REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Determination of Pseudomonas aeruginosa in sea water and sewage by the membrane filtration (MF) culture method

Reference Methods For Marine Pollution Studies No. 29 (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 (IS0) 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. 29 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 overall objectives of the Long-term Programme of Pollution Monitoring and Research in the Mediterranean Sea (MED POL Phase II), which constitutes the environmental assessment component of the Mediterranean Action Plan, adopted by the governments of the region in 1975, include the assessment, on a continuing basis, of the state of pollution of the Mediterranean Sea, the identification of the sources, pathways and effects of pollutants entering into it, and the establishment of temporal trends in pollution levels.

In order to assist laboratories participating in this activity and to ensure the necessary degree of intercomparison of data, a set of reference methods and guidelines has been developed by the Regional Seas Programme of UNEP in cooperation with the relevant specialized agencies of the United Nations system, as well as other organizations. In these reference methods and guidelines, the style used by the International Organization for Standardization (ISO) is followed as closely as possible.

In common with other microbiological methods and guidelines regarding effluents, coastal recreational waters and shellfish areas, the original version of this document was prepared by the World Health Organization within the framework of the MED POL Programme and issued by UNEP's Regional Seas Programme as part of its Reference Methods for Marine Pollution Studies series.

Actual preparation of methods and guidelines falling within the responsibility of WHO is entrusted to competent microbiologists with experience of the relevant characteristics of the Mediterranean marine environment. Following its preparation in draft form, each method is reviewed by international experts in the field, either individually or (normally) during consultation meetings. Following their issue in substantive form, methods and guidelines are distributed to Mediterranean laboratories and are periodically updated and/or revised on the basis of experience.

2. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of *Pseudomonas aeruginosa* in coastal bathing waters of temperate and sub tropical seas and in sewage effluents. It is designed to be used as a supporting parameter in the sanitary surveillance of bathing beaches.

This method employs a membrane filter procedure, which allows concentration of the bacteria prior to incubation, and is a considerable advantage in situations where low numbers of bacteria are to be estimated. It is less labour-intensive than the Multiple Tube Fermentation (MPN) method and is time-saving, as results are obtained in 24 to 48 hours. The mPA-E medium, which is the De Vicente *et al.* modification of the original m-PA medium facilitates colony counting after 24 hours. The majority of the background flora is inhibited by the antibiotics in the medium, and the organisms that do grow form microcolonies which do not obscure the typical colonies of *P. aeruginosa*. Incubation at 42 °C prevents growth of other fluorescent pseudomonads.

Pseudomonas aeruginosa is a common inhabitant of soil and has a worldwide distribution. It cannot replace faecal coliforms as an indicator of faecal contamination,

since it is found in only 10% of normal stools. However, recreational waters are a source of infection by contact rather than ingestion, and *P. aeruginosa* is a potential pathogen which has been increasingly implicated in ear, throat and skin infections through bathing in contaminated waters. It is therefore recommended as a supporting parameter in assessing the sanitary quality of bathing beaches.

3. DEFINITION

Pseudomonas aeruginosa are aerobic, non-sporeforming gram-negative rods that grow at 42 °C and produce oxidase and catalase. They do not ferment sugars. Glucose and several other sugars (xylose, L-arabinose, D-galactose) are metabolised by oxidation. Most strains are motile (1-2 flagella), hydrolyse casein and produce pyocianin.

4. PRINCIPLES

From seawater or sewage effluent samples taken under sterile conditions, a dilution series is set up according to the number of *P. aeruginosa* expected in the water sample. Known volumes of this dilution series are filtered through 0.45µ pore-size membrane filters. The membrane filters are placed on the surface of mPA-E medium contained in petri dishes and incubated at 36 ± 1 °C for 24-48 hours. Under conditions described in this document the colonies of *P. aeruginosa* are flat and more round (0.8-2.2 mm in diameter, with irregular edges. The *P. aeruginosa* colonies will appear as flat colonies with light outer rims and dark brown to greenish-black centres and dry, and its color varied between greenish grey with a drak center or black with or without a rim. Suspected and doubtful colonies can be tested for casein hydrolysis and pyocianin production by streaking on milk agar and King's A agar.

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

5. APPARATUS AND GLASSWARE

5.1 Sample bottles of dark coloured borosilicate glass for surface seawater or effluent, of 200-300 ml capacity, wide-mouthed and with ground-glass stoppers, or plastic containers of similar capacity with well fitting 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.

