REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Determination of feacal coliforms in sediments by the pour plate (PP) method

Reference Methods For Marine Pollution Studies No. 47 (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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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. 47 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

This recommended method has been 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). It is also being issued separately within UNEP's Regional Seas Programme Activity Centre's Reference Methods for Marine Pollution Studies 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 faecal coliforms in sediments of coastal bathing waters of temperate and tropical seas and is designed to be used in sanitary surveillance of bathing beaches and shellfish growing areas.

This method allows colonies determination, as the Membrane Filtration Culture Method, but the sample volume cannot be larger than 1 ml because the culture medium must be able to absorb the volume of the material inoculated and solidify without difficulty.

Faecal coliforms exhibit a highly specific positive correlation with faecal contamination from warm-blooded animals and, therefore, are good indicators for the sanitary quality of coastal waters and marine sediments. Since faecal coliforms die within hours when exposed to sunlight in sea water, their presence in the sea indicates only recent contamination by faecal material. Die-away rates (T-90) depend, among other parameter, on salinity, temperature and solar radiation, factors that must be taken into consideration when interpreting the results.

3. DEFINITION

Faecal coliforms are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing gas in less than 24 hours at 44.5 °C. They produce indole in peptone water containing tryptophane at 44.5 °C. Under the culture conditions described in this reference method, faecal coliforms appear as blue colonies.

4. PRINCIPLES

From sediment samples taken in sterile conditions, a dilution series is set up made according to the quantity of faecal coliforms expected in the sediment sample. A 1-ml aliquot volume of this dilution series are transferred to empty Petri dishes (5.16). Then m-FC agar (6.1) is poured in each Petri dish. Once the agar has solidified, the Petri dishes are incubated at 44.5 ± 0.2 °C for 24 hours. Lactose fermentation will turn faecal coliform colonies into their characteristic blue colour.

Suspect and doubtful colonies can be tested for acid and gas development with a confirmative test using the MacConkey broth or the Brilliant Green broth. In urban bathing areas where sewer outlets discharge rain water as well as wastewater, the confirmative test for indole production is necessary.

5. APPARATUS AND GLASSWARE

5.1 Sterile plastic bags (maximum capacity 1 litre).

5.2 Core sampling cylinders, metallic or borosilicate glass, of approximately 2-cm diameter and 15-cm length.

5.3 Borosilicate glass bottles, 250-ml capacity, to store prepared m-FC agar. These bottles must be sterilized before receiving the agar.

5.4 Thermo-insulated plastic boxes (camping equipment), with prefrozen cooling packs or similar cooling units, for transport and storage of sediment samples.

5.5 Thermometer, 0 to 50 °C, precision ± 1 °C, preferable of unbreakable plastic type, to be used for checking the temperature in plastic boxes.

5.6 Stainless steel homogenizer or blender with several blender vessels, sterilizable in a drying oven (5.10) or in an autoclave (5.9).

5.7 Water incubator for 44.5 ± 0.2 °C.

5.8 Stereoscopic microscope, magnification of 10 - 50 X, and/or darkfield colony counter.

5.9 Autoclave, maximum 2 atmospheres, electric or gas.

5.10 Drying oven for sterilization at 160 °C

5.11 pH meter, precision ± 0,1 pH units.

5.12 Sterile Pasteur pipettes, completed with cotton plugs.

5.13 Analytical balance, precision ± 1 mg.

5.14 Refrigerator, 4 ± 0.5 °C.

5.15 Electric vibrator (shaker) for mixing liquids in dilution tubes.

5.16 Borosilicate glass Petri dishes, diameter 8 to 9 cm, with stainless steel containers for sterilization, or plastic disposable pre-sterilized Petri dishes.

5.17 Erlenmeyer flasks of borosilicate glass for media preparation (capacity 1 and 2 litres), or media conservation (250 ml).

5.18 Borosilicate glass bacteriological culture tubes.

5.19 Total volume (blow-out) borosilicate glass pipettes of 1, 10 and 20 ml capacity, with stainless glass containers for sterilization.

