REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

# *Determination of Staphylococcus aureus in sea water and sewage by the multiple test tube (MPN) method*

*Reference Methods For Marine Pollution Studies No. 67*

**Prepared in co-operation with**

WHO

UNEP 1995

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# PREFACE

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

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

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

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

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

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

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

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

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

This first substantive issue of Reference Methods for Marine Pollution Studies No. 67 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 *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 bathing beaches.

This method is based on the Multiple-Tube Fermentation (MPN) test. An alternative method for such determination, i.e. the Membrane Filtration (MF) method, offers some advantages for examination of the microbial quality of the water, such as quicker results and less labour requirements. However, the MPN technique has its own advantages. Seawater often presents excessive turbidity, algal blooms, and dense growth of competitive flora, which inhibit the growth of the colonies and hinder the reading of the membranes.

All strains of coagulase positive *S. aureus* are potential pathogens, causing a wide range of infections, being found in nasal membranes, hair follicles and skin of warm-blooded animals. The presence of a high number of swimmers on a beach correlates with the increase of the densities of staphylococci. Being salt tolerant and

resistant to some bactericide factors, they survive in seawater and sand, constituting a potential hazard to bathers on crowded beaches. Thus, pathogenic staphylococci strains have been proposed as additional or alternative indicator organisms.

## 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.

## 4. PRINCIPLES

Quantification of *S. aureus* in seawater samples is accomplished in two phases: 1) multiple sample portions (5x5x5) are tested in modified m-*staphylococcus* broth for the presence of turbidity at 37 °C within 24 hours, and 2) positive findings in the first phase are spread on to Vogel - Johnson agar plates. After 48 hours incubation at 37 °C, typical colonies are tested for coagulase production.

## 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 clamp to hold the sampling bottle (Figure 1).

5.3 Subsurface samplers 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) of the appropriate capacity, for storage of samples.

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

5.6 Air incubator thermostatically controlled at 36 ± 1.0 °C.

5.7 Refrigerator thermostatically controlled at 4 ± 2 °C.

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

5.9 Drying oven for sterilization up to 170 °C.

5.10 pH meter, precision ± 0.1 pH units.

