REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Determination of total coliforms in sea water by the multiple test tube (MPN) method

Reference Methods For Marine Pollution Studies No. 21 (Rev.1)

Prepared in co-operation with

WHO

UNEP 1995

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources, and has requested the development of regional action plans. The Regional Seas Programme at present includes 12 regions and has over 140 coastal states participating in it (1), (2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity, and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies is being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared by, or in cooperation with, the relevant specialized bodies of the United Nations system as well as other organizations, and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions, the users are invited to convey their comments and suggestions to:

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GREECE

which is responsible for the development and preparation of microbiological and other health-related Reference Methods.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1, UNEP, 1982.

(2) P. HULM: A strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme for comprehensive support for regional and global marine pollution assessments. UNEP, 1990.

This revised issue of Reference Methods for Marine Pollution Studies No. 21 was prepared by the World Health Organization on the basis of a review of the Method during expert meetings and comments from individual scientists who tested the Method. The assistance of all those who contributed to this revised issue of the Reference Method is gratefully acknowledged.

CONTENTS

1. INTRODUCTION

The original version of this recommended method was prepared by the World Health Organization within the framework of the Long-term Programme of Pollution Monitoring and Research in the Mediterranean Sea (MED POL Phase II) and issued by the United Nations Environment Programme as Reference Method for Marine Pollution Studies No. 21 within UNEP's Regional Seas Programme Activity Centre's series.

The method is essentially based on already-existing recognized techniques, and also drawn on the experience of microbiologists in a number of Mediterranean laboratories. In the description, the style used by the International Organization for Standardization (ISO) is followed as closely as possible. While designed primarily with conditions prevailing within the Mediterranean Sea in mind, the method is also, to variable extents, suitable for other similar ecological regions.

The present version of this method incorporates a number of amendments, based on reviews during expert consultation meetings organized by WHO, and on comments received from Mediterranean laboratories using the method within the framework of their national or local marine pollution monitoring programme.

2. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of total coliforms in coastal bathing waters of temperate and tropical seas and is designed to be used in sanitary surveillance of bathing beaches.

It is based on the Multiple-Tube Fermentation (MPN) Test and can be employed as an alternative to the Membrane Filtration Culture Method. Whether the Membrane Filtration (MF) Culture Method is preferred to the MPN Test depends on local conditions and personal preferences. In general the MF method is less labour intensive and due to the preconcentration of the bacteria in the sample it is more suitable in situation where low numbers of coliforms are to be estimated. The MPN test should be given preference when the test sample contains high amounts of particulate matter which will hinder the reading of the MF's after incubation.

Since total coliforms die within hours when exposed to sunlight in seawater at temperatures above + 4° C, their presence in seawater indicates only recent contamination by faecal material and by soil in stormwater runoff. Die-away rates (T-90) depend on salinity, temperature, solar radiation, etc. and must be taken into consideration when interpreting the results.

3. DEFINITION

Total coliforms are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas at 35° C within 48 hours.

4. PRINCIPLES

Quantitation of total coliforms in marine (seawater) samples is accomplished by testing multiple sample portions in a lactose based medium for gas production at 35° C within 48 hours. Positive findings in the presumptive test phase are confirmed in a second medium for verification of coliform occurrence.

Five sample portions for each multiple tube dilution (10 ml, 1 ml, 0.1 ml, etc.) are inoculated into individual tubes of lactose broth. After 24 hours incubation, all lactose cultures are examined for gas production. Those that have visible evidence of gas production are confirmed by transferring a loop-full of culture from each positive tube to individual tubes of MacConkey broth or brilliant green broth and incubating at 35 ± 0.5° C. Presumptive tubes showing no gas production are re-incubated for an additional 24 hours, then inspected for gas production. Any new positive cultures are confirmed as previously described and incubated at 35 ± 0.5° C. All 48 hour negative presumptive tube results are recorded, then discarded. All confirmation cultures are inspected at 24 hours and 48 hours for gas production. These results are also recorded and the combination of confirmed positive reactions (gas development in brilliant green or gas + acid development in MacConkey broth at 35° C) in the different dilutions within 48 hours is used for the calculation of the most probable number (MPN) of total coliforms in the water sample.

5. APPARATUS AND GLASSWARE

5.1 Sample bottles of borosilicate glass or autoclavable plastic bottles with screw-cap tops, 200-250 ml capacity, wide-mouthed and with ground-glass stoppers are used to collect marine (seawater) samples.

