANAEROBES. It may be found, on testing to be unsuited to some spoiling bacteria and some pathogens.

2.3.2.4 Sterile Culture Medium

- can be recommended where any doubt exists regarding the suitability of other diluents. It ensures that the diluent is osmotically suitable for the organism and, in the case of anaerobes, provides the proper environmental conditions for maintenance of viability.

Where culture medium diluents are used, they should be (4°C) and all counting operatings should be completed within the generation time of the cells. This is most important. The inducement of a lag period (e.g. by the cold shock of the chilled diluent) aids the operation. Peptone water is a frequently used diluent falling into this category.

2.3.2.5 Sterile Ringer's Solution - is recommended for use with milk since it achieves a more uniform dispersion of milk solids than tap water or saline.

2.4 Dispersion of Clumped Cells

When organisms form aggregates such as chains, sarcina-forms, zoogleae, or simply coherent masses, the number of colonies which will develop is dependent on the degree to which the individual cells are separated.

The majority of Gram negative rod-shaped bacteria occur singly and present little trouble. The Gram positive organisms are particularly prone to form aggregates and, in the case of the genus *Mycobacterium*, the cells are, in addition, hydrophobic because of a waxey cell wall.

Dispersion of clumps can be achieved to a degree by shaking the suspension gently in a sealed bottle with glass beads. Progress can be followed by examination of smears microscopically. Violent agitation must be avoided since it will shatter the cells.
Dispersion of hydrophobic cells can be achieved by the addition to the diluent of a non-toxic wetting agent, such as 'Tween 80 (0.05% v/v).

2.5 Glassware

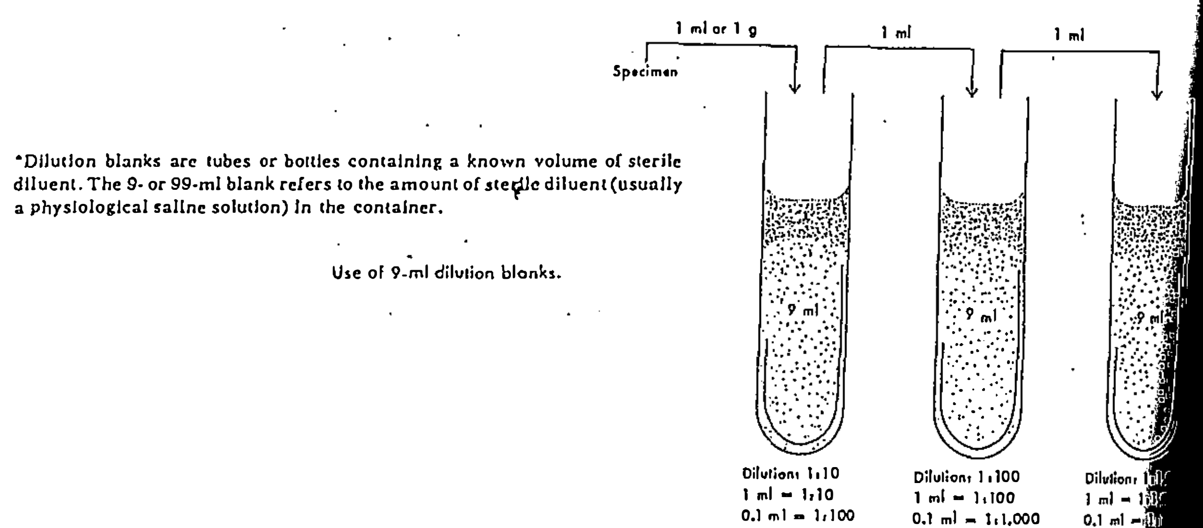
2.5.1 Petri dishes

are sterilized singly or in batches. Glass dishes by dry heat at 160°C for 1 hour. The trend now is to use disposable sterile plastic dishes.

2.5.2 Pipette Work

Where possible automatic pipettes should be used in all counting work. Where this is not possible the following notes should apply using plugged pipettes. A count requires the use of 1 x 10 ml graduated pipette and as many 1 ml graduated pipettes as there are dilution tubes. The 10 ml pipette, for maximum convenience, is sterilized in its own brown paper bag as are the 1 ml items. Again sterilization is at 160°C for 1 hour. All pipettes must have unbroken tips and be calibrated to deliver the stated volume. Tips of 1 ml pipettes should not be too broad.

NB Mouth pipetting should be avoided where possible. Mechanical devices will be provided in teaching laboratories.



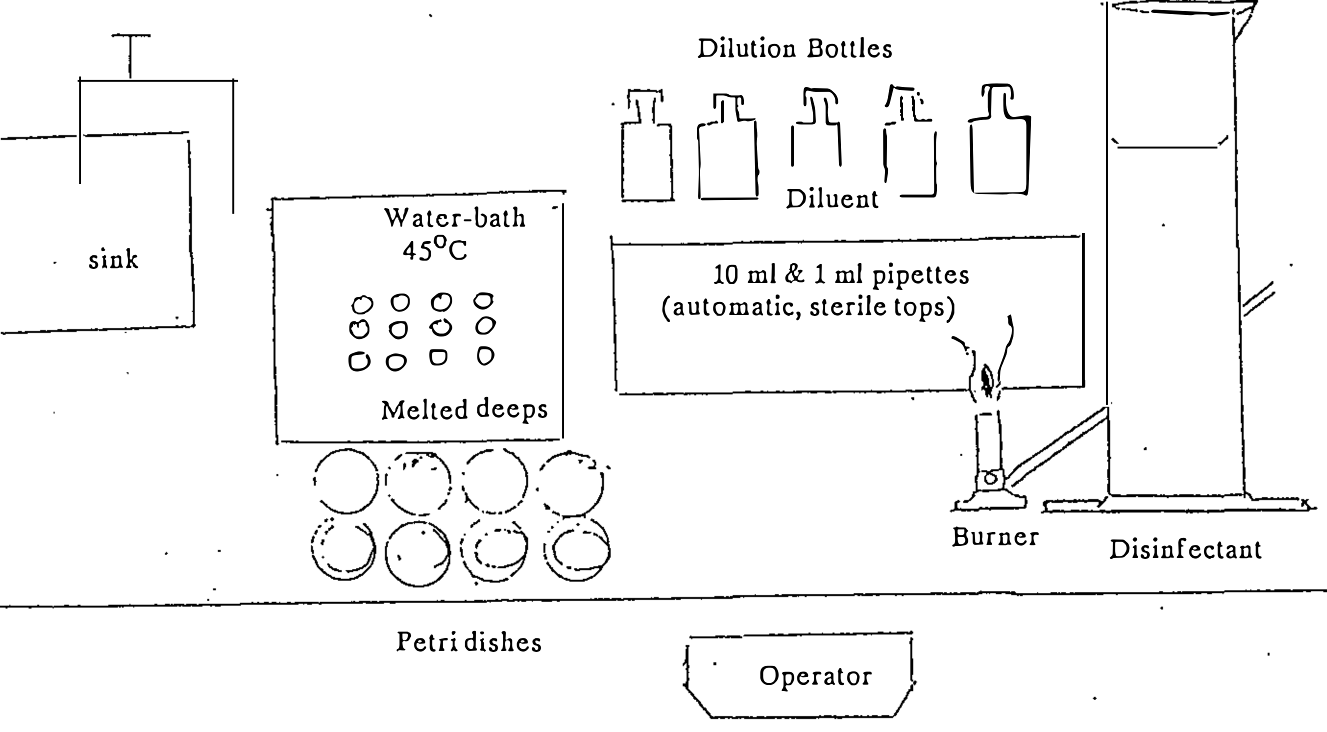
*Dilution blanks are tubes or bottles containing a known volume of sterile diluent. The 9- or 99-ml blank refers to the amount of sterile diluent (usually a physiological saline solution) in the container.