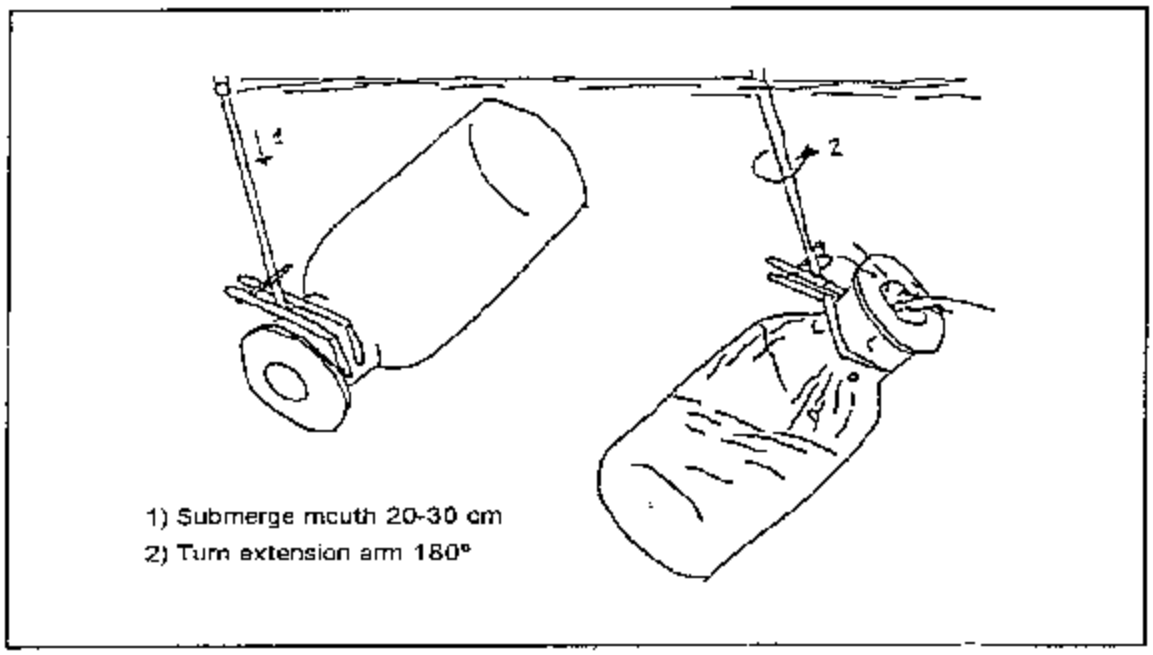


Figure 1. Subsurface sampling with extension arm.