5.2 Sample rod of non-corrosive material with a clamp to hold the sampling bottle (figure 1).

5.3 Subsurface sampler of the type shown in figure 2, or similar, complete with plastic rope and weight.

5.4 Thermo-insulated plastic container with prefrozen packs of chemical gel (camping equipment) for storage of samples.

5.5 Thermometer, 0 to 50° C, precision ± 1° C, to be used for checking temperature in sample collection containers (5.4). Thermometer, 0° to 50° C, precision ± 0.5° C, to be used for checking temperature during incubation.

5.6 Air or water incubator adjusted to 35 ± 0.5° C with suitable test tube racks for incubating multiple tube cultures (5.15).

5.7 Autoclave, maximum 2 atm., electric or gas.

5.8 Drying oven for sterilization of glass sample bottles and pipettes at 160° C for 2 hours.

5.9 pH meter, precision ± 0.1 pH units.

5.10 Balance for media preparation, precision ± 1 mg.

5.11 Refrigerator with temperature range of 2-8° C for storage of samples to be processed within 24 hours of collection.

5.12 Vibrator (shaker) for mixing sample aliquots in serial dilution (optional).

5.13 Ehrlenmeyer flasks of borosilicate glass for media preparation, capacity 1 and 2 litres.

5.14 Borosicilate glass bacteriological culture tubes with sufficient capacity to receive 20 ml of medium.

5.15 Small borosilicate glass tubes 6 x 50 mm ("Durham vials") to be inserted in culture tubes (5.15) for detection of gas formation in the multiple test tube.

5.16 Total volume (blow-out) borosilicate glass pipettes of 1, 9 and 10 ml capacity with stainless steel containers for sterilization.

Note: 9 ml capacity pipettes are optional.

5.17 Graduated borosilicate glass cylinders of 100, 500 and 1000 ml capacity with glass beakers, aluminium foil or wrapping paper for cover.

5.18 Bacteriological transfer loops made from 22-24 Chromel gauge, nichrome or platinum-iridium. Diameter of the loop: 3 mm.

5.19 Heavy wrapping paper or aluminium f%wl wrap.

5.20 Test tube racks, rust resistant, with capacity for 15 or 25 test tubes (preferably).

6. CULTURE MEDIA, REAGENTS 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 Preparation of Materials, Media, Reagents

All glassware and apparatus (5) should be washed with non-toxic detergent (6.9), followed by a thorough (complete) rinse with hot tap water and then rinsed at least three times with distilled water (6.7).

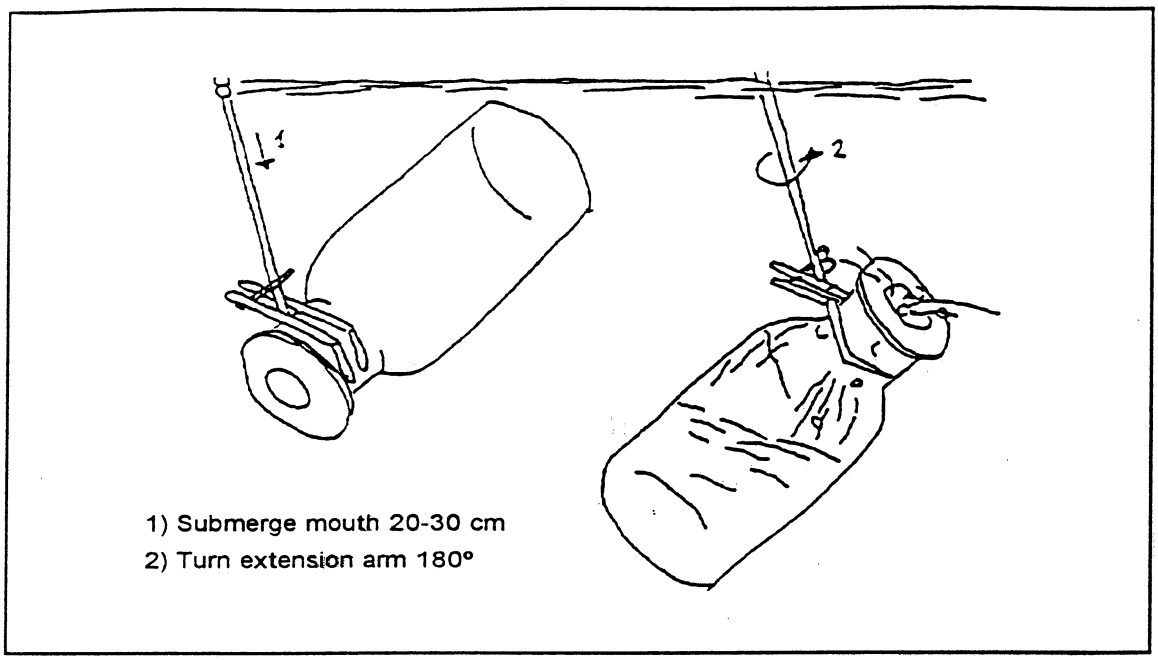

Figure 1. Subsurface sampling with extension arm.

