# BACTERIOLOGICAL METHODS MANUAL FOR TESTING DRINKING WATER IN RURAL AND ISOLATED INDONESIAN VILLAGES

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# 1.0 SAFETY AND HYGIENE IN THE LABORATORY

## 1.1 General

General and statutory considerations for safety in the laboratory, particular care and a high standard of personal hygiene are essential in microbiological work, if only because pathogenic organisms may always be encountered. In addition, media not necessarily designed for pathogens may support their growth so that great care is needed in the handling and disposal of all cultures. Sound technique is the basis of safe microbiological procedures and it is important that all individuals concerned with micrological work should have received adequate training. It is also important that the necessary laboratory equipment and facilities should conform to accepted codes of safety and good laboratory practice.

Certain precautions are essential in the laboratory not only to *prevent contamination* of samples and culture media but also to *avoid infection* hazards to personnel.

- ☐ Observe strictly, personal hygienic precautions. Keep finger nails short; use hair and beard protection if necessary
- ☐ Wash hands with soap and warm water before and after microbiological examinations as well as after use of the toilet
- ☐ Always wear protective clothing in the laboratory (i.e. lab-coat, eyeglasses)
- ☐ Do not eat, drink or smoke in the laboratory
- ☐ Report any accident, spillage or unusual occurrence to senior personnel or those in responsibility
- ☐ If in doubt, ask for advice from those in responsibility

# 2.0 METHODS OF ENUMERATION

## 2.1 General

Studies of microorganisms in natural waters involve enumeration as a general index of activity and as a measurement of bacteria in water and sediments. Cultural methods are most commonly used, even though they often yield only a small percentage of the microorganisms present.

The methods to be described in this manual will include viable organism enumeration by broth, and microplate techniques. When discussing viable counts, it must be remembered that they are only a fraction of the total count obtained by microscopic observations. The selective nature of synthetic media and atmospheric requirements preclude the growth of many bacteria, each having different growth requirements. Studies using the total of the counts obtained from several synthetic media have indicated these counts to be several times less than the total microscopic counts. This discrepancy may be partly attributable to the fact that many of the organisms observed are non-viable and that there is no single medium and condition of incubation which would be conducive to the growth of all viable organisms in a sample. Thus, selective and elective culturing techniques are used routinely in environmental microbiology. For selective culture, the goal is to inhibit the growth of organisms other than those of the group required and for elective culture, the aim is to develop a medium which allows the group desired to dominate the population which develops.

In the actual testing of a sample, the normal practice is to take a measured volume of sample with a pipette or graduated cylinder, and if the sample aliquot is less than 30 mL (membrane filtration techniques), thoroughly mix this aliquot with a known volume of diluent. A minor source of error may be caused by microbial cells adhering to the sides of the pipette, graduated cylinder, or the diluting flask and thus, not be transferred eventually to the agar medium or membrane filter. However, a far greater potential source of error is attributable to the adsorption of microorganisms on particulate matter. In water which contain little particular matter, most of the microorganisms may be free floating, but as most waters usually contain particulate matter it is advisable to standardize the sample preparation procedure. The water samples should always be thoroughly mixed and subsamples taken in a strict routine, related to time and techniques.

Aggregates of cells will behave like colonized particles and yield only one visible colony for the final count. It is not unusual to observe that plating of serial dilutions does not yield colony counts in proportion to the dilution. Increasing the dilution usually produces a disproportionately higher count. "Crowding" of the colonies on the plates is one explanation, but increasing dispersion of aggregates with increasing dilution can be another explanation.

The optimum growth temperature of many of the microorganisms found in natural waters, in temperate regions at least, is much below 35°C and indeed such waters have a high proportion of psychrophilic bacteria. The nature of the medium can influence the optimum and maximum temperature of growth but nevertheless, an incubation temperature of 15° to 25°C is always likely to be preferable to 35° C except for health oriented bacteria studies, i.e. fecal streptococci, fecal coliforms, *Escherichia coli, Pseudomonas aeruginosa, Legionella pneumophila*.

## 2.2 Scope

This section deals with specific technical procedures and does not include isolation techniques and specific media. The techniques to be described can be applied to water, wastewater, sludge or sediment samples, even though sample preparation may differ. The choice of technique depends on several factors, including the physical and chemical characteristics of the water as well as the nature of the microorganisms sought, their probable concentration, and the test precision required.

The requirements of regulations may also influence the choice of technique to be used by indicating, for example, the precision desired or whether presence or absence of an indicator organism in a specified test volume will be sufficient.

### 2.2.1 Sample Handling

In general, samples should be transported to the laboratory for examination as soon as possible. If there is likely to be a delay, the samples should be placed in a cool, insulated container containing melting ice and examined not later than 30 hours after collection.

- ☐ Before examination, mix the sample thoroughly by vigorous agitation to achieve uniform distribution of microorganisms and, depending on the nature of the water and the bacterial content anticipated, make any dilutions necessary at this stage.

- ☐ For 10-fold dilutions, measure out 90 mL or 9 mL of the diluent into sterile dilution bottles or tubes. Alternatively, use known volumes of pre-sterilized diluent in screw-capped bottles. Make one or more 10-fold dilutions by transferring one volume of water sample to nine volumes of diluent. With a fresh pipette, mix thoroughly and transfer one volume of this dilution to another nine volume of diluent. Repeat these steps as often as required. Prepare sufficient volume of each dilution for all the tests to be carried out on the sample. For dilutions other than ten-fold, adjust appropriately the volume of the diluent to test portion accordingly.

### 2.2.2 Criteria for the Choice of Enumeration Technique

The factors affecting the choice of procedure may be classified as follows:

a. Those concerned with the quality of the results (see section 2.3)
b. Those concerned with the nature of the water sample (see section 2.4)
c. Those concerned with the cost of the analysis (see section 2.5)

## 2.3 Factors Concerning the Quality of the Results

The quality of results obtained from any test can be defined in terms of three main characteristics: accuracy; precision; and limit of detection.

### 2.3.1 Accuracy

Errors in the accuracy, the difference between the true and the actual measured values of results can be quantitative or qualitative. Quantitative errors occur, for example, when the microorganisms enumerated do indeed belong to the group sought, but the result of the final enumeration underestimates the true number. Qualitative errors occur, for example, when a number of microorganisms not belonging to the group sought are considered and counted as such, so that the final value is greater than the true value. Both types of error can occur simultaneously.

- **Quantitative Errors.** Some errors are independent of the enumeration procedure. In the presence of suspended particles, the microorganisms may be adsorbed and become inseparable despite vigorous agitation; this invalidates the assumption that the formation of one colony or turbidity in broth inoculated with a small volume of the sample derives from a single microorganism.

  The action of a selective medium may be excessively inhibitory and thus interfere with the growth, not only of the accompanying microorganisms, but also some of the microorganisms to be enumerated.

  Other errors are attributable to the nature of the water sample. Physico-chemical constituents such as toxic substances or high concentrations of salts can affect the culture medium and thus inhibit growth. The effect is particularly important when large volumes of test portion are used compared with the volume of the medium (e.g. Most Probable Number (MPN) procedures in which double or multiple strength media are used or a dehydrated medium dissolved in the sample to be analyzed).

  Membrane filtration which separates the microorganisms from the sample may overcome this problem unless inhibiting substances are retained on the membrane. Biological constituents such as the accompanying microflora, can, through biological competition, interfere with the development of the microorganisms sought. This effect occurs especially in liquid media. In other procedures where the microorganisms form colonies separate from each other, this biological competition is limited, provided there is no spreading or overcrowding.

- **Qualitative Errors.** These occur when there are differences between the definition of the microorganisms sought and the identity of those isolated. When the definition is precise, as with the individual members of a genus or species, it is exceptional for a single observation to give accurate final results, and it would be impractical to

carry out all the further tests required. It is, therefore, important that the inoculation procedures and the media used should yield as much diagnostic information as possible.

### 2.3.2 Precision

This can be expressed as the closeness of agreement between results obtained by a particular method from the same sample, either in the same laboratory (repeatability) or in different laboratories (reproducibility). Numerous factors affect precision but only those connected with the random distribution of microorganisms in the samples are discussed.

Precision is often expressed in terms of confidence limits within which the true value of the result generally lies for a given degree of probability (usually 95%). Random distribution of the microorganisms in a sample causes imprecision in the results, this can be reduced by the use of a suitable inoculation system. With the MPN procedure, the precision is increased by increasing the number of replicate tubes inoculated with each series of test portion volumes. With the colony count procedure, precision improved as the number of plates or membranes cultured increases.

- **Precision of MPN Procedure.** The total precision for a given system varies according to the number of tubes inoculated with each dilution. The relative precision depends on the pattern of positive results obtained.

- **Precision of Bacterial Colony-Count Procedures.** With bacterial colony-count procedures, precision increases as the number of bacterial colonies increases.

### 2.3.3 Limit of Detection

The limit of detection of a procedure can be considered either as the volume in which a single microorganism sought can be detected or the smallest detectable number of microorganisms contained in a given volume, generally 1 mL or 100 mL of water.

- **Limit of Detection for Broth Culture Procedure**. When appropriate nutrient conditions are supplied, e.g. broth such as Presence/Absence medium for coliforms, or $H_2S$ paper strip test, one bacterium in the inoculated sample can be detected. If the bacterium is stressed or damaged, sometimes it still may be able to resusitate itself and rproduce in appropriate media.

- **Limit of Detection with the MPN Procedure**. The limit of detection depends on the volume of the sample actually examined. Increasing the total volume examined will therefore improve the limit of detection. With the MPN procedure, this may be

achieved either by increasing the number of tubes used for each dilution or by increasing the test portion volume for each tube.

- **Limit of Bacterial Detection of Colony-Count Procedures**. The limit of detection corresponds to the development of one bacterial colony in the test portion. It therefore depends on the volume of sample inoculated. There is, however, a limitation imposed by other accompanying microorganisms in the sample which are able to grow in the same test conditions as the one to be enumerated. If the bacterial colonies are numerous, they can overgrow the cultures. For example, on a membrane where no more than about 100 bacterial colonies can be accepted, the limit of detection will be reduced if the number of accompanying bacterial colonies is more than 100 times greater than the number of bacterial colonies sought. In such cases, and in the absence of satisfactory selective media, the MPN procedure may be preferable.

In practice, the limits of detection are as follows:

a. With membrane filtration, it is possible, in the case of clean water, to filter large volumes of the sample without difficulty, thus giving a limit of detection of one organism in the volume filtered.

b. With the pour-plate procedure, a volume of 5 mL is about the maximum volume of one microorganism in 5 mL. The limit of detection can be improved by increasing the number of test portions used, but in practice, inoculation of more than five plates is impractical and the limit of detection is therefore one microorganism in 25 mL.

c. With the spread-plate procedure, the maximum volume of sample usually used is 0.2 mL for each plate and thus, the limit of detection with five plates inoculated is one microorganism per mL. With very dry medium, test portions of sample water of 1 mL may be used for each plate, thus giving a limit of detection of one microorganism in 5 mL where five plates are inoculated.

## 2.4 Factors Concerning the Nature of the Water Sample

### 2.4.1 Nature of the Microorganisms

In addition to the microorganisms to be detected, growth of other microorganisms in the same culture may also influence the choice of method. For example, certain microorganisms are strict aerobes and their detection may be carried out by the broth culture surface-spread or membrane-filtration methods. Other microorganisms tolerate,

and sometimes prefer, a certain degree of anaerobiosis and the pour-plate method is preferable. If greater anaerobiosis is required to eliminate strict aerobes, then deep tubes of medium may be used.

Certain microorganisms cannot withstand thermal shocks, e.g. 44°C, and thus, elevated temperatures should therefore be avoided for their enumeration; this applies to numerous bacteria in lakes and other surface waters. However, to detect in such waters, microorganisms which normally live at a higher temperature (bacteria in the gut) and have greater resistance to thermal shock, the elevated temperature methods have some selective advantage. There is increasing evidence that some *E. coli* survive and reproduce in tropical waters which is not the finding in temperate climates.

