REGIONAL SEAS

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Determination of mesophylic aeromonads in sea water by the membrane filtration (MF) culture method

Reference Methods For Marine Pollution Studies No. 69

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.

The first substantive issue of Reference Methods for Marine Pollution Studies No. 69 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 mesophilic aeromonads in coastal bathing waters of temperate and tropical seas. 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.

Aeromonas species are known for their importance as pathogens for fish, reptiles and warm-blooded animals (Janda and Duffey, 1988). In recent years the significance of *Aeromonas* species is receiving increasing attention as human pathogens (Burke *et al.*, 1984). The aquatic environment is considered the major source of infection (Burke *et al.*, 1984; Joseph *et al.*, 1979). Reports from many parts of the world suggest that *Aeromonas* species cause an acute self-limiting diarrhoeal illness in man (Barer *et al.*, 1986; Mascher *et al.*, 1989).

Mesophilic aeromonads are widely distributed in aquatic environments (Joseph *et al.*, 1988), with densities depending on pollution, trophic state, and temperature (van der Kooij, 1988). The exclusive use of faecal bacterial indicators underestimate the risk of infection posed by opportunistic pathogens like motile aeromonads and it is thus advisable to monitor also these bacteria in bathing beaches.

3. DEFINITION

The taxonomy of aeromonads has been constantly changing (Popoff, 1984). Major taxonomic studies conducted over the last years have provided some clarification of the systematics of aeromonads with respect to the number of DNA hybridization groups (genospecies) and phenotypic species (phenospecies) which currently exist among aeromonads (Carnahan *et al.*, 1991).

Aeromonads are members of the family Vibrionaceae and share a number of properties with other genera in this family (Janda and Duffey, 1988): they are oxidase-positive, motile by means of a polar flagellum, facultatively anaerobes, and utilize D-glucose as their sole source of energy. Microscopically, *Aeromonas* species are gram-negative bacilli, usually 1-3 µm in length. Rods are generally straight in appearance and do not appear as curved bacilli, a trait more typically observed in *Vibrio* species (Janda and Duffey, 1988).

The optimum growth temperature for motile *Aeromonas* species is 28 °C (Popoff, 1984). Some strains can grow at 5 °C. The maximum temperature range at which growth occurs is usually 38-41 °C.

The following physiological tests are positive for motile *Aeromonas* species (Popoff, 1984): catalase, starch hydrolysis, lecithinase, phosphatase, ADH, hydrolysis of o-nitrophenyl-ß-D-galactopyranoside (ONPG), growth in nutrient broth without NaCl, and fermentation of trehalose, fructose, galactose and dextrin. The following tests are negative (Popoff, 1984): Gram stain, pectinase, tryptophan and phenylalanine deaminase, growth on cetrimide agar, growth in nutrient broth containing 5% NaCl, and acid production from sorbose, erythritol, and raffinose.

Mesophilic *Aeromonas* species exhibit a number of consistent biochemical features that aid in their identification and separation from other phenotypically similar organisms (Janda and Duffey, 1988): they are oxidase-positive and indole positive, they are facultative anaerobes, they grow on salt-free media, and they are resistant to vibriostatic agent 0/129. A battery of 10 to 15 tests should be performed to identify all *Aeromonas* species linked to human disease (Table 1) (Janda, 1991).

4. PRINCIPLES

Aeromonas can be isolated from environmental sources on numerous types of media with varying degrees of success. Generally it is not a difficult organism to culture, but its separation from other organisms in mixed populations and its eventual identification is more complex and difficult (Joseph *et al.*, 1988). Following growth on a suitable agar medium, suspect colonies should be subjected to a test procedure

hydrophila, *A. sobria* or *A. caviae*) is based on sugar and other biochemical reactions (Popoff, 1984). These can be performed using standard biochemical and bacteriological reactions, or the simplified AH medium (Kaper *et al.*, 1979).

From the seawater samples taken under sterile conditions, a dilution series is set up according to the number of mesophilic aeromonads expected in the water sample. Portions of this dilution series are filtered through 0.45 µm pore size sterile membrane filters. The membrane filters are placed on the surface of mADA/0129 agar (Alonso and Garay, 1989) contained in petri dishes and incubated at 30 °C for 24 hours. Dextrine fermentation will cause colonies of mesophilic aeromonads to exhibit a characteristic yellow or yellow-orange colour.

Suspect and doubtful colonies can be tested by using the following protocols, tests and media: oxidase, growth on TSI agar and *A. hydrophila* confirmation medium. To be considered as mesophilic aeromonads, strains must show the following biochemical features in AH medium: ornitine decarboxylation (-), H_2S (-), motility (+) and indole (+); those strains should be further tested for acid and gas production from glucose and esculine hydrolysis. The incubation temperature of all the identification tests is 30 °C, the readings being performed after 48 hours.

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 2).

