REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Isolation/enumeration of Salmonella from sea water and sewage

Reference Methods For Marine Pollution Studies No. 30 (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. 30 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 methods described are suitable for isolation and/or enumeration of salmonellae in coastal recreational and shellfish waters. A separate technique is given for *S. typhi*. The methods described have three main advantages:

1. They provide a high recovery rate of *Salmonella* organisms.
2. They inhibit efficiently the growth of competitive organisms and especially of the bacteria which produce *Salmonella*-like colonies on plating media, saving thus time and media.
3. Their enrichment media can be prepared in advance and then stored for at least three months, and they are cheaper than most of the media traditionally used.

Salmonellae are always pathogenic bacteria for humans and they are widely distributed in animals. Raw foods, especially of animal origin, are often contaminated with these organisms. Sewage, food industry effluents, river and drainage water entering the sea contain salmonellae, sometimes in high numbers.

The organisms do not survive in seawater and although their die-off rates (T90) have not been well studied, the T90 time for salmonellae seems to be similar to that of coliform bacteria. Solar radiation and temperature seem to be important factors for the die-off phenomenon. However, when interpreting results of the *Salmonella* isolation techniques, the following should be taken into account:

1. Salmonellae are bacteria of intestinal origin.
2. The isolation techniques are not 100% effective.
3. The organisms die rapidly in seawater, especially under strong sun radiation and high temperatures.

3. DEFINITION

The genus *Salmonella* is composed of Gram-negative rods that conform to the definition of the family of Enterobacteriaceae. They are oxidase-negative, catalase-positive, and do not either hydrolyze urea or deaminate phenylalanine. The majority of strains are motile, active H_2S producers, usually beta-galactosidase negative, form lysine decarboxylase, and grow in citrate, but not in KCN media. Salmonellae produce acid and gas (with a few exceptions for gas) from glucose and attack mannitol, usually dulcitol, and sorbitol. By biochemical reactions they divide into 4 subgenera. More than 2200 serovars have been recognized.

4. PRINCIPLES

Samples of seawater are collected in sterilized bottles or disinfected plastic containers. An appropriate sample volume or portions of decimal dilutions of the initial seawater sample are passed through 0.45 µm pore size membrane filters which are transferred into pre-enrichment media. After incubation, enrichment media are inoculated and incubated at the appropriate temperature. Selective agar plates are seeded from enriched media and, after incubation, typical *Salmonella* colonies are picked for subsequent biochemical and serological identification. Cultures with typical or suspect *Salmonella* reactions are shipped to a *Salmonella* Reference Centre for complete identification.

5. APPARATUS AND GLASSWARE

5.1 Sample bottles of dark coloured borosilicate glass for surface seawater of 2 litres capacity, wide-mouthed and with ground-glass stoppers, or plastic containers of 2 litres capacity with a well fitting stopper (occasionally of 5 litres capacity).

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.

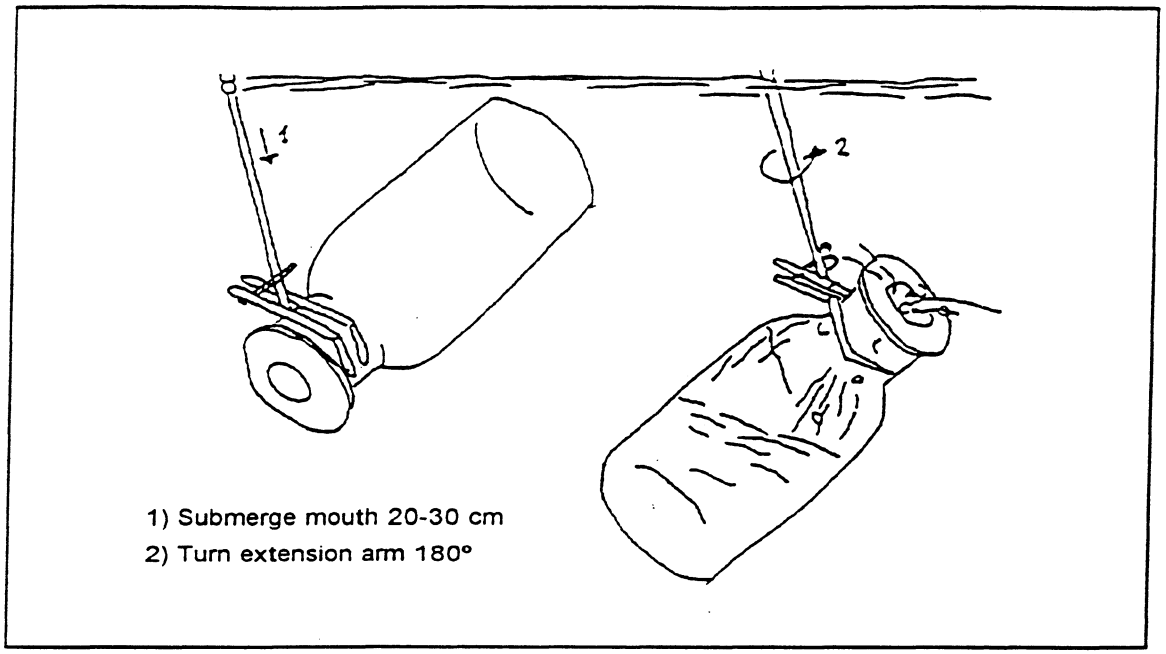

Figure 1. Subsurface sampling with extension arm.

