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REFERENCE METHODS FOR POLLUTION STUDIES IN THE MEDITERRANEAN

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Introduction

The Convention for the Protection of the Mediterranean Sea against Pollution (Articles 10 and 11) requires that pollution monitoring programmes be established and that exchange of data and other scientific information take place. The Protocol for the Protection of the Mediterranean Sea against Pollution from Land-Based Sources requires the formulation and adoption of common guidelines and, as appropriate, standards or criteria dealing in particular with the quality of sea-water for the protection of human health, living resources and ecosystems. For this purpose a set of Environmental Quality Criteria have been developed by UNEP in close co-operation with WHO and FAO (UNEP/WG.62/6), whose application requires that reference methods are available to follow the evolution of the environmental indicators selected.

WHO and FAO, in close collaboration with UNEP, have developed various reference methods, relevant to the proposed Environmental Quality Criteria, covering a number of indicators for assessing the quality of recreational and of shellfish growing waters as well as the mercury contents of fish and shellfish used for human consumption. These methods were developed and used during the Pilot Phase of the Mediterranean Pollution Monitoring and Research Programme (MED POL - PHASE I) by a large number of participating institutions and submitted to intercalibration exercises co-ordinated by the International Laboratory of Marine Radioactivity of the IAEA.

The methods, as they are formulated, may not be the best possible ones at the present time. Other, often more sophisticated, techniques, might result in higher accuracy or resolution. However, taking into account the diversity of technical capabilities of the institutions carrying out the monitoring, the present formulation has proven to be well adapted to the Mediterranean environment.

The Working Group on Scientific and Technical Co-operation is expected to review the proposed methods and make recommendations to UNEP and the Co-operating Agencies for their final editing before submission for adoption to the forthcoming meeting of the Contracting Parties. However, adoption of the present formulation should not prevent UNEP, the Co-operating Agencies and the Mediterranean institutions from developing further techniques and making proposals for their modification when it is deemed necessary. The reference methods here proposed will be edited individually in the form of a series on Mediterranean Reference Methods, which will be kept up-dated.

DETERMINATION OF TOTAL COLIFORMS IN SEA-WATER

BY THE MEMBRANE FILTRATION CULTURE METHOD

1. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of total coliforms in coastal bathing waters of the Mediterranean Sea and is designated to be used in sanitary surveillance of the Mediterranean bathing beaches.

One advantage of this method is that it avoids false positive readings of colonies from anaerobic bacteria such as Clostridium perfringens. Many non-coliforms will not grow on the test medium used. The non-coliforms that do grow have to be excluded after special tests. A disadvantage of the method is that it will also detect coliforms of non-faecal origin and is consequently not the test indicated for pollution by faecal material.

2. REFERENCES

- WHO (1975) Report of WHO/UNEP expert consultation on coastal water quality control programme in the Mediterranean. (Geneva, 15 to 19 December 1975) EHE/76.1. WHO, Copenhagen, 25 pp.
- WHO (1977a) Guidelines for health related monitoring of coastal water quality. Report of a group of experts jointly convened by WHO and UNEP (Rovinj, Yugoslavia, 23 - 25 February 1977). WHO, Copenhagen, 165 pp.
- WHO (1977b) Health criteria and epidemiological studies related to coastal water pollution. Report of a group of experts jointly convened by WHO and UNEP (Athens, 1 - 4 March 1977). WHO, Copenhagen, 33 pp.
- WHO (1977c) Mid-term review of the joint WHO/UNEP co-ordinated pilot project on coastal water quality control in the Mediterranean (MED VII). Report of a meeting of principal investigators of collaborating laboratories, jointly convened by WHO and UNEP (Rome, 30 May - 1 June 1977). WHO, Copenhagen, 23 pp.
- WHO (1978) Monitoring of recreational coastal water quality and shellfish culture areas. Report of a seminar jointly convened by WHO and UNEP (Rome, 4 - 7 April 1978). WHO, Copenhagen, 39 pp.

3. DEFINITION

Coliform bacteria are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Under the operational conditions described, the coliform colonies will appear as pink to dark red often with a metallic sheen. Lactose negative bacteria appear as colourless colonies. Cytochromoxidase - positive, gramme negative bacteria may be separated from coliforms by means of cytochromoxidase test.

These bacteria include E. coli type I which are of faecal origin and irregular types II and VI which may not be of faecal origin.

The faecal E. coli type I produces indole from tryptophan at 44°C; the other non-faecal coliforms do not produce indole (see Faecal Coliform test).

4. PRINCIPLE

From sea-water samples taken under sterilized conditions, a dilution series is set up according to the number of total coliforms expected in the water sample. Aliquots of this dilution series are filtered through 0.45 µ pore size membrane filters. The membrane filters are placed on liquid m-FC medium and incubated at 36°C ± 1°C for 24 hours. Lactose fermentation will cause coliform colonies to exhibit pink to dark red colonies often with metallic sheen. Residual chlorine, if present, is neutralized by adding thiosulphate to the sampling bottle before sterilization.

NOTE: Suspected and doubtful colonies can be tested with a confirmative test using MacConkey broth (acid and gas development).

5. SAMPLING

5.1 Sampling plan

Identify sources of pollution (outfall, river mouth, etc.) and mark them on a hydrographic map of the area. Select sampling points for surface samples about every 250 m along the coastline, 10 m offshore, choosing stations which are located in the major pollution sources and also outside them. In areas with tides, the sampling stations should be located 10 m offshore from the low-tide mark. If the water depth exceeds 5 m, take also one sample near the bottom (without disturbing the sediments) and one sample half-way between the bottom and the surface.

Establish one reference sampling point for every one km of coastline at one km offshore.

Sample all points at least twice a month during the swimming season and at three equally spaced intervals during the remainder of the year. In order to obtain an estimation on the sampling variability, collect double samples during the first three sampling periods and repeat the collecting of double samples in selected stations once every season for estimation of sampling error.

Revise sampling plan after analysis and interpretation of the results of three monthly surveys, and after two years of observations.

NOTE: After the first three series of samples have been analysed, the number of stations may be increased or reduced.

NOTE: The sampling should be programmed in such a way that, from the time of sampling to the time when samples are processed in the receiving laboratory, not more than six hours have passed. Start sampling at the furthest sampling station in order to reduce the sample storage time. Samples which arrive at the receiving laboratory six hours or more after collection must be discarded and replaced by new samples.

NOTE: Die-away rates (T-90) of two to three hours have been observed; therefore samples must be filtered as soon as possible.

5.2 Sampling procedure, sample transport and sample storage

5.2.1 Sampling of surface water

Attach clean sterilized sample bottle (9.2.1) to the clean sampling rod (6.2). 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 about half a metre under the water surface with the mouth of the bottle downwards, then turn the sample bottle upwards and take the sample (figure 1(a)). The sterilized sample bottle may also be filled directly by hand.

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 (6.4) with cooling pads at about 4°C, avoiding exposure to more than 10°C. Separate bottles from each other with clean wrapping paper (6.23) to avoid breakage. Check the temperature with a thermometer (6.5) every three hours. Report irregularities in the sampling protocol (6.26). Label sample bottles.

5.2.2 Sampling of subsurface water

Lower the sterilized subsurface sampler (9.2.2) after attaching it to a clean plastic rope, without letting the weight disturb the bottom sediments (figure 1 (b)). Release the messenger and after one minute retrieve the sampler and store it in a thermoisolated box (6.4). Proceed as for sampling of surface water (5.2.1).

NOTE: It is recommended that the label and standard forms developed in the MED POL VII pilot project should be used (see WHO, 1977a).

NOTE: Coliforms die in sea-water. Therefore storage must be minimized. If transport time is too long, in situ filtration should be considered.

6. APPARATUS AND GLASSWARE

- 6.1 Sample bottles for surface sea-water: 200-300 ml capacity, wide-mouthed, of borosilicate glass.

NOTE: For sea-water sampling an adequate boat may be needed.

- 6.2 Sampling rod of non-corrosive material with a clamp to hold the sampling bottle (figure 1 (a). Minimum number: three).

- 6.3 Subsurface sampler of the type shown in figure 1 (b), or similar, complete with plastic rope and weight.

- 6.4 Thermoisolated plastic boxes with cooling pads or similar cooling units.

- 6.5 Thermometer, 0 to 50°C, possibly unbreakable plastic type, used for checking temperature in plastic boxes (6.4).

- 6.6 Membrane filter filtration apparatus for 4.7 cm diameter filters consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material, complete with electric or water vacuum pump.

- 6.7 Air or water incubator for 36°C ± 1°C.

- 6.8 Binocular microscope, magnification 1.000X, stereomicroscope magnification 10 - 50X and/or Darkfield Quebec colony counter magnification 2 - 3X.

- 6.9 Autoclave, max 2 atm., electric or gas.

- 6.10 Drying oven for sterilization at 160°C.

- 6.11 pH meter ± 0.1 units.

- 6.12 Stainless steel forceps.

- 6.13 Analytical balance ± 1 mg.

- 6.14 Refrigerator 4°C ± 0.5°C.

- 6.15 Vibrator for mixing liquids in culture tubes.

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

- 6.17 Ehrlenmeyer flasks of borosilicate glass for media preparation, capacity 500 ml and 1 litre.
- 6.18 Borosilicate glass bacteriological culture tubes.
- 6.19 Total volume (blow-out) pipettes of borosilicate glass, graduated, of 1 and 10 ml capacity, in stainless steel containers for sterilization.
- 6.20 Graduated borosilicate glass cylinders with glass beakers for cover.
- 6.21 Small borosilicate glass vials ("Durham tubes") to be inserted in culture tubes (6.18).
- 6.22 Bacteriological loops made from 22-24 Chromel gauge, bichrome or platinum-iridium. Diameter of the loop: 3 mm.
- 6.23 Heavy wrapping paper.
- 6.24 Aluminium foil (household quality).
- 6.25 Membrane filters, pore size 0.45 u, diameter 4.7 cm, or similar fitting filtration apparatus (6.6).

NOTE: The 0.45 u pore size membrane filter (MF) 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, exhibiting a surface opening of 2.4 um, and a retention pore size of 0.7 um. Retention pore size is a measure of the smallest particle which is retained by the structure.

7. CULTURE MEDIUM AND CHEMICALS

7.1 M-endo-agar

Polypeptone	10.0 g
Thiopeptone	5.0 g
Casitone	5.0 g
Yeast extract	1.5 g
Lactose	12.5 g
Sodium chloride	5.0 g
Potassium dihydrogen phosphate	1.375 g

Dipotassium hydrogen phosphate	4.375 g
Sodium lauryl sulphate	0.05 g
Sodium deoxycholate	0.10 g
Sodium sulphite	2.10 g
Basic fuchsine	1.05 g
Distilled water	1,000 ml
Agar	15 g

Preparation: Rehydrate in 1 litre of distilled water containing 20 ml 95% ethanol. Heat to boiling point and promptly remove from heat. Cool to below 45°C. Do not sterilize by autoclaving. Adjust pH to 7.2. Dispense into petri dishes to provide minimal agar depth of 2 - 3 mm. Protect the medium from light. The finished medium should not be stored for more than 2 weeks even at 4°C.

NOTE: Finished media can be stored in the dark at + 4°C (6.14). Any unused media should be discarded after 96 hours. Check medium for sterility by incubating a blank, i.e. a Petri dish containing M-endo-agar medium together with the Petri dishes containing sample membrane filters (9.4).

7.2 MacConkey broth

	Single strength
Sodium taurocholate	5 g
Lactose	10 g
NaCl	5 g
Peptone	20 g
1 per cent bromo-cresol purple in ethanol (95 per cent)	2 ml

Glass-distilled or de-ionized water (7.5) 1 litre

Dissolve components by shaking. Adjust pH to 7.1 ± 0.1 with diluted HCl and add the bromo-cresol purple solution.

Distribute, 10 ml of the broth into culture test tubes (6.18) containing inverted vials (Durham tubes) and autoclave at 121°C for 15 minutes.

