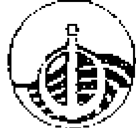

REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

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

Reference Methods For Marine Pollution Studies No. 28 (Rev.1)

Prepared in co-operation with

WHO

UNEP 1995

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PREFACE

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

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

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

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

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

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GREECE

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

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

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

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

This revised issue of Reference Methods for Marine Pollution Studies No. 28 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 isssue of the Reference Method is gratefully acknowledged.

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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 *Staphylococcus aureus* in coastal bathing waters of temperate and sub-tropical seas, and in sewage effluents. It is designed to be used as a supplementary parameter in sanitary surveillance of coastal waters.

This method employs a membrane filter procedure, which allows concentration of the bacteria prior to incubation, and is a great advantage in estimating low numbers of bacteria. The typical *S. aureus* colonies that develop on the medium after 24 hours incubation are coagulase positive (which is the main diagnostic test for *S. aureus*) and no confirmation is required. Hence the method is time and labour saving.

All strains of coagulase positive *S. aureus* are potential pathogens, causing a wide range of infections. They are found in the nasal membranes, hair follicles, skin and perineum of warm blooded animals. Their origin in bathing waters is undoubtedly human activity. They have been found to be shed by bathers under all conditions of swimming, and were recommended as an index of pollution from bathers in swimming

and resistant to some bacteriological factors, *S. aureus* may survive in the marine environment, constituting a potential health hazard to bathers in crowded beaches.

3. DEFINITION

Staphylococcus aureus are spheres, 0.5-1.0 µm in diameter. Cells occur singly and in pairs. Some uncommon strains produce cells with a capsule; they are Gram-positive, nonmotile, aerobes, facultative anaerobes, and catalase positive. The cell wall contains peptidoglycan and teichoid acid. Colonies are smooth, raised, glistening, circular, entire and translucent and single colonies may attain a size of 6-8 mm in diameter on non-selective media.

Under the conditions described in this document, the colonies of *S. aureus* will appear as grey to black dots surrounded by a slightly fluorescent zone on the filter and a white opaque precipitate in the agar underneath the colony.

4. PRINCIPLES

A dilution series of a seawater or sewage effluent sample, taken under sterile conditions, is set up according to the number of staphylococci expected in the water sample. Known volumes of this dilution series are filtered through 0.45 µm pore size membrane filters. The membranes are placed on the surface of 4-S agar and incubated at 42.0 ± 0.2 °C for 24 hours. Grey to black colonies above a zone of opaque precipitate in the underlying agar are coagulase-positive staphylococci.

Residual chlorine, if present, is neutralized by adding thiosulfate 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.

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.