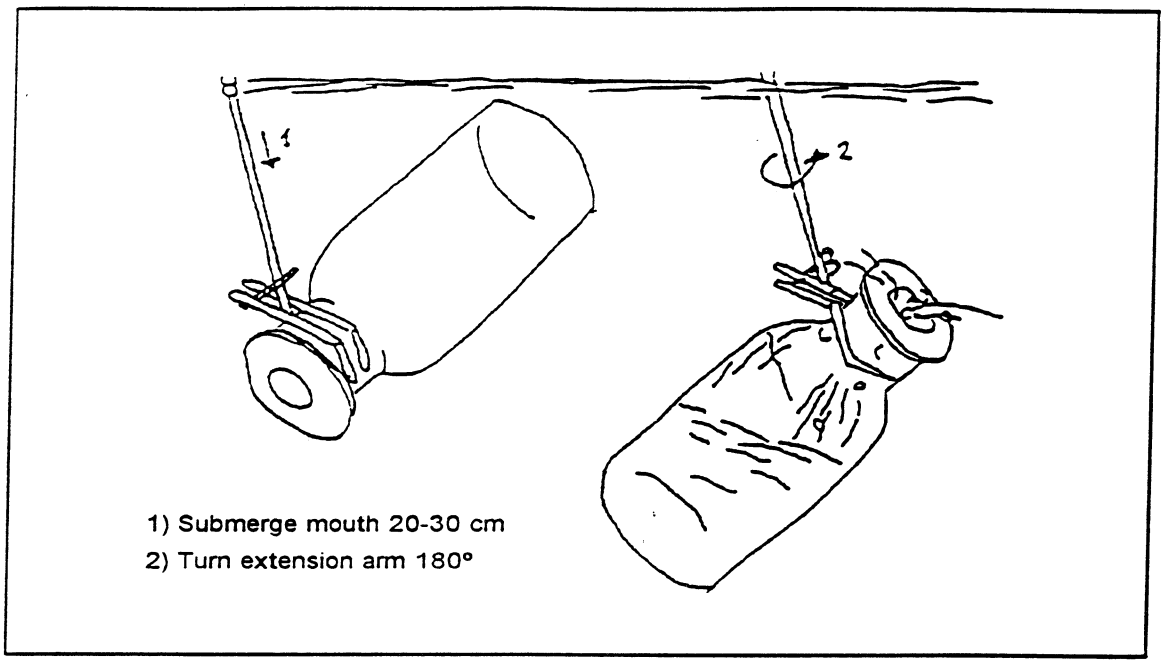

Figure 1. Subsurface sampling with extension arm.