Use of 9-ml dilution blanks.

3. METHODOLOGY

3.1 The Pour-Plate Method

3.1.1 Layout of Apparatus and Equipment



3.1.2 Materials (per bench)

Water-bath at 50°C with thermometer

Sterile Glassware: per pair

8 petri dishes
1 x 10 ml graduated pipette or automatic equivalent
6 x 1 ml graduated pipettes or automatic equivalent

Materials (per pair)

1 pipette discard cylinder

Media (per pair)

8 x 15 ml melted plate-count agar deeps at 45°C
5 x 9 ml peptone waters (in refrigerator at 4°C)

3.1 Procedure

1. Layout the apparatus as indicated in the illustration and see that the level of water in the bath is slightly higher than the level of the agar in the agar deeps. Check the temperature, °C.

2. At leat 20 minutes before use, melt the agar deeps and place them in the waterbath.

3. Label the petri dishes with the dilutions to be plated and any other relevant information. Lay them out in the sequence in which the are to be used, lid upper-most.

4. Ensure the availability of all the pipettes to permit easy access to them AT YOUR WORK STATION.

5. Arrange the required number of dilution bottles in a row.

6. Throughly mix the sample upon which the count is to be made (this is the bottle with swabs and glass beads in the case of "solid" samples.

7. Using a sterile 1 ml pipette transfer 1 ml of the sample to the first bottle of diluting fluid. In taking the sample only the tip of the pipette should be immersed and in discharging the material into the diluent it should be expelled with the tip of the pipette touching the inside of the bottle above the diluting fluid.

8. Discard the pipette tip into the disinfectant and mix the sample thoroughly. This is the 1/10 (i.e. 10^{-1}) dilution of the swab sample.

9. Continue making decimal dilutions in the same manner using a fresh pipette for each dilution, but take into account the following:

10. After preparing the lowest dilution which is to be plated out (e.g. 10^{-3}), move the plates which will receive the agar and sample to a position immediately in front of the operator.

11. Now transfer 1 ml to the next dilution bottle and one ml to each of the duplicate sterile petri plates (aseptic precautions please). Remove one of the melted agar deeps from the water-bath, dry the exterior of the bottle carefully and then pour the agar into the first plate. Do the duplicate immediately. Carefully and thoroughly, mix the sample into the agar by gently rotating the dish four times clockwise and then four times in an anticlockwise direction and then to and fro. Take care that the agar over the side of the dish. Push the dish aside to the back of the work area and allow to set.

12. Proceed as in 11 with subsequent dilutions

    (i) NB. If replicate plates of each dilution are being made, a sample of the particular dilution is transferred to each of the replicate plates before the pipette is discarded.

    (ii) It is poor technique to transfer all the dilutions to the respective plates and then pour all plates at once. This often results in adhesion of cells to the glass or plastic before the agar is added. Effective mixing is then impossible. On incubation, a mass of colonies appears in one area under the agar.

13. While the agars are setting discard all empty bottles into the discard tin or tray. plates until agar is set!!

14. INCUBATION:- Invert the fully labelled dishes and arrange in the incubator in stacks of more than 4 plates. Leave as much space as possible between stacks to facilitate good air circulation.

15. COUNTING: - Counting should be done with a Quebec type colony counter. Select plates i which the total number of colonies does not exceed 300. If the number of colonies is less 50, count the lot. If greater, divide the plate into sectors and count opposing sectors only multiply count by an appropriate factor.

To facilitate counting and eliminate "doubling-up" on the same colony, mark each colony with a pen on the plastic base of the petri plate. Tally counters are useful accessories provided attention is confined to the counting process. It is remarkably easy to keep pressing the counter out of time with the marking of colonies. Modern counting devices combine the marking with automatic tally counting so that a count is registered only when the marker's tip is pressed on the plate.

16. CALCULATION: The colony count for the plates are converted, normally by multiplication by the dilution and adjusted for unit area swabbed of weight of sample taken.

17. OBJECTIONS:

(i) The principal specific objection to the pour-plate method is that a single plate does not yield a statistically analysable sample. To obtain such, approximately 8 replicate plates should be prepared for each dilution. Costs become prohibitive.

(ii) The relationship between colony counts and the actual numbers of viable organisms in the first dilution bottle (swab bottle) depends on the extent of clumping and the degree of agitation of the sample. The correlation may be good for a Gram -ive rod but poor for a Staphylococcus.

(iii) In the case of mixed populations a number of criticisms can be raised.

(a) the medium selected may be suitable for only a proportion of the population.

(b) the pH, temperature and gas atmosphere may not be suitable for all organisms present.

(c) the time of incubation will vary with different organisms.

(d) a colony does not necessarily arise from only one cell type.

It must be remembered that in dealing with mixed cultures, no single medium is entirely suitable and medium selection is generally based on the purpose for which the count is being made, e.g. to count coliforms, staphylococci etc.

(iv) For swabs taken from a carcass:

(a) not ALL contaminants on the swabbed area will be removed on swabbing.

(b) Not all microbial cells will be eluted from the swab during shaking with the glass beads

(v) Keep in mind the uneven distribution of microorganisms on the surfaces means that the count obtained relates only to the region swabbed (e.g. bench tops, carcass surfaces etc.). Liquid samples will provide results for the material at that moment in time when sample is taken and may not represent the average count over a period of 24 hours. To obtain that sort of information, continuous and automatic sampling devices would be needed.

Application of Tests to Routine Examinations

The following basic considerations apply to the selection of the confirmed phase or the completed test in the examination of any given sample of water or wastewater. Schematic outlines of the tests are given in Figures 908:1a and 908:1b.