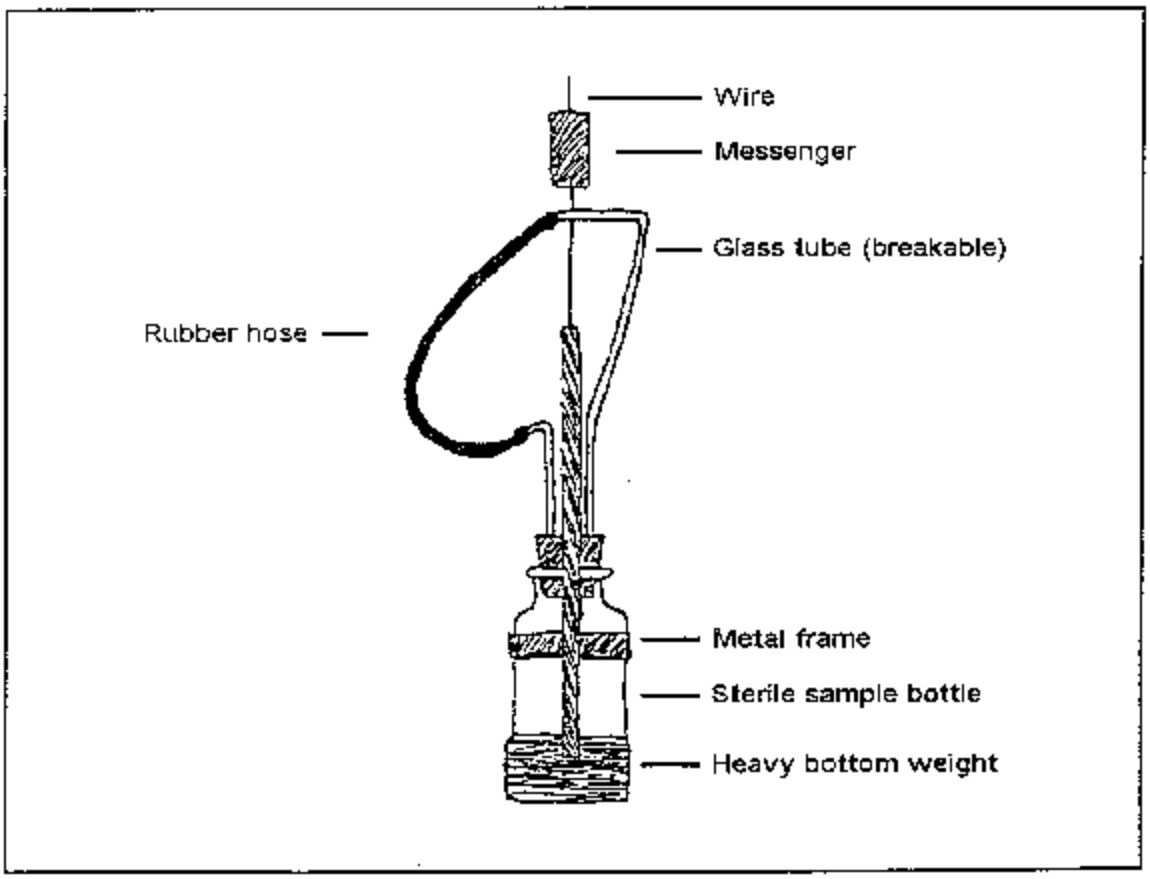


Figure 2. Sampler for sterile subsurface sampling

5.11 Analytical balance, precision ± 1 mg.

5.12 Bacteriological loops of 3 mm diameter.

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

5.14 Test tube racks, with capacity for 15 or 30 tubes.

5.15 Test tubes with cap (sterile), 17/18 mm diameter.

5.16 Petri dishes of borosilicate glass, 90 cm diameter, or disposable pre-sterilized plastic petri dishes.

5.17 Dark phase microscope.

5.18 Borosilicate glass bacteriological culture tubes.

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

5.20 Pipettes of borosilicate glass with total volume (blow-out) of 1, 10 and 20 ml capacity, with stainless steel containers for sterilization, or automatic pipettes of equivalent volume with appropriate sterile tips.

## 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 Buffered Peptone Water (BPW)

| | |
|---|---|
| Peptone | 10.0 g |
| Sodium chloride | 5.0 g |
| Disodium phosphate | 3.5 g |
| Potassium dihydrogen phosphate | 1.5 g |
| Distilled water | 1.0 litre |

Preparation: Dissolve the ingredients in 1 litre of distilled water. Distribute 10 ml portions into 100 ml capacity flasks. Sterilize at 121 °C for 10 minutes. Final pH after autoclaving should be 7.2 ± 0.2. Jars may be stored at room temperature for 1 to 3 months, provided screw caps are air tight.

### 6.2 m-*Staphylococcus* Broth (Modified)

#### 6.2.1 Single strength

| | |
|---|---|
| Tryptone | 10.00 g |
| Yeast extract | 2.50 g |
| Lactose | 10.00 g |

| | |
|---|---|
| Sodium chloride | 75.00 g |
| Sodium azide | 0.049 g |
| Distilled water | 1.0 litre |

Preparation: Dissolve the ingredients in 1 litre of distilled water. Distribute 10 ml portions into test tubes. Sterilize at 115 °C for 10 minutes. Final pH after autoclaving should be 7.0 ± 0.2. Tubes may be stored at room temperature for up to two weeks.

### 6.2.2 Double strength

| | |
|---|---|
| Tryptone | 20.0 g |
| Yeast extract | 5.0 g |
| Lactose | 4.0 g |
| Mannitol | 20.0 g |
| Sodium chloride | 70.0 g |
| Sodium azide | 0.098 g |
| Distilled water | 1.0 litre |

Preparation: Dissolve the ingredients in 1 litre of distilled water. Distribute 10 ml portions into appropriate tubes. Sterilize at 115 °C for 10 minutes. Final pH after autoclaving should be 7.0 ± 0.2. Tubes may be stored at room temperature for up to two weeks.

## 6.3 Vogel - Johnson Agar

| | |
|---|---|
| Tryptone | 10.0 g |
| Yeast extract | 5.0 g |
| Mannitol | 10.0 g |
| Dipotassium phosphate | 5.0 g |
| Lithium chloride | 5.0 g |
| Glycine | 10.0 g |
| Phenol red | 0.025 g |
| Agar | 16.0 g |
| Distilled water | 1.0 litre |

Preparation: Dissolve the ingredients in 1 litre of distilled water and bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 minutes. Final pH after autoclaving should be 7.1 ± 0.2. Cool to 50 °C and add 5.7 ml of sterile 3.5% potassium tellurite solution or 20 ml of 1% potassium tellurite solution. Plates may be stored at 4 °C for up to 7 days.