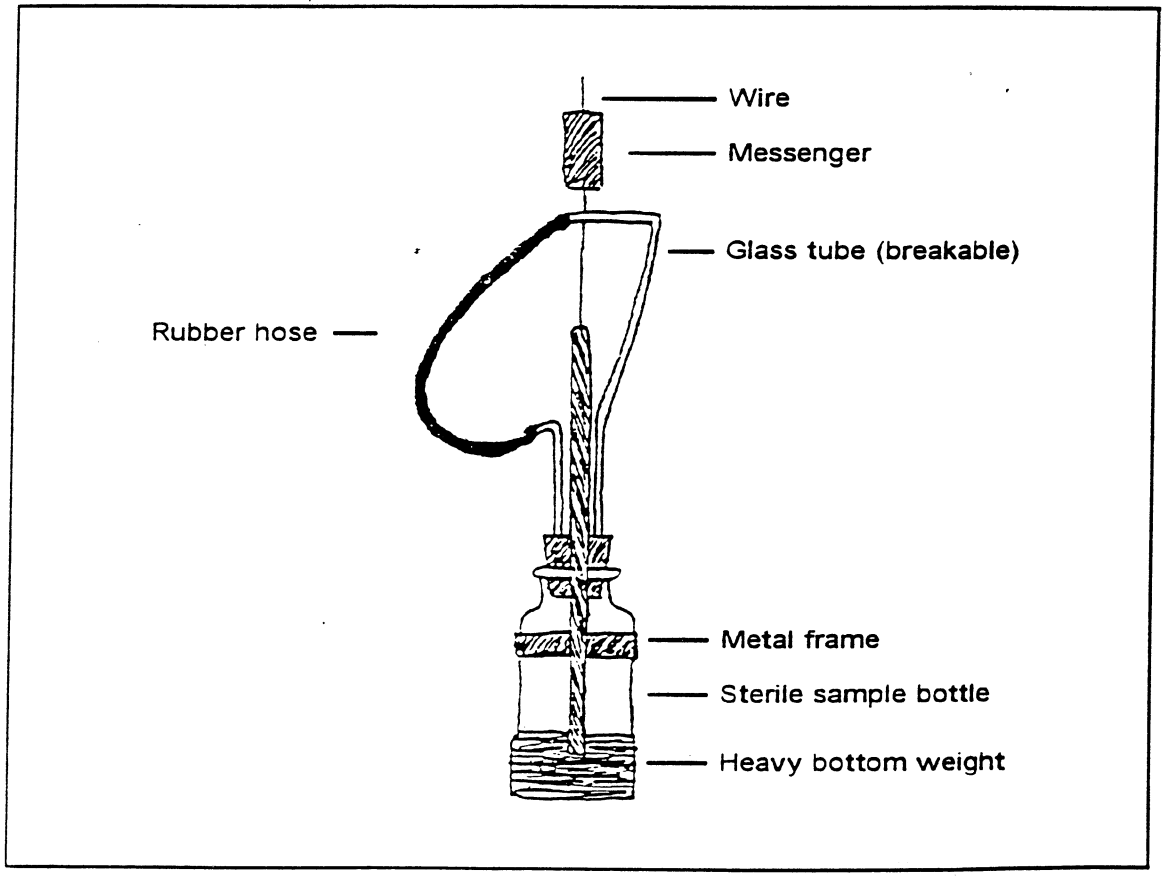

Figure 2. Sampler for sterile subsurface sampling.

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 ± 1 °C, preferably of unbreakable plastic, to be used for checking temperature in plastic boxes.

5.6 Filtration apparatus for 4.7 cm diameter membrane filters consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material (excluding metal filter holders and funnels), complete with electric or water vacuum pump.

5.7 Air incubator thermostatically controlled at 42.0 ± 0.2 °C, preferably with water jacket, and air incubator thermostatically controlled at 36 ± 1 °C

5.8 Stereomicroscope with a magnification 10-15x, or dark field colony counter, with a magnification 2-3x.

5.9 Autoclave, with maximum pressure of 2 atm, electric or gas.

5.10 Drying oven for sterilization up to 170 °C.

5.11 pH meter, precision ± 0.1 pH units.

5.12 Stainless steel forceps.

5.13 Analytical balance, precision ± 1 mg.

5.14 Refrigerator thermostatically controlled at 4 ± 2 °C.

5.15 Vibrator (Vortex type) for mixing liquids in culture tubes.

5.16 Petri dishes of borosilicate glass, 5 cm diameter, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic petri dishes.

5.17 Erlenmeyer flasks of borosilicate glass for media preparation, of 250 ml, 500 ml and 1 litre.

5.18 Borosilicate glass bacteriological culture tubes.

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

5.20 Graduated borosilicate glass cylinders of 100, 500 and 1000 ml capacity with glass beakers for cover.

5.21 Bacteriological loops of 3 mm diameter.

5.22 Heavy wrapping paper.

5.23 Aluminium foil (household quality).

5.24 Membrane filters, 0.45 µm pore size, 4.7 cm diameter.

Note: The 0.45 µm pore size membrane filters 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.

5.25 Filtration apparatus for preparing sterile solutions (Seitz filter or similar).

5.26 Blender with cups of sterilizable material.

5.27 Stomacher with plastics bags.

5.28 Filter paper.

6. CULTURE MEDIA, CHEMICALS AND STOCK CULTURE

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

6.1 mPA-E

6.1.1 Basal medium

L-lysin hydrochloride	5.0 g
Yeast extract	2.0 g
Xylose	2.5 g
NaCl	5.0 g
Sodium thiosulphate	5.0 g
Magnesium sulphate	1.5 g
Ferric ammonium citrate	0.8 g
Phenol red	0.08 g
Agar (Oxoid No. 4)	12.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by boiling and adjust the pH to 7.2, cool to about 55 °C, add antibiotic mixture (6.1.2) and pour 4-5 ml into each petri dish. After the agar has solidified in the petri dishes, invert them and store them in the refrigerator. The dishes with the prepared medium can be kept in a refrigerator for up to 30 days.