### 2.4.2 Constituents of Water

Chlorinated or disinfected drinking water may contain residual chemical activity and inhibit bacterial growth in culture media. In recreational waters and effluents, suspended matter causes interference, particularly in the membrane filtration procedure; clogging of the membrane limits filtration and for this reason, the sensitivity of the method. Partial clogging, sometimes unsuspected, can reduce nutrient exchange and prevent organisms on the membrane from forming colonies. This blockage may often be caused by living organisms, including microplankton or even bacteria which multiply in the pores. Sometimes large particles can be confused with colonies in the pour-plate procedure.

For very turbid waters, the MPN procedure may sometimes be the only one that can be used, if, as is often the case, the spread-plate method does not provide sufficient sensitivity. Soluble substances may interfere with the growth of microorganisms either by modifying the composition of the selected culture medium or because of their toxicity. This interference occurs in particular when the volume of the sample is relatively large in relation to that of the medium to be used. Sometimes substances in the sample can react with constituents of the culture medium and adversely affect the characteristic reactions of the microorganism sought, without interfering with their growth through the formation of a fermentable sugar not present in the original medium, followed by a change in pH value due to fermentation of this sugar. Membrane filtration can overcome such disadvantages.

## 2.5 Factors Concerning the Cost of the Analysis

- **Financial Requirements.** Comparison of the cost of these different procedures is fairly difficult and often dependent on local conditions. Nevertheless, knowledge of such cost factors may facilitate the choice of the method to be adopted.

# 3.0 DRINKING (POTABLE) WATER TESTS

## 3.1 General

Untreated or improperly treated drinking water may contain microorganisms that are pathogenic. Coliform bacteria are often associated with pathogenic organisms and thus, have become the principle index for the suitability of drinking water. Coliforms inhabit the intestinal tract of animals and humans and are discharged along with many other bacteria, e.g. *Clostridium* species, in great numbers in their feces. Therefore, coliform presence in potable waters indicates that these waters may be contaminated with feces and that the water is not microbiologically safe for human consumption as pathogenic organisms may also be present.

## 3.2 Hydrogen Sulphide ($H_2S$) Paper Strip Test

In 1975, Allen and Geldreich showed that the presence of coliforms in water was also associated with hydrogen sulphide ($H_2S$) producing organisms. Then, in 1982, Manja et al. reported on the use of a paper-strip method to screen for bacteriological contamination of potable waters. In this large study, they confirmed that the presence of $H_2S$ in the test samples was indicative of the presence of coliforms.

Bacteria can produce hydrogen sulphide through the anaerobic catabolism of cysteine, an amino acid containing the sulfahydryl group (-SH) or by the use of elemental sulphur or some oxidised sulphur compounds as the terminal electron acceptor in their metabolic processes. All members of the *Enterobacteriacae* and some other bacteria are capable of the former. The latter occurs in dissimilatory sulphur-reducing and dissimilatory sulphate-reducing bacteria (Buchanan and Gibbons, 1975). Other bacteria can reduce thiosulphate into hydrogen sulphide using thiosulphate as an electron acceptor in an anaerobic respiration process.

Using a medium with thiosulphate as a sulphur source and ferric ammonium citrate as an "indicator," only certain bacteria will produce hydrogen sulphide with the development of a black precipitate. The hydrogen sulphide would be produced by the reduction of thiosulphate and would then react with the ferric salt to form an insoluble black ferrous sulphide precipitate. The presence of a heavy metal such as iron salts as an indicator in the medium would also inhibit some bacteria from hydrogen sulphide production. Bacteria including, *Salmonella, Citrobacter* and *Proteus* are able to produce hydrogen sulphide in such a medium.

According to Manja et al. (1982) and Hazbun and Parker (1983), the $H_2S$ paper-strip test showed good agreement with the standard Most Probable Number (MPN) needed for determining the presence and number of coliforms per 100 mL. A number of field trials have been conducted in India, Phillipines, Solomon Islands, Indonesia, Hawaii, Peru, Chile

and Brazil, and all have indicated that the procedure works.

### 3.2.1 Preparation of Test Bottles ($H_2S$). Traditional Procedure

1. Any type of clean glass bottle with volume of 50 mL to 200 mL and which has a heat resistant cap/lid can be used.

2. The concentrated medium used in the test is prepared from the following, and the chemicals are dissolved into the distilled or dechlorinated tap water with stirring.

**$H_2S$ Media:**

| | |
|---|---|
| Bacteriological peptone | 40.0 g |
| Dipotassium hydrogen phosphate | 3.0 g |
| Ferric ammonium citrate | 1.50 g |
| Sodium thiosulphate | 2.00 g |
| Teepol | 2.0 mL |
| Water, distilled or boiled tap | 100.0 mL |

3. Taking tissue paper or non-toxic paper towelling, or Whattman filter papers, or absorbent pads from membrane filter packages, etc., place sufficient quantity into each bottle so that the paper will readily absorb 1 mL (20 mL test) or 2.5 mL (100 mL test) of the chemical solution.

4. Bottles can now be loosely capped and autoclaved for 15 minutes at 115°C and then, with the bottle caps still slightly loose, place into an oven at 55° to dry. Or, the loosely capped bottles can be placed in a hot air oven at 160°C for 60 minutes to sterilize and dry.

5. Once sterile, the prepared bottles can be marked with a line at the appropriate volume mark, e.g. 20 mL or 100 mL.

### 3.2.2 $H_2S$ Paper Strip Test Procedure. Traditional Procedure

1. For each sample, use either or both a 20 mL sample testing bottle and/or 100 mL sample testing bottle.

2. Premark the appropriate bottle at a 20 mL volume and a 100 mL volume mark. A marking pencil or tape can be used for this.

3. Label each bottle with the sample number. Record the date, name of location, such as home, facility, etc., and sample volume tested on a sample record sheet. (See Appendix 1)

4. a. If the sample is from a tap and the water source is *not chlorinated*, the sample may be collected directly into the appropriate bottles.

   b. Turn on the tap and let the water run freely for 30 seconds, then place the opened $H_2S$ paper strip test bottle under the tap and collect water up to the appropriate volume mark (20 mL or 100 mL).

   c. Put on the bottle cap/lid, shake well and incubate as soon as possible at 26° to 37°C; 35°C is the preferred temperature. Incubation should continue up to a maximum of threedays.

   d. Do not store the bottle with the sample water in the refrigerator or on ice.

5. a. If the sample being collected is from a *chlorinated water supply*, collect the sample in a sterile bottle (120 - 150 mL volume) containing 0.1 mL of a 3% solution of sodium thiosulphate ($Na_2S_20_3$). The sodium thiosulphate is used to neutralize the chlorine in the water supply. The thiosulphate solution should be added prior to sterilizing the sample collection bottle. The sample collection bottle can be sterilized by autoclaving (115°C for 15 minutes) or dry heat (160° for one hour). The sodium thiosulphate neutralizes the chlorine in the water supply.

   b. After sample collection, if possible, keep the bottle on ice or refrigerated until the water sample can be processed. Iced or refrigerated samples should be tested within six hours but may be maintained on melting ice up to 30 hours before testing. Non-refrigerated samples should be tested as soon as possible.

   c. In the laboratory, use either a sterile graduated cylinder to measure the required water volume prior to dispensing into the appropriate $H_2S$ paper strip bottle or carefully pour the water sample into the $H_2S$ paper strip bottle up to the appropriate volume mark (20 mL or 100 mL). In either case, flame the neck of the sample collection bottle before dispensing.

6. a. If the water sample is in a standing container or is a natural flowing water body, use the utensil that is normally used by the home-owner to collect the water sample, rinsing it several times before the sample is collected.

   b. After the sample has been collected, carefully pour the required sample volume into appropriate $H_2S$ paper strip bottles.

7. Check the incubated samples daily up to three days. If there is any blackening of the paper strip in the sample water, the test is positive and must be reported immediately. Record the results on a data sheet as shown in Appendix 1.

8. a. If the 100 mL $H_2S$ bottle becomes positive (black coloration), this suggests the presence of at least one indicator bacterium per 100 mL. However, if the

blackening occurs very quickly and intensively (<24 hours), this suggests the presence of larger numbers of indicator bacteria.

b. If the 20 mL $H_2S$ bottle becomes positive, this sugggests the presence of five or more indicator bacteria per 100 mL. Also, if the blackening occurs very quickly and intensively (<24 hours), our experience makes us believe there are >50 indicator bacteria per 100 mL of sample.

### 3.2.3 Preparation of 10 mL and 1 mL Sample Test Tubes for Indonesian Potable Water Testing

1. Use 16 x 150 mm screw capped tubes with heat resistant caps.

2. For tubes which are going to be used to test 10 mL water samples, add 10 mL of water to one tube and using a permanent marking pen make a mark on the tube at the maximum height of the added water. Using this mark as a guide, prepare as many tubes as needed with a 10 mL mark line.

3. For tubes which are going to be used to test 1 mL of water sample, add 9 mL of water to one tube and using a permanent marking pen make a 9 mL mark on the tube at the maximum height of the added water. Then add 1 mL of water to the 9 mL and make a mark at the total level (i.e. 10 mL level). Using these 9 mL and 10 mL marks as guides prepare as many tubes as needed (see Fig. 3.2.3 for details).

4. The concentrated medium used in the test is prepared from the following, and the chemicals are dissolved into distilled or boiled tap water with stirring.

**$H_2S$ Media:**

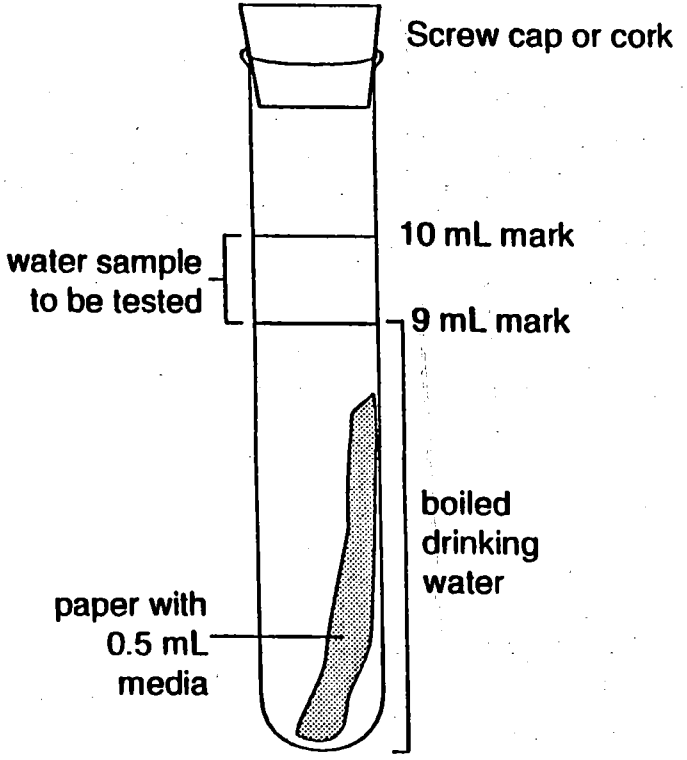
| | |
|---|---|
| Bacteriological peptone | 40.0 g |
| Dipotassium hydrogen phosphate | 3.0 g |
| Ferric ammonium citrate | 1.50 g |
| Sodium thiosulphate | 2.00 g |
| Teepol | 2.0 mL |
| Water, distilled or boiled tap | 100.0 mL |

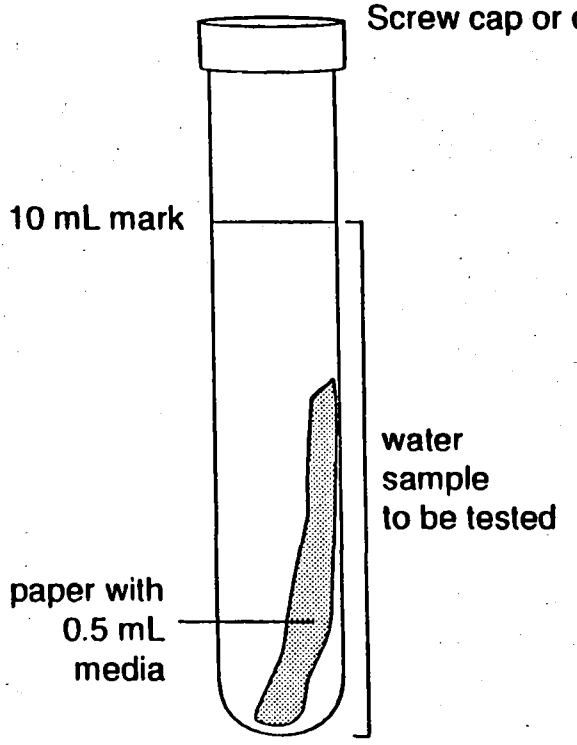
5. Taking strong non-toxic paper towels or Whattman filter paper #3 or absorbent pads from membrane filter packages, place sufficient quantity into each tube so that the paper will readily absorb 0.5 mL of the test chemical solution.