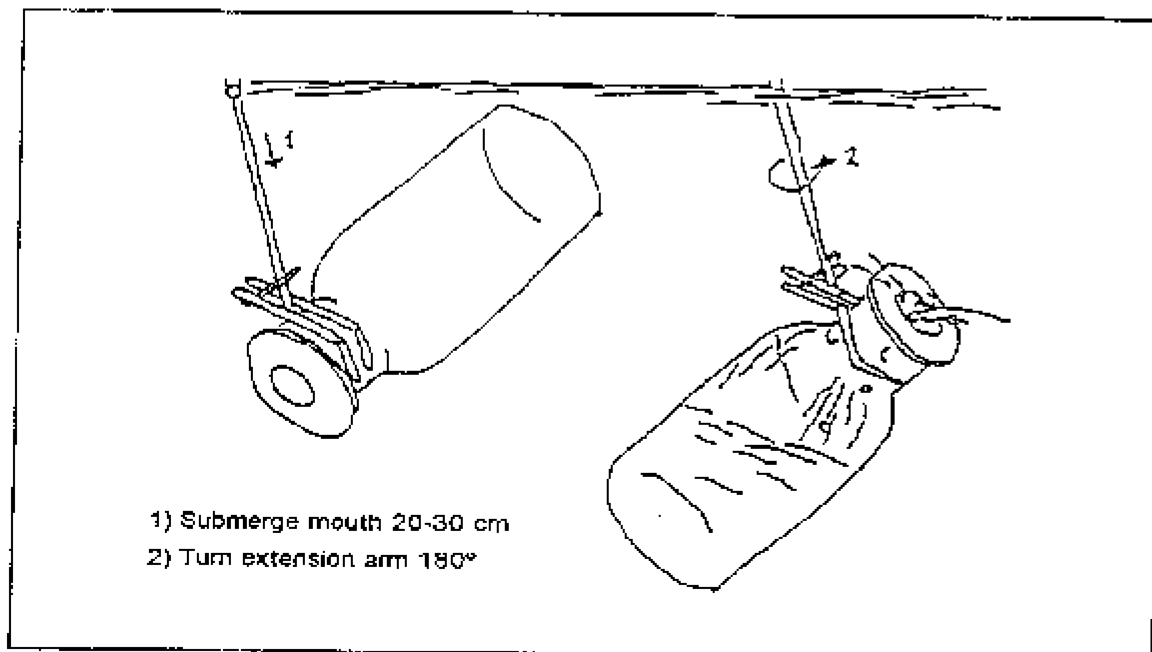

Figure 1. Subsurface sampling with extension arm.

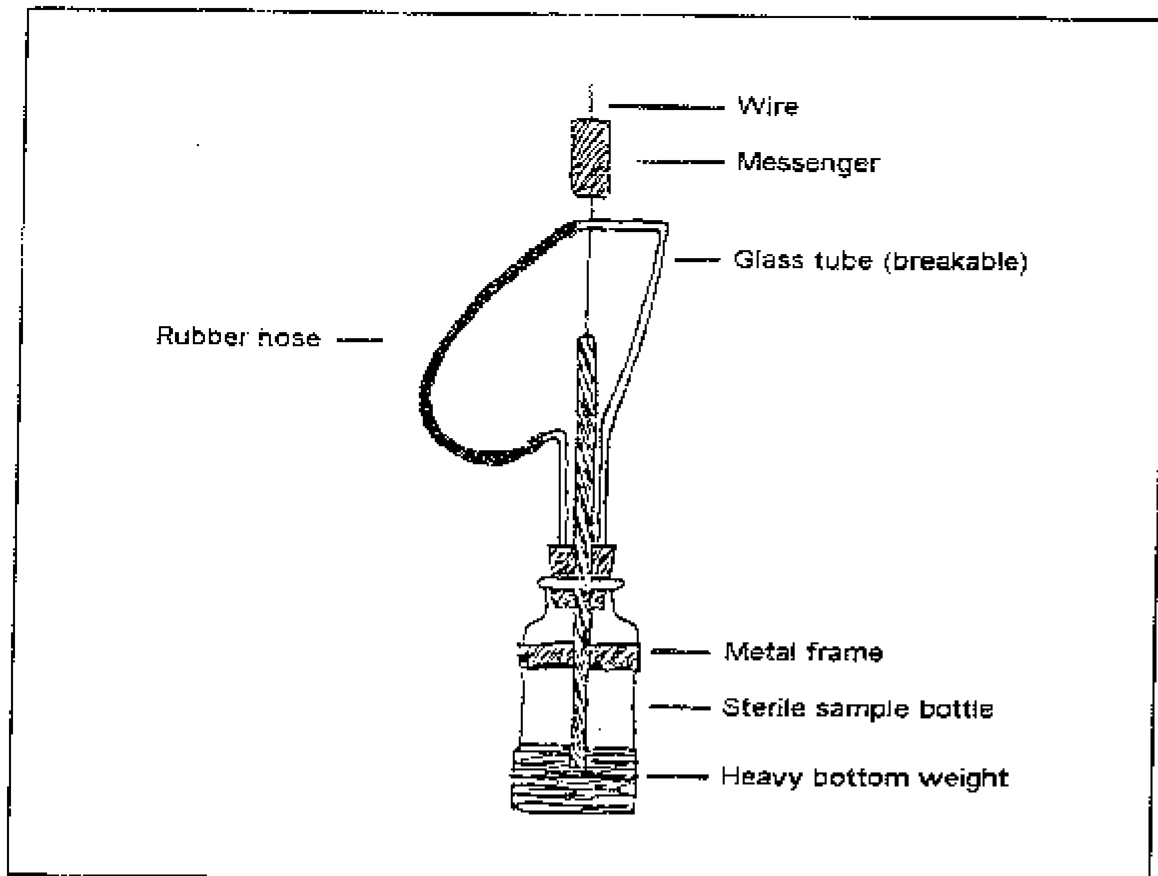

Figure 2. Sampler for sterile subsurface sampling.

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 a water jacket.

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

5.9 Autoclave, with a 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

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 amounts must be chosen accordingly.