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

5.20 Graduated borosilicate glass cylinders of 100, 500 and 1000 ml capacity with spout.

5.21 Small borosilicate glass vials ("Durham vials"), 6 x 50 mm, to be inserted in some culture tubes.

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

5.23 Heavy wrapping paper.

5.24 Aluminum foil (household quality).

5.25 Autoclavable wide-mouthed plastic bottles, 200 to 300 ml capacity.

5.26 Watertight metal boxes for incubating Petri dishes in a water bath (5.7).

5.27 Filter paper.

5.28 Water incubator for 44.5 ± 0.2 °C.

5.29 Stainless steel forceps.

6. CULTURE MEDIA AND REAGENTS

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 m-FC Agar

6.1.1 Medium

Tryptone	10.0 g
Proteose peptone no. 3	5.0 g

Yeast extract	3.0 g
NaCl	5.0 g
Lactose	12.5 g
Bile salts no. 3	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients of this agar in 1 litre of distilled water (6.7). Heat to boiling point until all ingredients are completely dissolved. If necessary, add 10 ml of 1% solution of rosolic acid (6.1.2), keep boiling for more than one minute and then cool. The final medium should have a pH of 7.4 ± 0.1. If necessary, adjust the pH with diluted analytical grade HCl. A volume of 200 ml of m-FC agar, with or without rosolic acid, is poured in each sterile 250 ml flask (5.3). For immediate use, these flasks are kept in a 50 °C water bath for a maximum of 3-4 hours. For later use, the flasks are kept in a refrigerator (5.14) for a maximum of 1 week.

Note: The addition of rosolic acid is optional and should only be added, if necessary, to suppress excessive growth of non-faecal coliforms.

Note: Do not autoclave this medium.

6.1.2 Rosolic acid

Prepare a small volume of 1% solution of rosolic acid by adding an adequate amount of rosolic acid in 0.2 N NaOH.

Note: The rosolic acid solution (initially in powder form) should be prepared freshly immediately before being added to the agar. The rosolic acid solution should must not be autoclaved.

6.2 MacConkey Broth

6.2.1 Medium

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

Preparation: Dissolve ingredients by shaking. Adjust pH to 7.1 ± 0.1 with diluted HCl and then add the bromo-cresol purple solution (6.2.2). Insert a small inverted Durham vial (5.21) in each clean culture tube (5.18, 8.1) and dispense sufficient medium into the culture tubes so that the inverted vials are at least partially covered after the entrapped air bubble in the vials has been driven out during autoclaving. Close the culture tubes with cotton plugs. Sterilize the culture tubes by autoclaving at 121 °C during 15 minutes.

6.2.2 Bromo-cresol purple solution

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

6.3 Brilliant Green Bile Broth

Oxgall, dehydrated	20.0 g
Lactose	10.0 g
Peptone	10.0 g
Brilliant green	13.3 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by shaking. Introduce a small inverted Durham vial (5.21) in each clean culture tube (5.18, 8.1) and dispense sufficient brilliant green broth into the culture tubes so that the inverted Durham vials are at least partially covered after the entrapped air bubble in the vials has been driven out during autoclaving. Close culture tubes with cotton plugs. Sterilize culture tubes by autoclaving at 121 °C (5.9), preferably for 12 minutes, but not exceeding 15 minutes. Final pH should be 7.2 ± 0.2. Before using this broth, test a sample of the finished product for performance using control stock cultures (6.10).

6.4 Indole Test Solutions

6.4.1 Tryptone water

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

Preparation: Dissolve the ingredients in distilled water (6.7). Dispense 5 ml into each test tube (5.18) and autoclave (5.9) at 121 °C for 15 minutes. Final pH should be 7.0 - 7.4. If necessary, adjust pH with diluted NaOH before sterilization.

6.4.2 Kovac's indole reagent

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

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

6.5 Phosphate Buffer (pH = 7.2)

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

Preparation: Dissolve the ingredients and autoclave (5.9) at 121 °C for 15 minutes

6.6 Commercial Tween 80

6.7 Distilled Water

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

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

6.8 Detergent for Cleaning Glassware and Apparatus

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

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

6.9 95% Ethanol per Analysis

6.10 Stock Cultures of *E.coli*

7. SAMPLING

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

When monitoring marine sediments, the settling characteristics of particles in sewage or in rivers may be of importance because the organisms adhere to or even constitute the settleable particles. The discharge of a river into the coastal zone may significantly influence the marine environment, so that several different monitoring regions must be distinguished, as salinity, temperature, turbidity and other parameters vary greatly among them.