6. Tubes can now be loosely capped and autoclaved for 15 minutes at 115°C. Or the loosely capped tubes can be placed in a hot air oven at 160°C for 60 minutes to sterilize and dry.

7. Once sterlized, the caps should be tightened and the tubes stored in a clean dark place.

# $H_2S$ Field Monitoring Qualitative Procedure




1 mL positive for $H_2S$ = 100 + coliforms /100mL
1 mL negative for $H_2S$ = < 100 coliforms /100mL

10 mL positive for $H_2S$ = 10 + coliforms/100 mL
10 mL negative for $H_2S$ = <10 coliforms/100 mL

Figure 3.2.3

### 3.2.4. 10 mL and 1 mL $H_2S$ Paper Strip Procedure. (Indonesian Waters)

1. For each sample use a 10 mL testing tube and a 1 mL (9+1) testing tube.

2. Label each tube with the sample number and record the date and location on the sample record sheet. (See Appendix)

3. For the 1 mL sample, take local boiled drinking water and carefully with a sterile technique add the boiled water to the 9 mL mark.

4. If the sample is from a tap, turn on the tap and let the water run freely for 30 seconds, then place the opened (9+1 mL) $H_2S$ paper strip test tube under the tap and collect 1 mL of water, up to the 10 mL mark (see figure 3,2,3). For the 10 mL sample fill the appropriate tube up to the 10 mL mark.

5. If the sample is from a well, spring, river, ditch etc. rinse out the collecting container at least three times in the water to be collected. To simplify transfer of sample from the large container to the 10 mL and 1 mL test tubes, pour water from the container into a cup/glass which has been sterilized by having boiled water poured into the cup/glass and let stand for 2-3 minutes. Pour out the boiled water, add the sample water and follow procedure for "tap" water.

6. Incubate the 10 mL and 1 mL sample tubes at 35°C for up to three days. If there is no incubator, place the tubes in the warmest part of the house, usually near the cooking area, for three days.

7. If there is any blackening of the paper strip in the sample water, the test is positive and must be reported immediately. Record the results on a data sheet as shown in Appendix 1.

7a. If the 10 mL tube becomes positive (black colouration) this suggests the presence of **at least 10** indicator bacteria per 100 mL. However, if the blackening occurs very quickly and intensively (<18 hours) this suggests the presence of larger numbers of indicator bacteria.

7b. If the 1 mL tube becomes positive (black colouration) this suggests th presence of **at least 100** or more indicator bacteria per 100 mL. Also, if the blackening occurs very quickly and intensively (<18 hours), this suggests the presence of larger numbers of indicator bacteria, >200/100 mL.

8. When using boiled water to make the 1 mL sample tube, a negative control, using 10 mL of the boiled water, should also be incubated along with the samples. One 10 mL tube of boiled water should be used for each different source of boiled water used.

## 3.3 Presence-Absence (P/A) Test

Dr. J. Clark, in 1968, working with the Ontario Water Resources Commission, developed a Presence-Absence (P/A) test, using a modified MacConkey broth for monitoring drinking water supplies. The P/A test is essentially a large volume procedure where a 20 mL or 100 mL volume of drinking water is inoculated into bottles containing Presence-Absence medium and incubated up to three days at 35°C. Research has shown that the test is functional at temperatures varying from 27° to 37°C.

International research projects using P/A media have reported: (1) More samples are positive compared to all traditional procedures (Most Probable Number [MPN] or Membrane Filter [MF]), particularly those with low coliform and high non-coliform densities; (2) Lower levels of pollution were detected; and (3) Supplementary water quality information can be provided with additional biochemical tests. The P/A procedure can test for other indicators of poor water quality, including *Escherichia coli*, fecal streptococci, pseudomonas, *Clostridia, Aeromonas* and staphylococci.

### 3.3.1 Preparation of Test Bottles, Presence-Absence (P/A) Test

1. Any type of clean glass bottle with volume of 50 to 200 mL and which has a heat resistant cap/lid can be used.

2. The medium used in this test is prepared from the following:

**P/A Media:**

| | |
|---|---|
| Bacto beef extract | 3.0 g |
| Peptone | 5.0 g |
| Lactose | 7.46 g |
| Tryptose | 9.83 g |
| Potassium phosphate dibasic | 1.35 g |
| Potassium phosphate monobasic | 1.35 g |
| Sodium chloride | 2.46 g |
| Sodium lauryl sulphate | 0.05 g |
| Brom cresol purple | 0.0085 g |

The medium is used in triple strength form. Therefore, three times the above ingredients (91.5 grams in total) are weighed out and added to one litre of distilled or dechlorinated tap water. The water and chemical ingredients are warmed gently to dissolve (almost to a boil).

3. Dispense 10 mL of the P/A broth into those bottles which will be used to test 20 mL water samples and 50 mL into those bottles which will be used to test 100 mL water samples.

4. Place caps/lids on bottles loosely and sterilize by autoclaving at 115°C for 15 minutes.

5. After autoclaving and bottles have cooled, tighten caps and store in a cool, dark place. Sample testing bottles can be stored for at least one year.

6. Prior to storage and using guide bottles, i.e. similar bottles containing 30 mL of fluid (10 mL P/A, plus 20 mL sample) and 150 mL of fluid (50 mL P/A, plus 100 mL sample), appropriate volume marks are placed on the sample collection bottles, e.g. 30 mL for 20 mL samples, and 150 mL for 100 mL samples.

### 3.3.2 Presence-Absence (P/A) Test Procedure

1. For each sample, use either or both a 20 mL sample testing bottle (50 mL size) and/or 100 mL sample testing bottle (200 mL size).

2. Premark the appropriate bottle at a 30 mL volume and a 150 mL volume mark. A marking pencil or tape can be used for this.

3. Label each bottle with the sample number and date, or name of location, such as home, facility, etc.

4. a. If the sample is from a tap and the water source is not chlorinated, the sample may be collected directly into the appropriate bottles containing P/A medium.

   b. Turn on the tap and let the water run freely for 30 seconds, then place the opened P/A medium bottle under the tap and collect water up to the appropriate volume mark (30 mL or 150 mL).

   c. Put on the bottle cap/lid, shake well and incubate as soon as possible at 26° to 37°C; 35°C is the preferred temperature. Incubation should continue up to five days.

   d. Do not store these bottles with samples in the refrigerator or on ice.

5. a. If the sample being collected is from a chlorinated water supply, collect the sample in a sterile bottle (120 - 150 mL volume) containing 0.1 mL of a 3% solution of sodium thiosulphate ($Na_2S_2O_3$). The sodium thiosulphate is used to neutralize the chlorine in the water supply. The sodium thiosulphate solution should be added prior to sterilizing the sample collection bottle. The sample collection bottle can be sterilized by autoclaving (115°C for 15 minutes) or dry heat (160° for one hour).

b. After sample collection, if possible, keep the bottle on ice or refrigerated until the water sample can be processed. Iced or refrigerated samples should be tested within six hours but may be maintained on melting ice up to 30 hours before testing. Non-refrigerated samples should be tested as soon as possible.

c. In the laboratory, after the water sample has been collected, using either a sterile graduate cylinder to measure the required water volume (20 mL or 100 mL) prior to dispensing into the appropriate P/A bottle or carefully pour the water sample into the P/A bottle up to the appropriate volume mark (30 mL or 150 mL).

6. a. If the water sample is in a standing container or is a natural flowing water body, use the utensil that is normally used by the home-owner to collect the water sample, rinsing it several times before the sample is collected.

b. After the sample has been collected, carefully pour the required sample volume into appropriate P/A bottles.

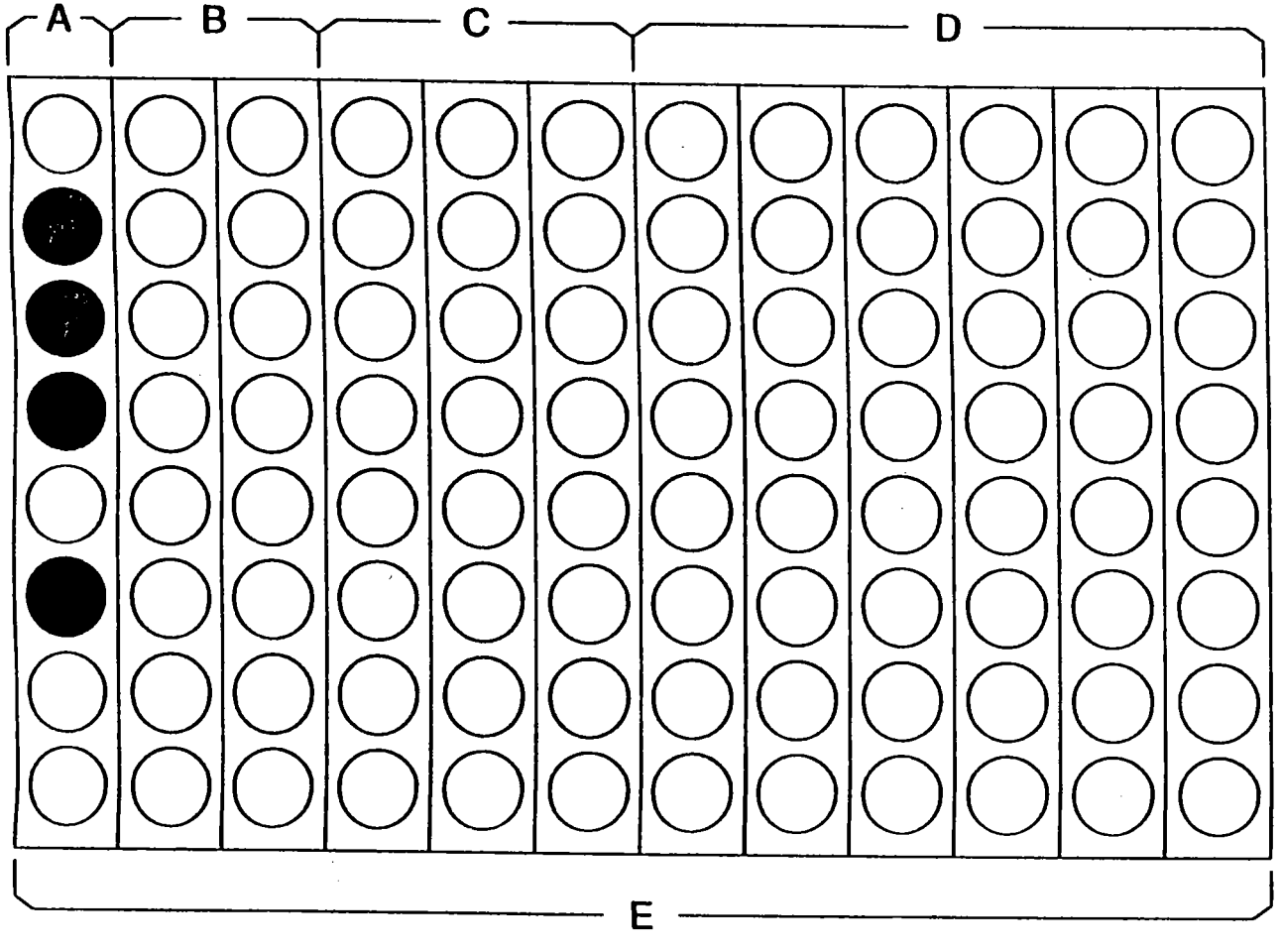
7. Check the incubated samples daily up to three days. If the purple medium changes to yellow, this is an indication that at least one type of pollution indicator is present. However, if this yellow medium is shaken and gas bubbles are noticed, then in all probability, coliforms and/or fecal coliforms are present in the bottle. The presence of acid (yellow colour) and gas does not exclude the presence of other indicator bacteria such as streptococci, *Clostridia,* staphylococci, or pathogenic bacteria. Record the results on a data sheet as shown in appendix.