6.1 4-S Agar

6.1.1 Agar base (modified)

Yeast extract	2.5 g
Pancreatic digest of casein (or tryptone)	5.0 g
Glucose	1.0 g
Agar	15.0 g
NaCl	50.0 g
Distilled water	1.0 litre

Note: Plate Count Agar can be used, instead of the first four ingredients, according to manufacturer directions.

Preparation: Dissolve the ingredients by boiling, and autoclave at 121 °C for 15 minutes (final pH about 7.0). Cool to about 55 °C and add aseptically 30 ml of egg yolk solution (6.1.2) and 3 ml of 1% sterile potassium tellurite (6.1.4), mix thoroughly 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 where they can be kept for 30 days, provided they are tightly closed in plastic bags. Check for sterility by incubating a blank 4-S plate without membrane filter.

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 Egg yolk solution

Mix aseptically equal volumes of egg yolk and sterile physiological saline solution (6.1.3) and homogenize by mixing for a few seconds in a sterile blender or stomacher. One egg yolk is 16-18 ml. This solution is commercially available.

6.1.3 Sterile physiological saline solution

NaCl	8.5 g
Distilled water	1.0 litre

Preparation: Dissolve sodium chloride by mixing and dispense desired amounts into erlenmeyer flasks. Autoclave at 121 °C for 15 minutes.

6.1.4 Potassium tellurite solution

Potassium tellurite	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve potassium tellurite by mixing, sterilize by filtration (5.25) and store in a refrigerator. Discard when the solution turns dark.

6.2 Phosphate Buffer (pH = 7.2)

K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
Distilled water	1.0 litre

6.2.1 P-buffer for filtration

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

6.2.2 P-buffer for dilutions

Preparation: Dissolve the ingredients and dispense 9 ml in the 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 sterile buffer (6.2.1) into sterile test tubes.

6.3 Thiosulfate 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.

6.4 Blood Agar Base (BA)

Beef heart muscle, infusion from	375.0 g
Tryptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients in 1 litre of distilled water and bring gently to the boil. Distribute 5 ml portions into test tubes. Sterilize at 121 °C for 10 minutes.

Cool tubes in a way to form agar slants. Final pH after autoclaving shoould be 7.4 ± 0.2. Slants may be stored at 4 °C up to 4 weeks.

6.5 Brain Heart Infusion Broth

Calf brain, infusion from	200.0 g
Beef (meat) heart, infusion from	250.0 g
Casein/meat (50/50) peptone	10.0 g
Glucose (dextrose)	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients in 1 litre of distilled water. Dispense 5 ml portions into test tubes. Autoclave at 121 °C for 15 minutes. Final pH after autoclaving should be 7.4 ± 0.2. Tubes may be stored at 4 °C for up to 6 months.

6.6 Rabbit Plasma

1-2 mg EDTA (ethylenediaminetetraacetate) per ml rabbit blood. (Check for antibiotic presence, interference with coagulase test).

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 of *S. aureus*.

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 *S. aureus*.

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

6.9 95% Ethanol for Analysis

6.10 Stock Culture of a *S. aureus* 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.

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.