Sediment sampling can normally be carried out only where the bottom consists of relatively soft and homogeneous sands, silts, clays or mud. A rocky bottom should not be sampled by the equipment described below, and a quantitative interpretation of any such results would be most difficult.

Core sampling is the only technique advised for fine sediments (mud). The grab samplers commonly used for other biological sampling seem inappropriate as they immediately mix the sediment surface with deeper sediments. The microbiological precipitate normally concentrates in the sediment surface, which is consequently where sampling should take place.

The Albrechtsen sediment sampler (Figure 1) has been used widely in Scandinavian waters and it is simple to produce as well as to use. The van Donsel-Geldreich bottom sampler (Figure 2) also seems adequate at least for areas where the sea bottom is soft.

In spite of the compatibility of data that can be achieved by using a single sampling device, it should be noted that due to lack of homogeneity in the microbiological density in the sediment, the comparability of results from different samplings will always be questionable. For this reason, the use of devices of different types would jeopardize the comparability of results.

The Albrechtsen sediment sampler, where it can be employed, is consequently recommended for common use. Use 200-300 ml wide-mouth sterile plastic bottles for collecting samples (5.25).

Samples of dry sand from recreational beaches can be collected with a shovel and kept into sterile plastic bags (5.1) or into 200-300 ml sterile plastic bottles (5.25). Approximately 100 g of sand should be collected for each sample.

7.1 Sampling Above Sea Level

7.1.1 Dry or lightly humid sand (recreational beaches)

The easiest way is to collect a sand sample of 100 g from the top 5-cm layer of the beach using a small clean shovel and to keep it into a plastic bag (5.1).

7.1.2 Very humid mud (shellfish growing areas)

Obtain a core sample from the top 5-cm layer using a metal or glass sterile cylinders (5.1). The core sediment sample is kept into a sterile plastic bottle (5.25).

7.2 Sampling Below Sea Level

Sampling procedures are more delicate.