NOTE: The air in the inverted vials will disappear after autoclaving.

7.3 Phosphate buffer (pH = 7.2)

K_2HPO_4	3 g
KH_2PO_4	1 g
Distilled water (7.5)	1 litre

Dissolve components and autoclave at 121°C for 15 minutes.

7.4 Thio-sulphate solution

10 per cent Na-thiosulphate solution.

7.5 Distilled water

Use only water distilled in all glass or all quartz distillation apparatus. De-ionized water is also acceptable if produced in non-toxic apparatus.

NOTE: Commercially available distilled water is often produced in copper and zinc apparatus and highly toxic.

Distilled water must contain <10 ug copper per liter.

7.6 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 E. coli (8).

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

7.7 95 per cent ethanol per analysis

8. PREPARATION OF CONTROL SAMPLE

Prepare a culture of E. coli strain from a type collection according to instructions supplied with the culture.

9. PROCEDURE

9.1 Washing of glassware and equipment

All glassware and apparatus (6) are washed with non-toxic detergent (7.6), first rinsed thoroughly with hot tap water and then rinsed at least three times with distilled water (7.5).

9.2 Sterilization of glassware and equipment.

9.2.1 Surface sample bottles (6.1). Clean sample bottles (9.1) are dried and sterilized in a drying oven (6.10) for two hours at 160°C. Before sterilization, place a small piece of filter paper in the neck of the 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 a pair of sterilized forceps (9.2.5) and fit the ground glass stopper securely into the neck of the bottle. Put the bottles into detergent-cleaned thermoisolated boxes (6.4). Separate the bottles from each other with clean filter paper to avoid breakage.

9.2.2 Subsurface sampler (6.3). Clean the subsurface sampler (9.1) with detergent (7.6), rinse with tap and distilled water (7.5). Enclose each sampler in heavy wrapping paper (6.23) and sterilize in an autoclave (6.9) for 15 minutes at 120°C.

NOTE: If residual chlorine is suspected in the water sample, add, aseptically, 0.1 ml of a 10 per cent thiosulphate solution (7.4) for each 100 ml of sample to the contents of the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

9.2.3 Clean Petri dishes (6.16) and pipettes (6.19), complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven (6.10) for three hours at 160°C.

NOTE: Disposable sterilized plastic Petri dishes may be more economical to use than reusable glass Petri dishes.

9.2.4 For sterilization of the MF-funnels (6.6), loosen the filter-holding assembly slightly and wrap the whole filter funnel in heavy wrapping paper (6.23) or aluminium foil (6.24). Sterilize in an autoclave (6.9) for 15 minutes at 120°C, or in a drying oven (6.10) for 3 hours at 160°C.

9.2.5 For sterilizing MFs (6.25) remove the paper separator (if present) and place 10 to 12 clean MFs into 9 cm diameter Petri dishes (6.16). Autoclave (6.9) for 15 minutes at 120°C. At the end of the sterilization let the steam escape rapidly in order to minimize the accumulation of condensate on the MFs. Sterilized MFs are commercially available in packings containing one or more filters.

9.2.6 Sterilize forceps (6.12) by dipping them into 95 per cent ethanol (7.7) and flaming them.

9.3 MF filtration procedure

9.3.1 Selection of sample size and dilution series

The MFs for counting should have from 20 to 80 colonies. If previous experience for planning the dilution series (see also figure 2) is not available, use the dilution series 100, 10, 1, 10^{-1} as a guide for clear sea-water. guide.

9.3.2 Preparation of the dilution series

Start the dilution series by taking 1 ml with a sterilized pipette (9.2.2) from the original sample (figure 2, dilution: D-0) and transfer this 1 ml into a clean, sterilized, culture tube (6.18). Add 9 ml of sterilized phosphate buffer (7.3) to make a 10 ml first dilution (D-1). Agitate the tube on a mixer (6.15) or shake it vigorously by hand.

9.3.3 Filtration procedure

Begin filtration with the highest dilution (D-1) in order to avoid contamination. Use a sterilized filtration funnel (9.2.4) for each dilution series. Place the sterilized MF (9.2.5) with flamed sterilized forceps (9.2.6) over the porous plate of the filtration apparatus (9.2.4). Carefully place the matching funnel unit over the receptacle and lock it in place. With a sterilized pipette (9.2.3), drop the 10 ml of the D-1 dilution into the funnel. Mix by blowing air with the pipette through the solution before filtration. Filter with a partial vacuum. Unlock and remove the funnel, immediately remove the MF with flamed sterilized forceps (9.2.6) and place it on the sterilized surface of an aliquot of M-endo-agar (7.1) contained in a sterilized Petri dish (9.2.3) with a rolling motion to avoid the entrapment of air. Before filtering the next lower dilution in the same manner, pass three 20 ml portions of sterilized buffer (7.3) through the assembled filtration unit. Aliquots filtered directly from the original sample bottle (i.e. 100 ml and 10 ml) do not need to be diluted with sterilized buffer.

NOTE: The aliquot of the M-FC-medium can be placed on a sterilized absorbent pad and the MF placed on top of it.

9.4 Incubation

The Petri dishes (9.3.3) containing the MF are incubated for 24 hours at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$, either in air or water. For water bath incubation, the Petri dishes are sealed and then placed horizontally inside clean plastic bags. These bags are then immersed in a water bath (6.7). Incubate also one blank (without MF) Petri dish with M-endo-agar (7.1) as a sterility check.

9.5 Interpretation

Count only colonies which appear as pink to dark red often with a metallic sheen. Test dubious colonies by cytochromoxidase test by means of commercially prepared paper strips. Report results for all dilutions on the form reproduced in table 1.

NOTE: Coliforms are cytochromoxidase negative. Positive reaction on paper strips occurs within 30 seconds (positive reaction = blue colour).

NOTE: If the number of dubious colonies is greater than 10 per cent of the total number of coliforms, test dubious colonies as follows:

9.6 Estimation of precision

Check the precision of the membrane filtration culture technique at the start of the project and at periodic intervals by preparing triplicate dilution series (9.3.2) of the same test sample and by triplicate membrane filtrations (9.3.3). Use an analysis of variance to determine the standard error of triplicate dilution series and the standard error of triplicate membrane filtrations. If the coefficient of variance is too high, determine the cause of the high variance, eliminate it and repeat the entire sampling procedure.

9.7 Quality control

Complete a quality control test according to the instructions given in the reference method on quality control of the membrane filtration culture methods.

9.8 Intercalibration

Complete a precision test according to the instructions given in a reference method on intercalibration and estimation of precision.

MacConkey broth test: With a flamed bacteriological loop (6.22) transfer the suspected colony from the MF into a culture tube containing MacConkey broth (7.2) and incubate at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 to 48 hours. Coliforms will develop gas which is trapped in the inverted vials, and acid which turns the violet-like colour of the original broth into a yellowish colour.

10. EXPRESSION OF RESULTS

10.1 Calculation of coliforms

- 10.1.1 Report the number of coliforms in terms of (total) coliforms per 100 ml of sample. Compute the count using MFs with 20 to 200 coliform colonies and not more than 200 colonies of all other types per MF, by the following equation:

(Total) coliforms/100 ml = (coliform colonies x 100/ (ml sample filtered)).

- 10.1.2 If two MFs corresponding to a certain dilution contain between 20 and 200 characteristic colonies, calculate the arithmetic mean of the number of colonies counted in the two MFs.

Retain only two significant figures, proceeding as follows:

- if the number is less than 100, round it to the nearest multiple of 5;
- if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;
- if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of coliforms per 100 ml. Express this result as a number between 1.0 and 9.9 multiplied by 10^n , n being the appropriate power of 10.

- 10.1.3 If there are MFs containing between 20 and 200 characteristic colonies at two consecutive dilutions, calculate the number of colonies for each dilution as specified in 10.1.1, and take as the result the arithmetic mean of the two values obtained, except when the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.

- 10.1.4 MFs that do not reach coliform numbers of 20 to 80 are also reported per 100 ml according to the formula above (10.1.1). For example, duplicated MFs had 5 and 3 coliform colonies for 50 ml sample filtered, equal to 8 colonies / 100 ml = $(5 + 3) \times 100 / (50 + 50)$ ml.

- 10.1.5 If there are fewer than 15 characteristic colonies on the MFs, report the results as:

- fewer than 15 coliforms per 100 ml.

If there are no characteristic colonies on the MFs report the result as:

- fewer than 1 coliform per 100 ml.

10.1.6 Record confluent growth or colonies other than coliform too numerous to count. Collect a new sample if too many coliforms or too many other colonies were in the MF of the highest dilution and select a higher dilution series for a new test.

Use for recording the data observed, and for further evaluation, the test report form reproduced in table 2.

10.2 Precision of the method

No information available.

11. TEST REPORT

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this reference method or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details required for the complete identification of the sample (table 2).

NOTE: For the assessment of the sanitary quality of coastal waters, other parameters are also taken into consideration, such as faecal coliforms, faecal streptococci, temperature, salinity, oxygen, etc.

Table I : microbiological laboratory examination

COASTAL MONITORING / LABORATORY EXAMINATION /

Institution
and country

Organism:

Area :

Year :

Sheet No :

Point no.

Date

Time h

Medium

Depth m

Temperature °C

Salinity ‰

Container no

Start incubation
d & h

Sample test amount unit:

g or ml

Number 50

of 10

Colonies 1

10⁻¹20 - 80 10⁻¹range 10⁻¹only 10⁻⁴10⁻⁵

Method

Date and time of cessation
of incubation

No. of colonies

Examiner's Signature

Notes: Per 100 ml for water, 100 g for shellfish, and per 1 g for sediments.

Remarks:

Table 2 : Test report on the determination of total coliforms in sea-water
by the membrane filtration culture method

1. General identification

1.1 Country _____ 1.2 Institute _____ 1.3 Area: Longitude _____ Latitude _____
1.4 Date: Time _____ Day _____ Month _____ Year _____ 1.5 Identification number or letter of sampling point _____

2. Bacteriological data:

Total coliforms colonies /100 ml _____

3. Operating conditions not specified in the reference method or regarded as optional as well as any circumstances that may have influenced the results.

DETERMINATION OF FAECAL COLIFORMS IN SEA-WATER
BY THE MEMBRANE FILTRATION CULTURE METHOD

1. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of faecal coliforms in coastal bathing waters of the Mediterranean Sea and is designated to be used in sanitary surveillance of the Mediterranean bathing beaches.

Faecal coliforms are specific, exhibiting a high positive correlation with faecal contamination from warm-blooded animals. Since faecal coliforms die when exposed to sea-water, this method will indicate recent faecal pollution. Die-away rates (T-90) of one to three hours depend on salinity, temperature, solar radiation etc.

In many instances faecal coliforms meet the requirement of a good indicator. The major problem consists in the short survival time in sea-water.

2. REFERENCES

- WHO (1975) Report of WHO/UNEP expert consultation on coastal water quality control programme in the Mediterranean. (Geneva, 15 to 19 December 1975) EHE/76.1. WHO, Copenhagen, 25 pp.
- WHO (1977a) Guidelines for health related monitoring of coastal water quality. Report of a group of experts jointly convened by WHO and UNEP (Rovinj, Yugoslavia, 23 - 25 February 1977). WHO, Copenhagen, 165 pp.
- WHO (1977b) Health criteria and epidemiological studies related to coastal water pollution. Report of a group of experts jointly convened by WHO and UNEP (Athens, 1 - 4 March 1977). WHO, Copenhagen, 33 pp.
- WHO (1977c) Mid-term review of the joint WHO/UNEP co-ordinated pilot project on coastal water quality control in the Mediterranean (MED VII). Report of a meeting of principal investigators of collaborating laboratories, jointly convened by WHO and UNEP (Rome, 30 May - 1 June 1977). WHO, Copenhagen, 23 pp.
- WHO (1978) Monitoring of recreational coastal water quality and shellfish culture areas. Report of a seminar jointly convened by WHO and UNEP (Rome, 4 - 7 April 1978). WHO, Copenhagen, 39 pp.