5.3 Subsurface sampler of the type shown in Figure 3, 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 (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 incubator thermostatically controlled at 30 ± 1 °C, preferably with a water jacket.

Table 1. Properties useful in the differentiation of mesophilic *Aeromona*

Property	*Aeromonas* species			
	A. hydrophila	*A. sobria*	*A. caviae*	
Esculin hydrolysis	+	-	+	
Growth in KCN broth	+	-	+	
Acid from salicin	+	-	+	
Acid from arabinose	+	-	+	
Gas from glucose	+	+	-	
Acetylmethylcarbinol	+	+	-	
Gluconate oxidation	+	+	-	
Arginine dihydrolase	+	+	+	
Lysine decarboxylase	+	+	-	
B-Hemolysis	+	+	-	
Lecithinase	+	+	-	
Elastase	+	V	-	

Note: (+) = positive; (-) = negative; NA = data not available; V = variable

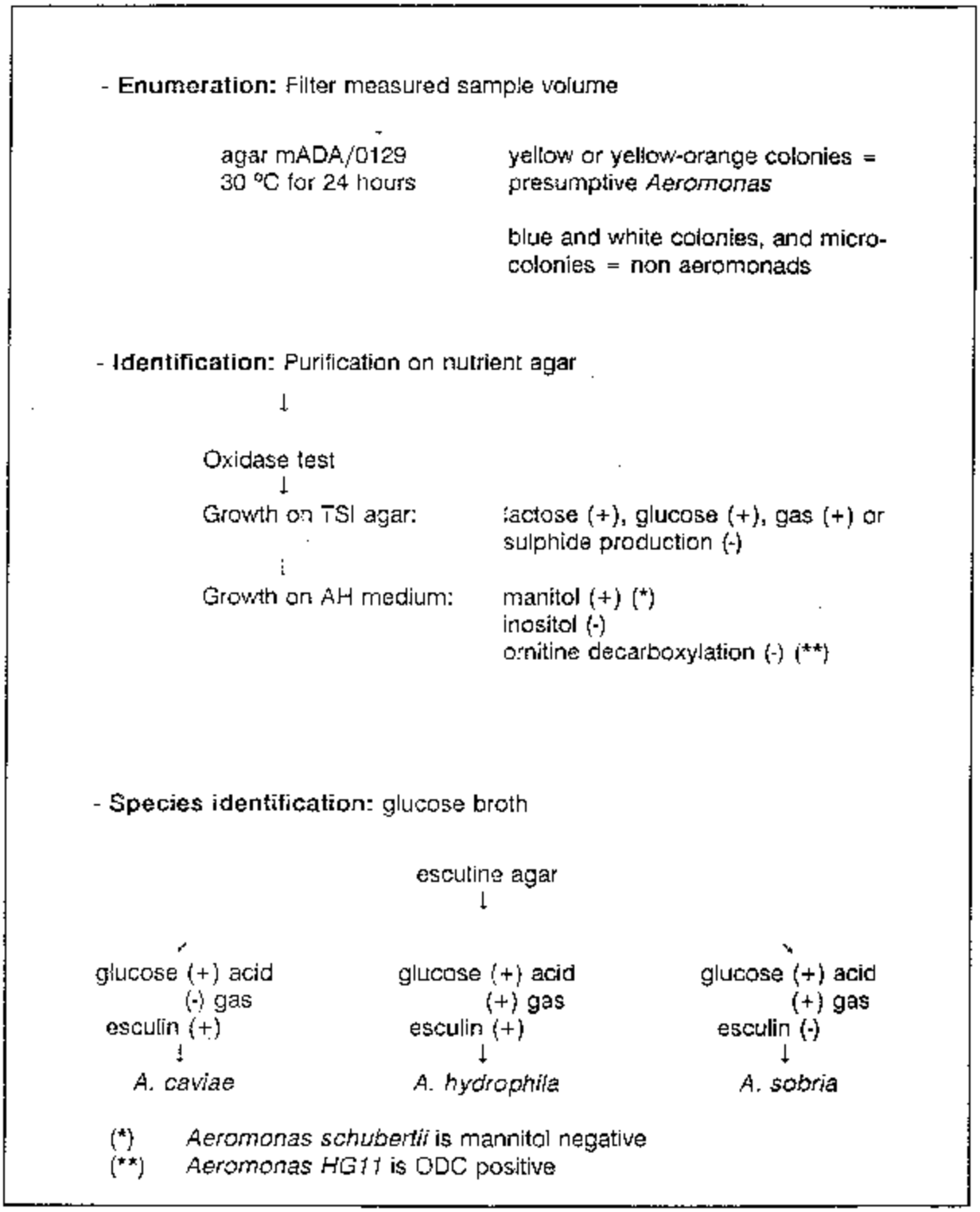

Figure 1. Schematic diagram for isolation, enumeration and identification of mesophilic aeromonads.

