



REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME

OCTOBER 1995

Determination of faecal coliforms in sea water by the membrane filtration (MF) culture method

Reference Methods For Marine Pollution Studies No. 3 (Rev.1)

Prepared in co-operation with



WHO

UNEP 1995

This document has been prepared by the World Health Organization (WHO) and issued by the International Atomic Energy Agency, Marine Environment Laboratory (IAEA-MEL) and the United Nations Environment Programme (UNEP) under project FP/MR/5101-93-03(3033).

For bibliographic purposes this document may be cited as:

UNEP/WHO: Determination of faecal coliforms in sea water by the membrane filtration (MF) culture method. Reference Methods for Marine Pollution Studies No. 3, Rev. 1 UNEP, 1995

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. DULM: | 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. 3 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.

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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. 3 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 faecal coliforms in coastal bathing waters of temperate and tropical seas. It is designed to be used in sanitary surveillance of bathing beaches.

It uses a membrane filter procedure which allows concentration of the bacteria prior to incubation. It can be employed in alternation with the Multiple-Tube Fermentation (MPN) Test (UNEP/WHO 1983). 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 labor-intensive and, due to the preconcentration of the bacteria in the sample, it is more suitable in situations 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 MFs after incubation.

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. Since faecal coliforms die within hours when exposed to sunlight in seawater at temperatures above $+4^{\circ}\text{C}$, their presence in seawater indicates only recent contamination by faecal material. Die-away rates (T-90) depend on salinity, temperature, solar radiation, etc. and 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 acid and gas, both at 36°C and 44.5°C , in less than 24 hours. They produce indole in tryptone water containing

tryptophan at 44.5° C. Under the conditions described in this reference method, the faecal coliforms appear as blue colonies.

4. PRINCIPLES

From sea-water samples taken under sterile conditions, a dilution series is set up according to the number of faecal coliforms expected in the water sample. Aliquots of this dilution series are filtered through 0.45µ pore size membrane filters. The membrane filters are placed on the surface of m-FC agar contained in Petri dishes and incubated at $44.5 \pm 0.2^\circ \text{C}$ for 24 hours. Lactose fermentation will cause colonies of faecal coliforms (3) to exhibit a characteristic blue colour.

Residual chlorine, if present, is neutralized by adding thiosulphate to the sampling bottle before sterilization.

Suspect and doubtful colonies can be tested for acid and gas development with a confirmative test using MacConkey broth or brilliant green bile broth. In areas where industries discharge polysaccharites (paper mills, sugar beet industries, etc.), further confirmation by the indole test may be necessary.

5. APPARATUS AND GLASSWARE

- 5.1 Sample bottles of borosilicate glass for surface seawater, 200-300 ml capacity, wide-mouthed and with ground-glass stoppers.
- 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 Thermoisolated plastic boxes with cooling pads or similar cooling units (camping equipment) for storage of samples.
- 5.5 Thermometer, 0 to 50° C, precision $\pm 1^\circ \text{C}$, preferably unbreakable plastic type, to be used for checking temperature in plastic boxes (5.4).
- 5.6 Filtration apparatus for 4.7 cm diameter membrane filters (MF) consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material, complete with electric or water vacuum pump.
- 5.7 Water incubator for $44.5 \pm 0.2^\circ \text{C}$.
- 5.8 Stereomicroscope, magnification 10-50X; and/or darkfield colony counter, magnification 2-3X.
- 5.9 Autoclave, max 2 atm., electric or gas.
- 5.10 Drying oven for sterilization at 160° C.