Note: Do not autoclave the medium.

Note: The agar surface should not become too dry, because that would prevent the membrane filter to adhere well to the agar surface and the nutrients to diffuse readily towards the organisms on the membrane. No colonies will develop in that case.

6.1.2 Antibiotic mixture

Kanamycin	85.0 mg
Nalidixic acid	37.0 mg
Distilled water	100.0 ml

Preparation: Dissolve the ingredients by mixing and distribute 10 ml portions into vials or plugged test tubes, and freeze. Thaw and add one 10 ml portion to one litre of basal medium. Smaller portions can be distributed according to the amount of medium required.

Note: If the antibiotics do not dissolve completely, mix thoroughly and constantly while withdrawing the separate portions.

6.2 Milk Agar

6.2.1 Agar base

Nutrient broth	12.5 g
NaCl	2.5 g
Agar	15.0 g
Distilled water	500.0 ml

Preparation: Dissolve the ingredients by boiling, and autoclave at 121 °C for 15 minutes.

6.2.2 Milk solution

Instant non-fat milk	100.0 g
Distilled water	500.0 ml

Preparation: Dissolve the instant milk in distilled water and autoclave at 121 °C for 15 minutes.

Preparation of milk agar: Cool both solutions (6.2.1 and 6.2.2) to about 55 °C, mix aseptically and pour into petri dishes (4-5 ml for 5 cm diameter, and 15 ml for 9 cm diameter plates). After the agar has solidified, invert the petri dishes and store them in the refrigerator.

Note: When instant non-fat milk powder is not available, 100 ml of sterile skim milk can be used but, in this case, the components of the agar base should be calculated for 600 ml instead of 1 litre, as given above.

6.3 King's A Agar

Peptone	20.0 g
KH_2PO_4	10.0 g
K_2SO_4	10.0 g
$MgCl_2$	1.4 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation: Dissolve the ingredients and adjust the pH to 7.2 and autoclave at 121° C for 15 minutes. Cool to 55° C and pour into petri dishes. After the agar has solidified, invest the petri dishes and store them in the refrigerator.

6.4 Phosphate Buffer for Filtrations (pH = 7.2)

K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients and autoclave at 121 °C for 15 minutes.

6.5 Peptone Saline Water for Dilutions

Peptone	1.0 g
Sodium chloride	9.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients and dispense 9 ml in test tubes used for preparation of the dilution series and autoclave at 121 °C for 15 minutes, or dispense aseptically with a sterile pipette 9 ml portions of the sterile saline solution (autoclaved at 121 °C for 15 minutes) into sterile test tubes.

6.6 Thiosulphate Solution

Preparation: Prepare a 10% (10 g/100 ml) sodium thiosulfate solution in distilled water and sterilize by filtering through a sterile membrane filter (5.25 or 8.5). The sterilization can be omitted if the solution is prepared every 2-3 weeks and kept in a refrigerator.

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 not releasing toxic substances.

Note: Commercially available distilled water is often produced in copper and zinc apparatus and is highly toxic for bacteria. Before using such water its toxicity should be checked with a stock culture (6.7).

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

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 *P. aeruginosa* (6.9).

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

6.9 95% Ethanol for Analysis.

6.10 Stock Culture of a *P. aeruginosa* Strain.

7. SAMPLING

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

7.1 Sampling of Surface Water

Attach a clean sterile bottle to a 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 sterile sample bottle may also be filled directly by hand (Figure 3).

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 with cooling pads at about 4 °C, avoiding exposure to more than + 10 °C. Separate bottles from each other with clean wrapping paper to avoid breakage. Check the temperature with a thermometer every 3 hours. Report irregularities in the test report. Label sample bottles indicating the sampling station, the time of sampling and other factors relevant to the interpretation of the results.