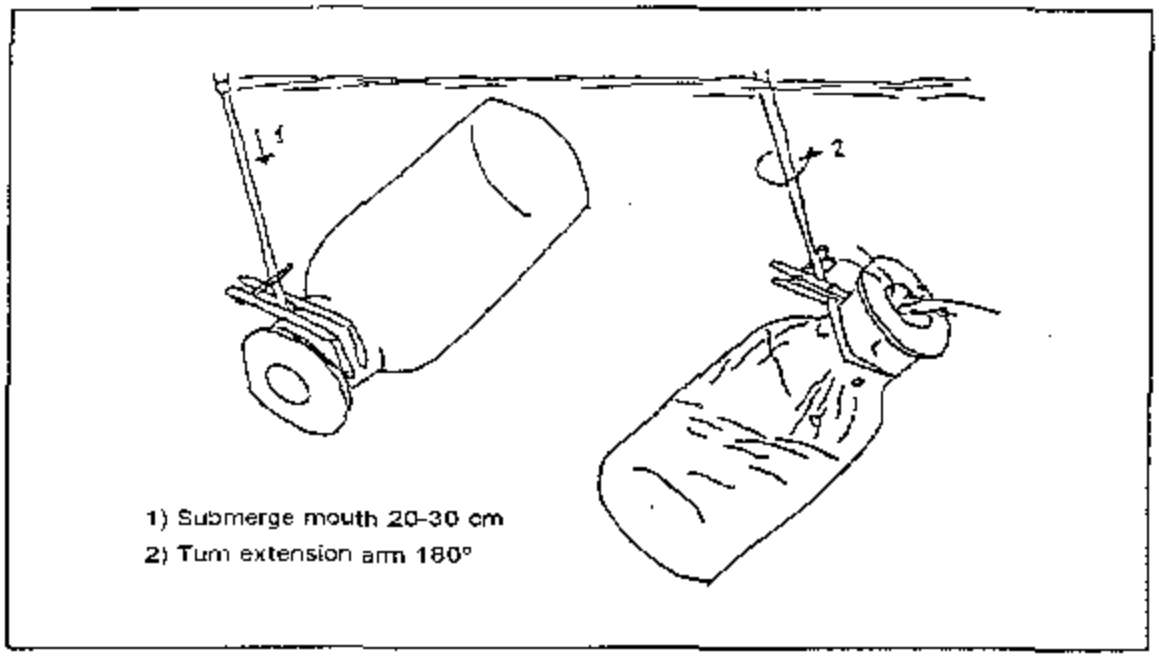

Figure 2. Subsurface sampling with extension arm.