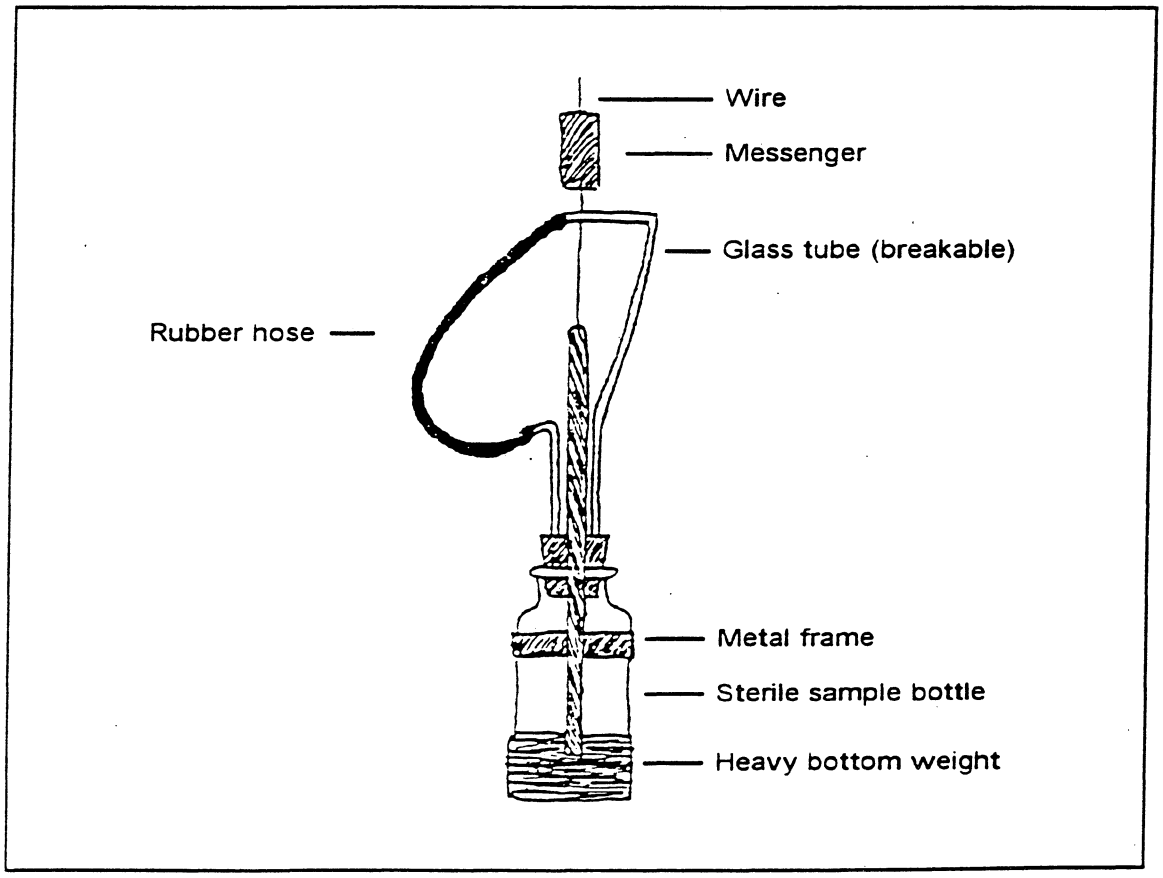

Figure 2. Sampler for sterile subsurface sampling.

5.5 Thermometer, 0 to 50 °C, precision ± 1 °C, preferably of unbreakable plastic, to be used for checking temperature in plastic boxes (5.4).

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 or water incubator thermostatically controlled at 36 ± 1 °C, and air incubator thermostatically controlled at 42.0 ± 0.2 °C, preferably with a water jacket.

5.8 Autoclave, with a maximum pressure of 2 atm, electric or gas, and sensitivity of 0.2 bars.

5.9 Drying oven for sterilization up to 170 °C.

5.10 pH meter, precision ± 0.1 pH units.

5.11 Stainless steel forceps.

5.12 Analytical balance, precision ± 1 mg.

5.13 Refrigerator thermostatically controlled at 4 ± 2 °C.

5.14 Petri dishes of borosilicate glass, 9 cm diameter, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic petri dishes.

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

5.16 Borosilicate glass bacteriological culture tubes.

5.17 Wide mouthed jars, of 100-120 ml and 350-500 ml, with stainless steel (or non-corrosive) screw-caps, which can be sterilized in an autoclave.

5.18 Pipettes of borosilicate glass with total volume (blow-out) of 1, 5 and 10 ml capacity, with stainless steel containers for sterilization.

5.19 Automatic pipettes for measuring 0.1 ml, with disposable pre-sterilized tips, or pasteur pipettes of 0.1 ml (approximately).

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

5.21 Durham tubes for collecting gas produced during the glucose fermentation test.

5.22 Screw-capped bottles, of 5 ml (bijout), 100 and 250 ml capacity.

5.23 Small glass tubes (10 x 95 mm).

5.24 Sterile 0.9% sodium chloride solution.

5.25 Slides for microscope.

5.26 Polyvalent-O and polyvalent-H Vi, 09 and Hd *Salmonella* agglutinating sera.

5.27 Bacteriological loops made of 3 mm diameter. A straight wire is also required.

5.28 Heavy wrapping paper.

5.29 Rubber stoppers size no. 0. To be sterilized in wrapped petri dishes at 121 °C for 15 minutes.

5.30 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.

5.31 Filters, 1.25 µm pore size (approximately), and 4.7 cm diameter.

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 90 ml (approximately) into 100 ml jars. Sterilize at 121 °C for 15 minutes. Final pH after autoclaving should be 7.2 ± 0.2. BPW can be stored at room temperature for 1-3 months, if screw-caps are air tight.

6.2 Rappaport-Vassiliadis Medium (RV)

Solution A:

Bacto-tryptone	0.5 g
Sodium chloride	0.8 g
Potassium dihydrogen phosphate	0.16 g
Distilled water	100.0 ml

Solution B:

Magnesium chloride $6H_2O$	40.0 g
Distilled water	100.0 ml

Solution C:

Malachite green oxalate	0.4 g
Distilled water	100.0 ml

Preparation of final medium:

Solution A	100.0 ml
Solution B	10.0 ml
Solution C	1.0 ml

Preparation: The final medium is distributed in test tubes in 10 ml portions (for immediate use) or in 50-100 ml portions (for storage) in screw-capped bottles and sterilized at 121 °C for 15 minutes.

Note: Solution B is prepared by dissolving the whole content of a bottle into the appropriate volume of distilled water, for example: 100 g of $MgCl_2$ $6H_2O$ are dissolved in 250 ml distilled water. Preferably analytical grade magnesium chloride, kept in well sealed bottles, should be used. Solution B can be stored for a long time at room temperature. Solution C can stored in a dark bottle protected from the light. The sterilized final medium can be stored in a refrigerator for at least 3 months. Commercially available dehydrated medium of the same composition can be used.

6.3 Modified Rappaport's Medium (R25)

Solutions A, B and C are prepared as for the RV medium.