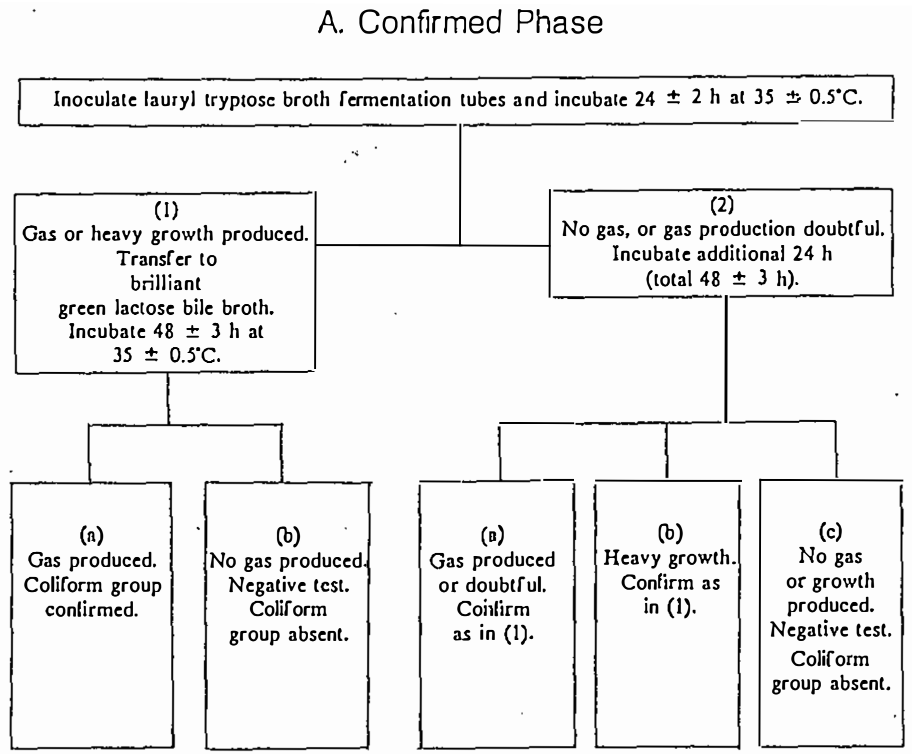


Figure 908:1a. Schematic outline of confirmed phase.

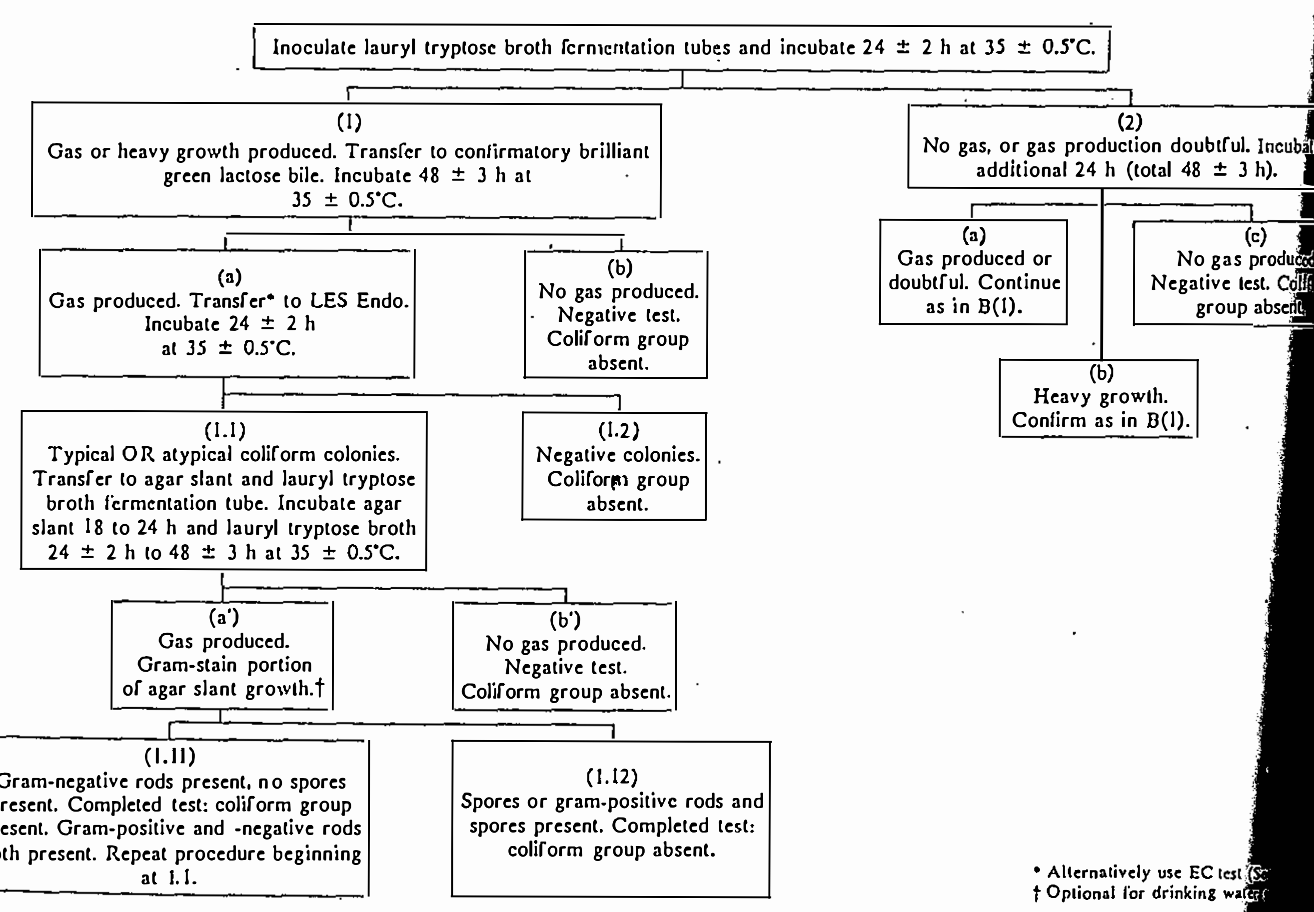


Figure 908:1b. Schematic outline of completed test.

ANNEX 29

DETERMINATION OF FAECAL COLIFORMS IN BIVALVES BY MULTIPLE TEST TUBE METHOD

1. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of faecal coliforms (3) in bivalve (shellfish) specimens from temperate and tropical seas. It is designed to be used in sanitary surveillance of sea-food.

Faecal coliforms are specific indicators, exhibiting a high positive correlation with faecal contamination from warm-blooded animals. Filter-feeding shellfish concentrates coliforms from its marine environment. The concentration of faecal coliforms in edible shellfish tissue gives an indication of the potential health hazard to consumers of shellfish due to pathogens of faecal origin which may have been present in the marine environment surrounding the shellfish.

2. REFERENCES

APHA (1981) Standard methods for the examination of water and waste water. American Public Health Association, Washington, D.C. (15th edition)

WHO/UNEP (1983) Consultation meeting on methods for monitoring selected pollutants in sewage effluents and coastal recreational water: WHO/UNEP joint project. Rome 24-26 November 1982. WHO regional office for Europe, Copenhagen.

UNEP/WHO (in preparation) Guidelines for monitoring the quality of coastal recreational and shellfish-growing waters. Reference Methods for Marine Pollution Studies No. 1, UNEP, Geneva.

3. DEFINITION

Faecal coliforms are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas, both at 36°C and at 44.5°C in less that 24 hours. At 44.5°C they produce indole in tryptone water containing tryptophan.

4. PRINCIPLES

After the shellfish have been washed and brushed in the laboratory, their soft tissue is separated under sterile conditions from the shell and transferred to a sterilized blender flask where the soft tissue is macerated and diluted by nine times its weight with phosphate buffer (or 0.1% peptone water). In this way a solution is obtained which contains 1 g mussel flesh per 10 ml of homogenate.