## 3.4 ColiPlate™-400 MPN Procedures for Total Coliforms and E.coli

The ColiPlate™-400 is a convenient test for the quantitative measure of total coliforms and E.coli. The test is designed to meet regulatory guidelines for surface water and raw drinking water, as well as recreational waters and wastewater. The full ColiPlate enables quantification of coliforms and E.coli densities ranging from <3 to 2400 colony forming units (cfu)/100 mL in a single test without dilution. (Fig. 3.4) The distinctive blue/green colouration of positive (coliforms) tested samples enables analysis of brownish, turbid or rust containing waters.

The ColiPlate test utilizes the proven X-Gal and MUG techniques to detect viable coliforms and/or E.coli. The Coliplate contains selective media to provide nutrients to stimulate the growth of coliforms and E.coli. The media also contains inducers and chromogenic/fluorogenic substrates. These substrates react with enzymes specific to coliforms (beta-D-galactosidase) and E.coli (beta-D-glucoronidase) to provide colour change to blue/green (coliforms) and fluorescence, (E.coli).

# Field Monitoring Quantitative Coliplate™-400 Procedure for E. Coli and Total Coliforms




Full plate range
< 3 – > 2424
with 0.40 mL addition
per cup

Combinations:

A - 8 wells = 1 strip = < 33 - > 619
B - 16 wells = 2 strips = < 16 - > 938
C - 24 wells = 3 strips = < 11 - > 1174
D - 48 wells = 6 strips = <5 - > 1696
E - 96 wells = 12 strips = < 3 - > 2424

Blue colour = coliform
fluorescing blue colour = *E. Coli*

Figure 3.4

Test results are recorded after 20-24 hours of incubation with the appearance of a blue/green colour in the wells containing coliforms. E.coli can be detected by the appearance of fluorescence under a long wavelength UV light, in some or all the wells with a blue/green colour. The ColiPlate can be used to confirm $H_2S$ paper strip results and establish E.coli densities (see Appendix 2).

### 3.4.1 ColiPlate™-400 Whole Plate Procedure

1. Using a pipette fill up all 96 wells (400 µl/well) of the microplate: **or**

1a. Dip the whole ColiPlate into the sample and fill up all 96 wells. This procedure works with well samples, where the water is collected in a bucket.

2. Cover microplate with lid; insert microplate into zip-loc bag (or other plastic bag which can be sealed) and incubate at 35°C for 20-24 hours. (Fig. 3.4.1)

3. After incubation, count the number of wells that have turned blue/green. Refer to 96 well MPN Table 1 to determine the Most Probable Number for total coliforms in 100 mL sample.

4. Place long wave UV(366 nm) light over the microplate and count the number of wells that have a blue fluorescence. Refer to 96 well MPN Table 1 to determine the Most Probable Number for E.coli in 100 mL sample.

5. For samples which are very polluted i.e. 1 mL $H_2S$ test is positive in 24 hours, it is recommended that only ½ of the microplate is used (48 wells). Thus 2 samples can be tested on the same plate. For MPN Total Coliforms and E.coli refer to the 48 well MPN Table 2.

# Inexpensive field incubator using insulated ice-chest

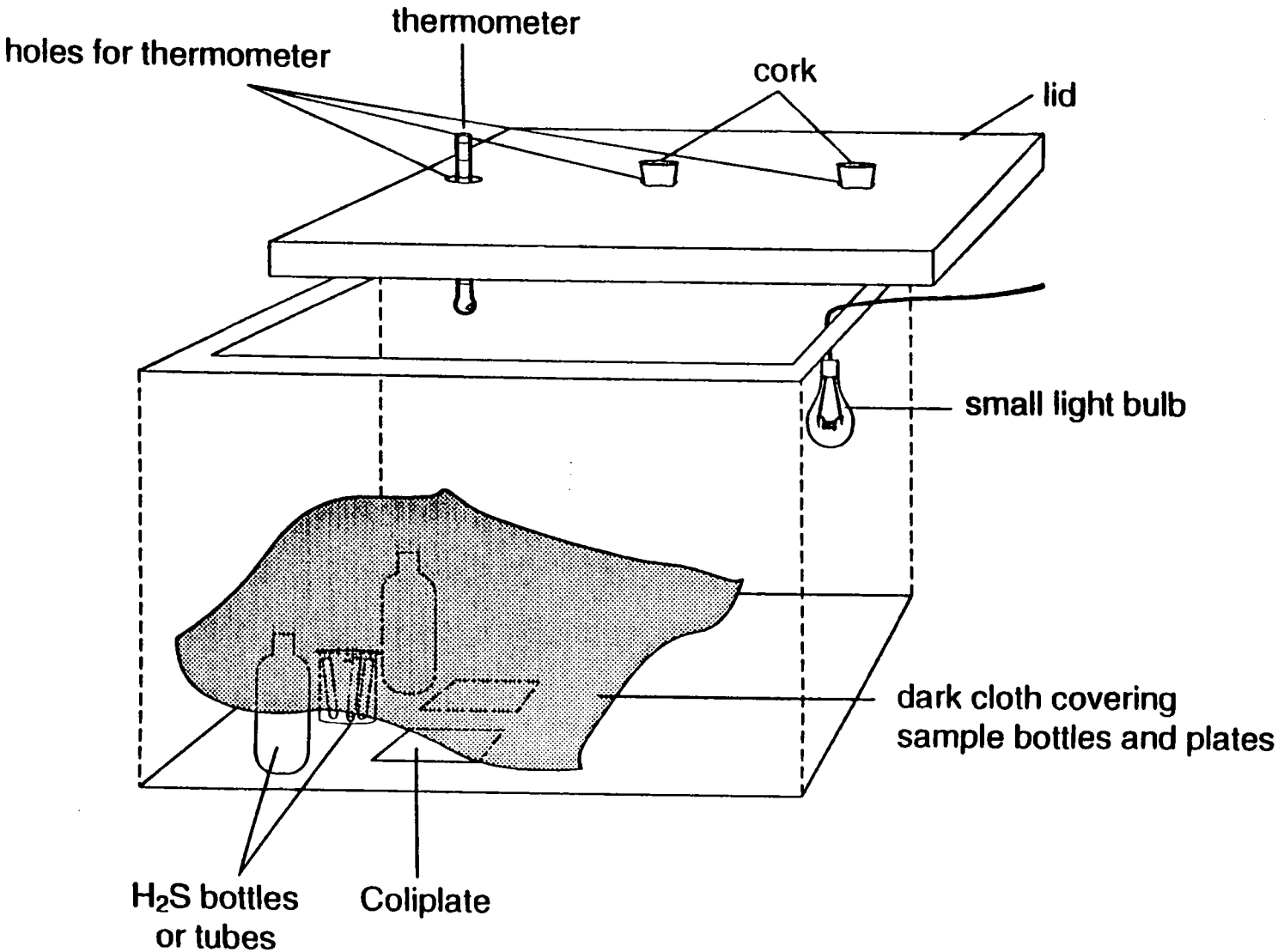



Figure 3.4.1

| TABLE 1: Microplate™-400 Total Plate MPN Values | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| No. Of Wells Giving Postive Reaction | | MPN per 100 ml Sample | No. Of Wells Giving Positive Reaction | | MPN per 100 ml Sample | No. Of Wells Giving Positive Reaction | | MPN per 100 ml Sample |
| | | | | | | | | |
| 0 | | <3 | | | | | | |
| 1 | | 3 | 33 | | 106 | 65 | | 298 |
| 2 | | 5 | 34 | | 110 | 66 | | 307 |
| 3 | | 8 | 35 | | 114 | 67 | | 317 |
| 4 | | 11 | 36 | | 119 | 68 | | 328 |
| 5 | | 13 | 37 | | 123 | 69 | | 339 |
| 6 | | 16 | 38 | | 127 | 70 | | 350 |
| 7 | | 19 | 39 | | 132 | 71 | | 362 |
| 8 | | 22 | 40 | | 136 | 72 | | 375 |
| 9 | | 25 | 41 | | 141 | 73 | | 388 |
| 10 | | 28 | 42 | | 146 | 74 | | 403 |
| 11 | | 30 | 43 | | 151 | 75 | | 418 |
| 12 | | 33 | 44 | | 156 | 76 | | 434 |
| 13 | | 33 | 45 | | 161 | 77 | | 451 |
| 14 | | 39 | 46 | | 166 | 78 | | 469 |
| 15 | | 43 | 47 | | 171 | 79 | | 489 |
| 16 | | 46 | 48 | | 177 | 80 | | 510 |
| 17 | | 49 | 49 | | 182 | 81 | | 534 |
| 18 | | 52 | 50 | | 188 | 82 | | 559 |
| 19 | | 55 | 51 | | 194 | 83 | | 587 |
| 20 | | 59 | 52 | | 200 | 84 | | 619 |
| 21 | | 62 | 53 | | 206 | 85 | | 654 |
| 22 | | 65 | 54 | | 213 | 86 | | 694 |
| 23 | | 69 | 55 | | 219 | 87 | | 740 |
| 24 | | 72 | 56 | | 226 | 88 | | 794 |
| 25 | | 78 | 57 | | 233 | 89 | | 858 |
| 26 | | 79 | 58 | | 240 | 90 | | 938 |
| 27 | | 83 | 59 | | 247 | 91 | | 1038 |
| 28 | | 87 | 60 | | 255 | 92 | | 1174 |
| 29 | | 90 | 61 | | 263 | 93 | | 1370 |
| 30 | | 94 | 62 | | 271 | 94 | | 1696 |
| 31 | | 98 | 63 | | 280 | 95 | | 2424 |
| 32 | | 102 | 64 | | 289 | 96 | | >2424 |

| TABLE 2: 48 Well Microplate™.400 MPN values for heavily contaminated samples | | | |
|---|---|---|---|
| No. Of Wells Giving Positive Reaction | MPN per 100 ml Sample | No. Of Wells Giving Postive Reaction | MPN per 100 ml Sample |
| 0 | <5 | 25 | 188 |
| 1 | 5 | 26 | 200 |
| 2 | 11 | 27 | 213 |
| 3 | 16 | 28 | 226 |
| 4 | 22 | 29 | 240 |
| 5 | 28 | 30 | 255 |
| 6 | 33 | 31 | 271 |
| 7 | 39 | 32 | 289 |
| 8 | 46 | 33 | 307 |
| 9 | 52 | 34 | 328 |
| 10 | 59 | 35 | 350 |
| 11 | 65 | 36 | 375 |
| 12 | 72 | 37 | 403 |
| 13 | 72 | 38 | 434 |
| 14 | 87 | 39 | 469 |
| 15 | 94 | 40 | 510 |
| 16 | 102 | 41 | 559 |
| 17 | 110 | 42 | 619 |
| 18 | 119 | 43 | 694 |
| 19 | 127 | 44 | 794 |
| 20 | 136 | 45 | 938 |
| 21 | 146 | 46 | 1174 |
| 22 | 156 | 47 | 1696 |
| 23 | 166 | 48 | >1696 |
| 24 | 177 | | |

### 3.4.2 ColiPlate™-400 Two Strip Quick Screening Procedure

1. Dip the double strip unit into the water sample until all wells are filled up completely.

2. Pull the strip unit back gently and place inside a plastic bag and seal, or

3. Using a pipette fill up 2 rows of wells (16 wells) of a 96 well microplate. Label. With this procedure 6 water samples can be tested with one 96 well microplate.