Final medium:

Solution A	100.0 ml
Solution B	10.0 ml
Solution C	2.5 ml

Preparation: The final medium is distributed in test tubes in 10 ml portions (for immediate use) or in 50-100 ml portions (for storage) in screw-capped bottles and sterilized at 121 °C for 15 minutes.

6.4 Selenite Cystine Broth (SCB)

Tryptone	5.0 g
Lactose	4.0 g
Disodium phosphate	10.0 g
Sodium acid selenite	4.0 g
L-cystine	0.01 g
Distilled water	1.0 litre

Preparation: Distribute the medium in sterile test tubes in 10 ml portions for immediate use, or in 50 - 100 ml portions in screw-capped bottles (for storage). Steam

for 10 minutes. **DO NOT AUTOCLAVE.** Final pH after preparation should be 7.0 ± 0.2. Store in a refrigerator. Old tubes or bottles in which even a slight evaporation has taken place should not be used. The medium is commercially available.

Important Note: This medium should not be handled by pregnant women.

6.5 Brilliant Green-Deoxycholate Agar (BGDA)

Lab. lemco powder	5.0 g
Bacteriological peptone	10.0 g
Yeast extract	3.0 g
Disodium hydrogen phosphate	1.0 g
Sodium dihydrogen phosphate	0.6 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red	0.09 g
Brilliant green	0.47 mg
Agar	12.0 g
Distilled water	1.0 litre

Preparation: Heat gently to dissolve completely the ingredients. Avoid unnecessary, prolonged heating. **DO NOT AUTOCLAVE.** Add 2.5 grams of sodium deoxycholate to 1 litre of medium. The final pH should be 6.9 ± 0.2. Cool and pour into petri dishes. Plates must be dried until no condensate water is present. Overdried plates should not be used. Plates can be stored in a refrigerator for 2-4 days, until they become excessively dry.

Note: Commercially available dehydrated medium of the same composition can be used.

6.6 Bismuth-Sulfite Agar (BSA)

Beef extract	5.0 g
Peptone	10.0 g
Dextrose	5.0 g
Disodium phosphate	4.0 g
Ferrous sulfate	0.3 g
Bismuth sulfate indicator	8.0 g
Agar	20.0 g
Brilliant green	0.025 g
Distilled water	1.0 litre

Preparation: Heat gently until the medium begins to boil and simmer for 30 seconds to dissolve the agar. Cool and, after efficient agitation for homogenization, pour thickly into plates. Final pH should be 7.6 ± 0.2. Dry the plates before use. Do not use plates that are overdried or more than 1 day old.

Note: The dehydrated medium is commercially available.

6.7 MacConkey Agar (MCA)

Peptone	20.0 g
Lactose	10.0 g
Bile salts	5.0 g
Neutral red	0.075 g
Agar	12.0 g
Distilled water	1.0 litre

Preparation: Heat to dissolve completely. Sterilize by autoclaving at 121 °C for 15 minutes. Mix well before pouring. Final pH should be 7.4 ± 0.2. Dry the surface of the gel before inoculation.

Note: Commercially available dehydrated media of MacConkey agar or MCA formulae can be used.

6.8 Hektoen Enteric Agar

Protesose peptone	12.0 g
Yeast extract	3.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
Bile salts No. 3	9.0 g
Sodium chloride	5.0 g
Sodium thiosulphate	5.0 g
Ammonium ferric citrate	1.5 g
Acid fuchsin	0.1 g
Bromothymol blue	0.065 g
Agar	14.0 g
Distilled water	1.0 litre

Preparation: Soak ingredients for 10 minutes. Heat gently and allow to boil for a few seconds to dissolve the agar. DO NOT AUTOCLAVE. Final pH 7.5 ± 0.2. Cool to 60° C and pour plates.

Note: The medium is commercially available.

6.9 Salmonella Shigella Agar

Lab-Lemco powder	5.0 g
Peptone	5.0 g
Lactose	10.0 g
Bile salts	8.5 g
Sodium citrate	10.0 g
Sodium thiosulphate	8.5 g
Ferric citrate	1.0 g
Brilliant green	0.00033 g
Neutral red	0.025 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Soak ingredients for 10 minutes. Bring to the boil with frequent agitation and allow to simmer gently to dissolve the agar. DO NOT AUTOCLAVE. Cool to about 60° C, mix, and pour in Petri dishes. Final pH 7.0 ± 0.2.

Note: The medium is commercially available.

6.10 Kligler Iron Agar (KIA)

Meat extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Lactose	10.0 g
Dextrose	1.0 g
Ferric citrate	0.3 g
Sodium thiosulfate	0.3 g
Sodium chloride	5.0 g
Phenol red	0.05 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by heating and dispense in tubes. Sterilize at 121 °C for 15 minutes. Final pH after autoclaving should be 7.4 ± 0.2. Allow to set as slopes with 2 cm (approximately) butts.

Note: The medium is commercially available.

6.11 Blood Agar Base (BAB)

Beef heart, infusion from	500.0 g
Bacto-tryptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients completely by heating. Distribute in small tubes (10 x 95 mm) and sterilize at 121 °C for 15 minutes. Final pH after autoclaving should be 7.0 ± 0.2. Sterile rubber stoppers replace the cotton plugs after sterilization. Small screw-capped bottles (bijout) can also be used instead of tubes. Caps should be kept loose during sterilization.

Well sealed tubes or bottles can be stored at room temperature until use for inoculation and shipping of cultures.

Note: The medium and similar blood agar base media are commercially available.

6.12 Simmons Citrate Agar

Magnesium sulfate	0.2 g
Monoammonium phosphate	1.0 g
Dipotassium phosphate	1.0 g

Sodium citrate	2.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Bromothymol blue	0.08 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by heating. Distribute in tubes or bottles and sterilize at 121 °C for 15 minutes. Final pH after autoclaving should be 6.8 ± 0.2. Allow to cool in semi-slant position.

Note: Dehydrate medium is commercially available.

6.13 Decarboxylase Test Media

Basal medium

Yeast extract	3.0 g
Dextrose	1.0 g
Bromcresol purple (1,6% aq. sol.)	1.0 ml
Distilled water	100.0 ml

Preparation: The basal medium is divided in 4 portions. L-lysine 0.5% (w/v) is added to 3 portions of the medium and all 4 portions are sterilized at 121 °C for 15 minutes. Final pH after autoclaving should be 6.1 ± 0.2. Prior to use, 3 ml of the complete medium are transferred to sterilized small test tubes (approx. 10 x 95 mm) with rubber stoppers. A tube with the basal medium (without L-lysine) should also be used in each series of test.