3. DEFINITION

Faecal coliforms are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas both at 36°C and 44.5°C in less than 24 hours. They produce indole in peptone water containing tryptophan at 44.5°C. Under the operational conditions described, the faecal coliforms appear as blue colonies.

4. PRINCIPLE

From sea-water samples taken under sterilized conditions, a dilution series is set up according to the number of faecal coliforms expected in the water sample. Aliquots of this dilution series are filtered through 0.45 µ pore size membrane filters. The membrane filters are placed on solidified and dried m-FC medium and incubated at 44.5°C ± 0.2°C for 24 hours. Lactose fermentation will cause coliform colonies to exhibit a characteristic blue colour. Residual chlorine, if present, is neutralized by adding thiosulphate to the sampling bottle before sterilization.

5. SAMPLING

5.1 Sampling plan.

Identify sources of pollution (outfall, river mouth, etc.) and mark them on a hydrographic map of the area. Select sampling points for surface samples about every 250 m along the coastline, 10 m sampling stations should be located 10 m offshore from the low-tide mark. If the water depth exceeds 5 m, take also one sample near the bottom (without disturbing the sediments) and one sample half-way between the bottom and the surface.

Establish one reference sampling point for every one km of coastline and one km offshore.

Sample all points at least twice a month during the swimming season and at three equally spaced intervals during the remainder of the year. Collect double samples during the first three sampling periods and repeat the collecting of double samples in selected stations once every season for estimation of sampling error.

Revise sampling plan after analysis and interpretation of the results of three monthly surveys, and after two years of observations.

NOTE: After the first three series of samples have been analysed, the number of stations may be increased or reduced.

NOTE: The sampling should be programmed in such a way that from the time of sampling to the time when samples are processed in the

receiving laboratory not more than six hours have passed. Start sampling at the furthest sampling station in order to reduce the sample storage time. Samples which arrive at the receiving laboratory six hours or more after collection must be discarded and replaced by new samples.

NOTE: Die-away rates (T-90) of two to three hours have been observed; therefore samples must be filtered as soon as possible.

5.2 Sampling procedure, sample transport and sample storage

5.2.1 Sampling of surface water

Attach clean sterilized sample bottle (9.2.1) to the clean sampling rod (6.2). 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 about half a metre under the water surface with the mouth of the bottle downwards, then turn the sample bottle upwards and take the sample (figure 1(a)). The sterilized sample bottle may also be filled directly by hand.

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 (6.4) with cooling pads at about 4°C, avoiding exposure to more than 10°C. Separate bottles from each other with clean wrapping paper (6.23) to avoid breakage. Check the temperature with a thermometer (6.5) every three hours. Report irregularities in the sampling protocol (6.26). Label sample bottles.

5.2.2 Sampling of subsurface water

Lower the sterilized subsurface sampler (9.2.2) after attaching it to a clean plastic rope, without letting the weight disturb the bottom sediments (figure 1(b)). Release the messenger and after one minute retrieve the sampler and store it in a thermoisolated box (6.4). Proceed as for sampling of surface water (5.2.1).

NOTE: It is recommended that the label and standard forms developed in the MED POL VII pilot project should be used (see WHO, 1977a).

NOTE: Faecal coliforms die in sea-water. Therefore storage must be minimized. If transport time is too long, in situ filtration should be considered.

6. APPARATUS AND GLASSWARE

- 6.1 Sample bottles for surface sea-water: 200-300 ml capacity, wide-mouthed, of borosilicate glass,

NOTE: For sea-water sampling an adequate boat may be needed.

- 6.2 Sampling rod of non-corrosive material with a clamp to hold the sampling bottle (figure 1 (a). Minimum number: three).
- 6.3 Subsurface sampler of the type shown in figure 1 (b), or similar, complete with plastic rope and weight.
- 6.4 Thermoisolated plastic boxes with cooling pads or similar cooling units.
- 6.5 Thermometer, 0° to 50°C , possibly unbreakable plastic type, used for checking temperature in plastic boxes (6.4).
- 6.6 Membrane filter filtration apparatus for 4.7 cm diameter filters consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material, complete with electric or water vacuum pump.
- 6.7 Water incubator for $44.5^{\circ} \pm 0.2^{\circ}\text{C}$.
- 6.8 Binocular microscope, magnification 100X, stereomicroscope, magnification 10-50X and/or Darkfield Quebec colony counter, magnification 2-3X.
- 6.9 Autoclave, max 2 atm., electric or gas.
- 6.10 Drying oven for sterilization at 160°C .
- 6.11 pH meter ± 0.1 units.
- 6.12 Stainless steel forceps.
- 6.13 Analytical balance ± 1 mg.
- 6.14 Refrigerator $4^{\circ} \pm 0.5^{\circ}\text{C}$.
- 6.15 Vibrator for mixing liquids in culture tubes.
- 6.16 Petri dishes of borosilicate glass, diameter 9 cm, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic Petri dishes.
- 6.17 Ehrlemeyer flasks of borosilicate glass for media preparation, capacity 500 ml and 1 litre.

- 6.18 Borosilicate glass bacteriological culture tubes.
- 6.19 Total volume (blow-out) pipettes of borosilicate glass, graduated, of 10 and 1 ml capacity, in stainless steel containers for sterilization.
- 6.20 Graduated borosilicate glass cylinders with glass beakers for cover.
- 6.21 Small borosilicate glass vials ("Durham tubes") to be inserted in culture tubes (6.18).
- 6.22 Bacteriological loops made from 22-24 Chromel gauge, bichrome or platinum-iridium. Diameter of the loop: 3 mm.
- 6.23 Heavy wrapping paper.
- 6.24 Aluminium foil (household quality).
- 6.25 Membrane filters, pore size 0.45 μ , diameter 4.7 cm, or similar fitting filtration apparatus (6.6).

NOTE: The 0.45 μ pore size membrane filter (MF) should be certified by the manufacturer to be free from substances which may hinder the growth and development of bacteria.

Maximum faecal coliform recoveries are obtained using membranes composed of mixed esters of cellulose exhibiting a surface opening of 2,4 μ m and a retention pore size of 0,7 μ m. Retention pore size is a measure of the smallest particle which is retained by the structure.

7. CULTURE MEDIUM AND CHEMICALS

7.1 m-FC-medium

Tryptose .	10.0 g
Proteose peptone No.3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salt No.3	1.5 g
Aniline blue	0.1 g

Distilled water (7.6) 1 litre

Diluted HCl (reagent grade for adjusting pH).

Preparation: Dissolve (dehydrate) the components of the medium in 1 litre of distilled water (7.6). Heat to boiling point until all components are completely dissolved. Add 10 ml of a 1 per cent solution of rosolic acid in 0.2N NaOH; keep boiling for more than one minute; then cool. The final medium should have a pH of 7.4.

Finished media can be stored in the dark at +4°C (6.14). Any unused media should be discarded after 96 hours. Check medium for sterility by incubating a blank, i.e. a Petri dish containing m-FC-medium together with the Petri dishes containing sample membrane filters (9.4).

NOTE: Difco m-FC broth base (No. 0883) or similar products may be used.

NOTE: Rosolic acid must be prepared freshly each time.

7.2 MacConkey broth

	Single strength
Sodium taurocholate	5 g
Lactose	10 g
NaCl	5 g
Peptone	20 g
1 per cent bromo-cresol purple in ethanol (95 per cent)	2 ml

Glass-distilled or de-ionized water (7.6) 1 litre

Dissolve components by shaking. Adjust pH to 7.1 ± 0.1 with diluted HCl and add the bromo-cresol purple solution.

Distribute 10 ml of the broth into culture test tubes (6.18) containing inverted vials (Durham tubes) and autoclave at 121°C for 15 minutes.

NOTE: The air in the inverted vials will disappear after autoclaving.

7.3 Indole test solutions

a) Peptone water

Tryptone	10.0 g
NaCl	5.0 g
Distilled water (7.6)	1 litre

Dissolve ingredients in distilled water and dispense 5 ml each into test tubes (6.18) and autoclave at 121°C for 15 minutes. The pH should be 7.0 - 7.4. If necessary adjust with diluted NaOH before sterilization.

b) Kovac's indole reagent

Paradimethyl-amino-benzaldehyde	5 g
Amyl alcohol	75 ml
Concentrated hydrochloric acid, HCl	25 ml

Dissolve the benzaldehyde in amyl alcohol and add hydrochloric acid. The reagent should be yellow.

Kovac's reagent is used to demonstrate indole production in peptone water (7.3a).

7.4 Phosphate buffer (pH = 7.2)

For injured bacteria addition of magnesium sulphate to phosphate buffer is recommended (0.250 g MgSO₄, 7H₂O per litre phosphate buffer).

K ₂ HPO ₄	3 g
KH ₂ PO ₄	1 g
Distilled water (7.6)	1 litre

Dissolve components and autoclave at 121°C for 15 minutes.

7.5 Thiosulphate solution

10 per cent sodium thiosulphate solution

7.6 Distilled water.

Use only water distilled in all glass or all quartz distillation apparatus. De-ionized water is also acceptable if produced in non-toxic apparatus.

NOTE: Commercially available distilled water is often produced in copper and zinc apparatus and highly toxic. Distilled water must contain < 10 ug copper per litre.

7.7 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 E. coli (8).

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

7.8 95 per cent ethanol per analysis.

8. PREPARATION OF CONTROL SAMPLE

Prepare a culture of E. coli strain from a national type collection according to instructions supplied with the culture.

9. PROCEDURE

9.1 Washing of glassware and equipment

All glassware and apparatus (6) are washed with non-toxic detergent (7.7), first rinsed thoroughly with hot tap water and then rinsed at least three times with distilled water (7.6).

9.2 Sterilization of glassware and equipment

9.2.1 Surface sample bottles (6.1). Clean sample bottles (9.1) are dried and sterilized in a drying oven (6.10) for two hours at 160°C. Before sterilization, place a small piece of filter paper in the neck of the 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 a pair of sterilized forceps (9.2.5) and fit the ground glass stopper securely into the neck of the bottle. Put the bottles into detergent-cleaned thermoisolated boxes (6.4). Separate the bottles from each other with clean filter paper to avoid breakage.

9.2.2 Subsurface sampler (6.3). Clean the subsurface sampler (9.1) with detergent (7.6), rinse with tap and distilled water (7.5). Enclose each sampler in heavy wrapping paper (6.23) and sterilize in an autoclave (6.9) for 15 minutes at 121°C.

NOTE: If residual chlorine is suspected in the water sample, add, aseptically, 0.1 ml of a 10 per cent thiosulphate solution (7.5) for each 100 ml of sample into the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

9.2.3 Clean Petri dishes (6.16) and pipettes (6.19), complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven (6.10) for 3 hours at 160°C.

NOTE: Disposable sterilized plastic Petri dishes may be more economical to use than reusable glass Petri dishes.

- 9.2.4 For sterilization of the MF-funnels (6.6), loosen the filter-holding assembly slightly and wrap the whole filter funnel in heavy wrapping paper (6.23) or aluminium foil (6.24). Sterilize in an autoclave (6.9) for 15 minutes at 120°C, or in a drying oven (6.10) for 3 hours at 160°C.
- 9.2.5 For sterilizing MFs (6.25) remove the paper separator (if present) and place 10 to 12 clean MFs into 9 cm diameter Petri dishes (6.16). Autoclave (6.9) for 15 minutes at 120°C. At the end of the sterilization let the steam escape rapidly in order to minimize the accumulation of condensate on the MFs. Sterilized MF's are commercially available in packings containing one or more filters.
- 9.2.6 Sterilize forceps (6.12) by dipping them into 95 per cent ethanol (7.8) and flaming them.

9.3 MF filtration procedure

9.3.1. Selection of sample size and dilution series

The MFs for counting should have from 20 to 80 colonies. If previous experience for planning the dilution series (see also figure 2) is not available, use the following dilution series 100, 10, 1, 10⁻¹ as a guide for clear sea-water.