7.2.1 A sediment sample can be obtained, using the technique 7.1.2, by skin-diving.

7.2.2 A sediment sample can be collected using a sediment samples (Albrechtsen or van Donsel-Geldreich) from a boat.

8. TEST PROCEDURE

8.1 Washing of Glassware and Equipment

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

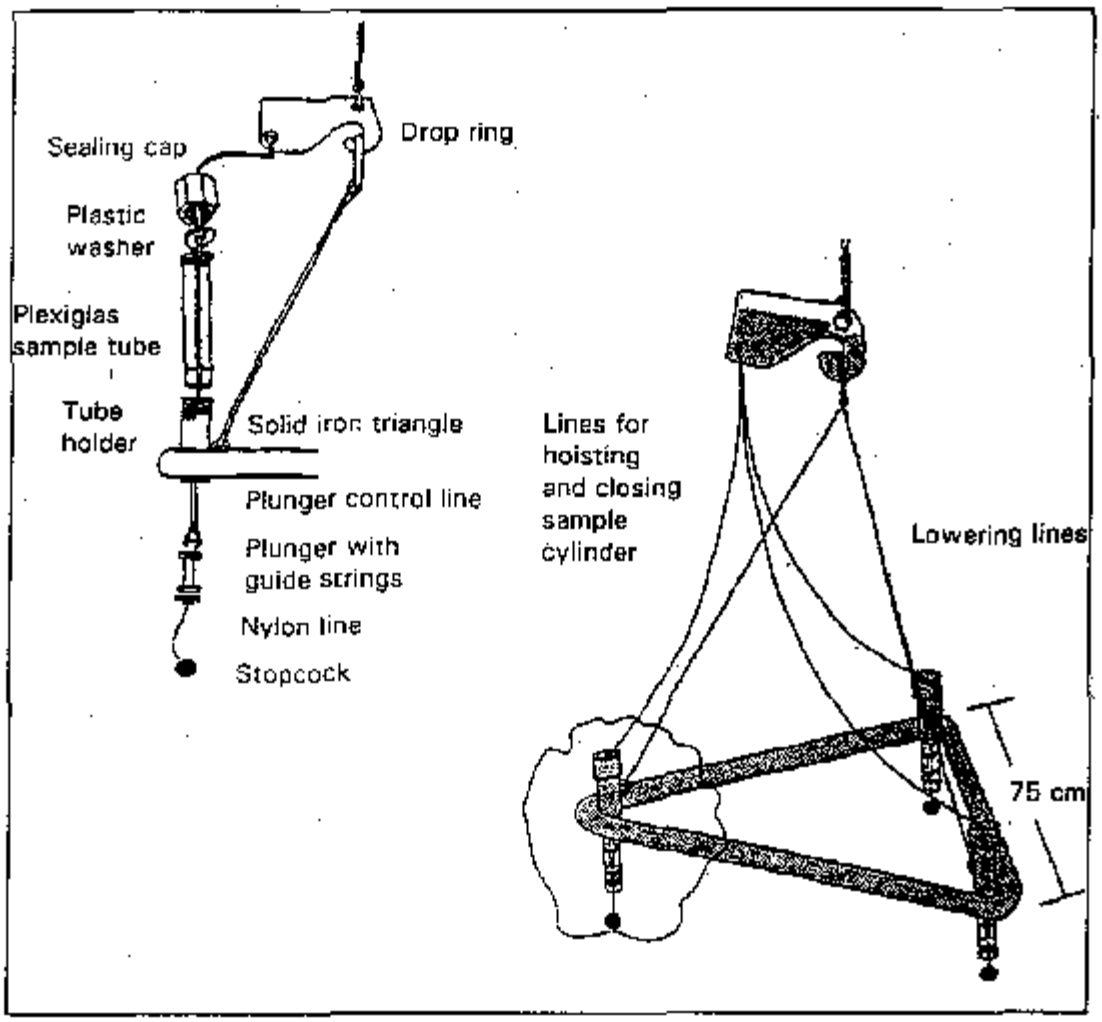

Figure 1. Three-core Albrechtsen bottom sampler.

This device is widely used for bottom sampling in marine waters in Denmark. It can be copied, modified and manufactured locally without permission from the inventor. Only some 50-60 grams of sediment are sampled in one operation, but the area coverage and the specificity as to layers are obvious benefits of its use. It is designed for use only in relatively soft or sandy bottoms. Taken from UNEP/WHO (1988).