6.14 Urea - Phenylalanine Broth (Urea-PPA)

(For the detection of urease and phenylalanine diaminase)

Yeast extract	1.0 g
Ammonium sulfate (cryst.)	2.0 g
Sodium chloride	3.0 g
Disodium phosphate	1.2 g
Sodium dihydrogen phosphate	0.8 g
DL-b phenylalanine	2.0 g
Distilled water	97.0 ml

Preparation: Dissolve by gentle warming. When solution cools to room temperature, add 5 g of urea and 3.5 ml of phenol red solution (0.5 g phenol red, 20 ml 0.1 N NaOH, and 230 ml distilled water). The complete medium is sterilized by filtration through a Seitz filtre, and distributed under sterile conditions in 10 ml portions, preferably in screw-capped bottles or well sealed sterile containers. To use the medium, prepare 1:10 dilutions (1 part of medium plus 9 parts sterile distilled water) and distribute in small test tubes (approx. 10 x 95 mm).

Note: The reagents for the PPA reaction appear in section 6.18.

6.15 Tryptone Water

Tryptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1.0 litre

Preparation: Distribute in test tubes in portions of no less than 5-6 ml. Sterilize at 121 °C for 15 minutes. Final pH after autoclaving should be 7.5 ± 0.2.

6.16 MR-VP Medium

Peptone	5.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1.0 litre

Preparation: Distribute in tubes and sterilize at 121 °C for 10 minutes. Final pH after autoclaving should be 7.5 ± 0.2.

6.17 Beta-Galactosidase or ONPG Test

It is recommended to use commercially available disks for ONPG test.

6.18 Carbohydrate Fermentation Media

Basal medium

Peptone	10.0 g
Beef extract	2.0 g
Sodium chloride	5.0 g
Bromothymol blue solution (1 gram bromothymol blue and 25 ml, 0.1 NaOH, in 475 ml distilled water)	12.0 ml
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by heating. Final pH after autoclaving 7.0 ± 0.2. Divide the basal medium into 4 portions and add to each the following sugars: glucose 1%, salicine 0.5%, and lactose 1%. After adding glucose, add the inverted durham tubes. Sterilize at 121 °C for 10 minutes.

6.19 Test Reagents

6.19.1 Kovac's indole reagent

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

Preparation: Dissolve the benzaldehyde in amyl alcohol and add hydrochloric acid. The reagent should be yellow. Only high grade analytical ingredients should be used.

6.19.2 Barrit's reagents for Voges-Proskauer test

1. Alpha naphthol in absolute alcohol	0.6%
2. Potassium hydroxide	40.0%

Test procedure: To 1 ml of a 48-h culture in MR-VP medium add 0.6 ml of solution no. 1 and 0.2 ml of solution no. 2. Shake well after the addition of each reagent. Read after 5 minutes.

6.19.3 Methyl red reagent

Methyl red	0.1 g
Ethyl alcohol	300.0 ml

Preparation: Dissolve the dye in the alcohol and then add distilled water to 500 ml.

Test procedure: Add 5-6 drops in 5 ml of a 24-h culture in MR-VP medium. Read immediately.

6.19.4 Phenylalanine reagents (for PPA test, 6.13)

Hydrochloric acid 0.1 N
Ferric chloride 10.0% (aqu. sol.)

Test procedure: After reading the urease test, add a few drops of 0.1 N HCl until colour appears. Add a few drops of 10% $FeCl_3$ and shake. A dark green colour indicates a positive reaction.

6.20 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 (5.30 or 9.2). The sterilization can be omitted if the solution is prepared every 2-3 weeks and kept in the refrigerator.

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.

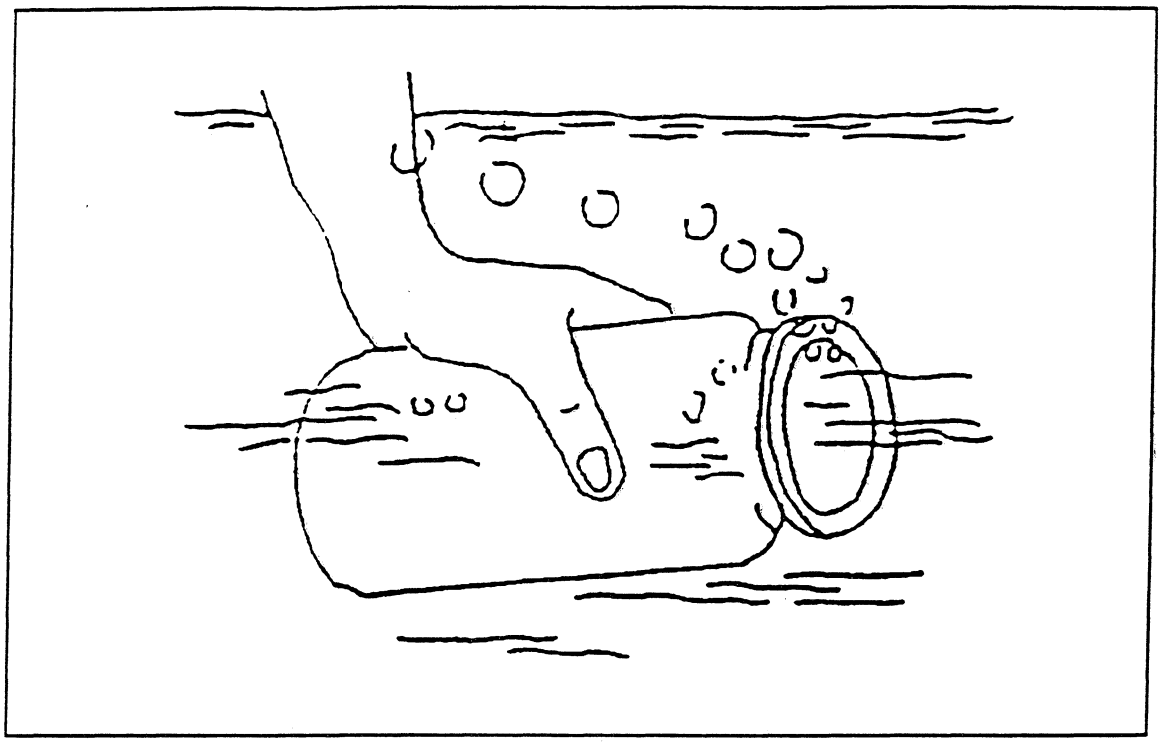

Figure 3. Subsurface sampling by hand.