9.3.2 Preparation of the dilution series.

Start the dilution series by taking 1 ml with a sterilized pipette (9.2.2) from the original sample (figure 2, dilution: D-0) and transfer this 1 ml into a clean, sterilized, culture tube (6.18). Add 9 ml of sterilized phosphate buffer (7.4) to make a 10 ml first dilution (D-1). Agitate the tube on a mixer (6.15) or shake it vigorously by hand.

9.3.3 Filtration procedure

Begin filtration with the highest dilution (D-1) in order to avoid contamination. Use a sterilized filtration funnel (9.2.4) for each dilution series. Place the sterilized MF (9.2.5) with flamed, sterilized forceps (9.2.6) over the porous plate of the filtration apparatus (9.2.4). Carefully place the matching funnel unit over the receptacle and lock it in place. With a sterilized pipette (9.2.3), drop the 10 ml of the D-1 dilution into the funnel. Mix by blowing air with the pipette through the solution before filtration. Filter with a partial vacuum. Unlock and remove the funnel, immediately remove the MF with flamed, sterilized forceps (9.2.6) and place it on the sterile surface of an aliquot of

m-FC-medium (7.1) contained in a sterilized Petri dish (9.2.3) with a rolling motion to avoid the entrapment of air. Before filtering the next lower dilution in the same manner, pass three 20 ml portions of sterilized buffer (7.4) through the assembled filtration unit. Aliquots filtered directly from the original sample bottle (i.e. 100 ml and 10 ml) do not need to be diluted with sterilized buffer.

NOTE: The aliquot of the m-FC medium can be placed on a sterilized adsorbent pad and the MF placed on top of it.

9.4 Incubation

The Petri dishes (9.3.3) containing the MF are sealed and then placed horizontally inside clean plastic bags. These plastic bags are then immersed in a water bath (6.7) and incubated for 24 hours at $44.5^{\circ} \pm 0.2^{\circ}\text{C}$. Incubate also one blank (without MF) Petri dish with m-FC-medium (7.1) as a sterility check.

9.5 Interpretation

Count only blue colonies. For counting use a binocular (6.8). Record results for all dilutions in table I.

NOTE: If the number of dubious colonies is greater than 10 per cent of the number of faecal coliforms, test dubious colonies as follows:

(a) MacConkey broth test

With a flamed bacteriological loop (6.22) transfer the suspected colony into a culture tube containing MacConkey broth (7.2) and incubate at $44^{\circ} \pm 0.2^{\circ}\text{C}$ for 24 hours. Coliforms will develop gas which is trapped in the inverted vials, and acid which turns the violet-like colour of the original broth into a yellowish colour.

NOTE: Colonies produced by faecal coliform bacteria are blue in colour. The non-faecal coliform colonies are grey to cream-coloured. Background colours on the membrane filter will vary from a yellowish cream to a faint blue, depending on the age of the rosolic acid salt reagent.

NOTE: Normally few non-faecal coliform colonies will be observed on m-FC medium because of the selective action of the elevated temperature and addition of the rosolic acid salt reagent.

9.6 Estimation of precision

Check the precision of the membrane filtration culture technique at the start of the project and at periodic intervals by preparing triplicate dilution series (9.3.2) of the same test sample and by triplicate membrane filtrations (9.3.3). Use an analysis of

variance to determine the standard error of triplicate dilution series and the standard error of triplicate membrane filtrations. If the coefficient of variance is too high, determine the cause of the high variance, eliminate it and repeat the entire sampling procedure.

9.7 Quality control

Complete a quality control test according to the instructions given in a reference method on quality control of the membrane filtration culture methods.

9.8 Intercalibration

Complete a precision test according to the instructions given in the reference method on intercalibration and estimation of precision.

10. EXPRESSION OF RESULTS

10.1 Calculation of faecal coliforms

10.1.1 Report the number of faecal coliforms in terms of faecal coliforms per 100 ml of sample. Compute the count using MFs with 20 to 200 coliform colonies and not more than 200 colonies of all other types per MF, by the following equation:

Faecal coliforms/100 ml = (Faecal coliform colonies x 100)/(ml sample filtered).

10.1.2 If two MFs corresponding to a certain dilution contain between 20 and 200 characteristic colonies, calculate the arithmetic mean of the number of colonies counted in the two MFs.

Retain only two significant figures, proceeding as follows:

- if the number is less than 100, round it to the nearest multiple of 5;
- if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;
- if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of coliforms per 100 ml. Express this result as a number between 1.0 and 9.9 multiplied by 10^n , n being the appropriate power of 10.

- 10.1.3 If there are MFs containing between 20 and 200 characteristic colonies at two consecutive dilutions, calculate the number of colonies for each dilution as specified in 10.1.1, and take as the result the arithmetic mean of the two values obtained, except when the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.
- 10.1.4 MFs that do not reach coliform numbers of 20 to 80 are also reported per 100 ml according to the formula above (10.1.1). For example, duplicated MFs had 5 and 3 coliform colonies for 50 ml sample filtered, equal to 8 colonies / 100 ml = $(5 + 3) \times 100 / (50 + 50)$ ml.
- 10.1.5 If there are fewer than 15 characteristic colonies on the MFs, report the results as:
- fewer than 15 coliforms per 100 ml.
- If there are no characteristic colonies on the MFs report the result as:
- fewer than 1 coliform per 100 ml.
- 10.1.6 Record confluent growth or colonies other than coliform too numerous to count. Collect a new sample if too many coliforms or too many other colonies were in the MF of the highest dilution and select a higher dilution series for a new test.

Use for recording the data observed, and for further evaluation, the test report form reproduced in table II.

10.2 Precision of the method.

No information available.

11. TEST REPORT

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this reference method or regarded as optional, as well as any circumstance that may have influenced the result.

The report shall include all details required for the complete identification of the sample.

NOTE: For the assessment of the sanitary quality of coastal waters other parameters are also taken into consideration, such as faecal streptococci, salmonella, vibrios, temperature, salinity, oxygen etc. It is recommended that the forms developed by the joint WHO/UNEP co-ordinated pilot project on coastal water quality control in the Mediterranean (MED PQL VII) should be used for reporting the microbiological and collateral parameters.

Table I : microbiological laboratory examination

COASTAL MONITORING / LABORATORY EXAMINATION /

Institution
and country

Organism:

Area :

Year :

Sheet No :

Point no.

Date

Time h

Medium

Depth m

Temperature °C

Salinity ‰

Container no

Start incubation
d & h

Sample test amount unit:

g or ml

Number 50

CF 10

Colonies 1

10⁻¹

10⁻¹

10⁻¹

10⁻⁴

10⁻⁵

Method

Date and time of cessation
of incubation

No. of colonies*

Examiner's Signature

Notes: *Per 100 ml for water, 100 g for shellfish, and per 1 g for sediments.

Remarks:

Table II: Test report on the determination of faecal coliforms in sea-water
by the membrane filtration culture method

1. General identification

1.1 Country _____ 1.2 Institute _____ 1.3 Area: Longitude _____ Latitude _____

1.4 Date: Time _____ Day _____ Month _____ Year _____ 1.5 Identification number or letter of sampling point _____

2. Bacteriological data:

Faecal coliforms (E. coli) colonies /100 ml _____

3. Operating conditions not specified in the reference method or regarded as optional as well as any circumstances that may have influenced the results.

DETERMINATION OF FAECAL STREPTOCOCCI IN SEA-WATER
BY THE MEMBRANE FILTRATION CULTURE METHOD

1. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of faecal streptococci in coastal bathing waters of the Mediterranean Sea and is designated to be used in sanitary surveillance of the Mediterranean bathing beaches.

The streptococci in the faecal streptococcal group are indicators of faecal pollution of water, in as much as the normal habitat of these organisms is generally the intestine of man and animals. Because recent studies indicate that streptococci similar to organisms in the faecal streptococcal group may be found in certain plants or plant products, it is possible that wastes from food-processing industries discharged into a body of water constitute the sources of some of these bacteria.

2. REFERENCES

- WHO (1975) Report of WHO/UNEP expert consultation on coastal water quality control programme in the Mediterranean. (Geneva, 15 - 19 December 1975) EHE/76.1. WHO, Copenhagen, 25 pp.
- WHO (1977a) Guidelines for health related monitoring of coastal water quality. Report of a group of experts jointly convened by WHO and UNEP (Rovinj, Yugoslavia, 23 - 25 February 1977). WHO, Copenhagen, 165 pp.
- WHO (1977b) Health criteria and epidemiological studies related to coastal water pollution. Report of a group of experts jointly convened by WHO and UNEP (Athens, 1 - 4 March 1977). WHO, Copenhagen, 33 pp.
- WHO (1977c) Mid-term review of the joint WHO/UNEP co-ordinated pilot project on coastal water quality control in the Mediterranean (MED VII). Report of a meeting of principal investigators of collaborating laboratories, jointly convened by WHO and UNEP (Rome, 30 May - 1 June 1977). WHO, Copenhagen, 23 pp.
- WHO (1978) Monitoring of recreational coastal water quality and shellfish culture areas. Report of a seminar jointly convened by WHO and UNEP (Rome, 4 - 7 April 1978). WHO, Copenhagen, 39 pp.

3. DEFINITION

Faecal streptococci are grampositive oblong-oval cocci occurring in pairs or in short chains, which after the prescribed time of incubation in the prescribed medium, grow colonies that by reduction of triphenyltetrazoliumchloride develop wholly or partially dark red or pink colour.

4. PRINCIPLE

From sea-water samples, taken under sterilized conditions, a dilution series is set up according to the number of faecal streptococci expected in the water sample. Aliquots of this dilution series are filtered through 0.45 μ membrane filters. The membrane filters are placed on M-enterococcus agar and incubated at $36^{\circ} \pm 1^{\circ}\text{C}$ for 48 ± 3 hours. Red to pink colonies are considered to be faecal streptococci. Residual chlorine, if present, is neutralized by adding thiosulphate to the sampling bottle before sterilization.

5. SAMPLING

5.1 Sampling plan.

Identify sources of pollution (outfall, river mouth, etc.) and mark them on a hydrographic map of the area. Select sampling points for surface samples about every 250 m along the coastline, 10 m offshore, choosing stations which are located in the major pollution sources and also outside them. In areas with tides, the sampling stations should be located 10 m offshore from the low-tide mark. If the water depth exceeds 5 m, take also one sample near the bottom (without disturbing the sediments) and one sample half-way between the bottom and the surface.

Establish one reference sampling point for every one km of coastline and one km offshore.

Sample all points at least twice a month during the swimming season and at three equally spaced intervals during the remainder of the year. Collect double samples during the first three sampling periods and repeat the collecting of double samples in selected stations once every season for estimation of sampling error.

Revise sampling plan after analysis and interpretation of the results of three monthly surveys, and after two years of observations.

NOTE: After the first three series of samples have been analysed, the number of stations may be increased or reduced.

NOTE: The sampling should be programmed in such a way that from the time of sampling to the time when samples are processed in the receiving laboratory not more than six hours have passed. Start sampling at the furthest sampling station in order to reduce the sample storage time. Samples which arrive at the receiving laboratory six hours or more after collection must be discarded and replaced by new samples.

NOTE: Die-away rates (T-90) of two to three hours have been observed as to faecal coliforms. Regardless, faecal streptococci are assumed to survive longer in sea-water than faecal coli. Samples must be filtered as soon as possible.

5.2 Sampling procedure, sample transport and sample storage

5.2.1 Sampling of surface water

Attach clean sterilized sample bottle (9.2.1) to the clean sampling rod (6.2). 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 about half a metre under the water surface with the mouth of the bottle downwards, then turn the sample bottle upwards and take the sample (figure 1 (a)). The sterilized sample bottle may also be filled directly by hand.