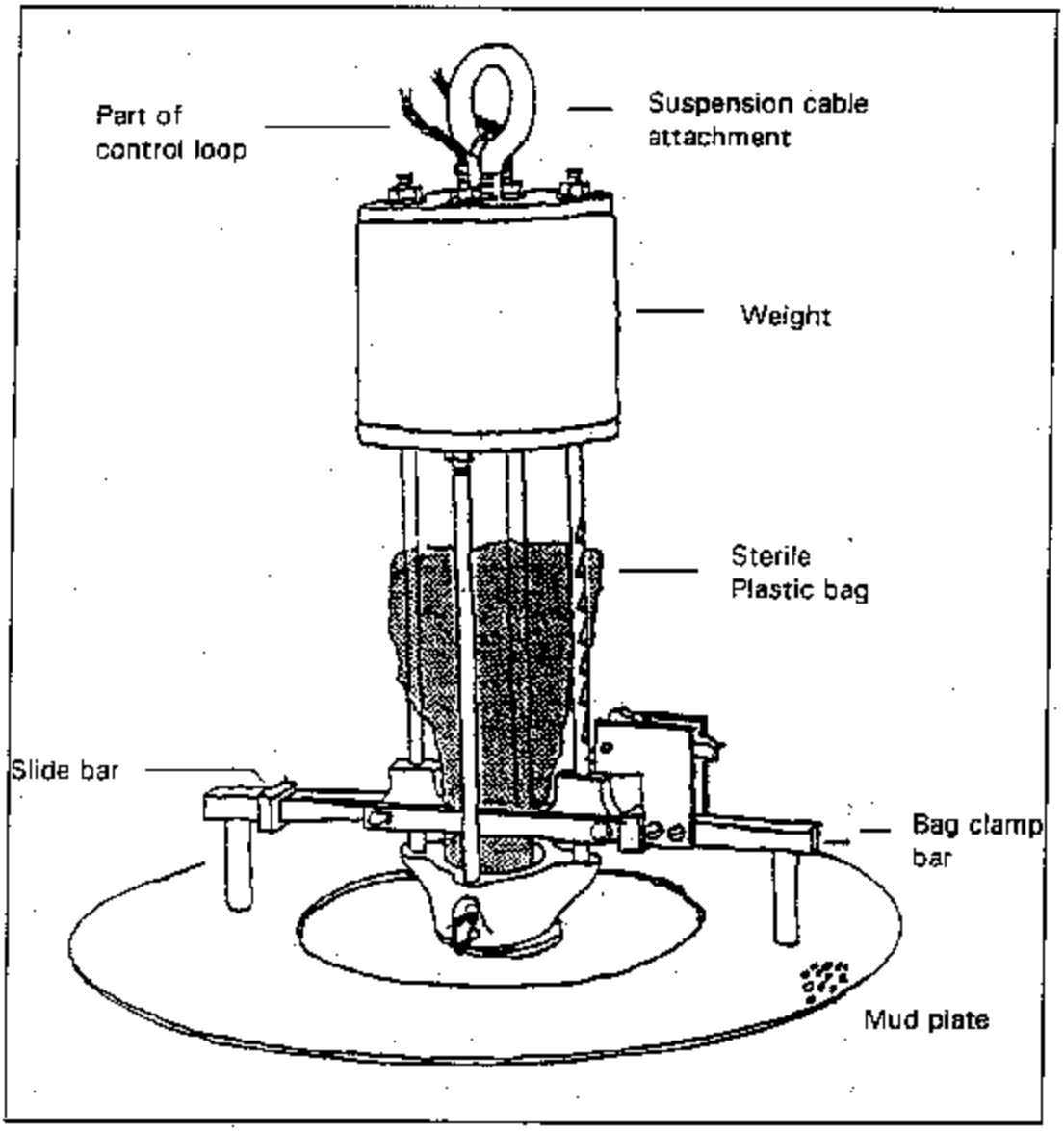

Figure 2. Van Donsel-Geldreich bottom sampler.
Taken from UNEP/WHO (1988).

8.2 Sterilization of Glassware and Equipment

8.2.1 Sampling bags and bottles

- plastic bags (5.1) and sterile plastic bottles (5.25) are disposable items.

- If these items are not available, similar size wide-mouthed glass bottles can be used. Then proceed as follows: clean sample bottles as described under 8.1. Dry and sterilize them in a drying oven (5.10) at 160 °C for 3 hours. Before sterilization, place a small piece of filter paper (5.27) in the neck of the bottle to prevent the glass stoppers from sticking after cooling. After cooling to ambient temperature in the drying oven, remove this filter paper with sterilized forceps (5.29) and fit the ground glass stopper securely into the neck of the bottle. Place the bottles into detergent-cleaned thermo-insulated boxes (5.4). Separate the bottles from each other with clean wrapping paper (5.23) to avoid breakage.

8.2.2 Glass Petri dishes (5.16) and glass pipettes (5.19)

Clean Petri dishes and pipettes, place a cotton plug in their mouth piece and put them into suitable stainless steel containers for sterilization in a drying oven at 160 °C for 3 hours.

Note: The m-FC agar for determination of faecal coliforms should not be sterilized.

8.3 Selection of Sample Size and Dilution Series

Inoculated Petri dishes should ideally present from 20 to 80 colonies after incubation. If previous experience for planning the dilution series for clean sediments is not available, inoculate dilutions D-1 to D-3. For more polluted sediments (as from estuaries and wastewater outfalls) inoculate dilutions D-2 to D-4.

8.4 Preparation of the Dilution Series (see Figure 3)

8.4.1 Preparation of dilution D-1 (stock solution)

Weigh 50 g of sediment and place them into a sterile bottle or flask with 447.5 ml of phosphate buffer (6.5) and 2.5 ml of Tween 80 (6.6). Homogenize the sediment suspension D-1 (stock solution containing 100 g sediment/litre). Use a sterilized laboratory blender (5.6). Homogenization must last for several minutes and must be repeated at least three times. Tween 80 is added to the phosphate buffer to promote the release of bacteria attached to sediment particles.