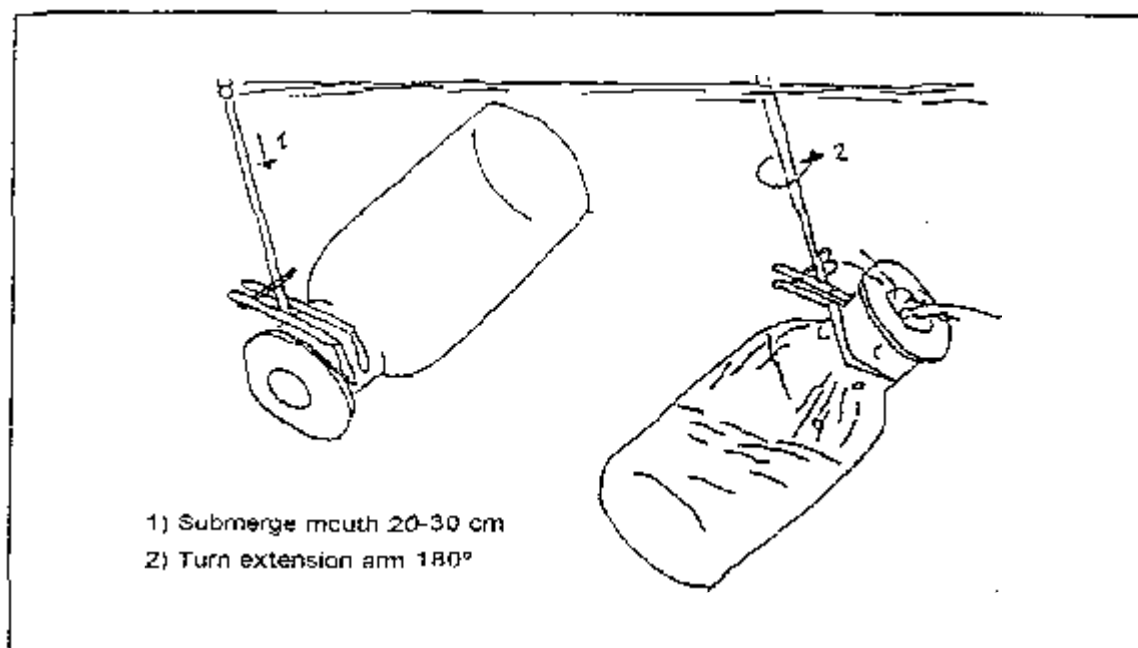


Figure 1. Subsurface sampling with extension arm.