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 (6.4) with cooling pads at about 4°C, avoiding exposure to more than 10°C. Separate bottles from each other with clean wrapping paper (6.24) to avoid breakage. Check the temperature with a thermometer (6.5) every three hours. Report irregularities in the sampling protocol (6.27). Label sample bottles.

5.2.2 Sampling of subsurface water

Lower the sterilized subsurface sampler (9.2.2) after attaching it to a clean plastic rope, without letting the weight disturb the bottom sediments (figure 1 (b)). Release the messenger and after one minute retrieve the sampler and store it in a thermoisolated box (6.4). Proceed as for sampling of surface water (5.2.1).

NOTE: It is recommended that the label and standard forms developed in the MED POL VII pilot project should be used (see WHO, 1977a).

NOTE: Faecal streptococci die in sea-water. Therefore storage must be minimized. If transport time is too long, in situ filtration should be considered.

6. APPARATUS AND GLASSWARE

- 6.1 Sample bottles for surface sea-water: 200-300 ml capacity, wide-mouthed, of borosilicate glass,

NOTE: For sea-water sampling an adequate boat may be needed.
- 6.2 Sampling rod of non-corrosive material with a clamp to hold the sampling bottle (figure 1 (a)). Minimum number: three.
- 6.3 Subsurface sampler of the type shown in figure 1 (b), or similar, complete with plastic rope and weight.
- 6.4 Thermoisolated plastic boxes with cooling pads or similar cooling units.
- 6.5 Thermometer, 0° to 50°C, possibly unbreakable plastic type, used for checking temperature in plastic boxes (6.4).
- 6.6 Membrane filter filtration apparatus for 4.7 cm diameter filters consisting of at least three filter funnels for simultaneous filtration, made of borosilicate glass or other non-toxic sterilizable material, complete with electric or water vacuum pump.
- 6.7 Air or water incubator for $36^{\circ} \pm 1^{\circ}\text{C}$.
- 6.8 Binocular microscope, magnification 1000X, stereomicroscope, magnification 10 - 50X, and/or Quebec colony counter, magnification 2 - 3X.
- 6.9 Autoclave, max 2 atm., electric or gas.
- 6.10 Drying oven for sterilization at 160°C.
- 6.11 pH meter ± 0.1 units.
- 6.12 Stainless steel forceps.
- 6.13 Analytical balance ± 1 mg.
- 6.14 Refrigerator $4^{\circ} \pm 0.5^{\circ}\text{C}$.
- 6.15 Water bath for preparation of culture media set at 50°C.
- 6.16 Vibrator for mixing liquids in culture tubes.
- 6.17 Petri dishes of borosilicate glass, diameter 9 cm, complete with stainless steel containers for sterilization, or disposable pre-sterilized plastic Petri dishes.
- 6.18 Ehrlenmeyer flasks of borosilicate glass for media preparation, capacity 500 ml and 1 litre.

- 6.19 Borosilicate glass bacteriological culture tubes.
- 6.20 Total volume (blow-out) pipettes of borosilicate glass, graduated, of 10 ml capacity, in stainless steel containers for sterilization.
- 6.21 Graduated borosilicate glass cylinders with glass beakers for cover.
- 6.22 Small borosilicate glass vials ("Durham tubes") to be inserted in culture tubes (6.19).
- 6.23 Bacteriological loops made from 22-24 Chromel gauge, bichrome or platinum-iridium. Diameter of the loop: 3 mm.
- 6.24 Heavy wrapping paper.
- 6.25 Aluminium foil (household quality).
- 6.26 Membrane filters, pore size 0.45 μ , diameter 4.7 cm, or similar fitting filtration apparatus (6.6).

NOTE: The 0.45 μ pore size membrane filter (MF) 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 exhibiting a surface opening of 2.4 μ m, and a retention pore size of 0.7 μ m. Retention pore size is a measure of the smallest particle which is retained by the structure.

7. CULTURE MEDIUM AND CHEMICALS

7.1 M-enterococcus agar

Tryptose	20.0 g
Yeast extract	5.0 g
D(+) Glucose	2.0 g
Dipotassium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$)	4.0 g 4.0 g
Sodiumazide (NaN_3)	0.4 g
Agar	10.0 g
Distilled water (7.4)	1,000 ml

After rehydrating in boiling water sterilize in a pressure cooker at 120°C for 15 minutes.

Add immediately before use 10 ml of a 1% solution of 1,3,5-triphenyltetrazoliumchloride prepared under aseptic conditions to each 1000 ml of liquefied and to 50°C cooled basis agar. The pH should be 7.2 after heating. After the agar has solidified in petri dishes (12-15 ml medium per petri dish) invert the petri dishes so that moisture can absorb into the agar. Use only well dried plates. The medium may be contained at between 45°C - 50°C for up to four hours before the plates are poured. Poured plates may be stored in the dark up to 30 days, when maintained at between 2°C and 4°C. Check for sterility by incubating a blank m-enterococcus agar plate containing a membrane filter.

NOTE: Dehydrated Difco-Oxoid-BBL of Merk m-enterococcus agar or equivalent may be used.

7.2 Phosphate buffer (pH = 7.2)

K ₂ HPO ₄	3 g
KH ₂ PO ₄	1 g
Distilled water (7.4)	1 litre

For injured bacteria addition of magnesium sulphate to phosphate buffer is recommended. (0,250 g MgSO₄·7H₂O per litre phosphate buffer).

Dissolve components and autoclave at 120°C for 15 minutes.

7.3 Thiosulphate solution

10 per cent sodium thiosulphate solution

7.4 Distilled water

Use only water distilled in all glass or all quartz distillation apparatus. De-ionized water is also acceptable if produced in non-toxic apparatus.

NOTE: Commercially available distilled water is often produced in copper and zinc apparatus and highly toxic.

7.5 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 E. coli (8).

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

7.6 Ethanol 95 per cent per analysis.

8. PREPARATION OF CONTROL SAMPLE

Prepare a culture of *Streptococcus* sp. strain from a type collection according to instructions supplied with the culture.

9. PROCEDURE

9.1 Washing of glassware and equipment

All glassware and apparatus (6) are washed with non-toxic detergent (7.5), first rinsed thoroughly with hot tap water and then rinsed at least three times with distilled water (7.4).

9.2 Sterilization of glassware and equipment

9.2.1 Surface sample bottles (6.1). Clean sample bottles (9.1) are dried and sterilized in a drying oven (6.10) for two hours at 160°C. Before sterilization, place a small piece of filter paper in the neck of the 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 a pair of sterilized forceps (9.2.5) and fit the ground glass stopper securely into the neck of the bottle. Put the bottles into detergent-cleaned thermoisolated boxes (6.4). Separate the bottles from each other with clean filter paper to avoid breakage.

9.2.2 Subsurface sampler (6.3). Clean the subsurface sampler (9.1) with detergent (7.6), rinse with tap and distilled water (7.5). Enclose each sampler in heavy wrapping paper (6.23) and sterilize in an autoclave (6.9) for 15 minutes at 121°C.

NOTE: If residual chlorine is suspected in the water sample, add, aseptically, 0.1 ml of a 10 per cent thiosulphate solution (7.3) for each 100 ml of sample into the sample bottle before sterilization. This amount is sufficient to neutralize about 15 mg of residual chlorine per litre.

9.2.3 Clean Petri dishes (6.17) and pipettes (6.20), complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven (6.10) for 3 hours at 160°C.

NOTE: Disposable sterilized plastic Petri dishes may be more economical to use than reusable glass Petri dishes.

9.2.4 For sterilization of the MF-funnels (6.6), loosen the filter-holding assembly slightly and wrap the whole filter funnel in heavy wrapping paper (6.24) or aluminium foil (6.25). Sterilize in an autoclave (6.9) for 15 minutes at 120°C, or in a drying oven (6.10) for 3 hours at 160°C.

9.2.5 For sterilizing MFs (6.26) remove the paper separator (if present) and place 10 to 12 clean-MFs into 9 cm diameter Petri dishes (6.17). Autoclave (6.9) for 15 minutes at 120°C. At the end of the sterilization let the steam escape rapidly in order to minimize the accumulation of condensate on the MFs.

9.2.6 Sterilize forceps (6.12) by dipping them into 95 per cent ethanol (7.6) and flaming them.

9.3 MF filtration procedure

9.3.1 Selection of sample size and dilution series

The MFs for counting should have from 20 to 80 colonies. If previous experience for planning the dilution series (see also figure 2) is not available, use the following dilution series 100, 10, 1, 10⁻¹, as a guide for sea-water.

9.3.2 Preparation of the dilution series.

Start the dilution series by taking 1 ml with a sterilized pipette (9.2.2) from the original sample (figure 2, dilution: D-0) and transfer this 1 ml into a clean, sterilized, culture tube (6.19). Add 9 ml of sterilized phosphate buffer (7.2) to make a 10 ml first dilution (D-1). Agitate the tube on a mixer (6.16) or shake it vigorously by hand.

9.3.3 Filtration procedure

Begin filtration with the highest dilution (D-1) in order to avoid contamination. Use a sterilized filtration funnel (9.2.4) for each dilution series. Place the sterilized MF (9.2.5) with flamed, sterilized forceps (9.2.6) over the porous plate of the filtration apparatus (9.2.4). Carefully place the matching funnel unit over the receptacle and lock it in place. With a sterilized pipette (9.2.3), drop the 10 ml of the D-1 dilution into the funnel. Mix by blowing air with the pipette through the solution before filtration. Filter with a partial vacuum. Unlock and remove the funnel, immediately remove the MF with flamed, sterilized forceps (9.2.6) and place it aseptically on the sterile M-enterococcus agar surface in a Petri dish (7.1) with a rolling motion to avoid the entrapment of air. Before filtering the next lower dilution in the same manner, pass three 20 ml portions of sterilized buffer (7.2) through the assembled filtration unit. Aliquots filtered directly from the original sample bottle (i.e. 100 ml and 10 ml) do not need to be diluted with sterilized buffer.

9.4 Incubation

The Petri-dishes (9.3.3) containing the MF are incubated for up to 48 hours at $36^{\circ} \pm 1^{\circ}\text{C}$, either in air or water. For water bath incubation, the Petri dishes are sealed and then placed horizontally inside clean plastic bags. These bags are then immersed in a water bath (6.7). Incubate also one blank (without MF) Petri dish with M-enterococcus agar (7.1) as a sterility check.

9.5 Interpretation

Count only colonies which are pink or dark red, eventually with a narrow whitish zone around the colonies. Uncoloured colonies are not counted. The size of the colonies will vary between 0,5-3 mm.

NOTE: It is a condition for the production of typical reducing colonies, that the medium is not overgrown. Reduction of triphenyltetrazoliumchloride to a red pigment (triphenylformazan) may fail if colonies lie too closely packed. Besides strongly reducing Streptococcus faecalis and its variants, the definition above (section 3), comprises the faint reducing Streptococcus faecium, Streptococcus bovis and Streptococcus equinus.

9.6 Estimation of precision

Check the precision of the membrane filtration culture technique at the start of the project and at periodic intervals by preparing triplicate dilution series (9.3.2) of the same test sample and by triplicate membrane filtrations (9.3.3). Use an analysis of variance to determine the standard error of triplicate dilution series and the standard error of triplicate membrane filtrations. If the coefficient of variance is too high, determine the cause of the high

9.7 Quality control

Complete a quality control test according to the instructions given in the reference method on quality control of the membrane filtration culture methods.

9.8 Intercalibration

Complete a precision test according to the instructions given in the reference method on intercalibration and estimation of precision.

10. EXPRESSION OF RESULTS

10.1 Calculation of faecal streptococci

- 10.1.1 Report the number of faecal streptococci in terms of faecal streptococci per 100 ml of sample. Compute the count using MFs with 20 to 200 streptococci colonies and not more than 200 colonies of all other types per MF, by the following equation:

$$\text{Faecal streptococci/100 ml} = (\text{Faecal streptococci colonies} \times 100) / (\text{ml sample filtered}).$$

- 10.1.2 If two MFs corresponding to a certain dilution contain between 20 and 200 characteristic colonies, calculate the arithmetic mean of the number of colonies counted in the two MFs.