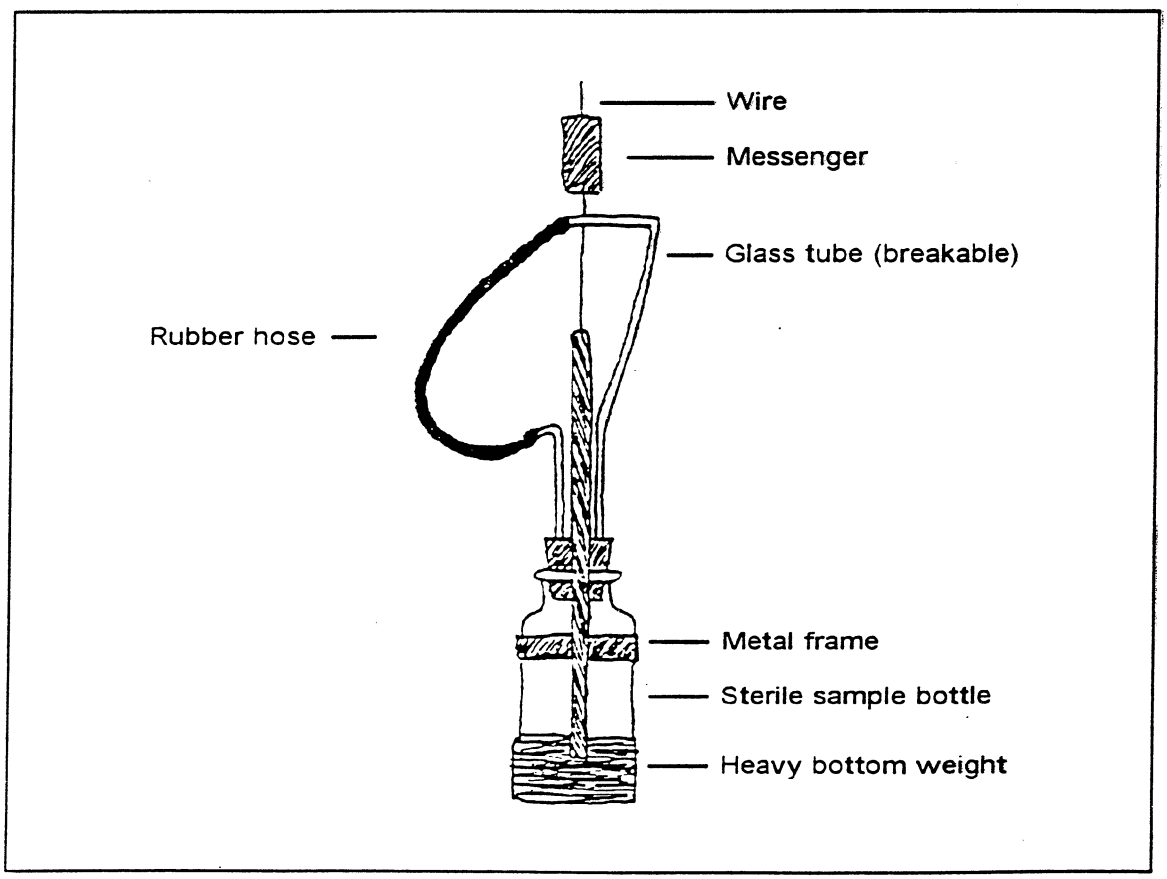

Figure 2. Sampler for sterile subsurface sampling.

6.2 Sterilization of Glassware and Equipment

6.2.1 Surface sample bottles (5.1). Clean all sample bottles as described under (6.1) and dry. Plastic sample bottles can only be sterilized in the autoclave (5.7) (121° C for 15 minutes). Be sure screw-cap tops are loose fitting during sterilization to avoid bottle collapse due to steam pressure in autoclaving. Sterilize glass sample bottles preferably in a drying oven (5.8) for two hours at 160° C. Before sterilization, place a small piece of filter paper in the neck of the bottle to prevent the ground glass stopper from sticking after cooling. The paper insert should be left in place until time of sample collection. At that time, discard the paper insert by flipping it out of bottle. Do not insert it into the sample or remove with fingers because of chance contamination. Put bottles into detergent-cleaned thermo-insulated boxes (5.4). Separate the bottles from each other with clean wrapping paper (5.19) to avoid breakage.