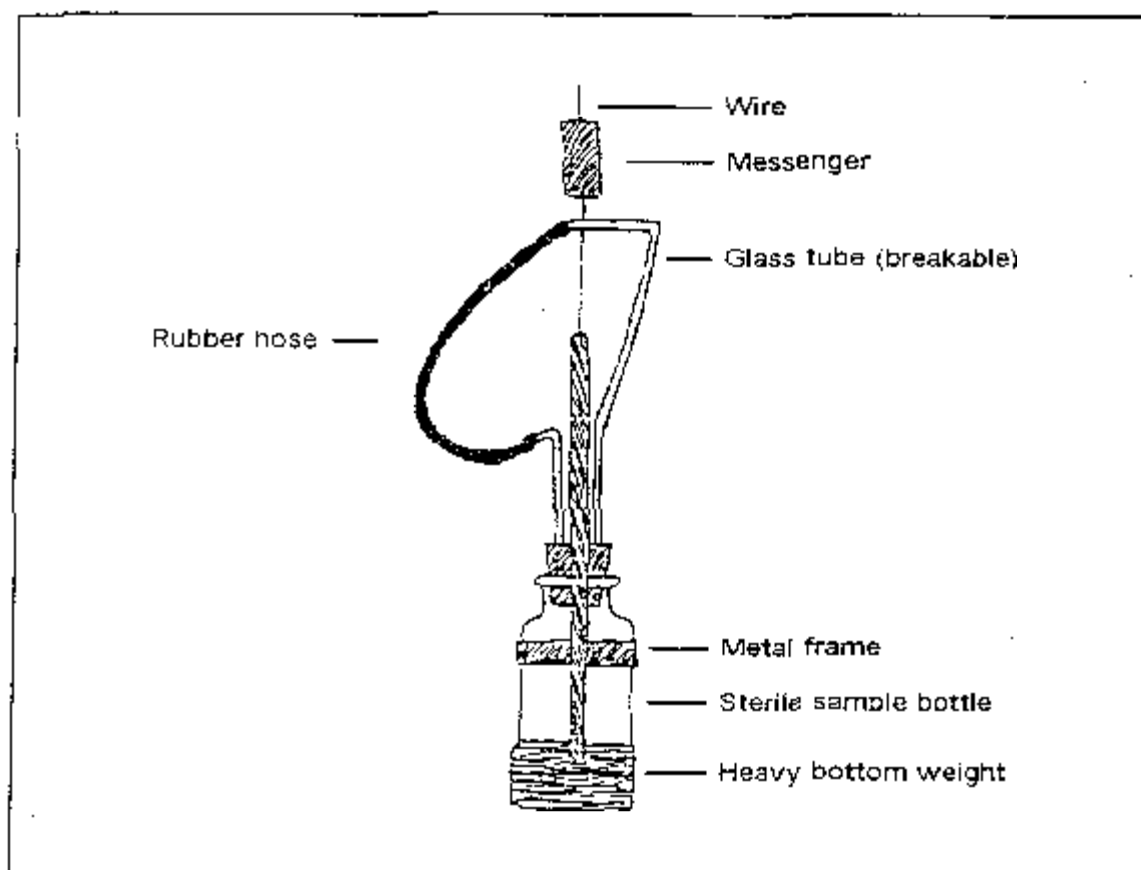


Figure 2. Sampler for sterile subsurface sampling.

- 5.12 Stainless steel forceps.
- 5.13 Analytical balance, precision ± 1 mg.
- 5.14 Refrigerator $4 \pm 1^\circ$ C.
- 5.15 Vibrator (shaker) for mixing liquids in culture tubes.
- 5.16 Petri dishes of borosilicate glass, diameter 5 cm, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic Petri dishes.
- 5.17 Ehrlenmeyer flasks of borosilicate glass for media preparation, capacity 1 and 2 litres.
- 5.18 Borosilicate glass bacteriological culture tubes.
- 5.19 Total volume (blow-out) borosilicate glass pipettes of 1, 9, 10 and 20 ml capacity, with stainless steel 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 glass beakers for cover.
- 5.21 Small borosilicate glass tubes 6 x 50 mm ("Durham vials") to be inserted in culture tubes (5.18).
- 5.22 Bacteriological loops made from 22-24 Chromel gauge, nichrome or platinum-iridium. Diameter of the loop: 3 mm.
- 5.23 Heavy wrapping paper.
- 5.24 Aluminium foil (household quality).
- 5.25 Membrane filters (MF), pore size 0.45μ , diameter 4.7 cm, or similar, fitting filtration apparatus (5.6), or membrane filter of similar diameter fitting the filtration apparatus.

Note: The 0.45μ pore size membrane filter (MF) should be certified by the manufacturer to be free from substances which may hinder the growth and development of bacteria. Maximum recoveries are obtained using membranes composed of mixed esters of cellulose.
- 5.26 Metal boxes which are watertight and suitable for incubating Petri dishes in the water bath (5.7).
- 5.27 Filter paper.
- 5.28 Water bath $44.5 \pm 0.2^\circ$ C.

6. CULTURE MEDIA, CHEMICALS AND STOCK CULTURE

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

6.1 M-FC Agar

6.1.1 Medium

Tryptone	10.0 g
Proteose peptone No. 3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salt No. 3	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Distilled water (6.7)	1.0 litre

Preparation: Dissolve the components of the medium in 1 litre of distilled water (6.7). Heat to boiling point until all components are completely dissolved. Add 10 ml of a 1 per cent solution of rosolic acid (6.1.2) keep boiling for more than one minute, then cool. The final medium should have a pH of 7.4 ± 0.1 ; adjust pH, if necessary, with diluted analytical grade HCl. Cool to about 45° C and pour 4-5 ml into each Petri dish. After the agar has solidified in the Petri dishes, invert the Petri dishes and store them in the refrigerator. The dishes with prepared medium can be kept in a refrigerator for 7 days.

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

Note: Do not autoclave the medium.

Note: The agar surface should not become too dry because if the agar is dry the MF will not adhere well to the agar surface (8.5) and hinder the diffusion of nutrients to the colonies on the MF.

6.1.2 Rosolic acid

Prepare a sufficient amount of 1% solution of rosolic acid in 0.2 N NaOH.

Note: Solution of rosolic acid should be prepared freshly each time. It should 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 components by shaking. Adjust pH to 7.1 ± 0.1 with diluted HCl and then add the bromo-cresol purple solution (6.2.2). Add inverted vials (5.21) to clean culture tubes (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 in these vials has been driven out during autoclaving and close the tubes with cotton plugs. Autoclave (5.9) the closed culture tubes at 121°C for 15 minutes.