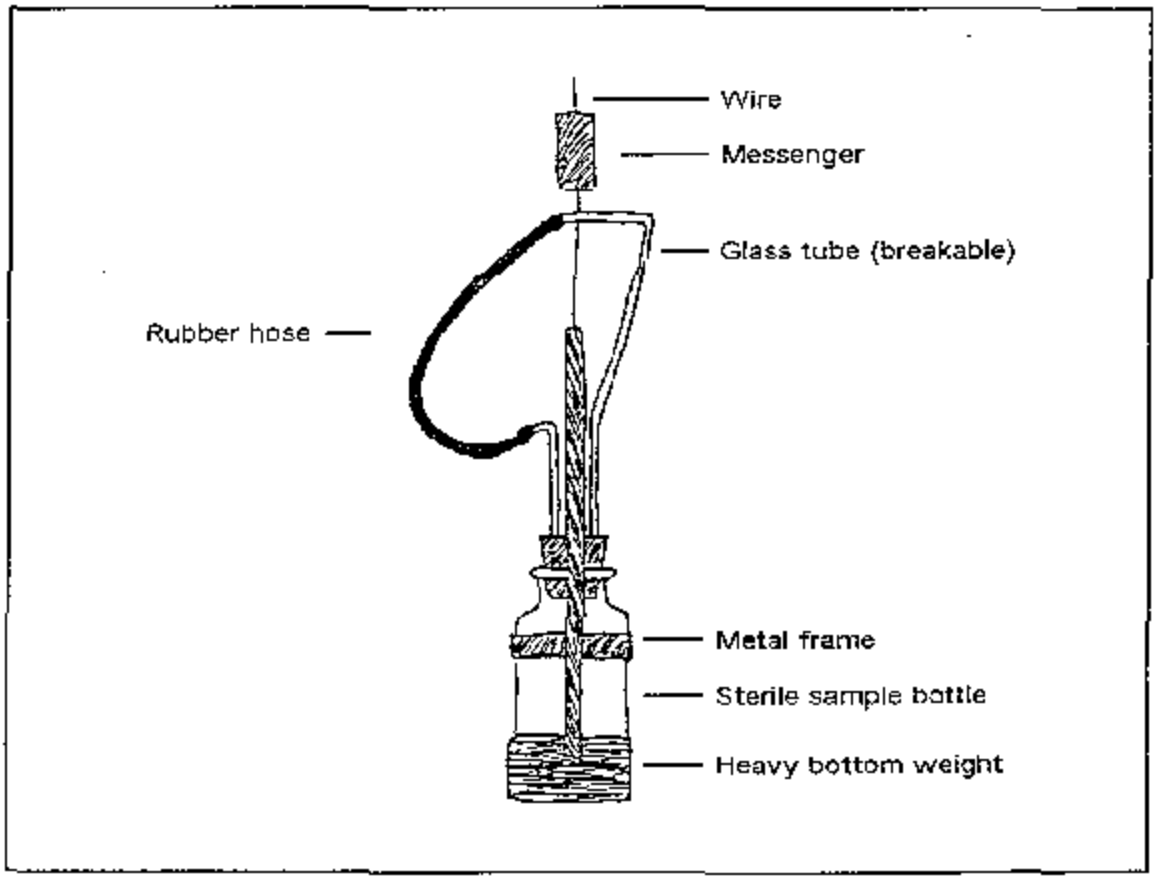

Figure 3. Sampler for sterile subsurface sampling.

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, 9, 10 and 20 ml capacity, with stainless steel containers for sterilization.

Note: 9 ml capacity pipettes are useful, but not essential.

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

5.21 Small borosilicate glass tubes 6 x 50 mm (Durham vials) to be inserted in culture tubes (5.18).

5.22 Bacteriological loops of 3 mm diameter.

5.23 Heavy wrapping paper.

5.24 Aluminium foil (household quality).

5.25 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.

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

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. Most of the following media are commercially available.

6.1 MADA/0129 Agar

6.1.1 Basal medium

Tryptose	5.0 g
Yeast extract	2.0 g
Dextrin weiss	10.0 g
NaCl	3.0 g
KCl	2.0 g
$MgSO_4.7H_2O$	0.2 g
$FeCL_3.6H_2O$	0.1 g
Bromothymolblue solution (6.1.2.)	8.0 ml
Agar	15.0 g
Distilled water	972.0 ml

Note: Different types of dextrin differ with respect to their solubility, fermentability and colony differentiation. Optimal results are obtained with Difco 161 and Merck 3006 (Havelaar and Vonk, 1988).

Preparation: Dissolve the ingredients (without the agar) in 972 ml of distilled water. Adjust the pH to 8.5 at 25 °C with 5 M NaOH, add the agar, and dissolve by gentle boiling. Distribute the medium in 196 ml portions and autoclave at 121 °C for 15 min. The basal medium may be stored for up to 1 month at 4-6 °C.

After autoclaving the basal medium and cooling to 45-50 °C in a water bath, add successively 2 ml of an ampicillin deoxycholate solution (6.1.3) and 2 ml of a vibriostatic agent 0/129 solution (6.1.4.) per bottle containing 196 ml of sterilized basal medium. The final medium should have a pH of 7.8 ± 0.2. Pour 4-5 ml of mADA/0129 agar into each petri dish. After the agar has solidified in the petri dishes, store them in the refrigerator in an inverted position. The dishes with prepared medium can be kept in a refrigerator for up to one week.

6.1.2 Bromothymolblue solution

Dissolve 1.0 g of bromothymolblue in 5 M NaOH and add distilled water up to a final volume of 100 ml.

6.1.3 Ampicillin deoxycholate solution

Dissolve 16 mg of sodium ampicillin and 100 mg of sodium deoxycholate in 10 ml of distilled water and sterilize by filtration through 0.45 µm pore size membrane filters.

6.1.4 Vibriostatic agent 0/129 solution

Dissolve 50 mg of 2,4 diamino-6,7-diisopropylpteridine (0/129) in 10 ml of an equal volume mixture (1:1) of acetone and distilled water, and sterilize by filtration through 0.45 µm pore size membrane filters.

6.2 Triple Sugar Iron Agar (TSI)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferric citrate	0.3 g
Sodium thiosulphate	0.3 g
Phenol red	0.024 g
Agar	12.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by gentle boiling and dispense in 6 ml portions in large size tubes (approx. 16 x 160 mm). Sterilize by autoclaving at 121 °C for 15 minutes. The pH after sterilization should be 7.4 ± 0.2. Allow the medium to solidify in a slant position to give a butt about 25 mm deep and a slope about 25 mm long. The medium may be stored in a cool dark place (2-8 °C) for at least 3 weeks, provided the tubes are tightly capped and there is no change in the appearance of the medium to suggest contamination, deterioration, or a pH alteration.

6.3 *A. hydrophila* Confirmation Medium (AH)

The reactions of AH medium are based upon the principles of the triple sugar iron agar, lysine iron agar, and motility-indole-ornitine medium (Kaper *et al.*, 1979).