## 6.4 Kranep Agar (KA)

| | |
|---|---|
| Peptone, from meat | 10.0 g |
| Sodium pyruvate | 8.2 g |
| Sodium chloride | 3.0 g |
| Disodium hydrogen phosphate | 2.0 g |
| Potassium thiocyanate | 25.5 g |
| Lithium chloride | 5.1 g |
| Sodium azide | 0.05 g |
| Actidione | 0.041 g |

| | |
|---|---|
| D(-)mannitol | 5.1 g |
| Agar | 13.5 g |
| also to be added: | |
| egg-yolk emulsion | 100.0 ml |

Preparation: Dissolve the ingredients (72.5 g) in 0.9 litre distilled water. Autoclave under mild conditions (20 minutes at 110° C). Add 100 ml 50% egg-yolk emulsion sterile at a temperature of approximately 50° C. Pour into plates. pH is 6.9 ± 0.1. The plates are turbid and yellowish.

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

### 6.6 Oxidation - Fermentation Medium (O/F)

| | |
|---|---|
| Tryptone | 10.0 g |
| Yeast extract | 1.0 g |
| Glucose | 10.0 g |
| Bromocresol purple | 0.04 g |
| Agar | 2.0 g |
| Distilled water | 1.0 litre |

Preparation: Dissolve the ingredients in 1 litre distilled water. Dispense 10 ml portions into test tubes. Autoclave at 121 °C for 15 minutes. Final pH after autoclaving should be 7.0 ± 0.2. Tubes may be stored for up to four weeks.

### 6.7 Brain Heart Infusion Broth

| | |
|---|---|
| Calf brain, infusion from | 200.0 g |
| Beef (meat) heart, infusion from | 250.0 g |
| Casein/meal (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.

### 6.8 Phosphatase Production Medium

| | |
|---|---|
| Evans peptone | 0.5 g |
| Lab-Lemco | 0.5 g |
| Sodium Chloride | 0.5 g |
| Agar | 1.5 g |
| Distilled water | 100.0 ml |

Preparation: Dissolve the ingredients in 100 ml of distilled water. Sterilize at 121 °C for 15 minutes. Allow to cool at 45 °C; add 1 ml of 1.0% phenolphthalein diphosphate and dispense into plates. Plates may be stored satisfactorily for up to 7 days.

### 6.9 Rabbit Plasma

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

### 6.10 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 stock culture of *S. aureus*.

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

### 6.11 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).

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.