6.2.2 Bromo-cresol purple solution

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

6.3 Brilliant Green Bile Broth

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

Preparation: Dissolve components by shaking. Add inverted vials (5.21) to clean culture tubes (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 in these vials has been driven out during autoclaving and close the tubes with cotton plugs. Sterilize by autoclaving (5.9) 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 the samples 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 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. The pH should be 7.0 - 7.4. If necessary adjust with diluted NaOH before sterilization.

6.4.2 Kovac's indole reagent

Paradimethyl-amino-benzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Concentrated hydrochloric acid, HCl	25.0 ml

Preparation: Dissolve the benzaldehyde in amyl alcohol and add hydrochloric acid. The reagent should be 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

6.5.1 P-buffer for filtration

Preparation: Dissolve components and autoclave (5.9) at 121° C for 15 min.

6.5.2 P-buffer for dilutions

Preparation: Dissolve components and dispense 9 ml in test tubes used for dilutions in the dilution series (8.4) and autoclave (5.9) at 121° C for 15 min.

6.6 Thiosulphate Solution

10 per cent sodium thiosulphate solution in distilled water (6.7) and sterilize by filtration (e.g. through a sterilized MF (8.2.4, 8.2.5)).

6.7 Distilled Water

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

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

6.8 Detergents for Cleaning Glassware and Apparatus

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

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

6.9 95 per cent Ethanol per Analysis.

6.10 Stock Culture of *E. coli*.

7. SAMPLING

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

7.1 Sampling of Surface Water

Attach clean sterilized sample bottle (8.2.1) to the clean sampling rod (5.2). Immediately before submerging the sample bottle, remove the ground glass stopper from the bottle without touching the stopper cone. 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 with the mouth of the bottle downwards, in order to avoid contamination by 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.

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 and store the samples in the clean thermoisolated box (5.4) with cooling pads at about 4° C. Keep samples in the dark avoiding exposure to more than +10° C. Separate bottles from each other with clean wrapping paper (5.23) to avoid breakage. Check the temperature with a thermometer (5.5) every three hours. Report irregularities in the test report. Label sample bottles indicating the sampling station, time of sampling and other factors relevant to the interpretation of the results.

7.2 Sampling of Subsurface Water

Lower the sterilized subsurface sampler (8.2.2) 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 thermoisolated box (5.4). Proceed as for sampling of surface water (7.1).

Note: It is known that the die-away rate of 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 filtered and incubated the same day. If this is not possible, the samples should be stored at 4° C and analyzed not later than 24 hours after sampling.

The water sample represents the test solution.

8. TEST PROCEDURE

8.1 Washing of Glassware and Equipment

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

8.2 Sterilization of Glassware and Equipment

8.2.1 Surface sample bottles (5.1). Clean the sample bottle as described under 8.1. Dry and sterilize it in a drying oven (5.10) for three hours at 160° C. Before sterilization, place a small piece of filter paper (5.27) in the neck of the bottle to prevent the ground glass stopper from sticking after cooling. After cooling to ambient temperature in the desiccator, the bottle is ready for use.

and fit the ground glass stopper securely into the neck of the bottle. Put the bottles into detergent-cleaned thermoisolated boxes (5.4). Separate the bottles from each other with clean wrapping paper (5.23) to avoid breakage.

Note: If residual chlorine is suspected in the water sample, add, aseptically, 0.1 ml of a 10 per cent thiosulphate solution (6.6) for each 100 ml sample to the contents of the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

8.2.2 Subsurface sampler (5.3). Clean the subsurface sampler as described under (8.1), rinse with tap and distilled water (6.7). Enclose each sampler in heavy wrapping paper (5.23) or aluminium foil (5.24) and sterilize in an autoclave (5.9) for 15 minutes at 121° C.

8.2.3 Petri dishes (5.15) and pipettes (5.19). Clean dishes and pipettes, complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven (5.10) for three hours at 160° C.

Note: Disposable pre-sterilized plastic Petri dishes may be more economical to use than reusable glass Petri dishes.

8.2.4 Filter funnels of filtration apparatus (5.6). Loosen the filter-holding assembly slightly and wrap the whole filter funnel in heavy wrapping paper (5.23) or aluminium foil (5.24). Sterilize in an autoclave (5.9) for 15 minutes at 121° C, or in a drying oven (5.10) for 3 hours at 160° C.