Proteose peptone	5.0 g
Yeast extract	3.0 g
Tryptone	10.0 g
L-ornitine hydrochloride	5.0 g
Manitol	1.0 g
Inositol	10.0 g
Sodium thiosulphate	0.4 g
Ferric ammonium citrate	0.5 g
Bromocresol purple	0.02 g
Agar	3.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients in 1 litre of distilled water and adjust the pH to 6.7. Heat the medium to a boil, dispense in 5 ml portions in tubes (13 x 100 mm), and autoclave at 121 °C for 12 minutes.

6.4 Phenol Red Basal Medium with Glucose (1%, wt/vol)

6.4.1 Basal medium

Protease peptone No. 3	10.0 g
Beef extract	1.0 g
Sodium chloride	5.0 g
Phenol red	0.018 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients in 1 litre of distilled water. Add inverted vials to clean culture tubes and dispense 9 ml of medium into culture tubes. Autoclave the closed culture tubes at 121 °C for 15 minutes. The final pH should be 7.4 ± 0.2. Once the tubes are cool, add aseptically 1 ml of glucose solution (6.4.2) to give a final concentration of 1%.

6.4.2 Glucose solution

Glucose	10.0 g
Distilled water	100.0 ml

Preparation: Dissolve 10 g of glucose in 100 ml of distilled water and sterilize by filtration through 0.45 µm pore size membrane filters.

6.5 Esculin Agar

Esculin	1.0 g
Ferric citrate	0.5 g
Heart infusion agar	40.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by gentle boiling. Dispense in tubes and sterilize at 121 °C for 15 minutes. The final pH should be 7.0. Allow the agar to solidify in a slant position.

6.6 Nutrient Agar

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1.0 litre

Preparation: Dissolve the ingredients by gentle boiling. Dispense in tubes and sterilize at 121 °C for 15 minutes. The final pH should be 6.8 ± 0.2. Allow the agar to solidify in a slant position.

6.7 Oxidase Reagent

Tetramethyl-p-phenylelenediamine	0.1 g
Distilled water	10.0 ml

6.8 Kovac's Indole Reagent

Para-dimethylaminobenzaldehyde	2.0 g
Isoamyl alcohol	30.0 ml
Hydrochloric acid, concentrated	10.0 ml

Preparation: Dissolve the dimethylaminobenzaldehyde in the isoamyl alcohol, and then add the concentrated hydrochloric acid while mixing well. Transfer to a clean brown bottle. Label the bottle, and mark it **Flammable**. Store at 2-8 °C. Renew the reagent monthly.

Note: Isoamyl alcohol is a highly flammable and toxic chemical, therefore it must be handled with care, and kept well away from an open flame.

Note: Concentrated hydrochloric acid is corrosive, therefore it must be handled with care. Do not mouth-pipette.

6.9 Phosphate Buffer (pH = 7.2)

K_2HPO_4	3.0 g
KH_2PO_4	1.0 g
Distilled waster	1.0 litre

6.9.1 P-buffer for filtration

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

6.9.2 P-buffer for dilutions

Preparation: Dissolve the ingredients, dispense 9 ml portions in the test tubes used for dilutions in the dilutions series, and autoclave them 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.

6.10 Thiosulphate Solution

Preparation: prepare a 10% (10 g/100 ml) sodium thiosulfate solution in distilled water and sterilize by filtering through a sterile membrane filter (5.26 or 8.5). The sterilization can be omitted if the solution is prepared every 2-3 weeks and kept in the refrigerator.

6.11 Distilled Water

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

Note: Commercially available distilled water is often produced in copper and zinc apparatus and is highly toxic for bacteria. Before using such water its toxicity should be checked with a stock culture (6.13).

6.12 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 (6.13).

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

6.13 Stock Culture of an Aeromonad 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 2). The sterile sample bottle may also be filled directly by hand (Figure 4).

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 3). 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.