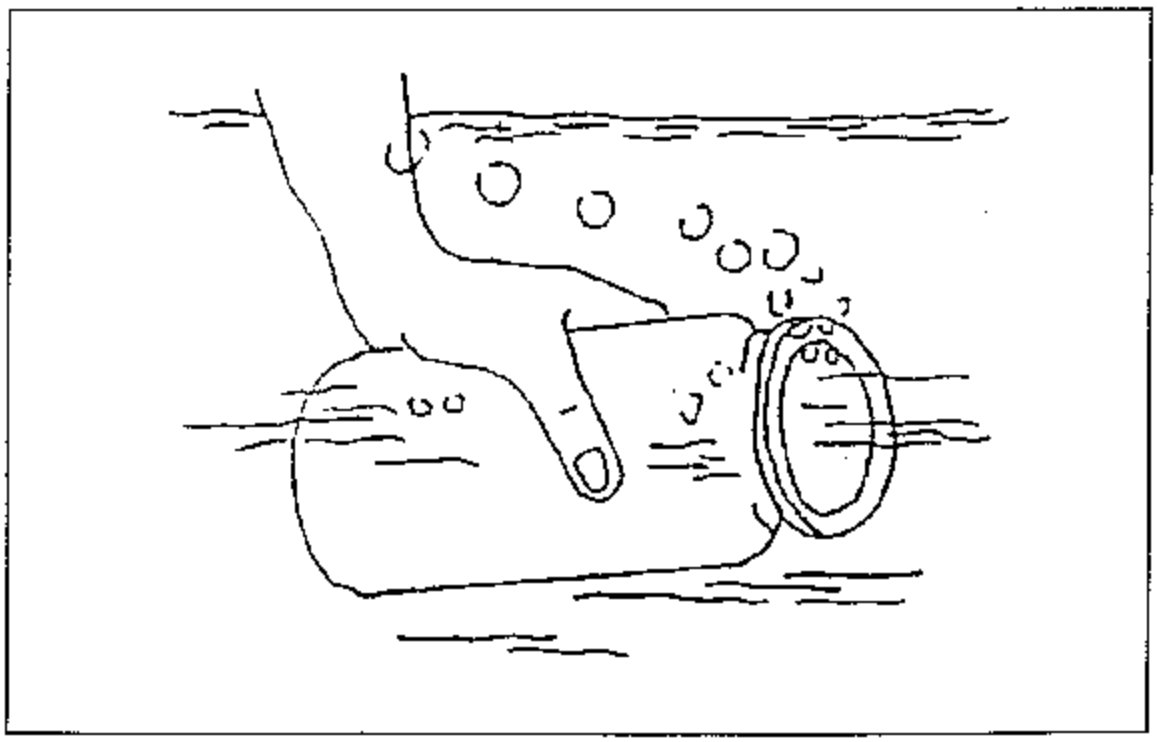

Figure 3. Subsurface sampling by hand.

## 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 3 times with distilled water.

### 8.2 Sterilization of Glassware and Equipment

#### 8.2.1 Surface sample bottles (5.1)

Clean sample 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)

Clean 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.

### 8.3 Selection of Sample Size and Dilution Series

Select a dilution series for each sample that will ensure positive tubes in the lowest dilution row and negative tubes in the highest dilution row. If previous experience for planning the dilution series is not available, transfer 9 ml of buffered peptone water into each of 5 sterilized test tubes using a sterilized pipette. If previous experience on

the level pollution is available, prepare an appropriate number of buffered peptone tubes.

## 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) transferring it into a culture tube containing 9 ml of buffered peptone water (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 buffered peptone water (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.