4. Incubate at 35$^0$C for 20-24 hours.

5. Count the number of wells that turned blue/green. Refer to 16 well Microplate™-400 MPN Table to determine the Most Probable Number for Total Coliforms in 100 mL sample.

6. Observe under long wave UV (366 nm) light and count the number of wells that have a blue fluorescence. Refer to 16 well Microplate™-400 MPN Table for E.coli in 100 mL sample.

**Double Strip (16 Wells) Microplate™-400 MPN Values For Screening Tests**

| No. Of Wells Giving Positive Reaction | MPN per 100 ml Sample |
|---|---|
| 0 | <16 |
| 1 | 16 |
| 2 | 33 |
| 3 | 52 |

| No. Of Wells Giving Positive Reaction | MPN per 100 ml Sample |
|---|---|
| 4 | 72 |
| 5 | 94 |
| 6 | 119 |
| 7 | 146 |

| No. Of Wells Giving Positive Reaction | MNP per 100 ml Sample |
|---|---|
| 8 | 177 |
| 9 | 213 |
| 10 | 255 |
| 11 | 307 |

| No. Of Wells Giving Positive Reaction | MPN per 100 ml Ssmple |
|---|---|
| 12 | 375 |
| 13 | 469 |
| 14 | 619 |
| 15 | 938 |
| 16 | >938 |

## 3.5 Multiple-Tube Fermentation Technique, MPN Test

It has been adequately demonstrated that even after the prescribed shaking, the distribution of bacteria in a sample may be irregular and in any given subsample may vary from none to a number much greater than the arithmetic average, based on the examination of the total volume. It is also quite probable that growth in any tube may be the result of one organism or many organisms; however, the principle of this test depends on the assumption that growth develops from one or more bacteria. As dilutions are made, the positive tubes representing the greatest dilutions before no growth occurs would be assumed to result from the presence of a single bacterium.

Data from aquatic microbiology studies indicate that, depending on the age of the

population being enumerated, the temperature of the water sample and the temperature-media-procedure combination used, only 40% to 90% of the population is enumerated.

### 3.5.1 Multiple-Tube Fermentation Technique, MPN Procedure

**Apparatus Required:**

- ☐ Glass tubes, 150 x 16 mm or 150 x 20 mm with caps and containing Durham tubes (glass vials)
- ☐ Wooden or metal racks to contain 150 x 16 mm or 150 x 20 mm tubes
- ☐ Double- strength and single-strength broths specific for tests
- ☐ 1 mL and 10 mL sterile, cotton-plugged pipettes, or automatic pipetting device, e.g. Oxford macro- and micro- system with sterile 1 mL and 10 mL disposable tips
- ☐ Dilution blanks, 90 mL and 9.0 mL
- ☐ China marking pencil
- ☐ Appropriate incubators or water baths
- ☐ Hand-held longwave, ultra-violet light

1. Clean and disinfect the working area with cleaning solution that leaves no residue.
2. Set out and label five replicate tubes of 10 mL double-strength broths.
3. Set out and label five replicate tubes of 10 mL single-strength broths for each mL or mL of sample dilution to be tested. Usually, two set of replicate 10 mL tubes are used containing 1 mL of sample and 1 mL of one-tenth dilution of sample.
4. Shake sample thoroughly 25 times. Make any dilutions required by starting with 10 mL of sample into one 90 mL dilution blank or 1 mL or sample into 9 mL dilution blank.
5. Pipette 10 mL of sample into each double-strength broth and 1 mL of sample or diluted sample into each set of five single-strength broths.
6. Incubate tubes for required time at required temperatures.
7. Repeat step 1.
8. Always add negative controls to each series of tests (dilution water and uninoculated media -- all sizes used).

*Confirmation. Most MPN procedures have confirmation requirements which are specific for each group of organisms being enumerated.*

## 3.6 Computing and Recording of Most Probable Numbers (MPNs)

The number of positive findings resulting from multiple-portion decimental dilution

planting should be computed as the combination of positives and recorded in terms of the Most Probable Number (MPN). Table 3 shows the MPN values for serial dilutions 10 mL, 1 mL and 0.1 mL. Table 3 can also be used in computing values where smaller portions of sample have been plated, e.g. samples 0.1, 0.01 and 0.001 mL would be recorded as 100 times the table values. Sediment bacteria are represented per gram wet or dry weight. When more than three dilutions are employed in a decimal series of dilutions, the results from o these are in computing the MPN. For example:

| | | | | | | |
|---|---|---|---|---|---|---|
| *5/5* | *5/5* | *2/5* | *0/5* | = | *5-2-0* | *x 10* |
| *5/5* | *4/5* | *2/5* | *0/5* | = | *5-4-2* | |
| *0/5* | *1/5* | *0/5* | *0/5* | = | *0-1-0* | |
| *5/5* | *3/5* | *2/5* | *1/5* | = | *5-3-1* | |
| *5/5* | *5/5* | *3/5* | *1/5* | = | *5-3-1* | *x 10* |
| *5/5* | *0/5* | *0/5* | *0/5* | = | *5-0-0* | |

The results are computed from the last dilution showing five positive tubes and the number of positive tubes in each of the two following consecutive dilutions. See Table 3.

**TABLE 3: Most Probable Number (MPN) Index per 100 mL when five 10 mL, 1mL and 0.1 mL replicate portions of each dilution are used**

| 10 | 1 | -.1 | MPN | 10 | 1 | 0.1 | MPN | 10 | 1 | 0.1 | MPN | 10 | 1 | 0.1 | MPN | 10 | 1 | 0.1 | MPN | 10 | 1 | 0.1 | MPN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | 2 | 1 | 0 | 0 | 2 | 2 | 0 | 0 | 5 | 3 | 0 | 0 | 8 | 4 | 0 | 0 | 13 | 5 | 0 | 0 | 23 |
| 0 | 0 | 1 | 2 | 1 | 0 | 1 | 4 | 2 | 0 | 1 | 7 | 3 | 0 | 1 | 11 | 4 | 0 | 1 | 17 | 5 | 0 | 1 | 31 |
| 0 | 0 | 2 | 4 | 1 | 0 | 2 | 6 | 2 | 0 | 2 | 9 | 3 | 0 | 2 | 13 | 4 | 0 | 2 | 21 | 5 | 0 | 2 | 43 |
| 0 | 0 | 3 | 5 | 1 | 0 | 3 | 8 | 2 | 0 | 3 | 12 | 3 | 0 | 3 | 16 | 4 | 0 | 3 | 25 | 5 | 0 | 3 | 58 |
| 0 | 0 | 4 | 7 | 1 | 0 | 4 | 10 | 2 | 0 | 4 | 14 | 3 | 0 | 4 | 20 | 4 | 0 | 4 | 30 | 5 | 0 | 4 | 76 |
| 0 | 0 | 5 | 9 | 1 | 0 | 5 | 12 | 2 | 0 | 5 | 16 | 3 | 0 | 5 | 23 | 4 | 0 | 5 | 36 | 5 | 0 | 5 | 95 |
| 0 | 1 | 0 | 2 | 1 | 1 | 0 | 4 | 2 | 1 | 0 | 7 | 3 | 1 | 0 | 11 | 4 | 1 | 0 | 17 | 5 | 1 | 0 | 33 |
| 0 | 1 | 1 | 4 | 1 | 1 | 1 | 6 | 2 | 1 | 1 | 9 | 3 | 1 | 1 | 14 | 4 | 1 | 1 | 21 | 5 | 1 | 1 | 46 |
| 0 | 1 | 2 | 6 | 1 | 1 | 2 | 8 | 2 | 1 | 2 | 12 | 3 | 1 | 2 | 17 | 4 | 1 | 2 | 26 | 5 | 1 | 2 | 64 |
| 0 | 1 | 3 | 7 | 1 | 1 | 3 | 10 | 2 | 1 | 3 | 14 | 3 | 1 | 3 | 20 | 4 | 1 | 3 | 31 | 5 | 1 | 3 | 84 |
| 0 | 1 | 4 | 9 | 1 | 1 | 4 | 12 | 2 | 1 | 4 | 17 | 3 | 1 | 4 | 23 | 4 | 1 | 4 | 36 | 5 | 1 | 4 | 110 |
| 0 | 1 | 5 | 11 | 1 | 1 | 5 | 14 | 2 | 1 | 5 | 19 | 3 | 1 | 5 | 27 | 4 | 1 | 5 | 42 | 5 | 1 | 5 | 130 |
| 0 | 2 | 0 | 4 | 1 | 2 | 0 | 6 | 2 | 2 | 0 | 9 | 3 | 2 | 0 | 14 | 4 | 2 | 0 | 22 | 5 | 2 | 0 | 49 |
| 0 | 2 | 1 | 6 | 1 | 2 | 1 | 8 | 2 | 2 | 1 | 12 | 3 | 2 | 1 | 17 | 4 | 2 | 1 | 36 | 5 | 2 | 1 | 70 |
| 0 | 2 | 2 | 7 | 1 | 2 | 2 | 10 | 2 | 2 | 2 | 14 | 3 | 2 | 2 | 20 | 4 | 2 | 2 | 32 | 5 | 2 | 2 | 95 |
| 0 | 2 | 3 | 9 | 1 | 2 | 3 | 12 | 2 | 2 | 3 | 17 | 3 | 2 | 3 | 24 | 4 | 2 | 3 | 38 | 5 | 2 | 3 | 120 |
| 0 | 2 | 4 | 11 | 1 | 2 | 4 | 15 | 2 | 2 | 4 | 19 | 3 | 2 | 4 | 37 | 4 | 2 | 4 | 44 | 5 | 2 | 4 | 150 |
| 0 | 2 | 5 | 13 | 1 | 2 | 5 | 17 | 2 | 2 | 5 | 22 | 3 | 2 | 5 | 31 | 4 | 2 | 5 | 50 | 5 | 2 | 5 | 180 |
| 0 | 3 | 0 | 6 | 1 | 3 | 0 | 8 | 2 | 3 | 0 | 12 | 3 | 3 | 0 | 17 | 4 | 3 | 0 | 34 | 5 | 3 | 0 | 79 |
| 0 | 3 | 1 | 7 | 1 | 3 | 1 | 10 | 2 | 3 | 1 | 141 | 3 | 3 | 1 | 21 | 4 | 3 | 1 | 40 | 5 | 3 | 1 | 110 |
| 0 | 3 | 2 | 9 | 1 | 3 | 2 | 13 | 2 | 3 | 2 | 17 | 3 | 3 | 2 | 24 | 4 | 3 | 2 | 47 | 5 | 3 | 2 | 140 |
| 0 | 3 | 3 | 11 | 1 | 3 | 3 | 15 | 2 | 3 | 3 | 20 | 3 | 3 | 3 | 28 | 4 | 3 | 3 | 54 | 5 | 3 | 3 | 180 |
| 0 | 3 | 4 | 13 | 1 | 3 | 4 | 17 | 2 | 3 | 4 | 22 | 3 | 3 | 4 | 31 | 4 | 3 | 4 | 62 | 5 | 3 | 4 | 210 |
| 0 | 3 | 5 | 15 | 1 | 3 | 5 | 19 | 2 | 3 | 5 | 25 | 3 | 3 | 5 | 35 | 4 | 3 | 5 | 69 | 5 | 3 | 5 | 250 |
| 0 | 4 | 0 | 8 | 1 | 4 | 0 | 11 | 2 | 4 | 0 | 15 | 3 | 4 | 0 | 21 | 4 | 4 | 0 | 41 | 5 | 4 | 0 | 130 |
| 0 | 4 | 1 | 9 | 1 | 4 | 1 | 13 | 2 | 4 | 1 | 17 | 3 | 4 | 1 | 24 | 4 | 4 | 1 | 48 | 5 | 4 | 1 | 170 |
| 0 | 4 | 2 | 11 | 1 | 4 | 2 | 15 | 2 | 4 | 2 | 20 | 3 | 4 | 2 | 28 | 4 | 4 | 2 | 56 | 5 | 4 | 2 | 220 |
| 0 | 4 | 3 | 13 | 1 | 4 | 3 | 17 | 2 | 4 | 3 | 23 | 3 | 4 | 3 | 32 | 4 | 4 | 3 | 64 | 5 | 4 | 3 | 280 |
| 0 | 4 | 4 | 15 | 1 | 4 | 4 | 19 | 2 | 4 | 4 | 25 | 3 | 4 | 4 | 36 | 4 | 4 | 4 | 72 | 5 | 4 | 4 | 350 |
| 0 | 4 | 5 | 17 | 1 | 4 | 5 | 27 | 2 | 4 | 5 | 28 | 3 | 4 | 5 | 40 | 4 | 4 | 5 | 81 | 5 | 4 | 5 | 430 |
| 0 | 5 | 0 | 9 | 1 | 5 | 0 | 13 | 2 | 5 | 0 | 17 | 3 | 5 | 0 | 25 | 4 | 5 | 0 | 13 | 5 | 5 | 0 | 240 |
| 0 | 5 | 1 | 11 | 1 | 5 | 1 | 15 | 2 | 5 | 1 | 20 | 3 | 5 | 1 | 29 | 4 | 5 | 1 | 17 | 5 | 5 | 1 | 350 |
| 0 | 5 | 2 | 13 | 1 | 5 | 2 | 17 | 2 | 5 | 2 | 23 | 3 | 5 | 2 | 32 | 4 | 5 | 2 | 21 | 5 | 5 | 2 | 540 |
| 0 | 5 | 3 | 15 | 1 | 5 | 3 | 19 | 2 | 5 | 3 | 26 | 3 | 5 | 3 | 37 | 4 | 5 | 3 | 25 | 5 | 5 | 3 | 920 |
| 0 | 5 | 4 | 17 | 1 | 5 | 4 | 22 | 2 | 5 | 4 | 29 | 3 | 5 | 4 | 42 | 4 | 5 | 4 | 30 | 5 | 5 | 4 | 1,600 |
| 0 | 5 | 5 | 19 | 1 | 5 | 5 | 24 | 2 | 5 | 5 | 32 | 3 | 5 | 5 | 45 | 4 | 5 | 5 | 36 | 5 | 5 | 5 | |