7.2 Sampling of Subsurface Water

Lower the sterile subsurface sampler 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 the raw waste 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. WASHING AND STERILIZATION OF GLASSWARE AND EQUIPMENT

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 times 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 alcohol 95°. After adding an appropriate volume of alcohol to each bottle, they are thoroughly agitated, keeping its stopper in place. After 2-3 alcohol washings, the bottle is well drained and kept in an oven at 40-50 °C temperature until complete evaporation of the alcohol. The stopper is also placed on a sterile petri dish to dry.

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

Forceps are wrapped in paper and sterilized at 121 °C for 15 minutes. If they have plastic parts which do not tolerate repeat exposure to high temperature, or in case of emergency, boil them for at least 20 minutes in distilled water. Salmonellae and non-sporing organisms do not withstand such treatment.

8.2.7 Culture tubes

Close culture tubes for enrichment media with cotton plugs or metal caps, and sterilized them in an autoclave at 121 °C for 15 minutes, or in a dry oven at 160 °C for 2 hours, or at 170 °C for 1 hour. Follow the same procedure for sterilization of the small tubes (10 x 95 mm) used for urease and decarboxylase tests.

9. TEST PROCEDURE

9.1 Principles of Methodology

A direct isolation or counting of salmonellae from seawater is impracticable because:

a. It is not possible to inoculate large volumes of seawater on the surface of selective agar plates.

b. The number of salmonellae is so small compared to that of the non salmonellae enteric bacteria that it is impossible to develop and isolate characteristic on selective media.

c. Seawater is an hostile, inimical environment for enterobacteria. Consequently, salmonellae are very often sub-lethally injured and thus unable to grow on selective and even in enrichment media.

It has been observed that a pre-enrichment step in a non-selective medium, followed by an enrichment technique and plating on selective media, results in the highest recovery of salmonellae.

Isolation and counting of salmonellae by this improved technique requires inoculation of large volumes of seawater into the pre-enrichment media. Apart from the possible interference of seawater constituents during the pre-enrichment phase, the use of large volumes of media and high capacity jars and incubators is costly and time consuming. To overcome these difficulties and to obtain a more predictable result, a concentration technique can be used, by filtering an appropriate volume of seawater through a membrane filter of the appropriate pore size. After filtration, the membrane filter is transferred into small jars containing the pre-enrichment medium; by filtering decimal dilutions of seawater and culturing the corresponding membranes, the method

becomes quantitative. Similarly to the multiple test tube technique, the tables of the Most Probable Number can be used to calculate the concentration of salmonellae in a given volume of seawater.

From the numerous enrichment media available, the Muller-Kauffmann tetrathionate broth, incubated at 43 °C, was recommended as the medium of choice for *Salmonella* isolation from meat and meat products (Anon. 1975). However, a series of publications have shown that a slight modification of Rappaport's medium (Rappaport *et al.*, 1956), the so-called R25 medium (Vassiliadis *et al.*, 1970), incubated at 37 °C, was superior or at least equally efficient to the tetrathionate broth for isolation of *Salmonella*.

The more significant advantages of the R25 medium over the tetrathionate broth are: 1) it is easier to prepare, 2) it can be stored for at least 3 months, and 3) it is much more economical.

Further investigations by Vassiliadis and co-workers (Vassiliadis *et al.*, 1976, 1978, 1979a, 1979b, 1981, 1984; Papadakis and Efstratiou, 1980; Xirouchaki *et al.*, 1982) showed that a more drastic modification of Rappaport's medium, the so-called Rappaport-Vassiliadis (RV) medium, was definitely superior to Muller-Kauffmann tetrathionate broth for *Salmonella* isolation. These findings have been also confirmed by others (Alcaide *et al.*, 1982; van Schothorst and Renaud, 1983; Fricker *et al.*, 1983; Tongpin, Beumer and Kampelmacher, 1984).

An additional advantage of the RV medium over the other enrichment media, in addition to those mentioned for the R25 medium, is that the RV medium is more effective in inhibiting competing germs and especially those producing *salmonella*-like colonies on the selective plates (Vassiliadis, 1983).

9.2 Qualitative Test for Salmonellae in Seawater

One litre of seawater is filtered through one or more (in case of blockage) membrane filters of 0.45 µm pore size, using a sterilized filter apparatus. If seawater is turbid and blocks quickly the membrane filters, it is recommended to prefilter the sample through a filter pad.

The membrane filters, including those used for prefiltration, are transferred with sterile forceps into a jar containing 80 ml buffered peptone water for the pre-enrichment step. The membranes are completely immersed into the medium and the jar is incubated at 37 °C for 18-20 hours.

With a sterile (tip) automatic or pasteur pipette, 0,1 ml are transferred to 10 ml enrichment RV medium, which is incubated at 42° C for 24 and 48h. However if the water sample is contaminated with *Pseudomonas aeruginosa* incubation at 43° C is recommended or verification of great number of typical colonies is necessary. The colonies of *Ps. aeruginosa* are very similar to the *Salmonella* colonies, but at 43° C this organism is relatively inhibited though salmonellae grow freely (Vassiliadis *et al.* 1992).

After incubation, brilliant green-deoxycholate agar plates and Hectoen agar plates are streaked at 24 and 48 hours intervals. The plates are incubated at 37 °C for 24 hours.

Typical salmonellae colonies are 2-3 mm diameter, smooth, red coloured, and surrounded by bright red medium. Three to four of those colonies are inoculated into tubes with Kligler's medium and then incubated at 37 °C for 16-24 hours. The typical reactions of salmonellae on Kligler's medium are:

Butt = gas and blackening (H_2S production).
Slant = abundant growth without significant change of the original colour of the medium (red).

As a few strains of salmonellae may be anaerogenic or not H_2S producers, it is recommended not to discard those tubes without gas or without blackening.