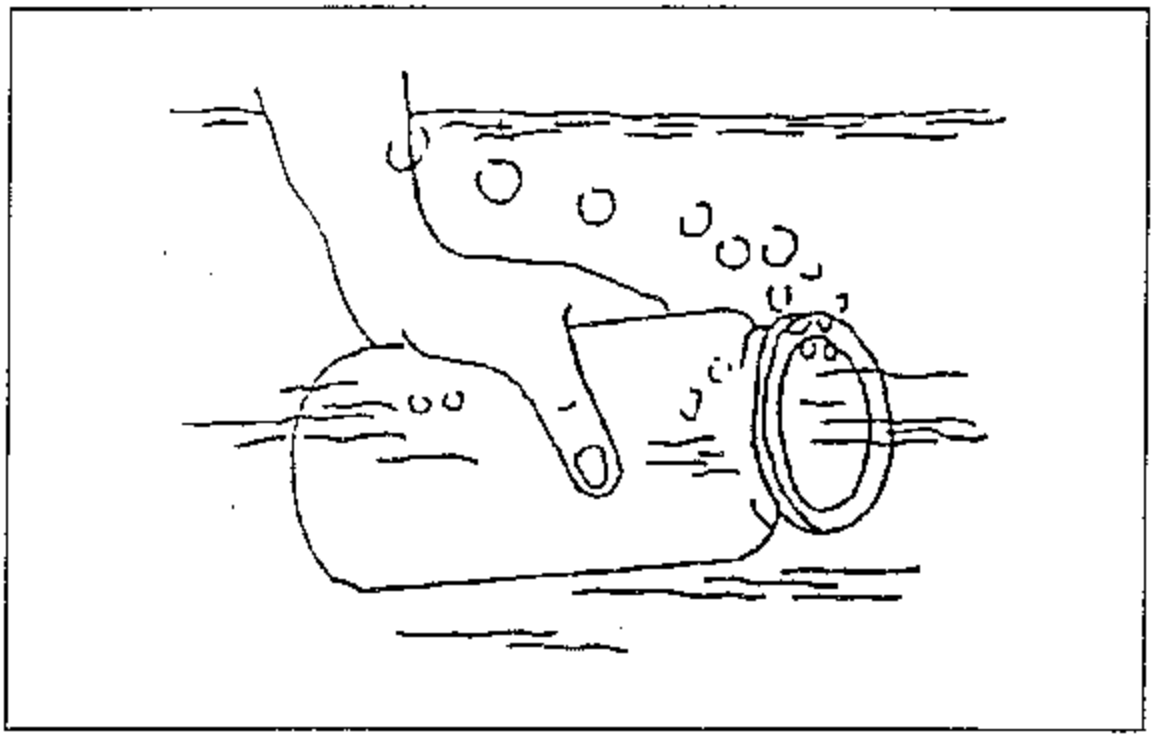

Figure 4. Subsurface sampling by hand.

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 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 for 2 hours at 160 °C, or 1 hour at 170 °C. 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, agitate them thoroughly, keeping its stopper in place. After 2-3 alcohol washings, the bottles are 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 (6.10) 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 for 2 hours at 160 °C, or 1 hour at 170 °C.

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

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 esterilization is completed, let the steam escape rapidly in order to minimize the accumulation of condensate on the membrane filters.

Note: Sterilized membrane filters are commercially available.

8.2.6 Forceps

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

8.3 Selection of Sample Size and Dilution Series

After incubation, membrane filters should ideally have from 20 to 80 colonies. If previous experience for planning the dilution series 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 5). Dilutions have to be greater for contaminated waters.

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 5, dilution D-0) and

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 (6.9.2) in order to obtain the second dilution (D-2). The desired dilution levels can be reached by a systematic application of this dilution process.