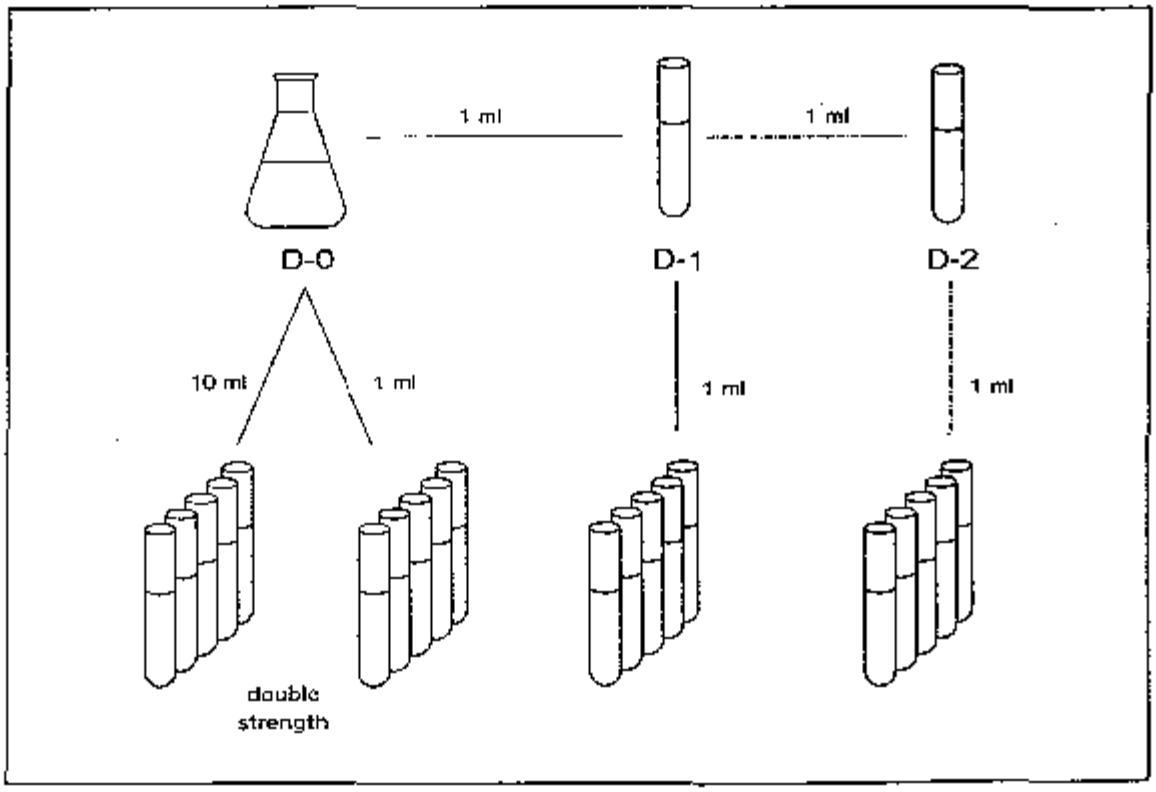


Figure 4. Preparation of dilution series.

## 8.5 Inoculation and Incubation in m-*Staphylococcus* Broth

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.

Using sterilized pipettes transfer 10 ml portions of the water sample into each of 5 sterilized culture tubes containing 10 ml double strength m-*Staphylococcus* broth (6.2.2). Next, transfer with a sterile pipette 1 ml sample portions of dilution D-1 into 5 sterilized culture tubes containing 10 ml single strengh m-*Staphylococcus* broth (6.2.1). Repeat this procedure with each of the subsequent dilutions to be tested.

Incubate the series of m-*Staphylococcus* broth culture tubes in an incubator at 37 °C for 24 hours.

## 8.6 Isolation and Confirmation of *S. aureus*

All m-*Staphylococcus* broth tubes showing turbidity after 24 hours must be subcultured onto Vogel-Johnson agar (6.3) or Kranep agar (6.4).

Label a plate for each positive obtained, noting the dilution series. Using a 3 mm flamed loop, transfer one loopful of each turbid m-*Staphylococcus* broth tube onto a Vogel-Johnson agar plate. Spread it across the plate using the streak plate technique.

Incubate the Vogel-Johnson or Kranep agar plates at 37 °C for 48 hours. Typical *S. aureus* colonies on Vogel-Johson agar are black and produce a yellow pigment, while those grown on Kranep agar show egg-yolk degradation.

### 8.6.1 Coagulase test

Each *S. aureus* typical colony 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 (6.6) and incubate the tubes for 18-20 hours at 37 °C. Transfer 0.1 ml (2 drops) to a tube containing 0.5 ml of dilute (1:3) rabbit plasma. Incubate at 37 °C and observe hourly for 3 hours. Clotting within the 3 hour incubation period constitutes a positive test for *S. aureus*.

### 8.6.2 Identification of *S. aureus* colonies (optional)

A simplified scheme of key characters is proposed for the identification of *S. aureus* typical colonies that produce a non-satisfactory or ambiguous coagulase reaction. *S. aureus* strains are non motile, ferment carbohydrates (glucose fermentation test), produce acid aerobically from lactose, maltose, mannitol and produce phosphatase (phosphatase test). Strains subcultured on Blood agar slants (6.4) should be used for all the above tests.

### 8.6.3 Glucose fermentation test

Before use, the Oxidation/Fermentation medium (6.5) is steamed for 10-15 minutes to remove dissolved oxygen. Tubes are immediately and heavily inoculated with a wire loop, making certain that the inoculum reaches the bottom of the tube. The surface of the tube is covered with a layer of a sterilized parafine oil 25 mm or greater. Incubate at 37 °C for 5 days. Production of acid, under anaerobic conditions, and the subsequent change to a yellow colour throughout the test tube indicate the presence of *Staphylococcus*. If there is no acid production, or acid is found only at the

surface of the test tube, it is indication of oxidative breakdown of the sugar, and the organism present is *Micrococcus*.

### 8.6.4 Phosphatase production test

Phosphatase production plates (6.7) are inoculated from BA slants and incubated at 37 °C for 3-5 days. The release of phenolphthalein is detected using a drop of 0.880 sp.gr. ammonia placed in the lid of each petri dish. Phosphatase producing colonies turn deep pink immediately on exposure to ammonia vapors.