8.4.2 Preparation of dilutions D-2, D-3 and D-4 (see Figure 3)

These are 1/10 dilutions prepared using phosphate buffer (6.5), but without Tween 80. For each sediment sample, prepare 4-5 tubes each with 9 ml of phosphate buffer and autoclave them. Prepare the dilution series by taking with a sterilized pipette (5.19), after vigorously shaking the sample, 1 ml from the bottle or flask D-1 and transfer this 1 ml into a culture tube containing 9 ml of phosphate buffer to make the D-2 dilution. Agitate the tube on a mixer (5.15) for at least a minute or shake it vigorously

by hand. Repeat agitation three times and then continue the preparation of the dilution series by taking 1 ml from the previous dilution (D-2) and mixing it in a new culture tube containing 9 ml of phosphate buffer in order to obtain the third dilution D-3. Proceed until reaching the chosen dilution level.

8.5 Inoculation Procedure (pour plate method)

During inoculation, or one hour before, liquify the m-FC agar previously prepared by immersing the flask in a water-bath at 55 °C (5.28). A 250 ml flasks should contain approximately 200 ml of agar. The m-FC agar should not be autoclaved. The agar can be kept liquified in a water bath at 48-50 °C for three hours maximum.

Place 9-cm diameter Petri dishes and mark on their back the sample number and the dilution number, using a soft pencil or an alcohol felt pen. Set up 2 Petri dishes for each dilution tube.

Begin inoculation with the highest dilution (for example D-4). With a sterilized pipette (5.12) transfer 1 ml of that dilution to the middle of the corresponding empty Petri dish. (Repeat this operation with a second dish for the same dilution). Using the same pipette, for example of 2-ml capacity, transfer 1 ml of the next smallest dilution bottle, D-3 in this example, into each of the two corresponding Petri dishes. Proceed in the same manner until arriving at the D-1 dilution that should be vigorously shaken again.

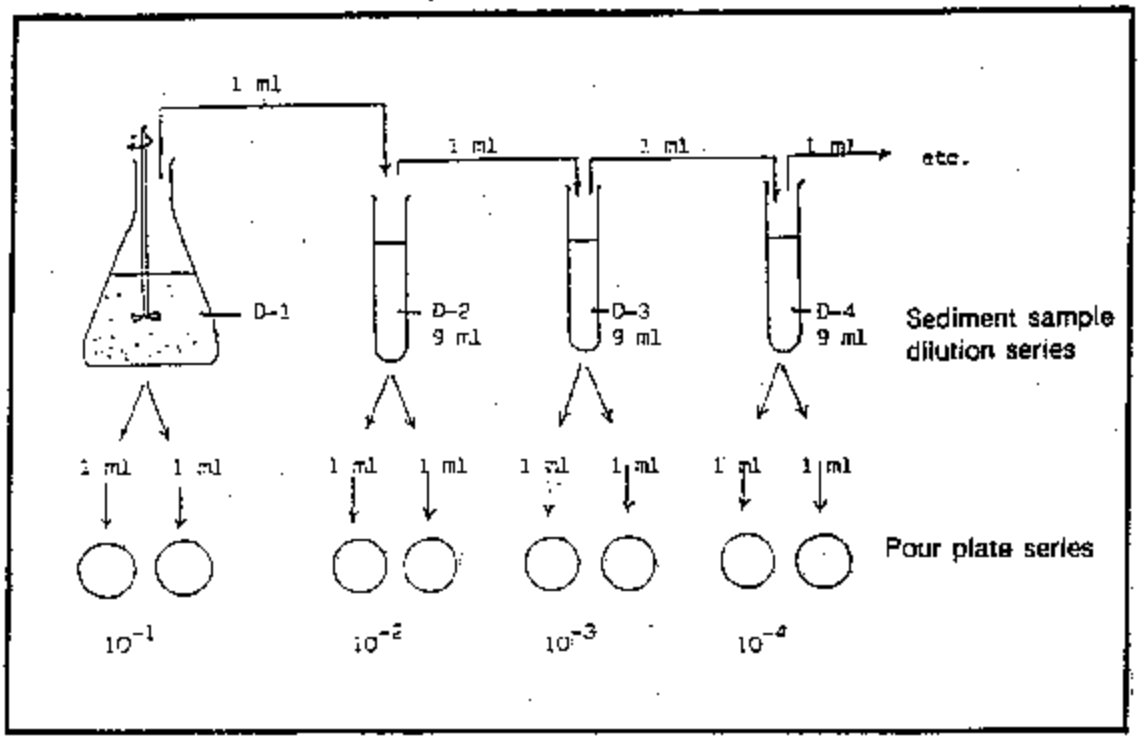

Figure 3. Working diagram for the determination of faecal coliforms

Once the inoculation procedure is complete, each dilution tube should correspond to two Petri dishes, each containing 1 ml of sediment dilution.