From this homogenate a first multiple test tube dilution series containing lactose broth is set up and incubated at 36 ± 1°C (presumptive test).

After 24 hours, one drop of all positive test tubes is transferred into a second multiple test tube dilution series containing MacConkey broth (or brilliant green broth) and incubated at 44.5 ± 0.2°C (first confirmed test).

At the same time, one drop of all positive test tubes is transferred into a third multiple test tube series containing tryptone water and incubated also at 44.5 ± 0.2°C (second confirmed test).

The frequency of positive reactions in the test tubes is used for the calculation of the most probable number (MPN) of faecal coliforms in the analytical test sample.

5. APPARATUS AND GLASSWARE

5.1 Thermoisolated plastic boxes (camping equipment) with cooling pads or similar cooling units for transport and keeping live mussel specimens.

5.2 Water incubator for 36 ± 1°C and for 44.5 ± 0.2°C.

5.3 Autoclave, max 2 atm, electric or gas.

5.4 Drying oven for sterilization of glassware and equipment at 160°C.

5.5 pH meter, precision ± 0.1 pH units.

5.6 Stainless steel forceps.

5.7 Balance for media preparation, precision ± 10 mg.

5.8 Refrigerator, 4 ± 2°C.

5.9 Vibrator for mixing liquids in culture tubes.

5.10 Ehrlenmeyer flasks of borosilicate glass for media preparation, capac 500 and 1000 ml.

5.11 Borosilicate glass bacteriological culture tubes with autoclavable rac

5.12 Small borosilicate glass tubes ("Durham vials").

5.13 Total volume (blow-out) borosilicate glass pipettes of 1, 5, 9 and 1[illegible] capacity for transfer of culture media in test tubes, with stainless [illegible] containers for sterilization.

NOTE: 9 ml capacity pipettes are useful, but not essential.

5.14 Graduated borosilicate glass cylinders of 100, 500 and 1,000 ml capacity with glass beakers for cover.

5.15 Stainless steel homogenizer or blender with several blender vessels, sterilizable in a drying oven (5.4) or autoclave (5.3).

5.16 Brush for cleaning shellfish shells.

5.17 Surgeon's scalpels or similar knives for opening mussels.

6. CULTURE MEDIA, CHEMICALS AND STOCK CULTURE

NOTE: The composition of the media is based on one litre solutions or similar units. Before preparation, the actual needs have to be established and adequate amounts must be chosen accordingly.

6.1 Lactose broth

	strength	
	single	double
Beef extract	3.0 g	6.0 g
Peptone	5.0 g	10.0 g
Lactose	5.0 g	10.0 g
Distilled water (6.7)	1.0 litre	1.0 litre

Preparation: Dissolve ingredients in the distilled water (6.7). pH should be between 6.8 and 7.0, but preferably 6.9 after sterilization (5.7).

Place in an autoclavable test tube rack 3 rows (more in case the expected MPN of faecal coliforms is high) of 5 clean culture tubes (5.11, 9.1) each. Then add inverted vials (5.12) to all culture tubes and dispense sufficient medium into the culture tubes so that the inverted vials are at least partially covered after the entrapped air in these vials has been driven out during autoclaving. Into the first row of these culture tubes transfer double strength broth (6.1). Into the second and third rows (and if necessary into successive rows) transfer single strength broth and close the tubes with cotton plugs. Autoclave (5.3) the closed culture tubes at 121°C for 15 minutes. Check if the pH is between 6.8 and 7.0. If not, adjust the pH of the remaining broth and prepare a new test tube series.

NOTE: Double strength broth is only necessary in the first row where 10 ml of test solution is added to the culture tubes to contract dilution. If higher than 10 ml inocula of the ~~seawater~~ test dilution sample are used the lactose broth has to be prepared in higher than double strength in order to avoid that the lactose broth is diluted below single strength after the addition of the inoculum.

6.2 MacConkey broth

6.2.1 Medium

Sodium taurocholate	5.0 g
Lactose	10.0 g
NaCl	5.0 g
Peptone	20.0 g
Distilled water (6.7)	1.0 litre

Preparation: Dissolve ingredients by shaking and adjust pH to 7.1 ± 0.1 with diluted HCl or NaOH. Add 2 ml bromo-cresol purple solution (6.2.2) to the MacConkey broth.

Place in an autoclavable test tube rack 3 rows (more in case the expected MPN of faecal coliforms is high) of 5 clean culture tubes (5.11, 9.1) each. Then add inverted vials (5.12) to all culture tubes and close the tubes with cotton plugs. Autoclave (5.3) the closed culture tubes at 121°C for 15 minutes. Check if the pH is between 7.0 and 7.4. If not, adjust the pH of the remaining broth and prepare a new test tube series.

6.2.2 Bromo-cresol purple solution

Preparation: Dissolve 1 g of bromo-cresol purple in 99 ml of 95% ethanol (6.9)

6.3 Brilliant green bile broth

Oxgall, dehydrated	20.0 g
Lactose	10.0 g
Peptone	10.0 g
Brilliant green	13.3 mg
Distilled water (6.7)	1.0 litre

Preparation: Dissolve the chemicals in one litre of distilled wa[illegible] (6.7). Then add inverted vials (5.12) to all the culture tubes and [illegible] sufficient medium into the culture tubes so that the inverted vials are a[illegible] partially covered after the entrapped air in the vials has been driven [illegible] during autoclaving. Close the tubes with cotton plugs. Steriliz[illegible] autoclaving at 121°C, preferably for 12 minutes, but not exceeding 15 min[illegible] After sterilization, cool the broth as quickly as possible. Final pH sho[illegible] 7.2 ± 0.2. Test the samples of the finished product for performance [illegible] control stock cultures (6.10).

6.4 Tryptone water

Tryptose	10.0 g
NaCl	5.0 g
Distilled water (6.7)	1.0 litre

Preparation: Dissolve the ingredients in distilled water (6.7).

Dispense into each of the 5 test tubes in the 3 rows (more in case the expected MPN of faecal coliforms is high) of the dilution series 10 ml of tryptone water. Autoclave (5.3) at 121°C for 15 minutes. The final pH should be between 7.2 and 7.4. If necessary, adjust the pH before sterilization.

6.5 Dilution solutions

6.5.1 Phosphate buffer (pH 7.2) for dilutions

K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
Distilled water (6.7)	1.0 litre

Preparation: Dissolve components and dispense 9 ml in test tubes used for dilutions in the dilution series (9.3) and autoclave (5.3) at 121°C for 15 min.

6.5.2 Peptone water for dilutions

Preparation: Dissolve a sufficient amount of peptone to obtain a 0.1% solution of peptone in distilled water (6.7). Dispense 9 ml in test tubes used for dilution series (9.3) and autoclave (5.3) at 131°C for 15 minutes.

6.6 Kovac's indole reagent

Paradimethyl amino-benzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Conc. HCl	25.0 ml

Preparation: Dissolve the benzaldehyde in amyl alcohol and add HCl. The reagent should be yellow.

6.7 Distilled water

Use only water distilled in all-glass or all-quartz distillation apparatus. De-ionized water is also acceptable if produced in apparatus not releasing toxic substances.