## 8.7 Interpretation of *S. aureus* Culture Plates

A plate containing typical, coagulase positive *S. aureus* colonies, constitute a positive test.

# 9. EXPRESSION OF RESULTS

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

If sample portions of 10 ml, 1 ml and 0.1 ml per test tube have been used, take the number of recorded positive plates and find the corresponding most probable number (MPN) from Table 3. When more than three dilutions are employed, the results of only three of these are used in computing the MPN. For MPN calculation purposes, select the highest dilution that gives positive results in all 5 plates tested (no lower dilution should give a negative result) and the next two higher dilutions that have some negative plates. Occasionally, positive plates may be encountered beyond the three serial dilutions. In those cases, and for purposes of calculating the MPN, include such additional results in the third row. These skip combinations of positive plates should occur with a frequency lower than 1% of the MPN tests performed. Higher frequencies would suggest laboratory error in pipetting sample dilutions.

Table 1 illustrates an example of the numbers of positive test obtained with a series of 5 consecutive dilutions: 5-5-3-2-1. For MPN calculations the highest dilution selected would be that using 1 ml of sample. The final combination of positive plates reported would be 5-3-3, instead of 5-3-2. The MPN value corresponding to the 5-3-3 combination would be 170 *S. Aureus*/100 ml (Table 3), and not the MPN value of 140 *S. aureus*/100 ml associated to the 5-3-2 combination.

The MPN density thus obtained should be adjusted for dilution by multiplying for as many powers of 10 as dilutions were performed with the first plate series considered below 10 ml. For example, a MPN value of 5-3-3 would be expressed as 170 *S. Aureus*/100 ml when the starting dilution is 10 ml, but it would be counted as 1 700 *S. aureus*/100 ml when the starting dilution is 1 ml, and 17 000 *S. aureus*/100 ml when the largest sample volume used was 0.1 ml.

Table 1. Example for computing the density of *S. aureus* in a water sample analyzed by the MPN method.

| Sample portion, ml | Positive tubes |
|---|---|
| 10 | 5 |
| 1 | 5 (x) |
| 0.1 | 3 (x) |
| 0.01 | 2 (x) |
| 0.001 | 1 |

Dilution factor: 10/1 = 10
MPN (5-3-3) = 170 x 10 = 1 700 *S. aureus*/100 ml
95% confidence limits:
lower: 80 x 10 = 800 *S. aureus*/100 ml
higher: 410 x 10 = 4 100 *S. aureus*/100 ml

## 9.2 Precision of Results

Select from Table 3 the 95% confidence limits associated to the MPN selected in calculating the density of *S. aureus* and adjust their values with the dilution factor. Enter the final results in the test report.

## 10. TEST REPORT

Table 2. *Staphylococcus aureus* in seawater 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 positive agar plates

| Dilution | ml of original sample inoculated | No. positive plates |
|---|---|---|
| D-0 | 10 | _____ |
| D-0 | 1 | _____ |
| D-1 | 0.1 | _____ |
| D-2 | 0.01 | _____ |
| D-3 | 0.001 | _____ |
| D-4 | 0.0001 | _____ |
| D-5 | 0.00001 | _____ |

9. Test results
   MPN of *S. aureus*/100 ml

   Combination _____ Dilution factor _____

   _____ *S. aureus*/100 ml

   95% confidence interval
   lower: _____ *S. aureus*/100 ml
   higher: _____ *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 3. MPN index and 95% confidence limits for various combinations of positive and negative results when five 10-ml portions, five 1-ml portions and five 0.1-ml portions are used.