Note: If residual chlorine is suspected in the water sample, add 0.1 ml of a 10 per cent sodium thiosulphate solution (6.8) for each 100 ml of sample to the contents of the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

6.2.2 Subsurface sampler (5.3). Clean the subsurface sampler as described under 6.1, rinse with tap and distilled water (6.7). Enclose each sampler in heavy wrapping paper (5.19) and sterilize in an autoclave (5.7) for 15 minutes at 120° C.

6.2.3 Pipettes (5.16). Clean pipettes as described under 6.1 then insert a cotton plug in the mouthpiece and place into suitable stainless steel containers prior to sterilization in a drying oven (5.8) for two hours at 160° C.

6.3 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	1.0 litre	1.0 litre

Preparation: Dissolve ingredients in distilled water (6.7). The pH of single strength broth should be between 6.8 and 7.0, but preferably 6.9 after sterilization (5.8). Double strength broth pH should be 6.7 ± 0.1.

Place 5 clean culture tubes (5.14) in each of 3 rows of a test tube rack (more in case the expected MPN of total coliforms is high). Then add inverted vials (5.15) to all culture tubes (5.14) and dispense 10 ml of medium into the culture tubes or sufficient amount so that the inverted vials (Durham vials) are at least partially covered after then entrapped air in these vials has been driven out during autoclaving. Use double strength lactose broth in the first row of these culture tubes. Into the second and third rows (and if necessary into successive tows) transfer single strength broth. Close all

prepared tubes with metal caps or cotton plugs. Autoclave (5.7) the prepared media tubes at 121° C preferably for 12 minutes but not to exceed 15 minutes. Check tubes of sterile medium for a final pH of 6.9 ± 0.1 after autoclaving. Test a sample of the sterilized medium for performance using control stock cultures (6.11). If the non-lactose fermenting control culture produces gas in 24 hours at 35° C, medium has been overheated in autoclaving and should be discarded.

Note: Double strength broth must be used in the first row when 10 ml of sample is added to the culture tubes. If sample portions to be tested are greater than 10 ml, prepare medium concentration such that after sample addition, the medium will approximate 1 x (normal) strength.

6.4 MacConkey Broth

Sodium taurocholate	5.0 g
Lactose	10.0 g
NaCl	5.0 g
Peptone	20.0 g
Distilled water	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 (6.4.2) to each litre of prepared MacConkey broth.

Place 5 clean culture tubes in each of 3 rows of a test tube rack. Then add inverted vials (5.15) 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. Close all prepared tubes with metal caps or cotton plugs. Autoclave the prepared medium in culture tubes at 121° C, preferably for 12 minutes, but not exceeding 15 minutes.

After sterilization, cool the broth as quickly as possible. Final pH of single strength medium should be 7.2 ± 0.2 after autoclaving. Test a sample of the sterilized medium for performance using control stock cultures (6.11). If the non-lactose fermenting control culture produces gas in 24 hours at 35° C, the medium has been overheated in autoclaving and should be discarded.

6.4.2 Bromo-cresol Purple Solution

Preparation: Dissolve 1 g of bromo-cresol purple in 99 ml of 95% pure ethanol (6.10).

6.5 Brilliant Green Bile Broth

Oxgall, dehydrated	20.0 g
Lactose	10.0 g
Peptone	10.0 g
Brilliant green	0.0133 g
Distilled water	1.0 litre

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

Place 5 clean culture tubes in each of 3 rows of a test tube rack. Then add inverted vials (5.15) to all culture tubes and dispense 10 ml of medium into the culture tubes, or sufficient amount, so that the inverted vials are at least partially covered after the entrapped air in these vials has been driven out during autoclaving. Close the tubes with metal caps or cotton plugs. Sterilize by autoclaving at 121° C, preferably for 12 minutes, but not exceeding 15 minutes. After sterilization, cool the broth as quickly as possible. Final pH should be 7.2 ± 0.2. Test a sample of the finished product for performance using control stock cultures (6.11). If the non-lactose fermenting control culture produces gas in 24 hours at 35° C, medium has been overheated in autoclaving and should be discarded.

6.6 Phosphate Buffered Water

Preparation: To prepare stock phosphate buffer solution, dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4), in 500 ml distilled water, adjust to pH 7.2 ± 0.5 with 1N sodium hydroxide (NaOH), and dilute to 1 litre with distilled water.