Although the technique described efficiently inhibits Proteus strains to flourish, it is recommended to inoculate Kligler's medium up to 5-10 mm from the top of the slant. Proteus strains causing the same reactions that Salmonellae, often swarm to the top end, a phenomenon easily recognized by careful observation.

From typical 24-hour cultures on Kligler's medium, a slide agglutination test is performed using polyvalent-0 and polyvalent-H *Salmonella* agglutinating sera. Cultures with typical reactions and positive sero-tests are streaked on MacConkey's agar plates, from where 1-2 isolated, smooth colonies are transferred to blood agar base slopes in small tubes, and incubated at 37 °C for 18-24 hours. The incubated tubes are shipped to the nearest *Salmonella* Reference Centre (or other laboratory) for further typing.

When suspect colonies give no typical reactions, then a series of differential tests can be applied (see Table 1).

9.3 Alternative Qualitative Test for Salmonellae in Seawater

The procedure is the same as described in the section above (9.2), except for the use of the R25 medium and a temperature of incubation of 37 °C for 24 and 48 hours.

Some authors (Harvey *et al.*, 1979) have found this technique better for *Salmonella* isolation from water. Moreover there is no need for an extra incubator at 43 °C. Nevertheless the use of RV medium and an incubation temperature of 43 °C always results in a better inhibition of competing microbes and especially those producing *Salmonella*-like colonies on selective plates.

9.4 Qualitative Test for *S. typhi* in Seawater

One litre of seawater is filtered according to the previously described technique (9.2). Selenite cystine broth (SCB) is used as enrichment medium, instead of RV medium. The broth is inoculated with 1 ml (instead of 0.1 ml) from the BPW jar and incubated at 37 °C.

After 24 and 48 hours of incubation the enrichment medium is subcultured using a loop onto bismuth sulphite agar (BSA) and/or SS agar plates. All plates are incubated at 37 °C for 24 hours, and suspect colonies are transferred to Kligler's tubes for differential diagnosis. If negative, BSA plates are incubated for another 24 hours.

S. typhi colonies grown on SS medium are colourless, non-lactose fermenters, and of butyric consistency. On freshly prepared bismuth sulfite agar (no more than 2 days old) *S. typhi* develops dark colonies with a surrounding halo of black or brown colour of the medium. On Kligler's medium, *S. typhi* is anaerogenic with a faint amount of blackening. For serology identification see the last section of this method.

9.5 Quantitative Tests for Salmonellae in Seawater

When counting small numbers of salmonellae per litre, the following portions of seawater can be filtered: 1 x 500 ml, 5 x 100 ml, and 5 x 10 ml.

After filtration all membranes are transferred to the properly labelled jars with 80 ml (approximately) BPW, and then incubated at 37 °C for 18-20 hours. Using an automatic or a pasteur pipette, transfer 0.1 ml from the BPW cultures to properly labelled tubes with 10 ml RV medium. The enrichment tubes are incubated at 42 °C, and after 24 and 48 hours brilliant green-deoxycholate agar plates are streaked and incubated at 37 °C for 24 hours (ISO 1990). However, when *Ps. aeruginosa* is present, especially in high numbers, the incubation of RV medium should be at 43° C. *Salmonella*-like colonies are subcultured into Kligler iron agar tubes for biochemical and serological identification. The water portions giving a positive result for salmonellae are recorded and the MPN of salmonellae per litre is calculated from the MPN tables (Annex 1). For example, if salmonellae were isolated from the 500 ml filtration, from 3 of the 100 ml portions, and from 1 of the 10 ml portions, then the MPN of salmonellae per 1 litre of seawater is 11 (Table 4, Annex 1).

The combination of water portions to be filtered depends on the laboratory facilities, the expected concentration of salmonellae in seawater, and the desired accuracy of the results. Tables 1 to 5 in Annex 1 can be used as a guideline. The combinations considered in Table 4 (11 filtrations) and Table 5 (9 filtrations) are generally appropriate.

Salmonellae determination in turbid seawater, with high concentration of suspended particles or algae, require a pre-filtration step using a good quality filter paper or a membrane filter. It is important that these pre-filters should be cultured together with the corresponding membrane filters of 0.45 µm porosity. If the water turbidity is low, the filtration step may be completed using 2-3 membrane filters (0.45 µm), avoiding the laborious pre-filtration step.

9.6 Alternative Quantitative Test for Salmonellae

The procedure is the same as that described in section 9.4 above, except for the use of the R25 medium and a temperature of incubation of 37 °C for 24 and 48 hours.

9.7 Quantitative Test for *S. typhi*

The procedure is the same as that described in section 9.3 above, except that several seawater portions will be filtered and analyzed. The seawater volumes to be filtered will vary according to the expected concentrations of *S. typhi*. Tables 1 to 5 in Annex 1 can be used as a guideline.

10. ISOLATION AND ENUMERATION OF SALMONELLAE FROM SEWAGE

10.1 Qualitative Test

As sewage is turbid and contains numerous suspended particles, a prefiltration step becomes usually necessary (9.3). The volume to be filtered depends on the expected density of salmonellae in the water analyzed and may vary from 10 to 1000 ml. All the filters used during the prefiltration and filtration steps should be introduced in the same BWP jar; the analytical procedure then proceeds as described in section 9.2, or in section 9.3 for the alternative enrichment technique.

The same filtration and pre-enrichment procedure is applied for *S. typhi* isolation. However, selenite cystine broth (SCB) is used as enrichment medium, instead of RV or R25 media, and incubation is done as described in section 9.4. Typical *S. typhi* colonies on selective plates are further identified as described in section 11.

10.2 Alternative Qualitative Test for *Salmonella*

As filtration of sewage is very often difficult, it may be necessary to use an alternative technique for *Salmonella* detection. Moor's swab has been proved efficient and it makes unnecessary to sample and transport large volumes of sewage into the laboratory, where filtration may be difficult. To prepare the swab, an ordinary gauze of 15 x 45 cm size is folded many times and tied up in the middle by a good quality string or wire. After it has been properly wrapped in a good quality paper, it is sterilized in an autoclave or an air sterilizer. To use it, the cover paper is torn up with care and the swab is fixed by the string or wire to the end of a good quality wire properly secured (a string is not recommended because it is attacked by rats or mice). An appropriate sinker (a lead weight, or even a suitable stone) is also fixed in order to keep the swab always immersed in the sewage, even in case of a strong current.