Retain only two significant figures, proceeding as follows:

- if the number is less than 100, round it to the nearest multiple of 5;
- if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;
- if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of streptococci per 100 ml. Express this result as a number between 1.0 and 9.9 multiplied by 10^n , n being the appropriate power of 10.

- 10.1.3 If there are MFs containing between 20 and 200 characteristic colonies at two consecutive dilutions, calculate the number of colonies for each dilution as specified in 10.1.1, and take as the result the arithmetic mean of the two values obtained, except when the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.

- 10.1.4 MFs that do not reach streptococci numbers of 20 to 80 are also reported per 100 ml according to the formula above (10.1.1). For example, duplicated MFs had 5 and 3 streptococci colonies for 50 ml sample filtered, equal to 8 colonies / 100 ml = $(5 + 3) \times 100 / (50 + 50)$ ml.

- 10.1.5 If there are fewer than 15 characteristic colonies on the MFs, report the results as:

- fewer than 15 streptococci per 100 ml.

If there are no characteristic colonies on the MFs, report the result as:

- fewer than 1 streptococcus per 100 ml.

10.1.6 Record confluent growth or colonies other than streptococci too numerous to count. Collect a new sample if too many streptococci or too many other colonies were in the MF of the highest dilution and select a higher dilution series for a new test.

Use for reporting the data observed, and for further evaluation, the test report form reproduced in table 2.

11. TEST REPORT

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this reference method or regarded as optional, as well as any circumstance that may have influenced the result.

The report shall include all details required for the complete identification of the sample.

Table I : microbiological laboratory examination

COASTAL MONITORING / LABORATORY EXAMINATION /

Institution
and country

Organism:

Area :

Year :

Sheet No :

Point no.

Date

Time h

Medium

Depth m

Temperature °C

Salinity ‰/oo

Container no

Start incubation
d & h

Sample test amount unit:

g or ml

Number 50

of 10

Colonies 1

in the 10⁻¹

20 - 80 10⁻¹

range 10⁻¹

only 10⁻⁴

10⁻⁵

Method

Date and time of cessation
of incubation

No. of colonies*

Examiner's Signature

Notes: *Per 100 ml for water, 100 g for shellfish, and per 1 g for sediments.

Remarks:

Table II : Test report on the determination of faecal streptococci in sea-water
by the membrane filtration culture method

1. General identification

1.1 Country _____ 1.2 Institute _____ 1.3 Area; Longitude _____ Latitude _____

1.4 Date: Time _____ Day _____ Month _____ Year _____ 1.5 Identification number or letter of sampling point _____

2. Bacteriological data:

Faecal streptococci (Enterococci) colonies /100 ml _____
streptococci(Enterococci) _____

3. Operating conditions not specified in the reference method or regarded as optional as well as any circumstances that may have influenced the results.

DETERMINATION OF FAECAL COLIFORMS IN SHELLFISH
(BIVALVES) BY THE MULTIPLE-TEST-TUBE METHOD (MPN)

1. SCOPE AND FIELD OF APPLICATION

The method described is suitable for the determination of total coliforms in shellfish specimens in culture areas of the Mediterranean Sea and is designated to be used in surveillance of the hygiene of sea-food in those areas.

Faecal coliforms are specific, exhibiting a high positive correlation with faecal contamination from warm-blooded animals. Filter-feeding shellfish will concentrate coliforms. The number of faecal coliforms per gram in edible shellfish tissue will give an indication of the potential health hazard to consumers of shellfish.

2. REFERENCES

- WHO (1975) Report of WHO/UNEP expert consultation on coastal water quality control programme in the Mediterranean. (Geneva, 15 to 19 December 1975) EHE/76.1. WHO, Copenhagen, 25 pp.
- WHO (1977a) Guidelines for health related monitoring of coastal water quality. Report of a group of experts jointly convened by WHO and UNEP (Rovinj, Yugoslavia, 23 - 25 February 1977). WHO, Copenhagen, 165 pp.
- WHO (1977b) Health criteria and epidemiological studies related to coastal water pollution. Report of a group of experts jointly convened by WHO and UNEP (Athens, 1 - 4 March 1977). WHO, Copenhagen, 33 pp.
- WHO (1977c) Mid-term review of the joint WHO/UNEP co-ordinated pilot project on coastal water quality control in the Mediterranean (MED VII). Report of a meeting of principal investigators of collaborating laboratories, jointly convened by WHO and UNEP (Rome, 30 May - 1 June 1977). WHO, Copenhagen, 23 pp.
- WHO (1978) Monitoring of recreational coastal water quality and shellfish culture areas. Report of a seminar jointly convened by WHO and UNEP (Rome, 4 - 7 April 1978). WHO, Copenhagen, 39 pp.

3. DEFINITION

Faecal coliforms are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas both at 36° and 44.5°C in less than 24 hours. They produce indole in peptone water containing tryptophan at 44.5°C.

4. PRINCIPLE

After the shellfish specimens have been washed and brushed in the laboratory, their soft tissue is separated aseptically from the shell and transferred to a sterilized blender flask. Then the soft tissue is macerated and diluted with four times its weight of 0.05 per cent of peptone water or phosphate buffer as used for examination of water. From this homogenate, multiple test tube dilution series (MPN) involving three tube series and coherent incubations are set up. First test tube series in this procedure is identical for total coliforms after cultivation in MacConkey broth at 36°C ± 1°C in 24 hours.

The second test tube series is prepared by transferring one drop from all positive readings at 36°C after 24 hours (as an optimal procedure also after 48 hours) in this first tube series, to new sterile test tubes in identical positions on the tube rack for the second incubation at 44.5°C ± 0.2°C in 24 hours. The third incubation series is prepared at the same time as the second by transferring one drop from positive tubes in first test tube series incubated at 36°C in 24 hours in identical positions on the third test tube rack with tubes containing approximately 5 ml of peptone water (7.4). Criteria for counting:

1. Total coliforms: Use MPN-table after determination of positive tubes (gas acid production) in first test tube series incubated at 36°C in 24 hours. This reading gives combination of positive tubes for MPN-calculation by means of MPN-table (table 2) referring to quintuple reading.
2. Faecal coliforms: Use MPN-table after determination of positive tubes (gas, acid production) in second test tube series, and positive tubes in third test tube series (display a red surface colour after addition of Kovac's reagent after 24 hours cultivation). This combined reading gives combination of positive tubes for MPN-calculation by means of MPN-table (table 2) referring to quintuple reading.

NOTE: Criteria for positive identification of faecal coliforms are:

- a) generation of acid and gas after 24 hours at 44.5°C.
- b) positive indole reaction after 24 hours at 44.5°C after addition of Kovac's reagent.

5. SAMPLING

5.1 Sampling plan

Mark the shellfish areas on a hydrographic map. Identify potential sources of pollution (outfalls, river mouth, etc.). Establish 20 specimens are collected during the regular routines of the local commercial shellfish producers.

Minimum sampling frequency is every three months. For areas which produce more than 500 tonnes per year, the sampling frequency is once monthly. During the peak consumption period the sampling frequency is once a week. Devise the sampling plan in such a way that the laboratory examination can be carried out within six hours of the sampling.

5.2 Sampling procedure

Select 20 specimens at random from the commercially harvested shellfish and place them in a clean plastic bag (6.16) and then in a container (6.1). Avoid immersion of the specimens in sea-water from the sampling area.

6. APPARATUS AND GLASSWARE

- 6.1 Thermoisolated plastic boxes with cooling pads or similar cooling units.
- 6.2 Water incubator for $44.5^{\circ} \pm 0.2^{\circ}\text{C}$.
- 6.3 Autoclave, max 2 atm., electric or gas.
- 6.4 Drying oven for sterilization at 160°C .
- 6.5 pH meter ± 0.1 units.
- 6.6 Stainless steel forceps.
- 6.7 Analytical balance $\pm 10\text{mg}$.
- 6.8 Refrigerator $4^{\circ} \pm 2^{\circ}\text{C}$.
- 6.9 Vibrator for mixing liquids in culture tubes.
- 6.10 Ehrlenmeyer flasks of borosilicate glass for media preparation, capacity 500 ml and 1 litre.
- 6.11 Borosilicate glass bacteriological culture tubes.
- 6.12 Total volume (blow-out) pipettes of borosilicate glass, graduated, of 10 ml capacity, in stainless steel containers for sterilization.
- 6.13 Graduated borosilicate glass cylinders with glass beakers for cover.

- 6.14 Stainless steel homogenizer or blender complete with several blender vessels.
- 6.15 Brush for cleaning shellfish shells.
- 6.16 Plastic bag for containment of shellfish samples.
- 6.17 Surgeon's scalpels or knives.

7. CULTURE MEDIUM AND CHEMICALS

7.1 MacConkey broth

	Single strength	Double strength
Sodium taurocholat	5 g	10 g
Lactose	10 g	20 g
NaCl	5 g	10 g
Peptone	20 g	40 g
Distilled water	1000 ml	1000 ml

Dissolve ingredients by shaking. Adjust pH to 7.1 and add 2 ml of a 1% alcohol solution (ethanol, 95%) of bromo-cresol purple to single strength, and 4 ml to the double strength medium. Distribute 10 ml of the broth into culture test tubes containing inverted vials (Durham tubes), and autoclave tubes at 121°C for 15 minutes.

7.2 Peptone water

Tryptone	10 g
NaCl	5 g
Distilled water (7.3)	1000 ml

Dissolve the ingredients in distilled water and dispense 5 ml into culture tubes. Autoclave the closed test tubes at 121°C for 15 minutes. Adjust pH to 7.0 - 7.4.

7.3 Kovac's indole reagent

Paradimethyl amino-benzaldehyde	5 g
Amyl alcohol	75 ml
Concentrated hydrochloric acid (HCl)	25 ml

Dissolve the benzaldehyde in amyl alcohol and add hydrochloric acid. The reagent should be yellow.

NOTE: Kovac's reagent is used to demonstrate indole production in peptone water (7.2). Distilled water must contain < 10 ug/l.

7.4 Peptone water (0.05%) for dilution:

Peptone	0.5 g
Distilled water	1000 ml

Dispense 9 ml into culture tubes determined for dilution of shellfish flesh. Autoclave the tubes at 121°C for 15 minutes. Adjust pH to 7.2.

NOTE: Concentration of peptone in diluents must never exceed 0.05%, while concentration of peptone in peptone water for indole reactions is 1%.

7.5 95 per cent ethanol per analysis

8. PREPARATION OF TEST SAMPLE

Weigh the sterilized blender vessel (6.14). Select 10 random bivalves (about 10 g in fresh weight) from the samples taken at each sampling station (5.2). Before opening the shells, carefully clean them with a brush (6.15) and alcohol (7.5). Then hold the closed bivalve with sterilized forceps (9.2.4) for a short time over a flame in order to dry the outside of the shells.

Next, cut the bivalve open with a sterilized knife or scalpel (9.2.4) by inserting the knife into the opening from which the byssus extrudes and cut the posterior adductor muscle by turning the knife as indicated in figure 2. Then cut in the other direction and open the bivalve with sterilized forceps (9.2.4).

NOTE: Do not try to break the bivalve open with the knife. If the two muscles are cut the shellfish will open easily.

Drain the liquor from the shells and transfer the flesh into the sterilized blender flask (6.14) with sterilized forceps. After transferring the soft parts of all specimens into the blender vessel, weigh the vessel and determine the fresh weight of the flesh sample.

Homogenize for two minutes and then dilute the sample with four times its weight of 1 per cent peptone water (7.1). The sample now contains about 10 g of flesh in about 50 ml.

9. PROCEDURE

9.1 Washing of glassware and equipment

All glassware and apparatus (6) are washed with non-toxic detergent (7.4), first rinsed thoroughly with hot tap water and then rinsed at least three times with distilled water (7.3).