Add 1.25 ml stock phosphate buffer solution and 5.0 ml magnesium chloride solution (38 g anhydrous $MgCl_2l^{-1}$ distilled water) to 1 litre distilled water. Dispense in amounts that will provide 99 ± 2.0 ml or 9 ± 0.2 ml after autoclaving for 15 minutes. Final pH should be 7.2 ± 0.2. If the anhydrous salt is not available use 81.8 $MgCl_2.6H_2O$ per 1 litre of distilled water.

Peptone water (alternative dilution water): Prepare a 10% stock solution of peptone in distilled water. Dilute a measured volume to provide a final 0.1% working solution. Dispense in amounts to provide 99 ± 2.0 ml or 9 ± 0.2 ml after autoclaving for 15 minutes. Final pH should be 6.8.

Do not suspend bacteria in any dilution water for more than 30 minutes at room temperature because death or multiplication may occur.

6.7 Distilled Water

Use only water distilled in all-glass or all-quartz distillation apparatus or reverse osmosis (RO) water. De-ionized water is also acceptable if produced in apparatus that does not release toxic substances.

Note: Commercially available distilled water is often produced in copper and zinc apparatus and is highly toxic for coliforms.

6.8 Thiosulphate Solution

Prepare a 10 per cent sodium thiosulphate ($Na_2S_2O_3$) stock solution. To a 120 ml sample bottle, add 0.1 ml of the 10% solution of $Na_2S_2O_3$ before sample bottle sterilization.

6.9 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*.

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

6.10 95 per cent Pure Ethanol (not denatured)

6.11 Stock Cultures for Quality Control

Stock cultures of *E. coli* for positive control (lactose fermentation) and *Proteus inirabilis* or a *Salmonella* strain for negative control (glucose fermentation).

7. SAMPLING

Details of a sampling plan are provided in Part I of these guidelines.

7.1 Sampling of Surface Water

Label sample bottle at collection site indicating the sampling station, time of sampling and other factors relevant to the interpretation of the results. Attach clean sterilized sample bottle (6.2.1) to the clean sampling rod (5.2). Immediately before submerging the sample bottle, remove the ground glass stopper from the bottle without touching the inner surface of the bottle and stopper or screw cap. Immerse the bottle from the bow of the boat or from the windward side while the boat is moving forward slowly. Push the bottle with the sampling rod 25 cm under the water surface film, then turn the sample bottle upwards and take the sample (figure 1). The sterilized sample bottle may also be filled directly by hand. Position bottle with neck pointing slightly upward and opening directed away from the hand. Plunge the bottle below the water surface with a sweeping motion against the current.

Retrieve the bottle and discard some water, if necessary, so that some air space remains in the closed bottle. This space is needed for homogenizing the water sample at the receiving laboratory. Replace the glass stopper or screw cap and store the samples in the clean, thermo-insulated sample bottle container (5.4) with prefrozen packs of chemical gel at about 4° C, avoiding exposure to more than + 10° C. Keep samples in the dark. Separate bottles from each other with clean wrapping paper (5.19) to avoid breakage.

7.2 Sampling of Subsurface Water

Label sample bottle at collection site indicating the sampling station, time of sampling and other factors relevant to the interpretation of the results. Lower the sterilized subsurface sampler (6.2.2) after attaching it to a clean plastic rope, without letting the weight disturb the bottom sediments (figure 2). Release the messenger and after one minute retrieve the sampler and store it in a thermo-insulated sample collection container (5.4). Proceed as for sampling of surface water (7.1).

Note: It is known that the die-away rate of total coliforms at ambient temperature in the presence of light is very high. Therefore, all efforts should be made so as not to collect more samples than can be analyzed and incubated the same day. If this is not possible, the samples should be stored at 4O° C and analyzed not later than 24 hours after sampling.