*APHA 1971, Standard Methods for the Examination of Water and Wastewater, 13th Edition, 662-677.

## 3.7 Fecal Coliform Enumeration Method

Fecal coliforms are defined as gram-negative, non-spore forming bacilli which are cytochrome oxidase negative and ferment lactose with the production of acid and gas at 44.5° ± 0.2°C within 24 hours.

### 3.7.1 Fecal Coliform MPN Media

**A-1 Broth:**

| | |
|---|---|
| Lactose | 5.0 g |
| Tryptone | 20.0 g |
| NaC1 | 5.0 g |
| Triton X-100 (Polyethylene glycol p-isooctylphenyl ether) | 1.0 mL |
| Salicin | 0.5 g |
| Water, distilled or dechlorinated | 1.0 L |

1. Add Durham tubes to tubes.

2. Double-strength broth (2 x) as above, except for 500 mL distilled water.

3. Heat to dissolve and dispense 10 mL of medium per tube, both single and double strength. Tubes should be of sufficient capacity to contain 1 mL inoculum + 10 mL single-strength broth and 10 mL inoculum + 10 mL 2x broth.

4. pH of medium should be approximately 6.9 after autoclave sterilization, 104 kPa/20 min. (121°).

5. Medium may be stored at least 30 days at 2 to 4° in humidified refrigerator or until colour or volume change is noted.

### 3.7.2 Multiple-tube Fermentation Technique (MPN)

1. Using tubes of A-1 broth containing Durham tubes, inoculate the series of tubes required with the sample and sample dilutions with appropriate sized pipettes.

2. Incubate at either:
   a. 4 hours at 35°C, followed by 18 to 20 hours at 44.5° ± 0.2°C
   b. 22 to 24 hours at 44.5° ± 0.2°C

3. Production of gas is considered a positive reaction for fecal coliforms.

4. Refer to Table 3 and 3.6 to establish MPN fecal coliform count/100 mL water sample.

## 3.8 *Escherichia coli (E. coli)* Enumeration Method

The genus *Escherichia* is composed of motile or non-motile bacteria that conform to the definitions of the family *Enterobacteriacae* and the tribe *Escherichieae* of which *E. coli* is the type species. *E. coli* are gram negative, oxidase negative bacteria which usually ferment lactose rapidly. Some strains utilize it slowly and some fail to ferment this substrate. When lactose is utilized, acid and gas are usually produced with 24 hours at 44.5°C. The enzyme ß-glucoronidase, unique to *E. coli* and some strains of Salmonella and Shigella, will metabolize MUG to produce 4-methylumbelliferone, which fluoresces under long-wave ultra-violet light.

### 3.8.1 *Escherichia coli (E. coli)* MPN Media

**A-1 Broth, plus MUG:**

| | |
|---|---|
| 4-methylumbelliferyl-ß-D-glucuronide (MUG) | 0.05 g |
| Lactose | 5.0 g |
| Tryptone | 20.0 g |
| NaC1 | 5.0 g |
| Triton X-100 (Polyethylene glycol p-isooctylphenyl ether) | 1.0 mL |
| Salicin | 0.5 g |
| Water, distilled or dechlorinated | 1.0 L |

1. Add Durham tubes to tubes, if fecal coliform results are also needed.

2. Double-strength broth (2x) as above, except for 500 mL distilled water

3. Heat to dissolve and dispense 10 mL of medium per tube, both single and double strength. Tubes should be of sufficient capacity to contain 1 mL inoculum + 10 mL single-strength broth and 10 mL inoculum + 10 mL 2x broth.

4. pH of medium should be approximately 6.9 after autoclave sterilization, 104 kPa/20 min. (121°C).

5. Medium may be stored at least 30 days at 2 to 4°C in humidified refrigerator or until colour or volume change is noted.

### 3.8.2 *Escherichia coli (E. coli)* Multiple Tube Fermentation Technique (MPN)

1. Using tubes of A-1 broth with MUG and/or containing Durham tubes, inoculate the series of tubes required with the sample and sample dilutions.

2. Incubate at either:
   a. 4 hours at 35°C, followed by 18 to 20 hours at 44.5°C ± 0.2°C.

   b. 22-24 hours at 44.5°C ± 0.2°C.

3. Production of a blue-green fluorescence under long-wave, ultra-violet light is considered a positive reaction and the tube is considered to contain *E. coli.*

4. Refer to Table 3 and 3.6 to establish MPN E.coli count/100 mL of water sample.

# 4.0 QUALITY ASSURANCE FOR MICROBIOLOGICAL ANALYSES OF WATER

## 4.1 General

Microbiological analyses are unique because they provide the most important data that describe the sanitary quality of water and wastewater, and that determine the public health risk from waterborne disease and toxic substances. Microbiological analyses are specified in various federal, provincial and state laws, and are performed by federal, provincial, state, municipal and private laboratories to:

- enforce discharge limits for municipal and industrial waste effluents
- monitor ambient water quality for recreational, agricultural, industrial and water supply uses
- assure the safety of drinking water
- trace the sources of bacterial pollutants
- evaluate new water resources

Because far-reaching decisions are based on these measurements, the resulting data must be technically sound and legally defensible. Therefore, it is important that strict control be exerted over all facets of microbiological laboratory and field procedures, and that personnel performance is evaluated.

## 4.2 Quality Assurance (QA) Program

All microbiology laboratories carry out some forms of quality control (QC), most of which were derived from principles of controlled experimentation that have evolved from

the development of microbiology as a science. Two types of QC are usually practiced by a competent laboratory:

- intralaboratory QC, the day-to-day checks made on internal operations
- interlaboratory QC, maintenance of minimal standards established among a group of participating laboratories

To establish an acceptable quality assurance (QA) program, the laboratory must incorporate these practices into a formal, documented plan and follow it continuously.

A QA program is the integration of intralaboratory and interlaboratory QC, standardized methods, written QA guidelines, standard reference materials, training courses and on-site laboratory evaluations into a formal, coordinated effort to ensure valid analytical results. The accuracy and reliability of analytical results cannot be guaranteed if the standard operating procedures of the laboratory do not include an effective, ongoing QA program. The QA program should be an integral part of the laboratory routine, but it must be practical and require a reasonable amount of time or it will be circumvented. As a general rule, about 15% of the laboratory effort is required for adequate QA practices. Compliance with a full QA program may be more difficult in small water laboratories that analyze few samples, operate on low budgets, have less opportunity for training and may experience a more rapid turnover of personnel.

## 4.3 Intralaboratory Quality (QC) Control

To establish a QC program that includes all factors that could influence the results, the microbiology laboratory should prepare and follow a written policy that consists of the basic elements outlined in this section.

In addition to the intralaboratory QC program described, laboratories should take part in available interlaboratory QC programs established to maintain minimal standards. Such a program includes QC activities that cannot be carried out by a single laboratory and require multiple laboratory participation.

*The U.S. EPA Certification Program for Laboratories Analyzing Drinking Water* is an example of a national interlaboratory QC program established to guarantee valid data from drinking water analyses. It consists of the following:

1. Formal collaborative method studies to establish the interlaboratory precision of selected methodology.

2. Specific criteria and standards for technical procedures and performance of

analysts. The criteria and procedures are described in the manual for the drinking water certification program.

3. Annual on-site inspections to verify that the established test procedures are followed.

4. Annual performance evaluation of analytical capabilities, using unknown samples.

5. Follow-up on problems detected in on-site inspection and performance evaluations.

## 4.4 Laboratory Personnel

The laboratory personnel must have adequate training and experience. As a minimum, the analyst should have a high school diploma, 30 days on-the-job training, and supervision or available consultation from an experience microbiologist. The supervisor should have a bachelor's degree in microbiology or a closely related field, at least two weeks of technical training in aquatic microbiology and one year of bench experience in sanitary microbiology.

## 4.5 Laboratory Facilities

The facilities should provide conditions for optimal operation of equipment, including good ventilation, freedom from dust and drafts, low humidity and temperature control. To minimize the potential for contamination, the design of the facility should limit through-traffic. Ideally, the glassware washing, media preparation and sterilization activities should be separated from the analytical laboratory. The laboratory must be kept clean. Walls should be covered with waterproof paint, enamel, tile or other surface material that provides a smooth finish which is easily cleaned or disinfected. Bench tops should be of stainless steel, epoxy plastic or other smooth impervious material which is inert and corrosion-resistant. Floors should be covered with good quality tiles or other similar heavy-duty material. A regular cleaning schedule should be maintained.

## 4.6 Laboratory Operations

Control must be maintained over all daily activities, from sample collection to data reporting.

- **Sample Collection**. The production of valid data begins with the collection of

representative samples, followed by sample analysis as soon as possible. Ideally, samples should be immediately iced or refrigerated after collection, and analyzed within six hours. However, drinking water samples may be held up to 30 hours at melting ice temperature if the collector cannot deliver them directly to the laboratory.