Flame the mouth of the flask containing the liquified m-FC agar.

Pour 10 to 14 ml of liquified m-FC agar in each Petri dish while conferring it a circular motion from right to left and then from left to right to ensure a thorough mixing and an even distribution of the inoculum in all the agar volume. Close two thirds of the dish surface with the dish top and let the agar solidify.

8.6 Incubation

Petri dishes are inverted to avoid condensation water forming inside Petri dish lid from running.

Inverted Petri dishes are placed horizontally inside clean metal boxes. These metal boxes (wrapped in tight plastic bags) are then placed in a water bath (5.28) and incubated immediately at 44.5 ± 0.2 °C for 24 hours. As a sterility test, incubate also one blank (without diluted sediment sample), i.e. a Petri dish containing agar only (6.1).

Note: The metal boxes must be suitably weighed to prevent them from floating.

Note: Water-bath incubation of Petri dishes is optional.

8.7 Interpretation

Count with a stereoscopic microscope or similar equipment (5.8) only colonies that appear as blue points and whose size vary from a pin head to a well formed colony. If the number of dubious colonies is higher than 10% of the total numbers of colonies on the Petri dish, test dubious colonies either with the MacConkey broth test (8.9.1) or the Brilliant Green Bile broth test (8.9.2) and confirm them with the indole test (8.9.3).

In urban bathing areas where sewer outlets discharge rain water as well as wastewater, the confirmative test for indole production is necessary (8.9.3).

Note: Colonies produced by faecal coliform bacteria are blue. The non-faecal coliform colonies are grey to cream-coloured. Background colours on the agar plate may vary from a yellowish cream to a faint blue, depending on the age of the rosolic acid. Normally, only few non-faecal coliform colonies will be observed on m-FC agar because of the selective action of elevated temperature, medium salts and the rosolic acid reagent.

Note: Possible precipitate accumulations on the agar should not be counted as colonies.

Note: If Petri dish reading (colony counting) must be suspended, place inverted Petri dishes in a refrigerator (5.14) at 4 °C for a maximum of 24 hours.

8.8 Estimation of Precision

Check the precision of the technique at periodic intervals (at least once every season) by preparing three independent series of dilutions (8.4) using the same sediment sample, i.e. repeating the dilutions steps described in 8.4 (see Figure 3). The sediment sample used should be collected during a routine monitoring programme at a coastal station typical of the area. The dilution series should be selected in such a way so that one dilution step yields three Petri dishes counts which satisfy the 20 to 80 colonies requirements expressed in section 9.1.

Inoculate each Petri dish by transferring the m-FC agar as described in 8.5. Incubate as described in 8.6. Report colony counts following the procedure described in sections 9.1 and 9.2, taking into account the interpretation method presented in section 8.7. Results should be reported in the test report (item 9 in Table 2).

Calculate the faecal coliforms concentration of the original sample for each of the replicate Petri dish according to section 9.3 and report the results in the test report (item 10 in Table 2).

For each dilution step having the three Petri dishes counts between 20 and 80 faecal coliform colonies, calculate: the mean concentration, the maximum and the minimum concentration, the standard deviation of the concentrations, and the coefficient of variation. Record those results in the test report (item 11 in Table 2).

If the sediment sample does not yield at least 20 colonies per Petri dish in one dilution, prepare a very diluted test solution from a stock culture and repeat the estimation of precision.