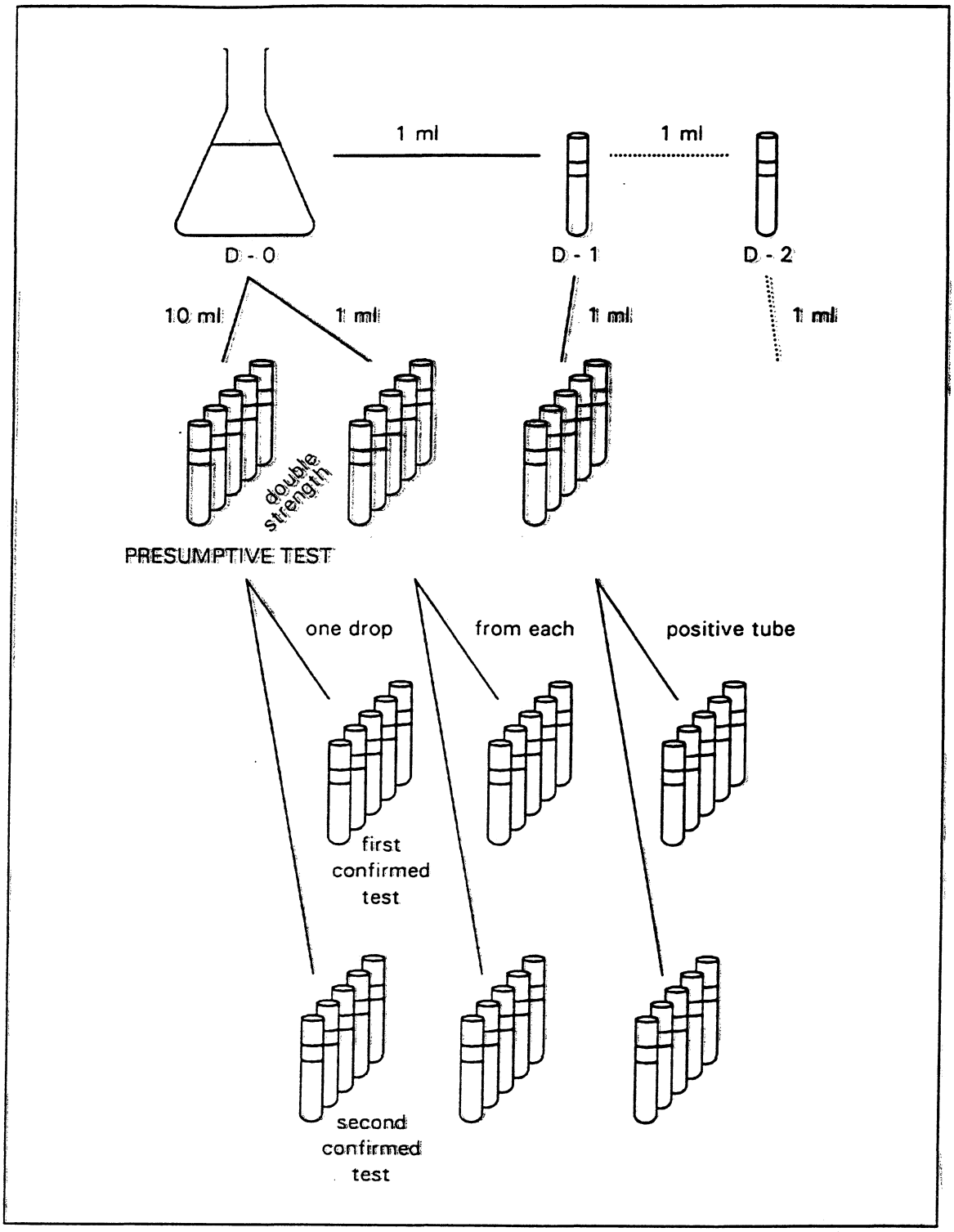

Figure 3. Scheme of preparation of dilution series.

8. TEST PROCEDURE

8.1 Selection of Sample Size and Dilution Series

Select a dilution series for each sample that will ensure positive tubes in the lowest dilution row and negative tubes in the higher dilution rows. If previous experience for planning the dilution series for clean seawater is not available, transfer 10 ml of the seawater to the first row containing 5 tubes of double strength lactose broth, 1 ml to the second row of 5 tubes and 0.1 ml to the third row of 5 tubes. All rows after the first row (if 10 ml sample portions are used) should contain only single strength lactose broth. In polluted seawater the dilution should be extended to 5 dilution rows (the first receiving 10 ml from the seawater sample into double strength lactose) or 5 dilution rows of single strength lactose broth into which the seawater sample is transferred after the appropriate serial (10 fold) dilutions (figure 3).

8.2 Incubation in Lactose Broth at 35 + 0.5° C for 48 hours (Presumptive test)

Using sterile pipettes (6.2.3) transfer 10 ml portions of the water sample (7) into each of 5 sterilized culture tubes containing double strength lactose broth (6.3).

Next transfer with a sterile pipette (6.2.3) 1 ml sample portions (7) into 5 sterilized culture tubes containing single strength lactose broth (6.3).

For preparing further dilutions shake sample again then transfer with a sterile pipette (6.2.3) 1 ml of water sample (7) into a test tube containing 9 ml of phosphate buffer (6.6) (dilution D-1). Mix vigorously by hand or optionally with vibrator (5.12). Transfer aseptically (6.2.3), using a sterile pipette, 1 ml of D-1 into each of the 5 culture tubes containing 10 ml single strength lactose broth (6.3).