NOTE: Commercially available distilled water is often produced in copper and zinc apparatus and is highly toxic for coliforms. Before using such water its toxicity should be checked with a stock culture of E. coli (6.10).

6.8 Detergents for cleaning glassware and apparatus

Use only detergents recommended by the supplier for bacteriological use. If such a detergent is not available, check normal household detergents with a biotest using a stock culture of E. coli (6.10).

NOTE: Never use toxic chromic-sulphuric acid mixture for cleaning glassware.

6.9 95 per cent ethanol per analysis.

6.10 Stock culture of E. coli.

7. SAMPLING

For a sampling plan, follow Reference Method No. 1 (UNEP/WHO, in preparation).

8. PREPARATION OF TEST SAMPLE

Weigh the sterilized blender vessel (9.2.1). Select 10 bivalves at random from the specimen samples collected at each sampling station (7). (For example, a mytilus of 4 cm shell length contains a soft part of about 0.5 g fresh weight (FW), hence 10 mussels of this size should yield about 5 g FW.) Before opening the shells, carefully clean them with a brush (5.16) and alcohol (6.9). Then hold the closed bivalve with sterilized forceps (9.2.3) for a short time over a flame in order to dry the outside of the shells.

Cut the bivalve open with a sterilized knife or scalpel (5.17, 9.2.3) by inserting the knife into the opening from which the byssus extrudes and cut the posterior adductor muscle by turning the knife as indicated in figure 1. Then cut in the other direction and open the mussel with sterilized forceps (9.2.3).

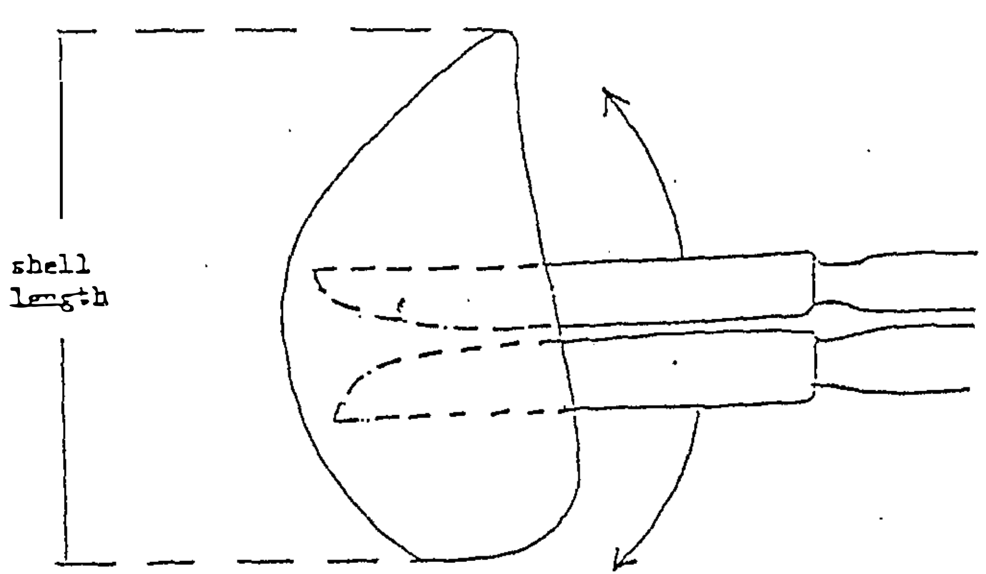


FIGURE 1.: CUTTING THE TWO ADDUCTOR MUSCLES

Do not try to break the mussel open with the knife. If the two muscles are cut the mussel will open easily.

Drain the liquor from the shells, discard it so that it will not be included in the sample and transfer the flesh (soft tissue) into the sterilized blender flask (9.2.1) with sterilized forceps (9.2.3). After transferring the soft parts of all 10 specimens into the blender vessel, weigh the vessel and determine the fresh weight of the flesh sample by subtracting the weight of the vessel plus soft parts from the predetermined weight of the empty blender vessel. Report the weight of the 10 soft parts in table 1, item 5. Then, (in order to arrive at a concentration of 1 g/10 ml) dilute the sample with 9 times its weight using phosphate buffer (6.5.1) or peptone water (6.5.2). (In our example, the sample is now composed of 5 g FW mussel flesh plus 45 ml of dilution solution resulting in a total of 50 ml of flesh solution homogenate).

Homogenize the flesh for 2 minutes. The homogenate now contains 1 g FW sample in 10 ml or 0.1 g FW/ml.

The homogenate represents the test sample (D-1 dilution).

TEST PROCEDURE

9.1 Washing of glassware and equipment

All glassware and apparatus (5) should be washed with non-toxic detergent (6.8), first rinsed thoroughly with hot tap water and then rinsed at least three times with distilled water (6.7).

9.2 Sterilization of glassware and equipment

9.2.1 Sterilize the stainless steel blender vessel (5.15) by heating it in a drying oven (5.4) for 3 hours at 160°C or in an autoclave (5.3) for 15 minutes at 121°C.

9.2.2 Place clean pipettes (5.13), complete with a cotton plug in the mouthpiece, into suitable stainless steel containers and sterilize them in a drying oven (5.4) for 3 hours at 160°C.

9.2.3 Sterilize forceps (5.6) and knives or scalpels (5.17) by dipping them into 95% ethanol (6.9) and by flaming them.

9.3 Incubation in lactose broth at 36 ± 1°C for 48 hours (Presumptive test)

Before taking aliquots from the original sample or from the dilutions these must be vigorously shaken in order to guarantee that representative aliquots are taken.

Using sterile pipettes (9.2.2) transfer 10 ml of the test sample (8) into five sterilized culture tubes containing double strength lactose broth (6.1). This test tube row contains now 1 g FW/tube (figure 2).

Next transfer with sterile pipettes (9.2.2) 1 ml of the test sample (8) into five sterilized culture tubes containing single strength lactose broth (6.1). This test tube row contains now 0.1 g FW/tube.

For preparing further dilutions transfer with a sterile pipette (9.2.2) 1 ml of test sample (8) (dilution D-1) into a test tube containing 9 ml of phosphate buffer (6.5.1) or peptone water (6.5.2) (dilution D-2). Mix vigorously by hand or with vibrator (5.13). Transfer aseptically (9.2.2) 1 ml of D-2 into each of the five culture tubes containing single strength lactose broth (6.1). This test tube row contains now 0.02 g FW/tube.

If necessary, for further dilutions transfer 1 ml of the D-2 dilution into a test tube containing 9 ml of phosphate buffer (6.5.1) or peptone water (6.5.2) to obtain dilution D-3, etc.

Incubate the series of culture tubes in an air or water incubator (5.2) at 36 ± 1°C for 48 hours.

After 24 hours, check for gas formation. The gas production is indicated by the broth turning turbid. The observation of gas formation in the Durham vials can be facilitated by slightly tapping on the walls of culture tubes. The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth must become cloudy. Active fermentation may be shown by the continued appearance of small bubbles of gas throughout the medium outside the inner vial when the fermentation tube is shaken gently. Record the number of tubes showing positive reactions (gas formation after 24 hours of incubation) in table 2, item 7 under (1a).