- **General Laboratory Practices**. Laboratory QC practices include proper operating, servicing and monitoring of autoclaves, regular production and testing of laboratory pure water, and checking of the effectiveness of glassware washing. The correct time and temperature for autoclave sterilization of frequently used materials and the recommended specifications for laboratory pure water are given in Tables 2 and 3, respectively.

**TABLE 2. Autoclave Sterilization Temperature and Time**

| MATERIAL | TEMPERATURE AND TIME |
|---|---|
| Membrane filters and pads | 121°C for 10 minutes |
| Carbohydrate-containing media (lauryl tryptose, BGB broth, etc.) | 121°C for 12 to 15 minutes |
| Contaminated materials and discarded cultures | 121°C for 30 minutes |
| Membrane filter assemblies (wrapped), sample collection bottles (empty) | 121°C for 15 minutes |
| Dilution water (99 mL in screw-cap bottles) | 121°C for 15 minutes |
| Rinse water volumes of 500 to 1,000 mL | 121°C for 30 minutes |
| Rinse water in excess of one litre | 121°C with time adjusted for volume; check for sterility |

## TABLE 3. Test Frequency and Criteria for Laboratory Pure Water Used in Microbiological Procedures

| TEST | MONITORING FREQUENCY | LIMIT |
|---|---|---|
| ROUTINE TESTS | | |
| Conductivity | Continuously or with each use | > 0.5 mΩ resistance or < 2 S/cm at 25° |
| pH | With each use | 5.5 to 7.5 |
| Standard Plate Count | Monthly | < 1,000 bacteria/mL |
| Water Use Test | Monthly and for a new source | calculated t-value < 2.78 |

*Table 3, continued*

| FOLLOWUP TESTS WHEN PROBLEMS ARE INDICATED | | |
|---|---|---|
| Total Organic, carbon, heavy metals, individually (Cd, Cr, Cu, Ni, Pb, Cn) | | < 1.0 mg/L<br>not > 0.5 mg/L |
| Heavy metals, total (Cd, Cr, Cu, Ni, Pb, Zn) | | equal to or < 1.0 mg/L |
| Ammonia/organic nitrogen, total chlorine residual, water suitability test | | < 0.1 mg/L<br>< detection limit ratio: 0.8 to 3.0 |

- **Glassware**. Glassware especially screw-capped dilution bottles and flasks, should be examined at each time of use for chipped or broken edges, and etched surfaces and discarded if necessary. After washing, the glassware should be carefully inspected; if water beads excessively on the cleaned surfaces, rewash. Each lot of cleaned glassware should be tested for acid or alkaline residues with a pH check. Glassware should be checked annually for detergent residue, and with changes in glassware-washing compounds.

- **Microbiological Culture Supplies**. To produce reliable data, microbiological supplies must be of high quality and should be tested for acceptability. Particular attention should be given to media, reagents, membrane filters and absorbent pads.

- **Media**. Cultural methods depend on correctly prepared media. The best available materials and techniques in media preparation, storage and application should be used. Commercially prepared media are preferable

whenever available, but may vary in quality among manufacturers, and even from lot-to-lot from the same manufacturer because many media components are prepared from natural sources. There may be variations in the peptones, bile derivatives, yeast and meat extracts, and other materials. Not more than a one-year supply of media should be ordered at one time.

Because storage conditions, such as temperature, light and moisture differ among laboratories, it is impossible to establish an absolute shelf-life for media in unopened bottles. A conservative estimate is two years at room temperature. Opened bottles of media should be used within six months after opening. The analyst should record type, amount, appearance, lot number, and dates received and opened. Media that are caked, discoloured, or show other deterioration must be discarded. After preparation, the media should be examined for unusual colour, darkening or precipitation, the pH should be checked and the observations recorded. Variations in sterilization time and temperature may cause such problems. If any of these occur, the medium must be discarded. Manufacturer's instructions for sterilization of specific media and materials should be followed.

A record should be maintained in a bound book of each batch of prepared medium with a name of preparer and date, name and lot number of medium, amount of medium weighed, volume of medium prepared, sterilization time and temperature, pH measurements and adjustments, and preparations of labile components. Sterile media should be prepared in amounts that will be used within the holding time limits given in Table 4.

**TABLE 4. Suggested Maximum Storage Times for Some Prepared Media**

| MEDIUM | HOLDING TIME LIMIT |
|---|---|
| ♦ Agar or broth in loose-cap tubes at $4^0$ | 4 weeks |
| ♦ Poured agar plates with loose-fitting cover in Sealed plastic bags at $4^0$ | 3 months |
| ♦ Large volume of agar in tightly closed, screw-Cap flask or bottle at $4^0$C | 12 months |

- **Reagents and Dyes**. Reagents used in microbiological analyses must be of high quality. Use only chemicals of American Chemical Society (ACS) or equivalent grade, because impurities can inhibit bacterial growth, provide nutrients, or fail to produce the desired reaction. Date chemicals and reagents when received and when first opened for use. Because microbiological dyes must be of proper strength and stability to produce correct reactions, use only those certified by the Biological Stain Commission.

- **Membrane Filters and Absorbent Pads**. Recommended specifications for membrane filters and absorbent pads are listed in Table 5. The quality and performance of membrane filters vary with the manufacturer, type, brand, and lot number. These variations result from differences in raw materials, manufacturing methods, quality control and storage conditions. The filters should be 0.45 µm pore size. The grid lines of the filters must be nontoxic, and filters and absorbent pads must be resistant to degradation by sterilization at 121°C for 10 minutes.

**TABLE 5. Specifications for Membrane Filters and Absorbent Pads.**

| PROPERTY | SPECIFICATION |
|---|---|
| MEMBRANE FILTER (MF) | |
| Diffusibility | Laboratory pure water should diffuse uniformly through the filters in 15 s, and the filters should have no dry spots when floated on water |
| Flow Rate | Flow rate should be at least 55 mL/min/cm$^2$ and a differential pressure of 70 cm mercury |
| Retention | Filters should retain at 100 mL suspension of *Serratia marcescens* containing 1 x 10$^3$ cells/mL |
| Inhibition | Filters should not inhibit growth of the last organism. When five aliquots of the same sample are analyzed by the MF and spread plate method, using the same agar medium, the arithmetic mean of the MF counts should be at least 90% of the arithmetic mean of the plate count |
| Extractables | Water extractables from an MF should not exceed 2.5% after boiling in 100 mL laboratory-pure water for 20 minutes drying, cooling and bringing to constant weight |
| Toxicity | Filters should be nontoxic and free of bacterial growth-inhibiting or growth stimulating substances |

| | |
|---|---|
| Sterility | Growth should not result when an MF is placed on a pad saturated with tryptone glucose extract (TGE) broth or TGE agar and incubated at 35°C for 24 hours |
| ABSORBENT PADS | |
| Absorptivity | 2.0 ± 0.2 mL Endo broth |
| Acidity | Pads should release less than 1 mg total acidity calculated as $CaCO_3$ when titrated to the phenolphthalein endpoint with 0.02 N NaOH |

- **Equipment**. Equipment, such as incubators, waterbaths, hot air ovens, refrigerators, freezers, balances, pH meters, ion exchange units, and stills must be routinely monitored and services. Table 6 gives monitoring procedures for major equipment items.

**TABLE 6. Monitoring Laboratory Equipment**

| EQUIPMENT | MONITORING PROCEDURE |
|---|---|
| Incubator (air/water-jacket) | • Check twice daily. If partially submersible glass thermometer is used, bulb and stem must be immersed in liquid/water to the mark on stem.<br>• Measure temperature twice daily on top and bottom shelves. Periodically, measure temperature on all shelves in use. |
| Incubator, *continued* | • Expand monitoring locations proportionately for walk-in incubators.<br>• Recording thermometer and alarm system are recommended. |
| Water bath | • Check and record temperature daily. Bath must maintain the uniform temperature needed for the test in use.<br>• Maintain accurate thermometer completely immersed in water bath.<br>• A recording thermometer and alarm system are recommended.<br>• Clean monthly |
| **Table 6 continued on next page.** | |

| | |
|---|---|
| Autoclave | • Record temperature and pressure for each run. Recording thermometer recommended.<br>• Verify that autoclave maintains uniform operating temperature.<br>• Check operating temperature with a minimum/maximum thermometer on a weekly basis.<br>• Test performance with spore strips or suspensions weekly. If evidence of contamination occurs, check until the cause is identified and eliminated.<br>• Procure semi-annual preventive maintenance inspections. |
| Hot air oven | • Test performance with spore strips or suspensions quarterly.<br>• Equip and monitor sterilization with a thermometer accurate in the 160 to 180°C range. |
| Refrigerator at 4°C | • Check and record temperature daily.<br>• Clean monthly.<br>• Require identification and dating of all material.<br>• Defrost unit and discard outdated materials in refrigerator and freezer compartment every three months. |
| Freezers | • Check temperature and record daily.<br>• Use of recording thermometer and alarm system recommended. Require identification and dating of all materials.<br>• Clean and defrost freezer every six months. Discard outdated materials. |
| Balance | • An analytical balance with a sensitivity of 1 mg or less at 10 g load should be used for weighing 2 g or less. For larger quantities, a balance with accuracy of 50 mg at 150 g load should be used.<br>• Check balance monthly with a set of certified Class S weights or weights of equivalent accuracy. Wipe balance and weights clean after each use. Protect weights from laboratory atmosphere and corrosion. Contract with a qualified expert for balance maintenance on an annual basis. |
| pH Meter | • Compensate for temperature with each use.<br>• Date standard buffer solutions when first opened and check monthly with another pH meter. Discard the buffer solution if the pH is more than ± 0.1 pH unit from the manufacturer's stated value or if it is contaminated with microorganisms. |
| pH Meter, cont'd. | • Standardize with at least two standard buffers (pH 4.0, pH 7.0, or pH 10.0) before each use.<br>• Do not re-use buffer solution.<br>• Have meters inspected at least yearly as part of maintenance contract. |

| Table 6 continued. | |
|---|---|
| Water Deionizer | • Monitor water for conductance daily. Monitor trace metals and other toxic or nutritive compounds annually.<br>• Replace cartridges as indicated by manufacturer or as indicated by analytical results.<br>• Monitor bacterial counts at exit point of unit. Replace the cartridges when standard plate count exceeds 500/mL. |
| Water Still | • Drain and clean monthly according to instructions from the manufacturer.<br>• Drain and clean distilled water reservoir quarterly.<br>• Check distilled water continuously or daily using a conductance meter.<br>• Conduct chemical tests on water to detect toxicity or stimulation effect annually.<br>• Conduct standard plate counts monthly on stored water and clean out reservoir if count > 500 mL. |

- **Data Reporting**. A careful check should be made to verify that computations were made correctly, results were transcribed accurately from the bench sheet to the report, and report forms were initialled by the analyst. If practical, a second person should check the computations and results recorded for the tests. If an information storage and retrieval system is used, data on the printouts should be double-checked.

## 4.7 Analytical Quality Control

In addition to the routine checks on laboratory operations, the analytical procedures must be controlled, including assurance of the sterility of test materials, verification of membrane filter (MF) colonies, completion of most probable number (MPN) tests, performance of duplication analyses and the use of positive and negative controls.

- **Sterility**. For MF tests, the sterility of media, membranes, dilution and rinse water, glassware and filtering apparatus is checked for each series of samples, using sterile water as the sample. In the MPN procedure, tube media, dilution water and glassware are checked for sterility by confirming that growth does not occur in sterility-control samples.

- **Verification of Membrane Filter Counts**. Periodically, typical colonies growing on the MF should be verified. When a new study or a different series of samples is initiated, it is recommended that at least ten colonies used in computing the final density are picked into fermentation or biochemical media and tested to verify that they are the target organisms. Verification is performed at least monthly.

- **Completion of Most Probable Number Tests**. At least 10% of positive samples should be carried through the completed tests to assure that target organism has been measured.

- **Duplicate Analyses.** Duplicate analyses should be performed on at least 10% of the samples and on at least one sample from each run. In laboratories with more than one analyst, each should conduct a similar number of parallel analyses.