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

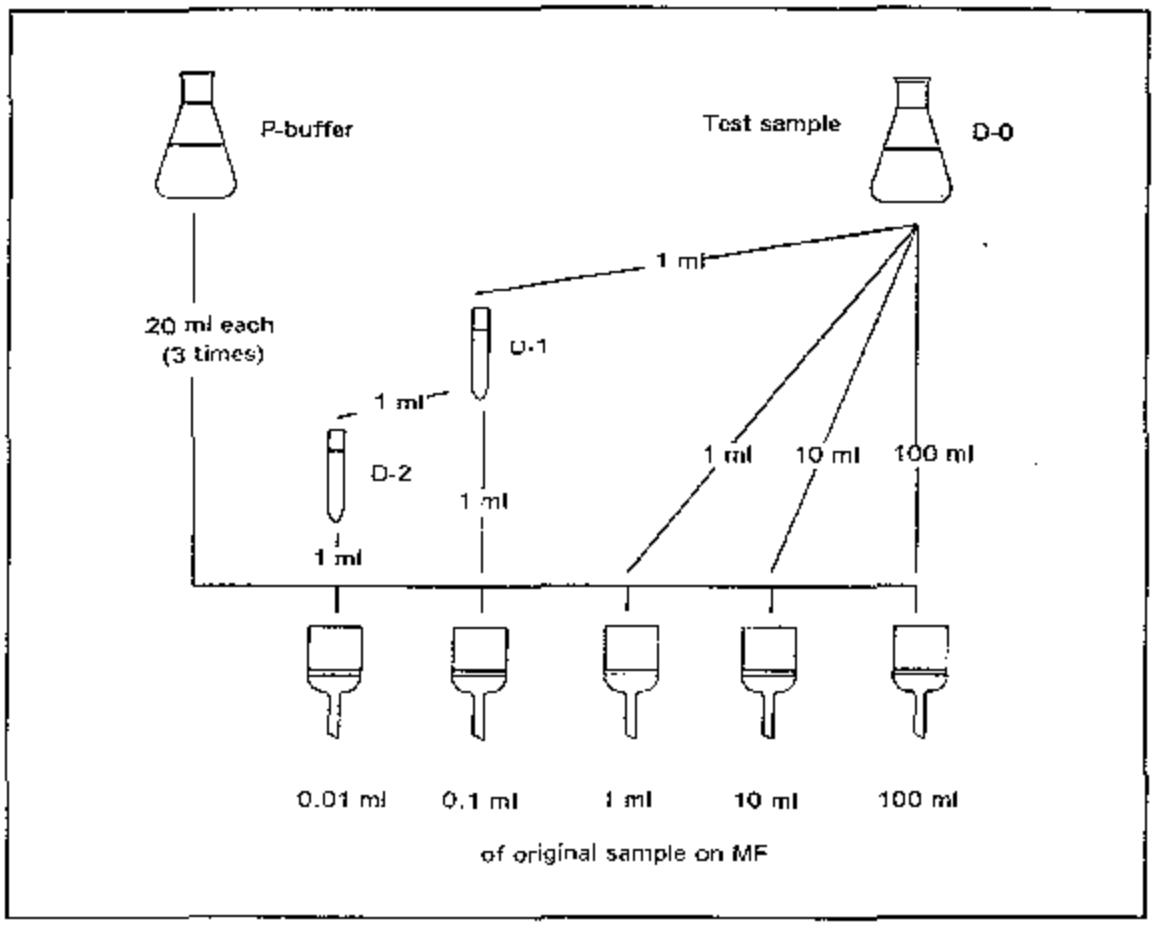

Figure 5. Preparation of dilution series and filtration procedure.

8.5 Filtration Procedure

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

Begin filtration with the highest dilution prepared (e.g. D-2) in order to avoid contamination from samples containing bacteria in higher concentrations. Use a sterilized filtration funnel for each dilution series. Place the sterilized membrane filters with flamed 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

vacuum. Wash the funnel walls two more times with 20 ml of buffer solution each time (6.9.1). Unlock and remove the funnel, immediately remove the membrane filters with flamed 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 (6.9.1) through the assembled filtration unit.

8.6 Incubation

The petri dishes containing the membrane filters on MADA/0129 agar (6.1.1) are sealed and incubated immediately at 30 ± 1 °C for 20 ± 4 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 (6.9.1).

8.7 Counting and Interpretation

Count with a stereomicroscope or similar magnifier only yellow or yellow-orange colonies. The size of those colonies may vary from 1 to 2 mm.

8.8 Verification of Colonies

8.8.1 Growth on TSI agar

Use a straight wire to inoculate TSI medium (6.2), first stabbing the butt and then streaking the slope in a zig-zag pattern. Incubate the tubes at 30 °C for 48 hours. The reactions of aeromonads are listed in Table 2.

Table 2. Typical reactions of aeromonads in triple sugar iron agar.

Organism	Butt	Slope	H_2S
Aeromonas spp.	A or AG	A	-
Escherichia coli	AG	A	-
Proteus vulgaris	AG	A	-
Shigella sonnei	A	NC or K	-
Proteus morganii	AG	NC or K	+
Salmonella enteriditis	AG	NC or K	+
Salmonella choleraesuis	AG	NC or K	-
Pseudomonas aeruginosa	K	K	-

Note: AG = acid (yellow) and gas formation; A = acid (yellow); NC = no change; K = alkaline (red); (+) = hydrogen sulphide (black); (-) = no hydrogen sulphide (no black).

Interpretation: Alkaline slant/alkaline butt (K/K) = non-fermenter; alkaline slant/acid butt (K/A) = glucose fermentation only; acid slant/acid butt (A/A) = glucose, sucrose, and/or lactose fermenter. Patterns K/A and A/A can be accompanied by black precipitate of ferrous sulphide and gas production.

8.8.2 Purification of colonies on nutrient agar

Cultures with typical aeromonads reaction in TSI medium are purified on nutrient agar (6.6). Use a sterile straight wire to streak the inoculum down the centre of the slope and then spread it in a zig-zag pattern. Incubate at 30 °C for 24 hours.

8.8.3 Oxidase test

Place a piece of filter paper in a clean petri dish and add 2-3 drops of freshly prepared oxidase reagent (6.7). A colony of the test organism, purified on nutrient agar, is then smeared on the filter paper. If the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour within 10 seconds.

Note: Ignore any blue-purple colour that develops after 10 seconds.

Controls: Positive oxidase control, *Aeromonas hydrophila*. Negative control, *Escherichia coli*.