8.2.5 Membrane Filters (MF) (5.25). Remove the paper separator (if present) and place 10 to 12 clean MFs into Petri dishes (5.16). Autoclave (5.9) for 15 minutes at 121° C. At the end of the sterilization let the steam escape rapidly in order to minimize the accumulation of condensate on the MFs.

Note: Sterilized MFs are commercially available.

8.2.6 Forceps (5.12). Sterilize forceps by dipping them into 95 per cent ethanol (6.9) and flaming them.

8.3 Selection of Sample Size and Dilution Series

The MFs should ideally have from 20 to 80 colonies after incubation. If previous experience for planning the dilution series for clean seawater is not available, filter the following volumes of the original sample: 100 ml, 10 ml, 1 ml and 0.1 ml (figure 3). For contaminated waters the dilutions have to be greater.

8.4 Preparation of the Dilution Series

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

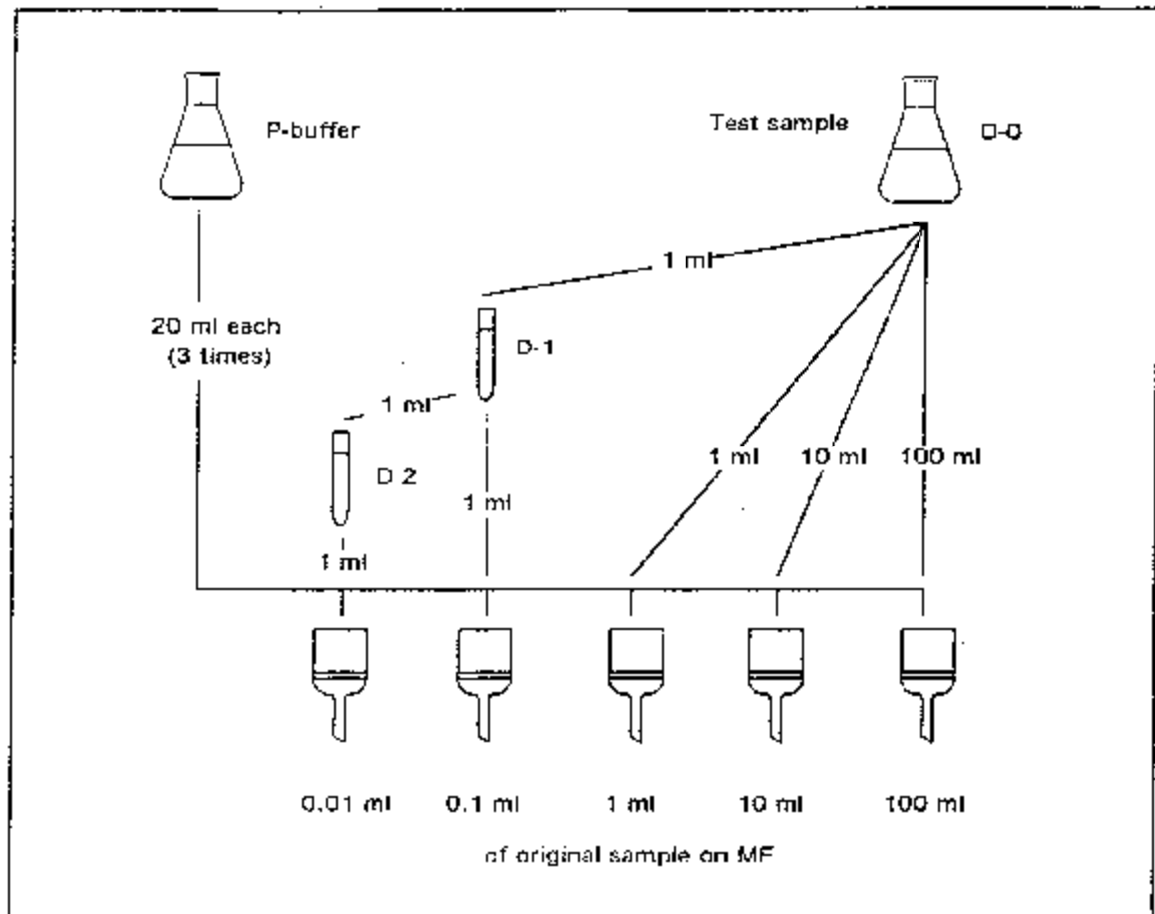


Figure 3. Preparation of dilution series and filtration procedure.