After 24 hours, and preferably after 48 hours, the swab is collected and detached from the wire and the sinker. The swab is left to drain (without squeezing it) and is then immediately transferred to the laboratory in an empty sterile jar of 350-500 ml. At the laboratory, 180-200 ml of BPW are added to the jar and it is incubated as described in section 9.3. For *S. typhi* selection, SCB is used as enrichment medium, instead of RV or R25 media, and incubation is done 37 °C for 24 and 48 hours, as described in section 9.4.

10.3 Quantitative test

The most probable number of salmonellae per litre of sewage can be found by filtering 3 (or 5) portions of 100, 10 and 1 ml and incubating the corresponding filters in separate cultures. As sewage usually contains numerous salmonellae, smaller

volumes (eg. 10, 1, 0.1) are normally recommended for use. In this case, the direct inoculation of sewage into a 10 times (approximately) larger volume of BPW is more simple method than the filtration technique. Three portion of 10 ml sewage are transferred to 3 jars containing 90 ml BPW, and 3 portions of 1 ml and 3 portions of 1 ml from a 1/10 dilution are inoculated into tubes containing 10 ml BPW. Incubation, subculture into enrichment media, and plating on selective media are the same as described in section 9.5. The same technique is applied to quantify *S. typhi*, but SCB is used as enrichment medium, as described in section 9.4.

11. BIOCHEMICAL AND SEROLOGICAL IDENTIFICATION OF SUSPECT CULTURES

11.1 Typical Biochemical and Serological Reactions

The majority of *Salmonella* strains give typical reactions on Kligler's medium. However it must be mentioned that anaerogenic strains (no gas in the butt) do occur, an important example being *S. typhi*. The same holds true for H_2S production. Some strains are not H_2S producers (blackening of the butt) among them *S. paratyphi A, S. pullorm, S. senftenberg*, and even a few *S. typhimurium*. For that reason, cultures with atypical reactions (no gas or no blackening) are examined using either an agglutination test against polyvalent-O and polyvalent-H *Salmonella* sera and/or further biochemical tests.

Moreover *Proteus* and *Citrobacter* strains may resemble *Salmonella* appearance on Kligler's medium. It is obvious that these cultures do not react with polyvalent-O and polyvalent-H antisera, although *Proteus* species agglutinate sometimes. *Citrobacter* cultures may also agglutinate with anti-Vi serum but the Kligler's medium provides an immediate recognition of *Proteus* cultures. A rapid positive urease test in the urea-PPA medium (it turns red within a few hours of incubation) and the PPA positive reaction after 24 hours are characteristic of *Proteus* species (Table 1). *Citrobacter* cultures may be excluded from *Salmonella* species by a negative lysine decarboxylase test and positive ONPG (some salmonellae are also positive) and occasionally positive urease and salicine tests. *Shigella* strains do not stand the techniques described for salmonellae, but rather occasionally they may pass through SCB and then grow on SS plates. These very rare cultures can be easily recognized by the different Kligler's appearance and the other tests cited in Table 1. *Escherichia, Klebsiella, Enterobacter* and *Serratia* give different, rather characteristic reactions on Kligler's medium, but usually do not show *Salmonella*-like colonies on selective plates. *Yersinia enterocolytica*, like *Shigella*, do not stand the enrichment media nor does it grow on BGDA and BSA. *Pseudomonas* colonies may be confused with salmonellae, but their Kligler's reaction and an oxidase test will differentiate them easily (see also Table 1).

11.2 Slide Agglutination Test

Typical or suspect salmonellae colonies must be examined by the slide agglutination test. Using a pasteur pipette, a drop of sodium chloride solution (5.24) is transferred onto a microscope slide. A thick suspension of the suspect culture is made with a straight wire from a 24 hour Kligler's slope. The progress of the agglutination process is observed after the addition of the serum, with the aid of a magnifying lens.

Freshly isolated *S. typhi* cultures may not agglutinate with polyvalent-O antisera, but they react with anti-Vi specific serum. A suspension of the culture is heated for 15 minutes in boiling water and, after it cools to room temperature, the agglutination test is repeated using polyvalent-O or specific anti-09 serum.

11.3 Shipment to Reference Centres

All positive or suspect *Salmonella* cultures are streaked on MCA, and 1-3 smooth typical colonies are transferred onto blood agar slopes in small test tubes with rubber stoppers. Simultaneously a new Kligler's agar tube is inoculated and all tubes are incubated at 37 °C for 20-24 hours. If Kligler's reaction is typical, meaning that no contamination has taken place during subculturing, the incubated tubes are packed properly and shipped to the nearest *Salmonella* Reference Centre, or other laboratory, for complete identification.

Important note: The methods described allow for the best recovery and enumeration of salmonellae. However by subculturing RV and R25 media only during 24 hours (and not 48 hours) the methods are abe to recover approximately 95% of *Salmonella* isolations that would be obtained when using 48 hours incubation (Vassiliadis, 1984; personal communication). Therefore laboratories with restricted facilities and staff can simplify the methods described by subculturing from enrichment RV and R25 media after 24 hours.

Table 1. Biochemical differentiation tests for salmonellae.

Species Genera	Reaction on Kligler's medium			Test											
	Colour Slant/Butt	Blackening	Gas	Indole	Urea	PPA	Lysine	ONPG	Citrate	Glucose *****	Lactose	Salicine	MR	VP	
S. typhi	Red/yellow	+(-)	-	-	-	-	+	-	-	-	-	-	+	-	
Other Salmonellae	Red/yellow	+ + +(-)	+(-)	-	-	-	+	-(+)	+(-)	+(-)	-	-(+)	+	-	
Citrobacter	Red/yellow	+ + +	+	-	-(+)	-	-	+	+	+	+/X	d	+	-	
Shigellae	Red/yellow	-	-	-/+	-	-	-	d	-	-	-	-	+	-	
Escherichiae	Yellow/yellow	-	+(-)	+	-	-	d	+	-	+	+	d	+	-	
Klebsiella	Yellow/yellow	-	+ +	-	X	-	d	+	+	+	+	+	-	+	
Enterobacter	Yellow/yellow	-	+ +	-	-	-	d	+	+	+	-/X	+	-	+	
Serratia	Red(Y)yellow	-	-	-	-	-	+	+	+	d	-/X	+	-	+	
Proteus	Red/yellow	+/-	+(-)	-(-)	+	+	-	-	d	+(-)	-	d	-	-(+)	
Yersinia	Red yellow or Yellow/yellow	-	-	-+	+	-	+	+	-	-	-		+	-	
Pseudomonas	Red/red	-	--	-	d	-	-	-	+	-	-	-	-	-	

Note: (+/-) = Positive or negative reactions, or vice versa; + (-) or - (+) = Positive reactions but occasionally strains negative, or vice versa; d = Different reactions; X = Delayed reactions.