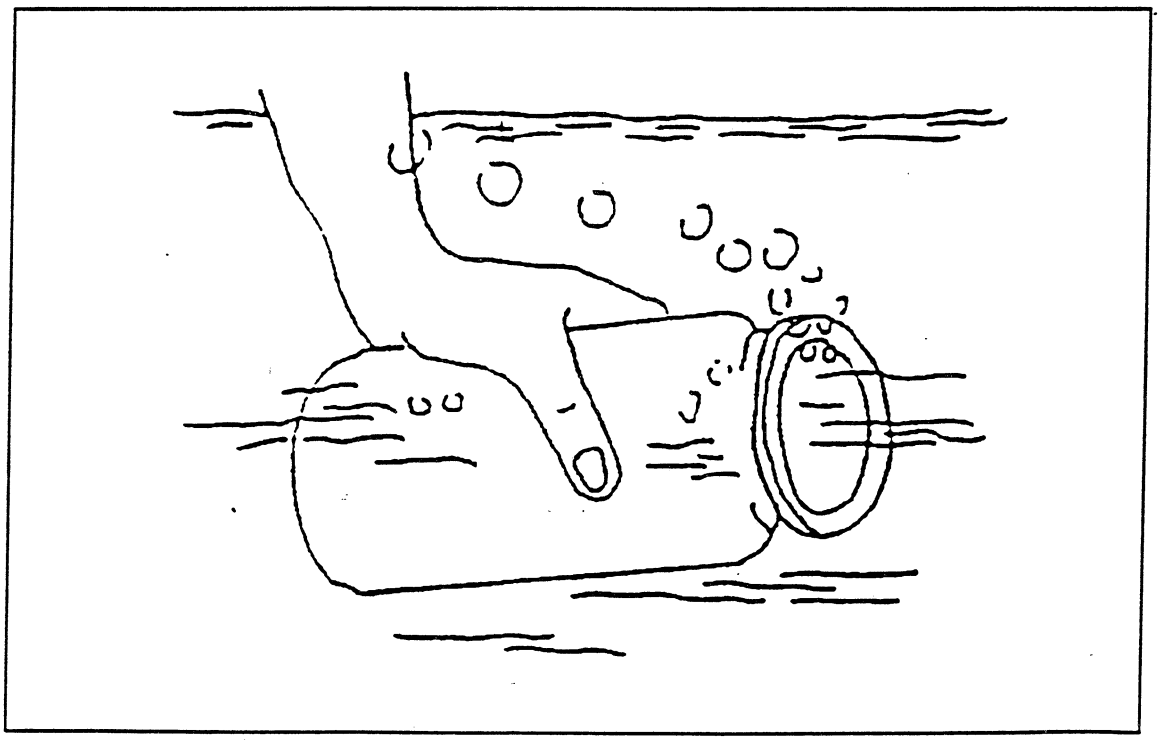

Figure 3. Subsurface sampling by hand.

7.2 Sampling of Subsurface Water

Lower the sterile subsurface sampler (5.3) 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. Proceed as for sampling of surface water (7.1).

7.3 Sampling of Sewage Effluents

Sampling of sewage is relatively difficult because raw wastewater varies both in composition and in flow. Sampling should be performed at points where there is a good mixture of the material to be sampled, unaffected by earlier deposits. Composite samples should be obtained when the aim is to measure average quality over a period not exceeding 24 hours. Sampling should be performed as described in 7.1 above. If the bottle is filled directly by hand, long rubber gloves, covering the hand and fore-arm should be worn.

The water sample represents the test solution.

Note: It is known that the die-away rate of bacteria at ambient temperature in the presence of light is very high. Therefore, all efforts should be made to collect only the number of 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.

8. TEST PROCEDURE

8.1 Washing of Glassware and Equipment

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

8.2 Sterilization of Glassware and Equipment

8.2.1 Surface sample bottles (5.1)

Wash glass bottles as described under 8.1. Dry and sterilize them in a drying oven at 160 °C for 2 hours, or at 170 °C for 1 hour. Before sterilization, place a small piece of filter paper in the neck of each bottle to prevent the ground glass stopper from sticking after cooling. After cooling to ambient temperature in the drying oven, remove this filter paper with sterilized forceps and fit the ground glass stopper securely into the neck of each bottle. Put the bottles into detergent-cleaned thermoisolated boxes. Separate the bottles from each other with clean wrapping paper to avoid breakage.

Wash plastic bottles as described under 8.1, then treat them with 95% alcohol. After adding an appropriate volume of alcohol to each bottle, shake them thoroughly, keeping its stopper in place. After 2-3 alcohol washings, the bottles must be well drained and kept in an oven at 40-50 °C temperature until complete evaporation of the alcohol. Stoppers are also placed on a sterile petri dish to dry.