Note: Coefficient of variation (%) = $\frac{\text{standard deviation} \times 100}{\text{mean}}$

8.9 Confirmatory Tests

8.9.1 MacConkey broth test

With a flamed bacteriological loop (5.22), or a flamed sterile Pasteur pipette (5.12), transfer the suspected colony from the Petri dish into a culture tube containing MacConkey broth (6.2.1) and incubate at 44.5 ± 0.2 °C for 24 hours. Coliforms will produce gas, that will be trapped as a big bubble in the inverted Durham vials, and acid, that will turn the violet-like colour of the original broth from into a yellowish and cloudy colour (if the tube becomes yellow with a greenish taint at the top and a surface veil, there is a presumptive evidence of Pseudomonas).

8.9.2 Brilliant Green Bile broth test

With a flamed bacteriological loop (5.22), or a flamed sterile Pasteur pipette (5.12), transfer the suspected colony from the Petri dish into a culture tube containing Brilliant Green Bile broth (6.3) and incubate at 44.5 ± 0.2 °C for 24 hours. Coliforms will produce gas that will be trapped in the inverted Durham vials.

Note: The MacConkey broth test is equivalent to the Brilliant Green Bile broth

8.9.3 Indole test

With a flamed bacteriological loop (5.22) transfer the suspected colony, a drop of a positive MacConkey broth or a drop of a positive Brilliant Green Bile broth into a culture tube containing tryptone water and incubate at 44.5 ± 0.2 °C in a water bath (5.28) for 24 hours. Then add 0,2 to 0,3 ml of Kovac's indole reagent (6.4.2) and shake. Let the tube stand for 10 minutes and observe the result.

A dark red colour in the amyl alcohol surface layer constitutes a positive indole test, the original colour of the reagent constitutes a negative test. An orange colour probably indicates the presence of skatole and may be reported as a positive reaction.

9. EXPRESSION OF RESULTS

9.1 Report the number of faecal coliform colonies on individual Petri dishes after the incubation has been completed and adjust this count according the results of the confirmatory test, if necessary. Count only dishes with a number of colonies between 20 and 80.

Indicate the results obtained for each Petri dish separately in the test report (Table 1, item 9).

9.2 Express the results in terms of faecal coliforms per gram of sediment, using the following equation:

$$\text{Faecal coliforms per gram of sediment} = \frac{\text{Adjusted number of faecal coliforms colonies}}{\text{grams of sediment collected}}$$

Indicate the results obtained for each dilution separately in the test report (Table 1, item 10). Report also the results obtained on Petri dishes with less than 20 coliform colonies per dish. If there are no faecal coliform colonies in any Petri dish, report the result as less than 1 colony at the lowest dilution. For example, if there is no colony in the two Petri dishes corresponding to the dilution D-1, the final result will be less than 1 faecal coliform per gram of sediment (< 1 FC/g sediment).

9.3 Compute the number of faecal coliforms per 1 g of collected sediment and report it as the final test result (Table 1, item 11). If there are Petri dishes containing between 20 and 80 characteristic colonies in two consecutive dilutions, calculate the mean on these dilutions and report it as the final test result.

9.4 Record in the test report (Table 1, item 12) anomalies observed in test procedures, such as confluent growth of colonies or deviations from temperature prescribed for sample storage and incubation.

NOTE: For routine tests, results are usually expressed in terms of the fresh weight or wet weight of sediment. For research tests, results are normally expressed in terms of dry weight of sediment together with its particle size description.

10. TEST REPORT

Table 1. Faecal coliforms in seawater sediment 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: _____
   Temperature at sampling depth: _____
   Salinity at sampling depth: _____
   Container number: _____
   Type of sediment: _____
   Duration of storage: _____
   (other factors which may influence the results should be reported under 12)

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

9. Number of faecal coliforms colonies per individual petri dish:

Dilution	original sample inoculated (ml)	weight of sediment(g)	colony count dish 1	colony count dish 2	mean colony count
D-1	1	0.5			
D-2	0.1	0.05			
D-3	0.01	0.005			
D-4	0.001	0.0005			

10. Number of faecal coliforms/g of sediment:
    Dilution D-1: ____ col./g; D-2: _____ col./g; D-3:_____ col./g; D-4: _____ col./g

11. Test results: _____ faecal coliforms/g of sediment

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

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

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

11. REFERENCES

UNEP/WHO (1983) Determination of total coliforms in seawater by the membrane filtration culture (MF) method. Reference Methods for Marine Pollution Studies No. 2 Rev. 1, UNEP, Geneva.

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