If necessary, for further dilutions transfer 1 ml of the D-1 dilution into a test tube containing 9 ml of phosphate buffer (6.6) to obtain dilution D-2, 1 ml of dilution D-2 into 9 ml of phosphate buffer (6.6) to obtain dilution D-3, etc. Shake each succeeding dilute aliquot prior to transferring dilute sample portions to culture tubes containing single strength lactose broth.

Incubate the series of lactose broth culture tubes in an incubator (5.6) at 35 + 0.5° C for 48 hours.

After 24 hours check for gas formation. 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 will 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 3, item 9, under (1a).

After 48 hours check again for gas formation in the same tubes and record the results in table 3, item 9, under (1b) (test report).

8.3 Incubation in MacConkey or Brilliant Green Bile Broth at 35° C for 24 hours (Confirmed test)

Three row lactose broth presumptive test: after incubation for 24 hours in lactose broth at 35° C (8.2) transfer with flame sterilized transfer loop (5.18) one loop-full from each lactose culture tube that provided a positive reading into the test tubes situated in identical positions on the tube rack containing MacConkey (6.4) or brilliant green bile broth (6.5).

Five row lactose broth presumptive test: after incubation for 24 hours in lactose broth at 35° C (8.2) select the highest dilution in which the row of 5 tubes gives positive readings in all 5 tubes (no negative readings should exist in lower dilutions) and all positive tubes in succeeding higher dilutions, then transfer with flame sterilized transfer loop (5.18) one loop-full from each lactose culture tube that provided a positive reading into test tubes situated in identical positions on the tube rack containing MacConkey (6.4) or brilliant green bile broth (6.5).

Incubate all confirmation cultures (MacConkey or brilliant green bile broth) at 35 + 0.5° C in an incubator (5.6) for 24 hours.

Repeat this confirmation procedure with all other culture tubes containing lactose broth which have become positive in the 24 - 48 hours interval.

At the same time, record the number of tubes showing positive reactions in table 3, item 9, under (2a) (test report). Coliforms will ferment lactose in either confirmatory medium, producing gas which is trapped in the inverted vials and if MacConkey broth is used, acid produced in fermentation will turn the violet-like colour of the medium to yellow.

After another 24 hours record the number of tubes showing positive reactions in table 3, item 9, under (2b) (test report).

9. EXPRESSION OF RESULTS

9.1 Calculation of Total Coliforms per 100 ml Seawater Sample

Based on the data in the test report (10) showing positive results in various serial dilutions, calculate the MPN (Most Probable Numbers) value using table 2.

If sample portions of 10 ml, 1 ml and 0.1 ml per test tube have been used, take the number of recorded positive readings from the 24-48 hour incubation in MacConkey or brilliant green bile broth at 35° C (8.3) i.e. the highest readings from either 2a or 2b in table 3, item 9, and find the corresponding most probable number (MPN) from table 2. Enter this value in the test report (table 3, item 10).

When more than three dilutions are employed, the results of only three of these are used in computing the MPN. For MPN calculation purposes, select the highest dilution that has all tubes positive and the next two following dilutions that will have some negative tubes.

Occasionally, positive plates may be encountered beyond the three serial dilutions. In those cases, and for purposes of calculating the MPN, include such additional results in the third row. These skip combinations of positive plates should occur with a frequency lower than 1% of the MPN tests performed. Higher frequencies would suggest laboratory error in pipetting sample dilutions. Select from either 2a or 2b in Table 3, item 9, the dilution in which the row of five tubes gives positive readings in all 5 tubes (no negative readings should exist in lower dilutions) and the two next succeeding higher dilutions. Find the MPN (Table 2) corresponding to the number of positive tubes in these three dilutions and adjust the value by multiples of 10 depending on the starting dilution below 10 ml.

Table 1 illustrates an example of the numbers of positive test obtained with a series of 5 consecutive dilutions: 5-5-3-2-1. For MPN calculations the highest dilution selected would be that using 1 ml of sample. The final combination of positive plates reported would be 5-3-3, instead of 5-3-2. The MPN value corresponding to the 5-3-3 combination would be 170 total coliforms/100 ml (Table 2), and not the MPN value of 140 total coliforms/100 ml associated to the 5-3-2 combination. The MPN density thus obtained should be adjusted for dilution by multiplying for as many powers of 10 as dilutions were performed with the first plate series considered below 10 ml. For example, a MPN value of 5-3-3 would be expressed as 170 total coliforms/100 ml when the starting dilution is 10 ml, but it would be counted as 1700 total coliforms/100 ml when the starting dilution, is 1 ml, and 17000 total coliforms/100 ml when the largest sample volume used was 0.1 ml.