9.2 Sterilization of glassware and equipment

9.2.1 Clean pipettes (6.12), complete with a cotton plug in the mouthpiece, are put into suitable stainless steel containers and sterilized in a drying oven (6.4) for three hours at 160°C.

9.2.2 Clean Ehrlenmeyer flasks (6.10) are covered with inverted beakers and sterilized in a drying oven (6.4) for three hours at 160°C.

9.2.3 Clean bacteriological culture tubes (6.11) with covers are sterilized either in an autoclave (6.3) for 20 minutes at 120°C or in a drying oven (6.4) for three hours at 160°C.

9.2.4 Sterilize forceps (6.6) and knives or scalpels (6.17) by dipping them into 95 per cent ethanol (7.5) and flaming them.

9.3 Transfer aseptically 5 ml of homogenized 1:4 diluted test sample into five sterilized culture tubes containing 5 ml MacConkey broth, double strength (see 7.1). This dilution is D-0, corresponding to 1 g of shellfish per tube (see figure 1).

For preparing dilution D-1, transfer aseptically 0.5 ml of homogenized 1:4 diluted test sample into five culture tubes, containing approximately 10 ml MacConkey broth, single strength (see 7.1).

This dilution is D-1, corresponding to 0.1 g of shellfish flesh per tube. For preparing dilution D-2, transfer 0.5 ml of a 1:9 dilution in 0.05% peptone water of homogenized 1:4 diluted test sample used for the preparation of D-0 and D-1, into five culture tubes containing approximately 10 ml MacConkey broth, single strength. This dilution is D-2, corresponding to 0.01 g shellfish flesh per tube.

For further dilutions if necessary transfer 1 ml of a 1:9 dilution of D-2 into five culture tubes containing approximately 10 ml MacConkey. Following this dilution technique, dilution D-3, D-4 etc., corresponding to 0.001 g, 0.0001 g shellfish flesh per tube may be produced.

NOTE: Using D-0, D-1 and D-2 as described above, faecal coli counts between 20 and 24,000 per 100 g flesh are possible. If all tubes are negative the results may be put down as < 20 per 100 g flesh.

NOTE: Using D-1, D-2 and D-3 as described above, faecal coli counts between 200 and 240,000 per 100 g flesh are possible.

If all tubes are negative the result may be put down as < 200 per 100 g flesh.

NOTE: Double amount of flesh added to the three dilution rows (2, 0.2 and 0.02 g respectively) cover the count interval between 10 and 12,000 per 100 g flesh.

NOTE: Inoculation directly from homogenized 1:4 diluted shellfish sample into MacConkey tubes cultivated at 44.5°C may be used, but give lower results than stepwise cultivation first at 36°C and then 44.5°C.

If first test tube series (see figure 1) is let out and total coliforms not estimated, the following procedure may be used:

- a) Inoculate directly from D-0, D-1 and D-2 into 3 X 5 tubes containing MacConkey broth as described above. Cultivate 24 hours at 44.5°C.
- b) Transfer after 24 hours a drop from positive tubes (acid and gas production) to peptone water for indole test, and cultivate 24 hours at 44.5°C. Add this cultivation 0.3 - 0.5 ml Kovac's reagent. Reading of tubes according to lines of direction given in point 4.

9.4 Incubation

Incubate the three dilution series in a water bath or in air at 44.5°C ± 2°C for 24 hours.

9.5 Indole test

Add 0.3 to 0.5 ml of Kovac's indole reagent (7.3) to each culture tube (9.3) and shake (6.9). Let the tube stand for about 10 minutes and observe the results.

A dark red colour in the amyl alcohol surface layer constitutes a positive indole test; the original colour of the reagent, a negative test.

9.6 Quality control and estimation of precision and accuracy

Complete a quality control test according to the instructions given in the relevant reference method.

10. EXPRESSION OF RESULTS

10.1 Record in table 1 the number of culture tubes which gave a positive reaction. Obtain the Most Probable Number (MPN) for coliforms and faecal coliforms from table 2, taking into account the sample test amount. Use table 3 for recording data and comments.

10.2 Precision of the result

Select the 95 per cent confidence limits from table 2.

11. TEST REPORT

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this reference method or regarded as optional, as well as any circumstance that may have influenced the result.

The report shall include all details required for the complete identification of the sample (table 3).

Reporting the data on reference points and multiple sampling points should be done on forms developed for MED POL - PHASE II (UNEP/WG.62/3, Appendix).

COASTAL MONITORING / LABORATORY EXAMINATION /

Institution
and country

Organism:

Area :

Year :

Sheet No :

Point no.

Date

Time h

Medium

Depth m

Temperature °C

Salinity ‰

Container no

Start incubation
d & h

Sample test amount unit:

g or ml

Number 50

of 10

Colonies 1

in the 10^{-1} 20 - 80 10^{-1} range 10^{-1} -only 10^{-4} 10^{-5}

Method

Date and time of cessation
of incubation

No. of colonies*

Examiner's Signature

Notes: *Per 100 ml for water, 100 g for shellfish, and per 1 g for sediments.

Remarks:

Table MPN for quintuple-test-tube series.

No. of tubes giving positive reaction out of 5 of 1 5 of 0.1 5 of 0.01 g each g each g each

MPN Index per 1 g

95% Confidence limits LOWER UPPER

No. of tubes giving positive reaction out of 5 of 1 5 of 0.1 5 of 0.01 g each g each g each

MPN Index per 1 g

95% Confidence limits LOWER UPPER

No. of tubes giving positive reaction out of 5 of 1	No. of tubes giving positive reaction out of 5 of 0.1	No. of tubes giving positive reaction out of 5 of 0.01	MPN Index per 1 g	95% Confidence limits LOWER	95% Confidence limits UPPER	No. of tubes giving positive reaction out of 5 of 1	No. of tubes giving positive reaction out of 5 of 0.1	No. of tubes giving positive reaction out of 5 of 0.01	MPN Index per 1 g	95% Confidence limits LOWER	95% Confidence limits UPPER
0	0	0	<0.2			4	2	1	2.6	0.9	7.8
0	0	1	0.2	<0.5	0.7	4	3	0	2.7	0.9	0.8
0	1	0	0.2	<0.5	0.7	4	3	0	3.3	1.1	9.3
0	2	0	0.4	<0.5	0.11	4	4	0	3.4	1.2	9.3
1	0	0	0.2	<0.5	0.7	5	0	0	2.3	0.7	7.0
1	0	1	0.4	<0.5	1.1	5	0	1	3.1	1.1	8.9
1	1	0	0.4	<0.5	1.1	5	0	2	4.3	1.5	11.0
1	1	1	0.6	<0.5	1.5	5	1	0	3.3	1.1	9.3
2	0	0	0.5	<0.5	1.3	5	1	1	4.6	1.6	12.0
2	0	1	0.7	0.1	1.7	5	1	2	6.3	2.1	15.0
2	1	0	0.7	0.1	1.7	5	2	0	4.9	1.7	13.0
2	1	1	0.9	0.2	2.1	5	2	1	7.0	2.3	17.0
2	2	0	0.9	0.2	2.1	5	3	0	7.9	2.5	19.0

No. of tubes giving positive reaction out of 5 of 1 5 of 0.1 5 of 0.01 g each	MPN Index per 1 g	95% Confidence limits		No. of tubes giving positive reaction out of 5 of 1 5 of 0.1 5 of 0.01 g each	MPN Index per 1 g	95% Confidence limits	
		LOWER	UPPER			LOWER	UPPER
2	1.2	0.3	2.8	5	11.0	3.1	25.0
3	0.8	0.1	1.9	5	14.0	3.7	34.0
3	1.1	0.2	2.5	5	18.0	4.4	50.0
3	1.1	0.2	2.5	5	13.0	3.5	30.0
3	1.4	0.4	3.4	5	17.0	4.3	49.0
3	1.4	0.4	3.4	5	22.0	5.7	70.0
3	1.7	0.5	4.6	5	28.0	9.0	85.0
3	1.7	0.5	4.6	5	35.0	12.0	100.0
4	1.3	0.3	3.1	5	24.0	6.8	75.0
4	1.7	0.5	4.6	5	35.0	12.0	100.0
4	1.7	0.5	4.6	5	54.0	18.0	140.0
4	2.1	0.7	6.3	5	92.0	30.0	320.0
4	2.6	0.9	7.8	5	160.0	64.0	580.0
4	2.2	0.7	6.7	5	240.0		

Source: (Standard Methods, American Public Health Ass. Wash. D.C.)

Table 3. Test report on the determination of faecal coliforms
in shellfish by the multiple-test-tube method

1. General information

1.1 Country 1.2 Institute 1.3 Area: Longitude
1.4 Date: Time Day Month Year

2. Bacteriological data:

Faecal coliforms (E. coli) MP/g shellfish flesh

3. Operating conditions not specified in the reference method or regarded as optional as well as any circumstances that may have influenced the results.

DETERMINATION OF TOTAL MERCURY IN EDIBLE TISSUE OF
FISH BY FLAMELESS ATOMIC ABSORPTION SPECTROPHOTOMETRY
AFTER LIQUID PRESSURE DECOMPOSITION OF THE ORGANIC MATERIAL

1. SCOPE AND FIELD OF APPLICATION

This reference method describes a flameless atomic absorption spectrophotometric method for the determination of total mercury in biological materials after the organic matter is decomposed by liquid pressure decomposition. Detection limit is 0.05 ug of mercury per kg of fresh weight.

2. REFERENCES

Bernhard, M. (1976), Manual of methods in aquatic environment research. Part 3. Sampling and analyses of biological material (Guidelines for the FAO(GFCM)/UNEP Joint Co-ordinated Project on Pollution in the Mediterranean) FAO Fish.Tech.Pap. (158): 124 pp.

FAO/UNEP(1975) Report of the FAO(GFCM)/UNEP Expert Consultation on the Joint Co-ordinated Report on Pollution in the Mediterranean, Rome, Italy, 23 June - 4 July 1975, Food and Agriculture Organization, Rome. Access No. H 0975:42 pp.

3. PRINCIPLE

After sampling and sample preparation of fish without contamination, an aliquot of the edible tissue is decomposed in a pressure container in the presence of nitric acid at 120° - 150°C. Then the mercuric ion is reduced with an excess of SnCl₂ to metallic mercury. The metallic mercury is then volatilized by aeration and the mercury determined as a monatomic vapour by flameless atomic absorption spectrophotometry (flameless AAS) at a wavelength of 253.7 nm.

4. REAGENTS

For this analysis use only distilled water and reagents of recognized analytical quality, with as low as possible mercury concentrations.

- 4.1 Demineralized distilled water or water of equivalent quality, free from mercury.
- 4.2 Sulphuric acid ($d_{20^{\circ}\text{C}} = 1.84 \text{ g/ml}$) diluted with an equal volume of water (4.1).

CAUTION - Add the acid to the water slowly and with constant stirring to avoid spattering of concentrated acid.

- 4.3 Nitric acid ($d_{20^{\circ}\text{C}} = 1.4$ g/ml, approximately).
- 4.4 Hydrochloric acid ($d_{20^{\circ}\text{C}} = 1.19$ g/ml).
- 4.5 Potassium permanganate solution (50 g/l).
- 4.6 Stannous chloride (50g/l $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$)/hydroxylamine sulphate solution. Prepare this solution daily by mixing 10 ml H_2SO_4 (4.2) with about 60 ml H_2O (4.1). After allowing to cool to room temperature, dissolve 3 g NaCl, 3 g hydroxylamine sulphate, and 5 g SnCl_2 in the diluted H_2SO_4 and bring the volume to 100 ml with H_2O .

NOTE: Instead of hydroxylamine sulphate an equivalent amount of hydroxylamine chloride can be used.