| No. of Tubes Giving Positive Reactions out of | | | MPN Index per 100ml | 95% Confidence Limits | |
|---|---|---|---|---|---|
| 5 of 10 ml each | 5 of 1 ml each | 5 of 0.1 ml each | | Lower | Upper |
| 0 | 0 | 0 | <2 | -- | -- |
| 0 | 0 | 1 | 2 | 1.0 | 10 |
| 0 | 1 | 0 | 2 | 1.0 | 10 |
| 0 | 2 | 0 | 4 | 1.0 | 13 |
| 1 | 0 | 0 | 2 | 1.0 | 11 |
| 1 | 0 | 1 | 4 | 1.0 | 15 |
| 1 | 1 | 0 | 4 | 1.0 | 15 |
| 1 | 1 | 1 | 6 | 2.0 | 18 |
| 1 | 2 | 0 | 6 | 2.0 | 18 |
| 2 | 0 | 0 | 4 | 1.0 | 17 |
| 2 | 0 | 1 | 7 | 2.0 | 20 |
| 2 | 1 | 0 | 7 | 2.0 | 21 |
| 2 | 1 | 1 | 9 | 3.0 | 24 |
| 2 | 2 | 0 | 9 | 3.0 | 25 |
| 2 | 3 | 0 | 12 | 5.0 | 29 |
| 3 | 0 | 0 | 8 | 3.0 | 24 |
| 3 | 0 | 1 | 11 | 4.0 | 29 |
| 3 | 1 | 0 | 11 | 4.0 | 29 |
| 3 | 1 | 1 | 14 | 6.0 | 35 |
| 3 | 2 | 0 | 14 | 6.0 | 35 |
| 3 | 2 | 1 | 17 | 7.0 | 40 |
| 3 | 3 | 0 | 17 | 5.0 | 46 |
| 4 | 0 | 0 | 13 | 5.0 | 38 |
| 4 | 0 | 1 | 17 | 7.0 | 45 |
| 4 | 1 | 0 | 17 | 7.0 | 46 |
| 4 | 1 | 1 | 21 | 9.0 | 55 |
| 4 | 1 | 2 | 26 | 12 | 63 |
| 4 | 2 | 0 | 22 | 9.0 | 56 |
| 4 | 2 | 1 | 26 | 12 | 65 |
| 4 | 3 | 0 | 27 | 12 | 67 |

Table 3. (Continued) MPN index and 95% confidence limits for various combinations of positive and negative results when five 10-ml portions, five 1-ml portions and five 0.1-ml portions are used.

| No. of Tubes Giving Positive Reactions out of | | | MPN Index per 100 ml | 95% Confidence Limits | |
|---|---|---|---|---|---|
| 5 of 10 ml each | 5 of 1 ml each | 5 of 0.1 ml each | | Lower | Upper |
| 4 | 3 | 1 | 33 | 15 | 77 |
| 4 | 4 | 0 | 34 | 16 | 80 |
| 5 | 0 | 0 | 23 | 9.0 | 86 |
| 5 | 0 | 1 | 30 | 10 | 110 |
| 5 | 0 | 2 | 40 | 20 | 140 |
| 5 | 1 | 0 | 30 | 10 | 120 |
| 5 | 1 | 1 | 50 | 20 | 150 |
| 5 | 1 | 2 | 60 | 30 | 180 |
| 5 | 2 | 0 | 50 | 20 | 170 |
| 5 | 2 | 1 | 70 | 30 | 210 |
| 5 | 2 | 2 | 90 | 40 | 250 |
| 5 | 3 | 0 | 80 | 30 | 250 |
| 5 | 3 | 1 | 110 | 40 | 300 |
| 5 | 3 | 2 | 140 | 60 | 360 |
| 5 | 3 | 3 | 170 | 80 | 410 |
| 5 | 4 | 0 | 130 | 50 | 390 |
| 5 | 4 | 1 | 170 | 70 | 480 |
| 5 | 4 | 2 | 220 | 100 | 580 |
| 5 | 4 | 3 | 280 | 120 | 690 |
| 5 | 4 | 4 | 350 | 160 | 820 |
| 5 | 5 | 0 | 240 | 100 | 940 |
| 5 | 5 | 1 | 300 | 100 | 1300 |
| 5 | 5 | 2 | 500 | 200 | 2000 |
| 5 | 5 | 3 | 900 | 300 | 2900 |
| 5 | 5 | 4 | 1600 | 600 | 5300 |
| 5 | 5 | 5 | ≥1600 | --- | --- |

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