- **Positive and Negative Controls**. Known positive and negative cultures are inoculated into each lot of test medium to make certain that the media perform correctly for the organism under test. Table 7 (next page) gives examples of test cultures for the commonly used parameters.

## 4.8 Procedures Manual

A manual of in-house laboratory procedures is basic to the operation of a QA program. The manual is a written protocol developed by the microbiology and toxicology staff that includes detailed descriptions of the analytical methods and the QA program used in the laboratory. A copy of the manual is provided to each analyst and his responsibilities carefully reviewed.

**TABLE 7. Control Cultures For Standard Microbiological Tests**

| PARAMETER | CONTROL CULTURE | |
|---|---|---|
| | POSITIVE | NEGATIVE |
| Total Coliforms | *Escherichia coli*<br>*Enterobacter aerogenes* | *Staphylococcus aureus*<br>*Pseudomonas sp.* |
| Fecal Coliforms | *E. coli* | *Escherichia aerogenes*<br>*Streptococcus faecalis* |
| Fecal streptococci | *S. faecalis* | *Staphylococcus aureus*<br>*E. coli*<br>*Streptococcus mutans* |

## 4.9 Quality Control Records

Operational QC checks such as those on laboratory pure water, media sterilization and pH, autoclave time and temperature, incubator temperature and analytical QC tests,

such as colony verification, sterility checks and MPN confirmation must be thoroughly documented. These records are held for three years and should be readily available for internal use and on-site inspection.

## 5.0 APPARATUS/SUPPLIES REQUIRED

- ☐ Autoclave
- ☐ Broths, Double-strength and single-strength, specific for tests
- ☐ Cooling container(s)
- ☐ Dilution blanks, 90 mL and 9.0 mL
- ☐ Durham tubes, 16 x 150 mm and 20 x 150 mm test tubes
- ☐ Graduated (sterile) cylinders, 100 mL, and 1,000 mL
- ☐ Incubators appropriate
- ☐ Marking pencil wax and permanent marking pen
- ☐ Pipettes, 1 mL and 10 mL sterile, cotton-plugged
- ☐ Refrigerator
- ☐ Sample collection bottles, 250 mL
- ☐ Sample testing bottles: 20 mL, 100 mL, 200 m
- ☐ Water deionizer or still (not essential
- ☐ Triple beam balance or other scale
- ☐ Hot plate or heat source
- ☐ Beakers, 400 mL, 2,000 mL
- ☐ Metal baskets for test tubes, test tube racks
- ☐ Test tube cleaning brushes
- ☐ Paper towels

## 6.0 CHEMICALS

- ☐ Absolute ethanol ..... mL
- ☐ Bacteriological peptone ..... g
- ☐ Bacto beef extract ..... g
- ☐ Brom cresol purple ..... g
- ☐ Dipotassium hydrogen phosphate ($K_2HPO_4$) ..... g
- ☐ Distilled, deionized water or boiled tap water ..... mL
- ☐ Ferric ammonium citrate ..... g
- ☐ Lactose ..... g
- ☐ MUG (methylumberlliferyl-β-D-Glucuronide) ..... g
- ☐ NaCl ..... g
- ☐ Peptone ..... g
- ☐ Potassium phosphate dibasic ..... g
- ☐ Potassium phosphate monobasic ..... g
- ☐ Salicin ..... g

- ☐ Sodium chloride ..... g
- ☐ Sodium lauryl sulphate ..... g
- ☐ Sodium thiosulphate ($Na_2S_2O_3$) ..... g
- ☐ Teepol ..... mL
- ☐ Triton X-100 (Polyethylene glycol p-isooctylphenyl ether) ..... mL
- ☐ Tryptone (pancreatic digest of casein) or equivalent ..... g

# 7.0 GENERA AND SPECIES OF ENTEROBACTERIACEAE

| GENUS | SPECIES | COMMENTS |
|---|---|---|
| *Budvicia** | *aquatica* | Found in drinking water and surface water. Isolated from human feces. |
| *Buttiauxella* | *agrestis* | Found in water, not associated with human disease |
| *Cedecea** | *davisae*<br>*lavagei*<br>*neteri*<br>(others) | Isolated from humans, primary respiratory tract and wounds |
| *Citrobacter* | *amalonaticus*<br>*diversus*<br>*freundii*<br>(others) | Isolated from humans, wound infections, urine, sepsis; fecal flora. Also insulated from environment. |
| *Edwardsiella** | *hoshinae*<br>*ictaluri*<br>*tarda*<br>(others) | Isolated from humans and animals; associated with diarrhea, wound infections, sepsis. |
| *Citrobacter** | *amalonaticus*<br>*diversus*<br>*freundii*<br>(others) | Isolated from humans, wound infections, urine, sepsis; fecal flora. Also isolated from environment |

| | | |
|---|---|---|
| *Enterobacter* | *aerogenes** *agglomerans** *amnigenus** *asburiae* *cloacoe** *dissolvens* *gergoviae** *intermedium* *nimipressuralis* *sakazakii** *taylorae** (others) | Very common, norman fecal flora; isolated from wounds, respiratory tract, urine, blood. *E. agglomerans* also in animals, others found in environment. *F. cloacae* most clinincally significant. *F. agglomerans* and *E. sakazakii* yellow-pigmented. |
| *Erwinia* | amylovora (others) | Associated with diseases of plants. |
| *Escherichia* | *blattae* *coli** *fergusonii** *hermannii** *vulneris** | Isolated from humans and animals. *E. coli* most common human pathogen. *E. Blattae* found only in cockroaches. *E. hermannii* and *E. vulneris* isolated from wounds. Others normal stool flora. |
| *Hafnia** | *alvei* (others) | Isolated from humans, respiratory tract, other sources. No environmental source known. |
| *Klebsiella* | *oxytoca** *ozaenae** *planticola** *pneumoniae** *rhinoscleromatis** *terrigena* | Normal stool flora, some strains found in environment. Isolated from respiratory tract, urine, wounds, blood. *K. terrigena* found only in water. |
| *Kluyvera** | *ascorbata* *cryocrescens* | Probably normal stool flora. Isolated from human infections, respiratory tract, blood, urine. |
| *Koserella* | *trabulsii* | Isolated from human feces, wounds, respiratory tract, knee joint. (Synonym for *Yokenella regensburgei*.) |
| *Leclercia** | *adecarboxylata* | Isolated from humans, respiratory tract, blood, urine, wounds; also found in food and water. |
| *Leminorella** | *grimontii* *richardii* | Isolated from human feces. $H_2S$ positive, not known to be pathogenic for humans |

| | | |
|---|---|---|
| *Moellerella** | *wisconsinsis* | Isolated from human stool and natural water. Not known to be pathogenic for humans. Resistant to colistin. |
| *Morganella** | *morganii* (others) | Normal stool flora. Isolated from human infections (urine, blood, others). |
| *Obsesumbacterium* | *proteus* | Isolated from brewery yeast. Not known to be pathogenic for humans. |
| *Proteus* | *mirabilis** *myzofaciens* *penneri** *vulgaris* | Most species normal fecal flora. Isolated from human infections, urine, wounds, blood, others. *P. Myxofaciens* isolated from moths only. *P. Penneri* resembles *P. Vulgaris*, including spreading growth, but is in dole-negative and chloramphenicol-resistant. |
| *Providencia* | *alcalifaciens** *heimbachae* *rettgeri** *rustigiannii** *stuartii** | Most species are normal fecal flora. Isolated from human infections, urine, wounds, blood. *P. rustigianii* not known to be pathogenic for humans |
| *Rhanella** | *aquatilis* | Isolated from water. Only one human isolate reported. Not known to be pathogenic for humans. |
| *Salmonella** | *(enterica)* >2,000 serovars | Widely distributed in humans and animals. Cause of gastroenteritis and enteric fever. Genus *Arizona* now included in *Salmonella.* Nomenclature still undecided. |
| *Serrattia** | *ficaria* *"fonticola"* *liquefaciens* *marcescens* *odorifera* *plymuthica* *rubidaea* (others) | Isolated from humans, water, rarely animals. Some species important human pathogens. Isolated from respiratory tracks, wounds, blood, urine, others. *S. ficaria, odoriferia, phymuthica, rubidaea* not known to be human pathogens. *S. fonticola* probably not a true member of *Serratia* genus. |
| *Shigella** | *hoydii* *dysenteriae* *flexneri* *sonnei* | Human reservoir only, not normal flora. Cause of gastroenteritis and bacteria dysentery. Genetically identical to *E. coli.* |

| | | |
|---|---|---|
| *Tatumella** | *ptyseos* | Isolated from humans, respiratory tract, urine, blood. Unlike other *Enterobacteriaceae, Tatumella* has polar flagella; susceptible to penicillin; poor grower, better at 25°C. |
| *Xenorhabdus* | *luminescens nematophilus* | Isolated from nematodes only. Grow only at 25°C, not at 35°C. No human isolates. |
| *Yersinia** | *aldovae enterocolitica frederiksenii intermedia kristensenii pestis pseudotuberculosis ruckeri* | Isolated from humans, animals, environment. Some important pathogens. Agents of plague, gastroenteritis, other infections. Isolated from stool, blood, urine, wounds. *Y. ruckerii* not found in humans, cause of "red mouth" disease of fish. *Y. ruckeri* probably not true member of genus *Yersinia.* |

Bailey and Scott's Diagnostic Microbiology, 8th Edition, 1990

# 8.0 DRINKING WATER GUIDELINES

**TABLE 8. World Health Organization (WHO) Guidelines Values for Bacteriological Drinking-Water Quality (1986)**

| | Number per 100 mL | |
|---|---|---|
| | Fecal coliforms | Coliform Organisms |
| Piped water supplies: | | |
| 1. Treated water entering the distribution system | 0 | 0 |
| 2. Untreated water entering the distribution system | 0 | 0 ( in 95% of samples)<br>3 (occasionally) |
| 3. Water in the distribution system | 0 | 0 ( in 95% of samples)<br>3 (occasionally) |
| Unpiped water supplied | 0 | 10 (occasionally) |
| Bottled drinking water | 0 | 0 |
| Emergency Water supplies | 0 | 0 |

Application of the WHO Guidelines is promoted by the World Health Organization in a parallel two-track approach: (1) development, adoption and implementation of suitable drinking-water quality standards in member states; and (2) establishment of appropriate programs for the control of drinking-water quality, particularly in rural areas. The development of standards is meaningful primarily in those countries which have already built or are committed to building the necessary infrastructure which guarantees implementation and enforcement. In predominantly rural areas, however, where adequate and safe drinking-water surfaces are still lacking, prevention, surveillance and control programs with heavy reliance on community involvement would be more appropriate.

Suitability of the WHO Guidelines was field-tested in pilot projects in countries such as Indonesia, Peru, Tanzania, Vanuatu and Zambia. Surveillance programs were based on two complementary activities, sanitary inspection and bacteriological testing for fecal coliforms. The latter results showed rather common violations of the strict WHO guideline values (Table 8) and an alternative classification system with class limits of 0, 10 and 50 fecal coliforms per 100 mL, was introduced. The pilot projects also demonstrated that the health risks estimate from sanitary surveys corresponded rather well with the results of the bacteriological field analyses.

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## VILLAGE WATER SAMPLING RECORD

**Appendix 1.**

| Date | Sample No. | Source | $H_2S$ 10 mL | | | $H_2S$ 1 mL | | |
|---|---|---|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | Day 3 | Day 1 | Day 2 | Day 3 |
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## COLIPLATE CONFIRMATION OF $H_2S$ PAPER STRIP TEST RESULTS

**Appendix 2.**

| Date | Sample No. | Source | | | Coliplate Results | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $H_2S$ positive results | | Coliforms | | | E. coli | | |
| | | | Volume | # Days | # + wells | # - wells | MPN | # + wells | # - wells | MPN |
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