After 48 hours, check again for gas formation in the same tubes and reco[illegible] the results in table 2, item 7 under (1b).

9.4 Incubation in MacConkey or brilliant green broth at 44.5°C for 48 hours (First confirmed test)

After incubation for 24 hours in lactose broth at 36°C (9.3) a second te[illegible] tube series (6.2.1) is prepared by transferring with sterile pipettes (9.2[illegible] one drop from each test tube that provided a positive reading into test tu[illegible] situated in identical positions on the tube rack containing MacConkey br[illegible] (6.2) or brilliant green broth (6.3).

Incubate this second test tube series at 44.5 ± 0.2°C in a water incub[illegible] (5.2) for 24 hours.

After 24 hours record the number of tubes showing positive reactio[illegible] table 2, item 7 under (2a). Coliforms will develop gas which is trapped i[illegible] inverted vials (brilliant green broth) and acid which turns the violet colour of the original MacConkey broth into a yellowish colour.

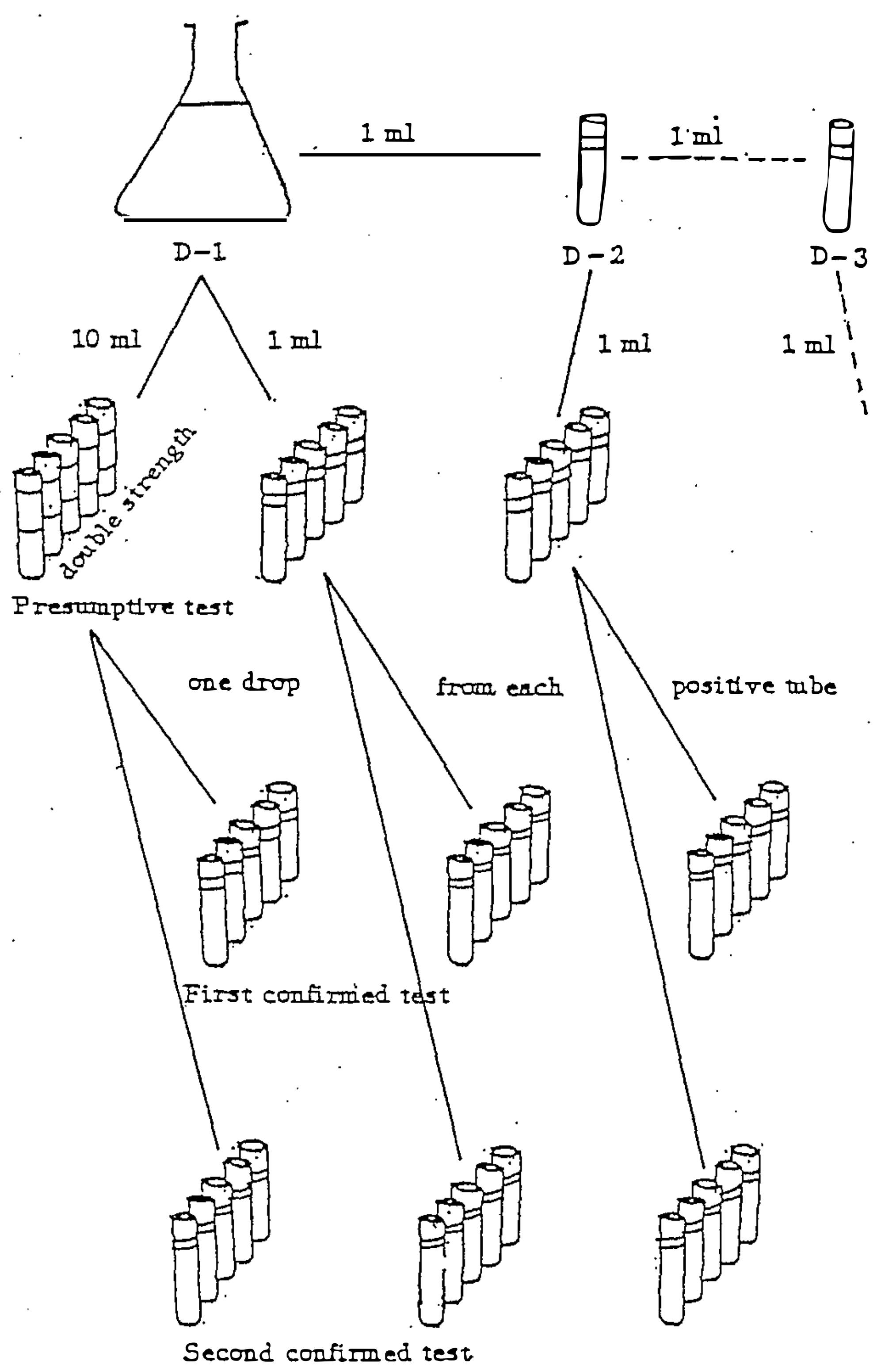


FIGURE 2 : SCHEME FOR THE PREPARATION OF DILUTION SERIES

After incubation for 48 hours in lactose broth at 36°C (9.3) with steril pipettes (9.2.2) one drop from each previously negative test tube, that after hours provides a positive reading, is transferred into test tubes situated identical positions on the tube rack containing MacConkey broth (6.2) brilliant green broth (6.3).

After another 24 hours record the number of tubes showing positi reactions (acid and gas production) in table 2, item 7 under (2b).

9.5 Incubation in tryptone water at 44.5 C for 24 hours (Second confirmed te

At the same time when the first confirmed test (9.4) is prepared, transf aseptically (9.2.2) a drop from each test tube which provided a positive reaction aft incubation for 24 hours in lactose broth at 36°C (9.4) into the third series test tubes situated in identical positions of a test tube rack contain tryptone water (6.4).

Incubate this third test tube series at 44.5 ± 0.2°C in a water incubato (5.2) for 24 hours.

After 24 hours add approximately 1 ml of Kovac's reagent (6.6) into ea of the test tubes and shake. Within 10 minutes positive reactions are indicat by a red colour of the amyl alcohol surface layer in the test tubes. Record th results in table 2, item 7 under (2c).

Repeat this procedure (9.5) with the tubes from the presumptive test (9.3) which have become positive during the 24-48 hours interval and record the results in table 2, item 7 under (2d).

10. EXPRESSION OF RESULTS

10.1 Calculation of faecal coliforms per gram of shellfish flesh

If the dilutions 1 g, 0.1 g, and 0.01 g of flesh per test tube have be used, take the highest number of recorded positive readings from the incubati in MacConkey broth or brilliant green at 44.5°C (9.4) and in peptone wat (9.5), i.e. the highest readings from either 2a, 2b, 2c or 2d in table 2, item and find the corresponding most probable number (MPN) from table 1.

When more than three dilutions are employed, the results of only three these are used in computing the MPN. Select the dilution in which the row 3 ~~five~~ tubes gives positive readings in all five tubes (no negative readin should exist in lower dilutions) and the two next succeeding higher dilutio from either 2a, 2b, 2c or 2d in table 2, item 7. Determine how many times the highest dilution is smaller than 1 g, find the MPN corresponding to t number of positive tubes in these three dilutions and multiply the MPN fou with number of times of 10. Enter this value in the test report (table 2, it 8).