Prepare the dilution series by taking with a sterilized pipette (8.2.3), after vigorously shaking the sample (7), 1 ml from the original sample (figure 3, dilution: D-0) and transfer this 1 ml into a culture tube containing 9 ml of P-buffer (6.5.2) to make the first dilution (D-1). Agitate the tube on a mixer (5.15) or shake it vigorously by hand. Continue the preparation of the dilution series by taking 1 ml from the first dilution (D-1) and mixing it in a new culture tube containing 9 ml of phosphate buffer (6.5.2) in order to obtain the second dilution (D-2), etc.

8.5 Filtration Procedure

Begin filtration with the highest dilution (e.g. D-2) in order to avoid contamination from samples containing bacteria in higher concentrations. Use a sterilized filtration funnel (8.2.4) for each dilution series. Place the sterilized MF (8.2.5) with flamed sterilized forceps (8.2.5) over the porous plate of the filtration apparatus (8.2.4). Carefully place the matching funnel unit over the receptacle and lock it in place. Add into the funnel about 20 ml of buffer solution (6.5.1). With a sterilized pipette (8.2.3) add 1 ml of the D-2 dilution into the buffer solution in the funnel. Filter with a partial vacuum. Wash the funnel walls with approximately 20 ml of buffer solution (6.5.1).

Filter with a partial vacuum. Wash the funnel walls two more times with 20 ml of buffer solution (6.5.1). Unlock and remove the funnel, immediately remove the MF with flamed sterilized forceps (8.2.6) and place the MF on the agar surface of the medium contained in the Petri dish (6.1.1) with a rolling motion to avoid the entrapment of air. Before filtering the next dilution (D-1) in the same manner, pass 20 ml of buffer solution (6.5.1) through the assembled filtration unit.

8.6 Incubation

The Petri dishes containing the MFs on agar (8.5) are sealed and immediately placed horizontally inside clean metal boxes. These metal boxes are then placed in a water bath (5.7) and incubated immediately for 24 hours at $44.5 \pm 0.2^\circ \text{C}$. As a sterility check, incubate also one blank (without MF), i.e. a Petri dish containing agar (6.1.1) only.

Note: Metal boxes must be suitably weighted to prevent them from floating.

8.7 Interpretation

Count with a stereomicroscope or similar (5.8) only colonies which appear as blue coloured. If the number of dubious colonies is greater than 10 per cent of the total number of colonies, test dubious colonies either by MacConkey broth test (8.9.1) or brilliant green bile broth test (8.9.2).

In areas where industries discharge polysaccharites (paper mills, sugar beet industries, etc.), further confirmation by the indole test may be necessary (8.9.3).

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

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 sample, i.e. repeating the four last consecutive dilution steps described under 8.3 and 8.4 (figure 3). The water sample used should be collected during the 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 MF counts which satisfy the 20 to 200 colonies requirements expressed in section 9.1.

Filter each individual dilution following procedure 8.5. Incubate according to procedure 8.6. Report MF counts following the procedure described in sections 9.1 and 9.2 taking into consideration interpretation method of section 8.7. Results should be reported in the test report (table 2, item 9).

Calculate the faecal coliform concentrations of the original sample for each of the replicate results, according to section 9.3 and report the results in the test report (table 2, item 10).

For each dilution step having the three MF counts between 20 and 200 faecal coliform colonies, calculate: the mean concentration, the concentration range, the standard deviation of the concentrations, and the coefficient of variation of the concentrations, and record them in the test report (table 2, item 11).

If the sample does not yield at least 20 colonies per membrane filter in one dilution, prepare a test solution from a stock culture (6.10) and repeat the estimation of precision.

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

8.9 Confirmatory Test

8.9.1 MacConkey broth test: With a flamed bacteriological loop (5.22) transfer the suspected colony from the MF into a culture tube containing MacConkey broth (6.2.1) and incubate (5.28) at $44.5 \pm 0.2^\circ \text{C}$ for 24 hours. Coliforms will develop gas which is trapped in the inverted Durham tubes, and acid which turns the violet-like colour of the original broth into a yellowish colour.