Note: If residual chlorine is suspected in the water sample, add 0.1 ml of a 10% thiosulfate solution (6.3) 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)

Wash the subsurface sampler as described under (8.1), rinse with tap and distilled water. Enclose each sampler in heavy wrapping paper or aluminium foil and sterilize them in an autoclave at 121 °C for 15 minutes.

8.2.3 Petri dishes and pipettes

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 at 160 °C for 2 hours, or at 170 °C for 1 hour.

Note: Disposable pre-sterilized plastic petri dishes may be more economical to use than re-usable 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 or aluminium foil. Sterilize in an autoclave at 121 °C for 15 minutes, or in a drying oven at 160 °C for 2 hours, or at 170 °C for 1 hour.

8.2.5 Membrane filters

Remove the paper separator (if present) and place 10 to 12 clean membrane filters into petri dishes. Autoclave them at 121 °C for 15 minutes. Once sterilization is completed, let the steam escape rapidly in order to minimize the accumulation of condensate on the membrane filters.

Note: Sterilized membrane filters are commercially available.

8.2.6 Forceps

Sterilize forceps by dipping them into 95% ethanol and flaming them. Let them cool before taking the membrane filters.

8.3 Selection of Sample Size and Dilution Series

After incubation, membrane filters should ideally have from 20 to 80 colonies. If previous experience for planning the dilution series for clean seawater samples is not available, filter the following volumes of the original sample: 100 ml, 10 ml, 1 ml and 0.1 ml (Figure 4). Dilutions have to be greater for contaminated waters and sewage effluents.

8.4 Preparation of the Dilution Series

Prepare the dilution series by taking with a sterilized pipette, after vigorously shaking the sample, 1 ml from the original sample (Figure 4, dilution D-0) and transfering it into a culture tube containing 9 ml of peptone water (6.4) to obtain the first dilution (D-1). Mix vigorously by hand or optionally with a vibrator (5.15). 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 peptone water (6.4) to obtain the second dilution (D-2). The desired dilution levels can be reached by a systematic application of this dilution process.

Before transferring any water volume, the original sample and the dilutions prepared with it must be vigorously shaken in order to insure that the water portions are representative of the original water sample.