Example:	dilution 1 g:	5 positive tubes	
	dilution 0.1 g:	5 positive tubes	(X)
	dilution 0.01 g:	3 positive tubes	(X)
	dilution 0.001 g:	2 positive tubes	(X)
	dilution 0.0001 g:	1 positive tube	

dilution 10/1 = 10
MPN (532) = 14
(14 x 10)g FW = 140 faecal coliforms/g FW flesh
95% confidence limits: lower (3.7 x 10 = 37)
higher (34 x 10 = 340)

10.2 Precision of results

Select the 95 per cent confidence limits from table 1 and enter them in the test report (table 2, item 8).

Table 1 : MPN index and 95% confidence limits for various reactions of positive and negative results when five 10-ml portions, five 1-ml portions and five 0.1-ml portions are used.

No. of Tubes Giving Positive Reactions out of 5 tubes containing -- 1 g	0.1 g	0.01 g	MPN Index per 1 g	95% Confidence Limits Lower	Upper	No. of Tubes Giving Positive Reactions out of 5 tubes containing -- 1 g	0.1 g	0.01 g	MPN Index per 1 g	95% Confidence Limits Lower	Upper
0	0	0	<0.2			4	2	1	2.6	0.9	7.8
0	0	1	0.2	<0.05	0.7	4	3	0	2.7	0.9	8
0	1	0	0.2	<0.05	0.7	4	3	1	3.3	1.1	9.3
0	2	0	0.4	<0.05	1.1	4	4	0	3.4	1.2	9.3
1	0	0	0.2	<0.05	0.7	5	0	0	2.3	0.7	7
1	0	1	0.4	<0.05	1.1	5	0	1	3.1	1.1	8.9
1	1	0	0.4	<0.05	1.1	5	0	2	4.3	1.5	11
1	1	1	0.6	<0.05	1.5	5	1	0	3.3	1.1	9.3
1	2	0	0.6	<0.05	1.5	5	1	1	4.6	1.6	12
2	0	0	0.5	<0.05	1.3	5	1	2	6.3	2.1	15
2	0	1	0.7	0.1	1.7	5	2	0	4.9	1.7	13
2	1	0	0.7	0.1	1.7	5	2	1	7	2.3	17
2	1	1	0.9	0.2	2.1	5	2	2	9.4	2.8	22
2	2	0	0.9	0.2	2.1	5	3	0	7.9	2.5	19
2	3	0	1.2	0.3	2.8	5	3	1	11	3.1	25
3	0	0	0.8	0.1	1.9	5	3	2	14	3.7	34
3	0	1	1.1	0.2	2.5	5	3	3	18	4.4	50
3	1	0	1.1	0.2	2.5	5	4	0	13	3.5	30
3	1	1	1.4	0.4	3.4	5	4	1	17	4.3	49
3	2	0	1.4	0.4	3.4	5	4	2	22	5.7	70
3	2	1	1.7	0.5	4.6	5	4	3	28	9	85
3	3	0	1.7	0.5	4.6	5	4	4	35	12	100
4	0	0	1.3	0.3	3.1	5	5	0	24	6.8	75
4	0	1	1.7	0.5	4.6	5	5	1	35	12	100
[illegible]	1	0	1.7	0.5	4.6	5	5	2	54	18	140
[illegible]	[illegible]	[illegible]	2.1	0.7	6.3	5	5	3	92	30	320
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	7.8	5	5	4	160	64	580
						5	5	5	240	[illegible]	[illegible]

TEST REPORT

Fill in the test report (table 2) giving full details in every column.

Table 2: Test Report on faecal coliforms in bivalves

1. Sampling area

   1.1 country: ________________ 1.2 area: ____________________

2. Sampling point (station)

   2.1 type of sampling point (e.g. fish market): __________________

   2.2 description of the location of the sampling point: ___________

   ____

   2.3 code number: _____________ 2.4 longitude: _________________

   2.5 latitude: _______________

3. Time of sampling

   3.1 hour: ____ 3.2 day: _____ 3.3 month: _____ 3.4 year: ______

4. Sampling and environmental conditions

   4.1 depth of sampling: ______________________________________

   4.2 storage procedure (e.g. + 2°C): ___________________________

   4.3 duration of storage: ___________ hours

   4.4 temperature at sampling depth: _____°C

   4.5 salinity at sampling depth: _______‰

5. Test sample

   5.1 Number of mussels per sample: ______________________________

   5.2 Weight of all soft parts: ________g FW

6. Incubations

6.1 Lactose broth at 36°C

Date and hours of start: ______________________

Date and hours of end: ______________________

6.2 MacConkey at 44.5°C

Date and hours of start: ______________________

Date and hours of end: ______________________

6.3 Brilliant green at 44.5°C

Date and hours of start: ______________________

Date and hours of end: ______________________

6.4 Tryptone water at 44.5°C

Date and hours of start: ______________________

Date and hours of end: ______________________

7. MPN in single dilutions

Aliquots transferred	number of positive reactions					
gr	lactose		MacConkey or Brilliant		tryptone	
	24 h (1a)	48 h (1b)	24 h (2a)	48 h (2b)	24 h (2c)	48 h (2d)
1						
0.1						
0.01						
0.001						
0.0001						

8. Test results:

MPN after 48 h in MacConkey, brilliant green or tryptone water at 44.5°C

_______________ faecal coliform / g shellfish flesh

_______________ 95% confidence limits

9. Anomalies observed during the test procedure:

____

____

____

____

10. Full address of the institution which carried out the test:

____

____

____

____

11. Name(s) and signature(s) of the person(s) who carried out the test:

____

____

Date: _______________

PURE-CULTURE TECHNIQUES

A prerequisite to the characterization of a microbial species is that it be available for study as pure culture. Recall from Exercise 2 that although mixed cultures exist in natural environments, it is impossible to characterize individual species. The term ***pure culture*** denotes that all the cells in the culture had a common origin and are simply descendants of the same cell. It is possible to obtain a pure culture by transferring a single cell to a sterile medium. This can be accomplished by using a micromanipulator in conjunction with a microscope. However, indirect methods are almost always used to obtain a pure culture from a mixture of bacteria; for example, agar plate cultures are inoculated in such a way that isolated colonies develop.

The assumption is made that the microbial population of a colony develops from a single cell and hence represents a pure culture. This may not always be the case because some cells tend to clump together and cannot be separated easily. Therefore, it may be necessary to examine what is presumed to be a pure culture by additional cultural and microscopic tests. For example, one can restreak the colony to get isolated colonies that give the same colonial morphology; Gram-stained cells from one colony should look alike.

Actually the term ***axenic culture*** may be more appropriate than *pure culture* because an axenic culture is one in which an organism is grown in an environment free of any other living organism. Essentially, the term *pure culture* implies genetic purity, while *axenic culture* does not.