8.8.4 Growth on AH medium

Use a sterile straight wire to inoculate the AH medium (6.3). Stab through the centre of the medium, taking care to withdraw the wire along the line of inoculum without making further stab lines. Incubate at 30 °C for 24 hours. Table 3 indicates the reactions of aeromonads in AH medium. A very distinct, typical combination of reactions is observed for aeromonads, i.e., a yellow butt with a purple band at the top, since this species ferment mannitol, but not inositol, and do not decarboxylate ornithine. The colour of the butt varies from bright yellow to yellowish grey. Motility is shown by a spreading turbidity from the stab line throughout the medium, as compared with an inoculated tube. After reading the reactions, add 3-4 drops of Kovac's reagent (6.8) to each tube. The appearance of a pink to red color indicates the production of indole. A yellow colour means that indole is not present.

Note: If reading is difficult because of weak reactions, incubation is continued for another 24 hours. Motility is recorded but not used for identification purposes because many false negative results are observed. *A. veronii* decarboxylates ornithine.

8.8.5 Acid and gas production form glucose

Inoculate the test organism on a phenol red basal medium containing 1% (wt/vol) glucose (6.4.2). Incubate at 30 °C for 48 hours. A colour change from red to yellow in the fermentation broth indicates that acidification has taken place. Gas production can be checked by inspecting the inverted Durham tube.

Controls: *A. caviae* produces acid and no gas. *A. hydrophila* and *A. sobria* produce acid and gas.

8.8.6 Esculin hydrolysis

Inoculate the test organism on a esculin agar slant (6.5). Incubate at 30 °C for 48 hours. Blackening of the agar slant is considered a positive result. No blackening of

Controls: *A. caviae* and *A. hydrophila* give positive results, and *A. sobria* give negative results.

Table 3. Typical reactions of aeromonads in AH medium.

Organism	Reaction				
	Top	Butt	Motility	H_2S	Indole
Aaeromonas spp.	K	A	+	-	+
K. pneumoniae	A	A	-	-	-
K. oxytoca	A	A	-	-	+
E. coli	K	K or A	+ or -	-	+
Salmonella spp.	K or A	K or A	+	+	-
Enterobacter spp.	K or N	K or N	+	-	-
Proteus spp.	R	K or A	+	+ or -	+
Y. enterocolitica	K or N	K or N	-	-	+ or -
Citrobacter spp.	K	A or K	+	+	-
Serratia spp.	N or K	N or K	+	-	-

Note: K = alkaline reaction; A = acid reaction; R = red; N = bleached neutral color due to destruction of indicator; (+) = 90% or more positive; (-) = 90% or more negative.

9. EXPRESSION OF RESULTS

9.1 Calculation of Mesophilic Aeromonads Density per 100 ml of Sample

Report the number of mesophilic aeromonads 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 aeromonads and non-aeromonads. Retain only two significant digits of the number of aeromonads colonies counted in each filter. Indicate the results obtained for each filter separately in the test report (Table 4, item 8).

Express the results in terms of mesophilic aeromonads per 100 ml of sample using the following equation:

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

Indicate the results obtained for each dilution separately in the test report (Table 4, item 9). Report also the results obtained on membrane filters with less than 20 mesophilic aeromonad colonies per filter. If there are no *Aeromonas* colonies on the filter through which 100 ml have been filtered report the results as " < 1 mesophilic aeromonads/100 ml ".

Compute the number of mesophilic aeromonads per 100 ml sample and report it as the final test result (Table 4, item 10). If there are membrane filters containing between 20 and 80 characteristic colonies in two consecutive dilutions, calculate the mean of these dilutions (total number of colonies counted/total volume in ml of sample filtered) and report it as a final test result.

Record in the test report (Table 4, 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 5). The water sample used should be collected during the routine monitoring programme at a coastal station typical of the area. The dilution series should be selected in such a way so that one dilution step yields 3 membrane filter counts which satisfy the 20 to 80 colonies requirements expressed in section 8.3.

Filter each individual dilution following procedure 8.5. Incubate according to procedure 8.6. Report membrane filter counts following the procedure described in section 9.1 taking into consideration the interpretation method described in section 8.7. Results should be reported in the test report (Table 5, item 8).

Calculate the mesophilic aeromonads 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 5, item 9).

For each dilution step having the three membrane filter counts between 20 and 80 mesophilic aeromonad 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 5, item 10).

If the sample does not yield at least 20 colonies per membrane filter in one dilution, prepare a test solution from a stock culture (6.13) and repeat the estimation precision.

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

10. TEST REPORT

Table 4. Mesophilic aeromonads 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 colonies per individual filter

Dilution	ml of original sample filtered	Mesophilic aeromonads
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. Mesophilic aeromonads/100 ml

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

10. Test result

_____ aeromonads/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 5. Precision estimation for mesophilic aeromonads determination.

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	mesophilic aeromonads replica 1st	2nd	3th

9. Mesophilic aeromonads/100 ml

Dilutions	col./100 ml

10. Results (aeromonads/100ml)
mean: _____ range: _____
std. dev.: ____ coef. var.: __ %

11. Anomalies observed in the test procedure:

_____
_____
_____
_____

12. Full address of the institut on 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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