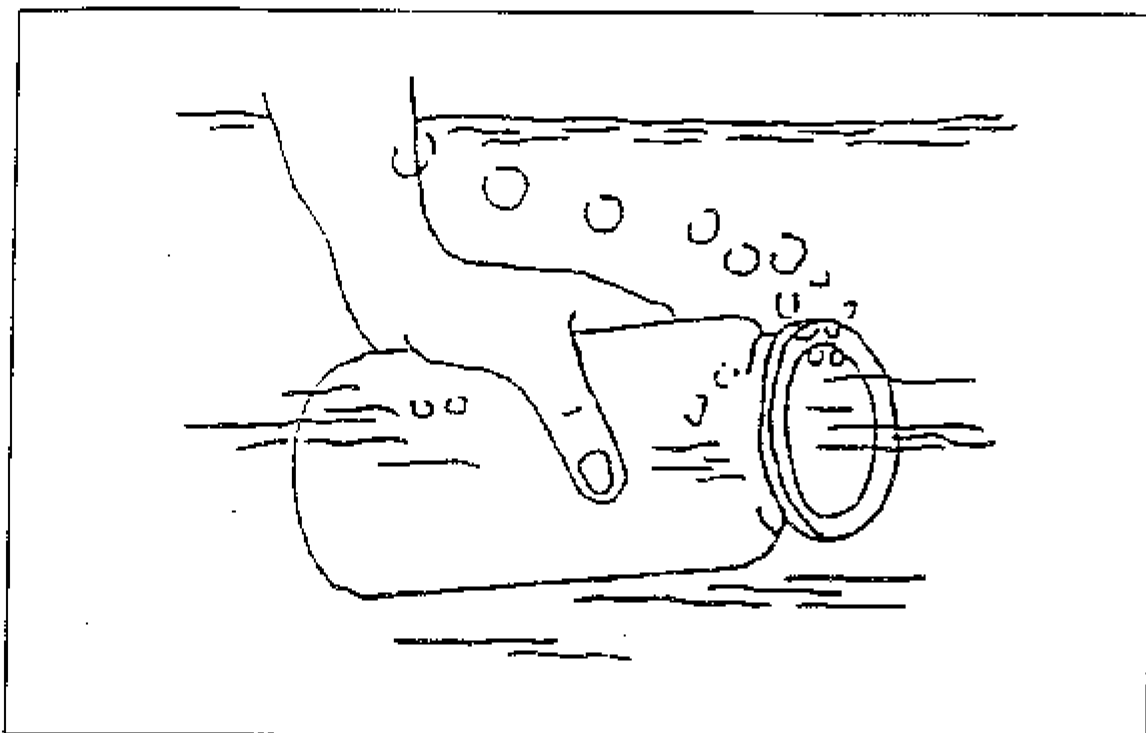

Figure 3. Subsurface sampling by hand.

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

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 60 colonies and no more than 150 colonies. If previous experience for planning the dilution series with 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.