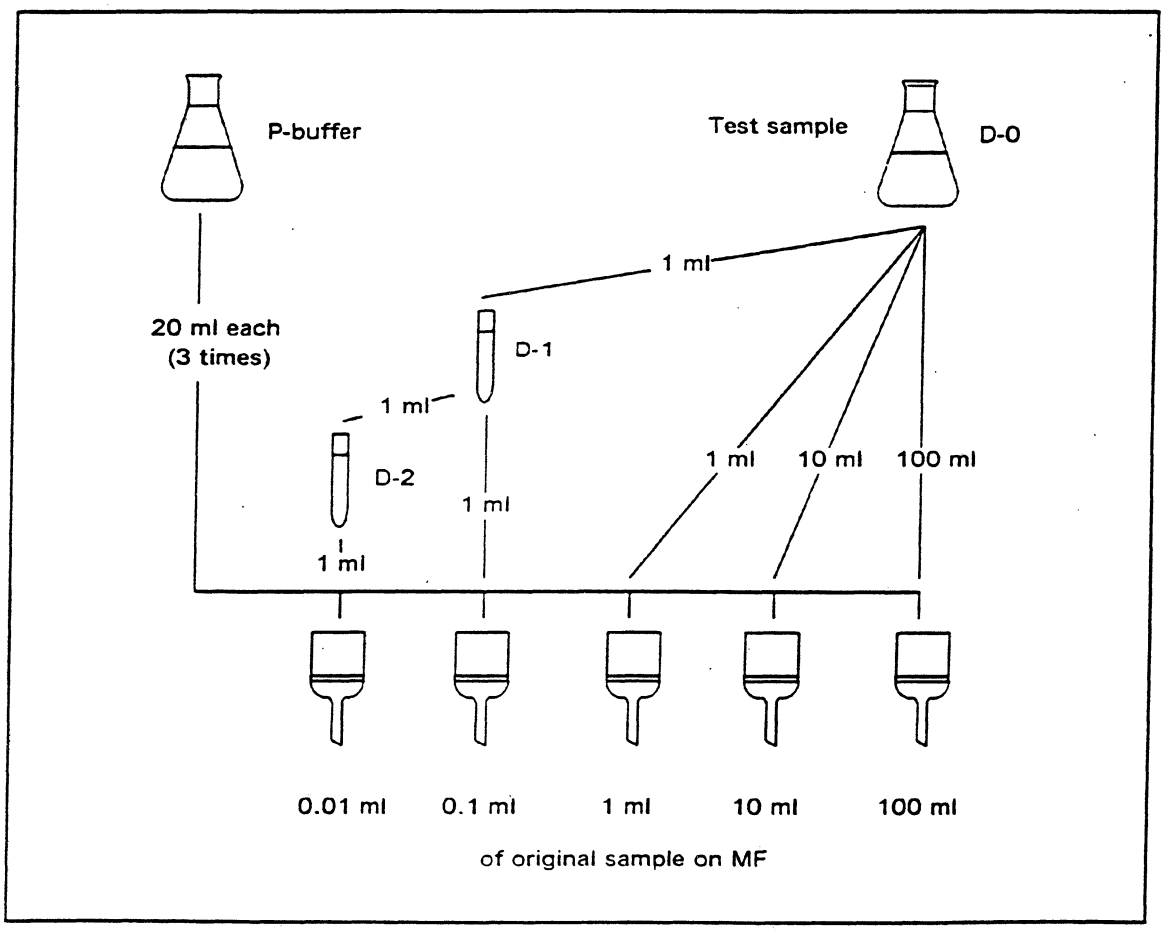

Figure 4. Preparation of dilution series and filtration procedure.

8.5 Filtration Procedure

Each water sample and all dilutions prepared with it must be vigorously shaken to insure a uniform dispersion of bacteria in each sample portion tested.

Begin filtration with the highest dilution prepared (e.g. D-2) in order to avoid contamination from samples containing bacteria in higher concentrations. Use a sterilized filtration funnel for each dilution series. Place the sterilized membrane filters with flame sterilized forceps over the porous plate of the filtration apparatus. Carefully place the matching funnel unit over the receptacle and lock it in place. Add into the funnel about 20 ml of P-buffer solution (6.2.1). With a sterilized pipette add 1 ml of the D-2 dilution into the P-buffer solution in the funnel. Filter with a partial vacuum. Wash the funnel walls with approximately 20 ml of P-buffer solution (6.2.1). Filter with a partial vacuum. Wash the funnel walls two more times with 20 ml of buffer solution each time (6.2.1). Unlock and remove the funnel, immediately remove the membrane filters with flame sterilized forceps andd place the membrane filter on the agar surface of the medium contained in a petri dish 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 P-buffer solution (6.2.1) through the assembled filtration unit.

In the case of sewage effluents, as the sample would be sometimes turbid due to numerous suspended particles, a blending step is generally necessary. If the turbidity of the water sample precludes filtration through the membrane filter, the Most Probable Number method provides a valuable alternative. The volume of effluent to be filtered will depend on the expected density of *Pseudomonas* and may vary between 10 ml and 1000 ml.

8.6 Incubation

The petri dishes containing the membrane filters on mPA-E agar (6.1.1) are sealed and incubated immediately at 37° ± 1 °C for 48 hours. As a sterility check, incubate also a petri dish containing the medium (6.1.1), but without a membrane filter, and another petri dish containing the medium and a filter washed with 20 ml of P-buffer solution.

8.7 Counting and Interpretation

After 24 hours of incubation, count with a stereomicroscope or similar magnifier only colonies with a brown or greenish-black center and light rim. If the number of dubious colonies is greater than 10% of total number of colonies, test dubious colonies on milk agar.

Incubate petri dishes with filters for another 24 hours and observe the appearence of any additional colonies. Confirm the additional colonies on milk agar.

Note: The final number of colonies is usually obtained after 24 hours of incubation.

8.8 Confirmation Test

With a flamed and cooled bacteriological loop touch the suspected colony and streak a straight line, 2-4 cm long, on milk agar (6.2) and on King's A agar (6.3) and incubate at 37 ± 1 °C for 24 hours. On milk agar, *P. aeruginosa* hydrolyses casein, resulting in a clear transparent zone around the streak, and produces pyoverdin. On King's A agar, *P. aeruginosa* produces pyocianin, a blue greenish diffusible, but not fluorescent pigment.