12. TEST REPORT

Table 2. Salmonellae 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 pre-filtration hour: ___ . ___ day: ___ / ___ / ___
6. Time of filtration hour: ___ . ___ day: ___ / ___ / ___

8. Qualitative and Quantitative Test for Salmonellae (including *S. typhi*)

Culture medium: _____

		Sample volume	Positive reactions
Start pre-enrich.:	hour: ____ day: __/__/__		
End pre-enrich.:	hour: ____ day: __/__/__	500	___
Start incub.:	hour: ____ day: __/__/__	100	___ ___ ___ ___ ___
End incubation:	hour: ____ day: __/__/__	10	___ ___ ___ ___ ___
Kligler incub.:	hour: ____ day: __/__/__		
End incubation:	hour: ____ day: __/__/__		

Number of positive cultures: _____

Test result:
_____ Salmonellae/litre

Serological and differential tests:
_____
_____

95% confidence interval:
(_____ ; _____)

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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ANNEX 1

TABLES FOR DETERMINING THE MOST PROBABLE NUMBER (MPN) OF *SALMONELLA* ORGANISMS PRESENT IN 1000 ML OF SEAWATER

These tables indicate the estimated number of salmonellae per 1000 ml of water, corresponding to various combinations of positive and negative results in the portions used for the test.

Table 1. MPN and 95% confidence limits for various combinations of positive and negative results when five 100-ml portions are used.

No. of tubes giving positive reactions out of	MPN	95% Confidence limits	
5 of 100 ml		Lower	Upper
0	< 2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	> 16.0	8.0	Infinite

Table 2. MPN and 95% confidence limits for various combinations of positive and negative results when one 500-ml portion and five 100-ml portions are used.

No. of tubes giving positive reactions out of		MPN	95% confidence limits	
1 of 500 ml each	5 of 100 ml each		Lower	Upper
0	1	1	<0.5	4
0	2	2	<0.05	6
0	3	4	<0.5	11
0	4	5	1	13
1	0	2	<0.5	6
1	1	3	<0.5	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40

Table 3. MPN and 95% confidence limits for various combinations of positive and negative results when five 100-ml portions, five 10-ml portions, and five 1-ml portions are used.

No. of tubes giving positive reactions out of			MPN	95% confidence limits	
5 of 100 ml each	5 of 10 ml each	5 of 1 ml each		Lower	Upper
0	0	0	2	<0.5	7
0	1	0	2	<0.5	7
0	2	0	4	<0.5	11
1	0	0	2	<0.5	7
1	0	1	4	<0.5	11
1	1	0	4	<0.5	11
1	1	1	6	<0.5	15
1	2	0	6	<0.5	15
2	0	0	5	<0.5	13
2	0	1	7	1	17
2	1	0	7	1	17
2	1	1	9	2	21
2	2	0	9	2	21
2	3	0	12	3	28
3	0	0	8	1	19
3	0	1	11	2	25
3	1	0	11	2	25
3	1	1	14	4	34
3	2	0	14	4	34
3	2	1	17	5	46
3	3	0	17	5	46
4	0	0	13	3	31
4	0	1	17	5	46
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	3	0	27	9	80
4	3	1	33	11	93
4	4	0	34	12	96
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	114
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	154
5	2	0	49	17	126
5	2	1	70	23	168
5	2	2	94	28	219
5	3	0	79	25	187
5	3	1	109	31	253
5	3	2	141	37	343
5	3	3	175	44	503
5	4	0	130	35	302
5	4	1	172	43	486
5	4	2	221	57	698
5	4	3	278	90	849
5	4	4	345	117	999
5	5	0	240	68	754
5	5	1	348	118	1005
5	5	2	542	180	1405
5	5	3	918	303	3222
5	5	4	1609	635	5805

Table 4. MPN and 95% confidence limits for various combinations of positive and negative results when one 500-ml portion, five 100-ml portions, and five 10-ml portions are used.

No. of tubes giving positive reactions out of			MPN	95% confidence limits	
1 of 500 ml each	5 of 100 ml each	5 of 10 ml each		Lower	Upper
0	0	1	1	<0.5	4
0	0	2	2	<0.5	6
0	1	0	1	<0.5	4
0	1	1	2	<0.5	6
0	1	2	3	<0.5	8
0	2	0	2	<0.5	6
0	2	1	3	<0.5	8
0	2	2	4	<0.5	11
0	3	0	3	<0.5	8
0	3	1	5	<0.5	13
0	4	0	5	<0.5	13
1	0	0	1	<0.5	4
1	0	1	3	<0.5	8
1	0	2	4	<0.5	11
1	0	3	6	<0.5	15
1	1	0	3	<0.5	8
1	1	1	5	<0.5	13
1	1	2	7	1	17
1	1	3	9	2	21
1	2	0	5	<0.5	13
1	2	1	7	1	17
1	2	2	10	3	23
1	2	3	12	3	28
1	3	0	8	2	19
1	3	1	11	3	26
1	3	2	14	4	34
1	3	3	18	5	53
1	3	4	21	6	66
1	4	0	13	4	31
1	4	1	17	5	47
1	4	2	22	7	69
1	4	3	28	9	85
1	4	4	35	12	101
1	4	5	43	15	117
1	5	0	24	8	75
1	5	1	35	12	101
1	5	2	54	18	138
1	5	3	92	27	217
1	5	4	161	39	>450

Table 5. MPN and 95% confidence limits for various combinations of positive results when three 100-ml portions, three 10-ml portions, and three 1-ml portions are used.

No. of tubes giving positive reactions out of			MPN	95% confidence limits	
3 of 100 ml each	3 of 10 ml each	3 of 1 ml each		Lower	Upper
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	149
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	379
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	2	1100	150	4800

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