Table 1. Example for computing the density of total coliforms in a water sample analyzed by the MPN method.

Sample portion ml	Positive tubes
10	5
1	5 (X)
0.1	3 (X)
0.01	2 (X)
0.001	1

dilution factor: = 10

MPN (5-3-3) = 170 x 10 = 1700 total coliforms/100 ml

95% confidence limits:
lower: 80 x 10 = 800 total coliforms/100 ml
higher: 410 x 10 = 4100 total coliforms/100 ml

9.2 Precision of Results

Select the 95 per cent confidence limits for the MPN selected in (9.1) from table 2 and enter them in the test report (table 3, item 10).

Table 2. MPN index and 95% confidence limits for various combinations 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			MPN Index per 100 ml	95% Confidence Limits	
5 of 10 ml each	5 of 1 ml each	5 of 0.1 ml each		Lower	Upper
0	0	0	<2	–	–
0	0	1	2	1.0	10
0	1	0	2	1.0	10
0	2	0	4	1.0	13
1	0	0	2	1.0	11
1	0	1	4	1.0	15
1	1	0	4	1.0	15
1	1	1	6	2.0	18
1	2	0	6	2.0	18
2	0	0	4	1.0	17
2	0	1	7	2.0	20
2	1	0	7	2.0	21
2	1	1	9	3.0	24
2	2	0	9	3.0	25
2	3	0	12	5.0	29
3	0	0	8	3.0	24
3	0	1	11	4.0	29
3	1	0	11	4.0	29
3	1	1	14	6.0	35
3	2	0	14	6.0	35
3	2	1	17	7.0	40
3	3	0	17	5.0	46
4	0	0	13	5.0	38
4	0	1	17	7.0	45
4	1	0	17	7.0	46
4	1	1	21	9.0	55
4	1	2	26	12	63
4	2	0	22	9.0	56
4	2	1	26	12	65
4	3	0	27	12	67
4	3	1	33	15	77
4	4	0	34	16	80
5	0	0	23	9.0	86
5	0	1	30	10	110
5	0	2	40	20	140
5	1	0	30	10	120
5	1	1	50	20	150
5	1	2	60	30	180
5	2	0	50	20	170
5	2	1	70	30	210
5	2	2	90	40	250
5	3	0	80	30	250
5	3	1	110	40	300
5	3	2	140	60	360
5	3	3	170	80	410
5	4	0	130	50	390
5	4	1	170	70	480
5	4	2	220	100	580
5	4	3	280	120	690
5	4	4	350	160	820
5	5	0	240	100	940
5	5	1	300	100	1300
5	5	2	500	200	2000
5	5	3	900	300	2900
5	5	4	1600	600	5300
5	5	5	≥1600	--	--

10. TEST REPORT

Table 3. Total coliforms in seawater samples.

1. Sampling area
country: _____
area: _____

2. Sampling point (station)

code number: _____
longitude: _____
latitude: _____

3. Time of sampling hour: ___.___ day: _____ month: ____ year: ____

4. Sampling and environment conditions
Sampling depth: _____ Container number: _____
Temperature at sampling depth: _____
Salinity at sampling depth: _____ Duration of storage: _____
(other factors which may influence the results should be reported under 11)

5. Time of inoculation hour: ___.___ day: ___/___/___
6. Start of incubation hour: ___.___ day: ___/___/___
7. End of incubation hour: ___.___ day: ___/___/___
8. Confirmatory test MacConkey: _____ Brilliant green: _____

9. Number of positive reactions at 35.0° C

Volume in ml transferred	Lactose 24h (1a)	Lactose 48h (1b)	MacConkey/BG 24h (2a)	MacConkey/BG 48h (2b)
10	____	____	____	____
1	____	____	____	____
0.1	____	____	____	____
0.01	____	____	____	____
0.001	____	____	____	____
0.0001	____	____	____	____

10. Test results:

10.1 MacConkey broth:
_____ T. colif./100 ml
95% confidence limits:
_____;_____ T. colif./100 ml

10.2 Brilliant Green:
_____ T. colif./100 ml
95% confidence limits:
_____;_____ T. colif./100 ml

11. Anomalies observed in the test procedure:
_____
_____
_____

12. Full address of the institution which carried out the analysis:
_____
_____
_____
_____

13. Name(s) and signature(s) of the person(s) who carried out the analysis:
_____
_____
_____
Date: _____

11. REFERENCES

APHA (1985) Standard methods for the examination of water and waste water. American Public Health Association, Washington, D.C. (16th edition).

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