8.9.2 Brilliant green bile broth test: With a flamed bacteriological loop (5.22) transfer the suspected colony from the MF into a culture tube containing brilliant green bile broth (6.3) and incubate (5.28) at $44.5 \pm 0.2^\circ \text{C}$ for 24 hours. Coliforms will develop gas which is trapped in the inverted Durham vials.

Note: The MacConkey broth test is equivalent to the brilliant green bile broth test. Either can be used for confirmation.

8.9.3 Indole test: With a flamed bacteriological loop (5.22) transfer the suspected colony into culture tubes containing tryptone water (6.4.1) and incubate at $44.5 \pm 0.2^\circ \text{C}$ in a water bath (5.28) for 24 hours. Then add 0.2 - 0.3 ml of Kovac's indole reagent (6.4.2) and shake. Let the tube stand for about 10 minutes and observe the results.

A dark red colour in the amyl alcohol surface layer constitutes a positive indole test; the original colour of the reagent, 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 coliform colonies on individual MFs after the incubation has been completed and adjust this count after the confirmatory tests, if necessary, have been made. Use only MFs with a total number of colonies (i.e. coliforms plus non-

coliforms) between 20 and 200. Retain only two significant digits of the counted number of coliform colonies per filter.

Indicate the results obtained for each filter separately in the test report (table 1, item 9).

9.2 Express the results in terms of faecal coliforms per 100 ml of sample, using the following equation:

$$\text{faecal coliforms per 100 ml sample} = \frac{\text{adjusted number of coliform colonies}}{\text{ml of sample filtered}} \times 100$$

Indicate the results obtained for each dilution separately in the test report (table 1, item 10). Report also the results obtained on MFs with less than 20 coliform colonies per filter. If there are no coliform colonies on the filter report the results as "< 1 coliform per 100 ml".

9.3 Compute the number of faecal coliforms per 100 ml sample and report it as the final test result (table 1, item 11). If there are MFs containing between 20 and 200 characteristic colonies in two consecutive dilutions calculate the mean of these dilutions and report it as final test result.

9.4 Record in the test report (table 1, item 12) anomalies observed in test procedure (confluent growth of colonies, deviation from temperature prescribed for sample storage and incubation, etc.).

10. TEST REPORT

Table 1. Faecal coliforms in seawater samples.

1. Sampling area country: _____ area: _____	2. Sampling point (station) _____	code number: _____ longitude: _____ latitude: _____
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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 12)				

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

9. Number of colonies per individual filter			10. Colonies of F. coliforms/100 ml	
Dilution	ml of original sample filtered	Faecal coliforms colonies	Dilutions	col./100 ml
D-0	100	_____	_____	_____
D-0	10	_____	_____	_____
D-0	1	_____	_____	_____
D-1	0.1	_____	11. Test result _____ F. coliforms/100 ml	
D-2	0.01	_____		
D-3	0.001	_____		
D-4	0.0001	_____		

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: _____

Table 2. Precision estimation for faecal coliforms determination.

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 12)
---	--

5. Time of filtration hour: ____ day: ____ 6. Start of incubation hour: ____ day: ____ 7. End of incubation hour: ____ day: ____	8. Confirmatory test MacConkey: _____ Brilliant green: _____ Indole test: _____
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10. Colonies of F. coliforms/100 ml	11. Results (F. coliforms/100 ml)																																													
<table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left;">Dilution</th> <th style="text-align: left;">ml of original sample filtered</th> <th style="text-align: center;">F. coliforms colonies replica</th> <th style="text-align: left;">Dilutions</th> <th style="text-align: left;">col./100 ml</th> </tr> <tr> <th></th> <th></th> <th style="text-align: center;">1st 2nd 3rd</th> <th></th> <th></th> </tr> </thead> <tbody> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> <tr><td>_____</td><td>_____</td><td>_____</td><td>_____</td><td>_____</td></tr> </tbody> </table>	Dilution	ml of original sample filtered	F. coliforms colonies replica	Dilutions	col./100 ml			1st 2nd 3rd			_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	_____	mean: _____ range: _____ std.dev.: _____ coef.var.: _____ %
Dilution	ml of original sample filtered	F. coliforms colonies replica	Dilutions	col./100 ml																																										
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12. Anomalies observed in the test procedure: _____ _____ _____	
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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: _____
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11. REFERENCES

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United Nations Environment Programme

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