Note: Several colonies can be inoculated on one plate, by keeping a proper separation between them.

9. EXPRESSION OF RESULTS

9.1 Calculation of *P. aeruginosa* Density per 100 ml of Sample

Report the number of *P. aeruginosa* colonies on individual membrane filters after the incubation has been completed. Use only membrane filters with a total of 20 to 80 colonies of *P. aeruginosa*. Retain only two significant digits of the number of *P. aeruginosa* colonies counted in each filter. Indicate the results obtained for each filter separately in the test report (Table 1, item 8).

Express the results in terms of *P. aeruginosa* per 100 ml of sample using the following expression:

$$\textit{P. aeruginosa} \text{ per 100 ml sample} = \frac{\text{number of } \textit{P. aeruginosa} \text{ colonies}}{\text{ml of sample filtered}} \times 100$$

Indicate the results obtained for each dilution separately in the test report (Table 1, item 9). Report also the results obtained on membrane filters with less than 20 *P. aeruginosa* colonies per filter. If there are no *P. aeruginosa* colonies on the filter through which 100 ml of sample have been filtered, report the results as " < 1 *P. aeruginosa*/100 ml ".

Compute the number of *P. aeruginosa* per 100 ml sample and report it as the final test result (Table 1, item 10). If there are membrane filters containing between 20 and 80 characteristic colonies in two consecutive dilutions, calculate the mean of these dilutions (total number of colonies counted/total volume in ml of sample filtered) and report it as a final test result.

Record in the test report (Table 1, item 11) any anomalies observed in the test procedure, such as confluent growth of colonies, and deviation from the temperatures prescribed for sample storage and incubation.

9.2 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 4). 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 3 membrane filter counts which satisfy the 20 to 80 colonies requirements expressed in section 8.3.

Filter each individual dilution following procedure 8.5. Incubate according to procedure 8.6. Report membrane filter counts following the procedure described in sections 9.1, taking into consideration the interpretation method described in section 8.7. Results should be reported in the test report (Table 2, item 8).

Calculate the *P. aeruginosa* concentration of the original sample for each of the filtrations performed, according to section 9.1, and report the results in the test report (Table 2, item 9).

For each dilution step having the three membrane filter counts between 20 and 80 *P. aeruginosa* 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 10).

If the sample does not yield at least 20 colonies per membrane filter in one dilution, prepare a test solution from a stock culture (6.9) and repeat the precision estimation process.

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

10. TEST REPORT.

Table 1. *P. aeruginosa* in seawater and sewage samples.

1. Sampling area
   country: _____
   area: _____

2. Sampling point code number: _____
   (station) longitude: _____
   latitude: _____

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

4. Sampling and environmental 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 filtration hour: ___ . ___ day: ___ / ___ / ___
6. Start of incubation hour: ___ . ___ day: ___ / ___ / ___
7. End of incubation hour: ___ . ___ day: ___ / ___ / ___

8. Number of colonies per individual filter

Dilution	ml of original sample filtered	*P. aerugionsa* colonies
D-0	100	
D-0	10	
D-0	1	
D-1	0.1	
D-2	0.01	
D-3	0.001	
D-4	0.0001	

9. Colonies of *P. aeruginosa*/100 ml

Dilutions	col./100 ml

10. Test result

_____ *P. aeruginosa*/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: _____

Table 2. Test report on precision estimation.

1. Sampling area
country: _____
area: _____

2. Sampling point code number: _____
(station) longitude: _____
latitude: _____

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

4. Sampling and environmental 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 filtration hour: ___ . ___ day: ___ / ___ / ___
6. Start of incubation hour: ___ . ___ day: ___ / ___ / ___
7. End of incubation hour: ___ . ___ day: ___ / ___ / ___

8. Number of colonies per individual filter

Dilution	ml of original sample filtered	*P. aeruginosa* colonies replica 1st	2nd	3th
_____	_____	_____		
_____	_____	_____		
_____	_____	_____		
_____	_____	_____		
_____	_____	_____		
_____	_____	_____		
_____	_____	_____		

9. Colonies of *P. aeruginosa*/100 ml

Dilutions	col./100 ml
_____	_____
_____	_____
_____	_____
_____	_____

10. Test result (*P. aeruginosa*/100 ml)
mean: _____ range: _____
std. dev.: ____ coef. var.: __ %

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

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