Once a pure-culture isolation has been made, it is desirable to maintain the culture, without change in its characteristics, in a viable condition for varying periods of time ranging from weeks to years. For short-term preservation of a culture (generally between 1 and 3 months), one simply makes periodic transfers to a fresh medium, for example, *nutrient-agar slants,* which are test tubes containing molten agar that have been left to solidify at an angle, or *cystine-trypticase-agar deep tubes.* Such cultures, with screw caps to minimize desiccation, are stored in the refrigerator at 4 to 10°C. Long-term preservation of a pure culture is best accomplished by ***lyophilization,*** that is, the culture species is dehydrated while in a frozen condition and then sealed under vacuum.

Transferring a pure culture of bacteria from one nutrient medium to another must be done without introducing unwanted microorganisms, or ***contaminants.*** This technique of transfer without the introduction of contaminants is carried out ***aseptically.*** The observance of aseptic technique must become second nature to any microbiologist. (Review Exercise 3.)

The Streak-Plate Method for Isolation of Pure Cultures

OBJECTIVE

To learn how to streak bacteria on the surface of an agar medium to obtain isolated colonies.

OVERVIEW

Practically all specimens of material obtained from natural environments contain a mixed population of microorganisms. Before one can make a detailed study of the characteristics of the individual species constituting the mixture, it is imperative that each species be isolated in pure culture. The *streak-plate technique* provides a simple and practical procedure for this purpose [FIGURE 17.1]. It is essentially a dilution technique that spreads a loopful of culture over the surface of an agar plate. Although there are many ways to streak a plate to isolate colonies, the "four-way method" is described here [FIGURE 17.2].

REFERENCES

MICROBIOLOGY, Chap. 6, "Cultivation and Growth of Microorganisms."
MM, Chap. 8, "Enrichment and Isolation."

MATERIALS

4 sterile Petri dishes
4 tubes nutrient agar
Diluted broth cultures of *Serratia marcescens*, *Micrococcus luteus*, and *Arthrobacter globiformis*
Mixed culture of preceding bacteria

PROCEDURE

1 Pour each tube of melted and cooled (45 to 50°C) nutrient agar into separate Petri dishes.
2 Allow the agar to become firmly solidified. Never attempt to streak a plate until the medium is firm.

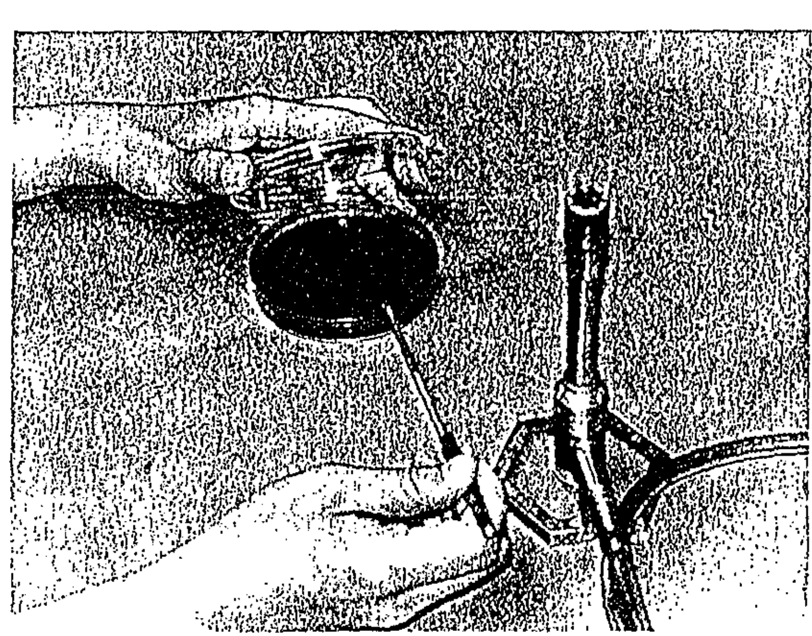
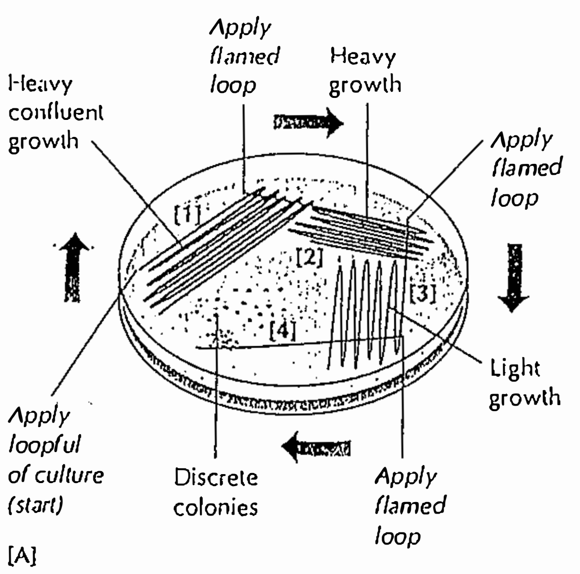


FIGURE 17.1
Techniques for streaking an agar medium for isolating colonies.

3 Streak each of the three bacterial suspensions on a separate plate as shown in FIGURE 17.2A. To begin, streak the initial inoculum over an area corresponding to 1. Flame the loop, and then make a single streak through area 1 to the side of the plate, streaking several times through area 2. Do not allow the loop to reenter area 1. Again flame the loop, make a single streak through area 2 to the side of the plate, and continue making streaks in area 3, being careful not to reenter area 2. Repeat the process for area 4. In each step, after the loop has been flamed, it is advisable to cool the loop in the air briefly prior to the streaking procedure.

4 The fourth plate is to be streaked with a mixture of all three species. Place a small loopful of the mixed-culture suspension on this plate in position 1. Proceed to streak this plate as for other cultures.

5 Label the plates with your name and date, and incubate them at 25°C, or room temperature, for 48 h. **An inoculated plate is always incubated in an inverted position to prevent condensation from falling onto the surface and interfering with discrete colony formation.**



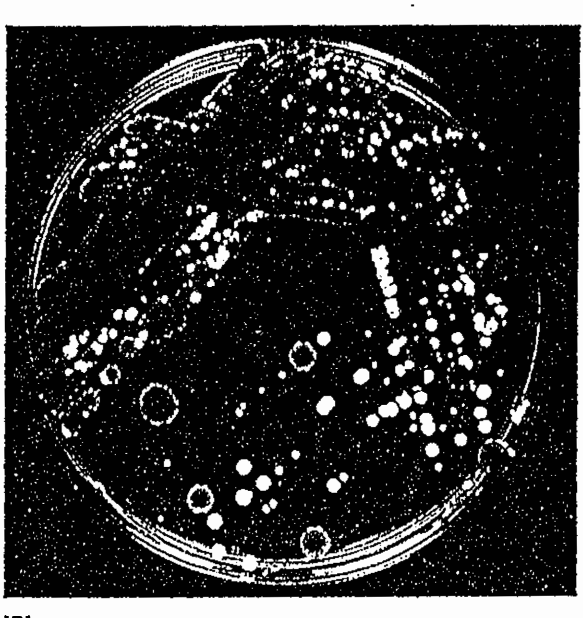


FIGURE 17.2
[A] Four-way streak-plate inoculation and [B] resultant isolated colonies.