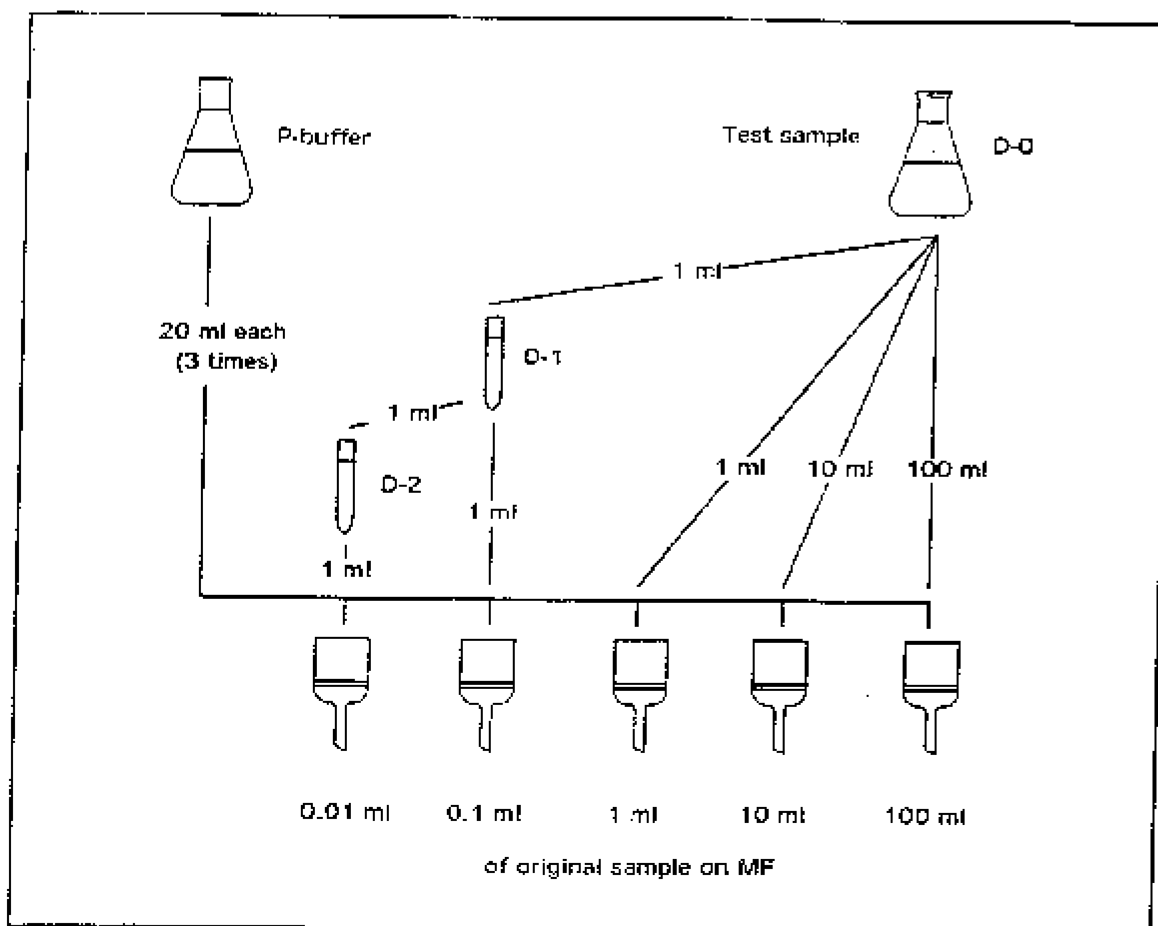

Figure 4. Preparation of dilution series and filtration procedure.

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 3, dilution D-0) and transferring it into a culture tube containing 9 ml of P-buffer (6.2.2) to obtain the first dilution (D-1). Mix vigorously by hand or optionally with a vibrator. 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 P-buffer (6.2.2) 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 to insure that the water portions obtained are representative of the original water sample.

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 and 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 *Staphylococcus* and may vary between 10 ml and 1000 ml.

8.6 Incubation

The petri dishes containing the membrane filters on 4-S agar (6.1.1) are sealed and incubated immediately at 42.0 ± 0.2 °C for 24 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 membrane filter washed with 20 ml of P-buffer solution.

8.7 Counting and Interpretation

Count with a stereomicroscope or similar magnifier only grey to black colonies with a white opaque zone underneath them. In the sunlight the colonies on the filter have a light fluorescent zone around them and the opaque precipitate is seen well in the agar layer when the filter is lifted with sterile forceps. For reevaluation it is possible to remove the filter and count the number of opaque circles on the medium, which corresponds to the number of colonies on the filter.

> **Note:** Dubious colonies can be transferred to nutrient agar, incubated for 24 hours at 37 ± 1 °C and then checked for coagulase production and Gram stain. This is usually unnecessary.

8.8 Coagulase Test

Dubious *S. aureus* colonies should be tested for coagulase production. Inoculate each *S. aureus* typical colony onto a Blood agar (6.4) slant. Incubate the BA slants at 37 °C for 24 hours. From each BA slant inoculate a Brain Heart Infusion broth tube (6.5) and incubate the tubes at 37 °C for 18-20 hours. Transfer 0.1 ml (2 drops) to a tube containing 0.5 ml of dilute (1:3) rabbit plasma (6.6). Incubate at 37 °C and

observe hourly for 3 hours. Clotting within the 3 hour incubation period constitutes a positive test for *S. aureus*.

9. EXPRESSION OF RESULTS

9.1 Calculation of *S. aureus* Density per 100 ml of Sample

Report the number of *S. aureus* colonies on individual membrane filters after the incubation has been completed. Use only membrane filters with a total number of colonies from 20 to 60, including both *S. aureus* and any other microorganisms. Retain only two significant digits of the number of *S. aureus* 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 *S. aureus* per 100 ml of sample using the following expression:

$$S.\ aureus \text{ per } 100 \text{ ml} = \frac{\text{number of } S.\ aureus \text{ colonies}}{\text{ml of sample filtered}} \times 100$$

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 *S. aureus* colonies per filter. If there are no *S. aureus* colonies on the filter through which 100 ml of sample have been filtered, report the results as " < 1 *S. aureus*/100 ml ".

Compute the number of *S. aureus* 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 60 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 60 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 section 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 *S. aureus* concentration of the original sample for each of the replicate results, 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 60 *S. aureus* 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 of *S. aureus*, and repeat the precision estimation process.

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

10. TEST REPORT

Table 1. *Staphylococcus aureus* 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	*S. aureus* 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 *S. aureus*/100 ml

Dilutions	col./100 ml
______	______
______	______
______	______
______	______

10. Test result

______ *S. aureus*/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. Precision estimation for *S. aureus* 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 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	*S. aureus* colonies replica 1st	2nd	3th

9. Colonies of *S. aureus*/100 ml

Dilutions	col./100 ml

10. Results (*S. aureus*/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: ____________

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