- 4.7 Sulphochromic mixture containing 4 g potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) in 1 litre of sulphuric acid (4.2).
- 4.8 Mercury standard solutions
 - 4.8.1 Mercury stock solution: 1 g Hg/l. Weigh 1.354 g mercuric chloride (HgCl_2) to the nearest mg. Transfer to a 1 000 ml volumetric flask. Prepare an approximate 1 % solution of nitric acid (4.3) with distilled water (4.1) and bring the volume to 1 000 ml with this 1 % nitric acid.
 - 4.8.2 Mercury standard solution: From the stock solution (4.8.1) prepare, by appropriate dilutions using micropipettes (5.20), a mercury standard solution which in 0.1 ml contains the lowest amount of mercury standard to be used for the standardization (7.4.1). Prepare this standard solution frequently using 1 ml nitric acid (4.3), diluted with 250 ml distilled water (4.1) for the appropriate dilutions.

NOTE: The concentration of the Hg standard depends on the Hg levels anticipated in the samples to be analysed.

NOTE: All reagents must be checked for Hg contamination by running blanks.

- 4.9 Matrix substandard: prepare a sufficiently large number of fillets to last for several digestions (e.g. 300 g FW) according to 7.1. Homogenize with a stainless steel blender or other homogenizer (5.28).

Test for homogeneity by running 5 mercury determinations following 7.3 and 7.4. If the standard deviation is acceptable the substandard is ready for use. Otherwise homogenize the substandard again or prepare a new substandard.

NOTE: This matrix substandard should be prepared from specimens of the same species to be analysed.

NOTE: Select for the preparation of the substandard the smallest specimens available. They will have low mercury content since often mercury concentration increases with size.

5. APPARATUS

- 5.1 Plastic thermoisolated boxes during warm periods, refrigerator (+1°C to +4°C) or ice for cooling
 - 5.2 Deep-freezer (- 18°C), if sampling trips take more than 48 hours.
 - 5.3 High density polyethylene bags.
 - 5.4 Plastic length-measuring board or a Pyrex dish with centimetre scale attached underneath for small and medium-size fish. For large fishes adequate length-measuring scale.
 - 5.5 Two to four plastic knives made out of high density polyethylene, or similar, of high purity (quartz knives are an alternative).
 - 5.6 Pyrex dishes, plastic sheets or similar.
 - 5.7 Two to three pairs of plastic tweezers (see annex II).
 - 5.8 High density, high quality, polyethylene bags and airtight plastic containers with screw caps for preservation of samples in deep freezer cleaned with detergents and rinsed with distilled water (4.1).
 - 5.9 High density polyethylene sheets to cover the bench.
 - 5.10 Smaller polyethylene sheets to be used as "weighing paper".
 - 5.11 Balance (100-200 g) with a precision of 0.001 g, or better, for weighing specimens and subsamples; preferably a "top-loading" balance
 - 5.12 Plastic washing bottle with glass-distilled water (4.1).
 - 5.13 Distilled water (4.1) in a plastic dispenser or clean sea-water.
 - 5.14 Five to nine digestion vessels (Teflon) of at least 25 ml capacity, either single or in a "digestion block".
- NOTE: If smaller digestion vessels are used, the amounts digested in procedure (7.3.3) must be accordingly reduced to avoid explosions.
- 5.15 Oven or hot plate for digestion at 120° - 150°C.

- 5.16 Five to nine 10 ml and two 50 ml volumetric flasks (borosilicate glass).
- 5.17 Weighing bottle with a ground stopper.
- 5.18 Desiccator.
- 5.19 Analytical balance (0.0001 g) for weighing reagents and samples.
- 5.20 Micropipettes to deliver accurately 0.1 ml.
- 5.21 Drying oven with thermometer.

NOTE: A freeze-drier for fish samples containing large amounts of oil and lipids may be needed.

- 5.22 Atomic absorption spectrophotometer provided with a hollow cathode mercury lamp and background correction; alternatively a mercury vapour analyser may be used.

NOTE: Background correction may not be necessary if it can be shown that the matrix effect is negligible.

- 5.23 A recorder, a maximum signal indicator, or similar.
- 5.24 A tall-form aeration flask with a volume suitable for the sample size and compatible with the rest of the apparatus, bearing a calibration mark corresponding to the optimum filling level, the dead volume of which has been reduced to a minimum and through which the gas current flows under optimum conditions. For this, the aeration tube should be fitted either with a finely drawn-out point, a ball pierced with holes, or a glass frit (porosity between 100 and 250 μm). It is necessary to confirm that a series of different flasks all give the same result. After each use, treat the aeration flask with the sulphochromic solution (4.7) to oxidize any traces of Sn(II) that it may contain.
- 5.25 Measuring cell with windows (e.g. quartz) transparent to UV(253.7 nm), the length and the diameter of which are suitable for the spectro-photometer being used.
- 5.26 Equipment which eliminates any condensation of water vapour in the measuring cell. Any appropriate system may be adapted for this: an infra-red lamp, an electrical heating element, a rod heater, by passing some air around the cell, etc.
- 5.27 Equipment to absorb mercury vapour leaving the measuring cell (e.g. 0.25 per cent iodine in 3 per cent potassium iodide solution).

NOTE: Figure 1 shows an example of the way in which the various components are assembled and the principle of the operation of the apparatus in the case of a measured system using an open circuit. Any other satisfactory arrangement may be utilized. The

entrainment gas may be air, nitrogen or argon, and an absorbing solution other than that given in "J" may be used. It is possible to use an aeration system based on a closed circuit system though this system is less sensitive than the open circuit one. Commercially available kits can also be found.

NOTE: Glassware that is used for the first time in the analytical procedure (7.4) must be thoroughly cleaned, e.g. by the following procedure:

- wash with diluted 10 % nitric acid (4.3);
- wash with a mixture of 4 volumes sulphuric acid (4.2) and 1 volume potassium permanganate solution (4.5) prepared in the container just before using it;
- wash with a hydroxylamine sulphate solution to remove all deposits of manganese dioxide;
- finally, wash several times with distilled water (4.1) or leave the glassware in sulpho-chromic acid (4.7) for several hours, rinse with distilled water (4.1) and leave in about 10 % nitric acid until the glassware is needed. Then rinse with distilled water (4.1).

5.2.8 Stainless steel homogenizer or other tissue homogenizer made from glass or Teflon.

6. SAMPLING

6.1 Sampling plan

Design a sampling plan in accordance with the objective of the investigation.

For surveys, collect fish monthly or seasonally. Make plans to obtain an unsorted fish sample of the species of interest in order to obtain a size frequency distribution of the specimens caught and record the length (and possibly also the weight) of all specimens in this unsorted sample. For analysis, plan to select from this sample specimens of all sizes.

NOTE: Mercury concentrations in fishes are known to correlate with size. Body weights are more suitable than length for correlating mercury concentration with size, but for simplicity of field operations length measurements are sufficient. With a length/weight curve of the species, the length measurements can be approximately transformed into body weights.

NOTE: The size frequency distribution of the specimens of the sample under investigation is needed for the estimation of the Hg intake rates by consumers.

NOTE: The minimum number for a composite sample is six specimens.

6.2 Presampling preparation

Clean thermoisolated boxes (5.1), high density polyethylene bags (5.3), and length-measuring board (5.4) with detergent and rinse with distilled water (4.1) or clean sea-water from the sampling zone.

6.3 Sampling procedure for surveys

Determine the length (fork length, figure 2) of all specimens in the entire catch if small, or of a representative subsample of the total catch, for a length frequency distribution of the catch. Proceed similarly for a length frequency distribution of the fish specimens offered on a market.

Then select from this sample a subsample of (6 to 20) specimens with intact skin representing all sizes encountered in the sample. Place these specimens in a clean plastic bag (5.3). Squeeze out the air and close the bag with a knot or thermoseal, or close similarly. The bag is then placed along with the sample identification note in a second plastic bag and closed in the same way as the first bag. If necessary, the sample is then placed in a refrigerator (+1° to + 4°C) or in a thermoisolated box (5.1) cooled with ice. Care must be taken that no water from the ice enters the plastic bags containing the fish, to avoid contamination.

NOTE: Large specimens from species such as tuna and swordfish are most conveniently subsampled from specimens caught by commercial fishermen. These subsamples may be obtained when the specimens are being sold, during their preparation for food processing, etc. In many cases in the course of these operations parts of the specimen are discarded and from these parts samples may be obtained. Every effort should be made to obtain sections or pieces at least 3 to 5 cm thick in order to allow the cutting off of dirty parts of the sample during sample preparation (7.1).

NOTE: If transport to the receiving laboratory requires more than 48 hours it is better to deep-freeze (-18°C) (5.2) the specimens

NOTE: Fish with strong fins, rays and spines will easily puncture the plastic bags. Special care is therefore needed to avoid such damage.

7. PROCEDURE

7.1 Sample preparation

7.1.1 Preoperational preparations

Clean knives (5.5), dishes (5.6), tweezers (5.7), length-measuring board (5.4) and polyethylene sheets (5.10) with detergent, rinse with distilled water (4.1). Cover the working area with pre-cleaned plastic sheets (5.9).

7.1.2 Sample preparation procedure

Clean hands carefully with detergent and rinse them with water. Determine the fork length (see figure 2) to the nearest mm on the length-measuring board (5.4).

Weigh (5.11) the fish on a clean plastic sheet (5.10) to the nearest 0.1 g and calculate the fresh weight (FW).

Rinse the fish with distilled water (4.1) and place it on the clean surface of a glass dish (5.6).

Remove the pectoral fin and cut the skin of the fish with a knife (5.5) near the dorsal fins, starting from the head to the tail as shown in figure 2. Then cut near the gills across the body, along the ventral edge from the gills to the tail and finally across the body near the tail. These four cuts should be carried out first on one side taking care not to cut too deep into the fillet in order to avoid cutting into the viscera. It is advisable that a second person hold the fish by the head and tail during the operation.

Pull the skin from the flesh with a pair of tweezers, taking care that the outer skin does not contaminate the flesh.

With a second clean knife (5.5), cut the fillet from the vertebral column starting from the cut near the gills, while a second person is holding the head and tail of the fish. Lift the fillet with a second pair of tweezers (5.7), so that the fillet will not touch the Pyrex vessel or other parts of the fish.

Weigh a clean piece of plastic "weighing paper" (5.10), note the weight and place the first fillet on it.

If one fillet does not yield enough material put the fish, skin side upwards, on a still clean surface of the Pyrex dish (5.6) or on a second clean Pyrex dish and remove the second fillet from the backbone as described above.

Add the second fillet to the first on the balance (5.11) and determine the weight of the two fillets. Note the value and calculate the FW.

Determine sex by examining the gonads.

Composite sample: In a composite sample it is necessary to reduce the weight of the two fillets to that of the smallest fish. Therefore arrange specimens according to size and start the sample preparation with the smallest specimen.

Store fillets temporarily under clean conditions. When the number of fillets from 6 or more fishes have reached the required amount of material weight the fillets and note the weight in the protocol.

Then homogenize the fillets in a stainless steel blender (5.28) and transfer the homogenate into a suitable clean container (5.8) which has been weighed empty. Weigh the container with the homogenate and note the weight in the protocol.

Close the container, label it and deep freeze it.

NOTE: Male and female specimens should be homogenized separately and placed in different containers.

NOTE: If the composite samples will be analysed also for other metals a tissue homogenized made from glass or Teflon should be used.

Single specimen sample: Place one or both fillets in an airtight container (5.8). Identify the container with a code number. Then deep-freeze.

Record all data in the protocol.

NOTE: Deep-frozen samples are partially thawed before sample preparation by placing them overnight in a refrigerator at -2° to -4°C .

NOTE: Partially frozen samples are easier to cut than fresh or completely thawed ones.

7.1.3 Sample preparation procedure for large-size fish

Partially thaw if necessary.

Rinse the slice with distilled water (4.1).

Place the slice on a clean Pyrex dish (5.6) and remove the skin and bones that may be present. If the tissue sample has been cut with a metal knife during the sampling of the fish, cut thin slices from both surfaces with a plastic knife (5.5) in order to obtain a clean uncontaminated surface.

Weigh a pre-cleaned plastic bag (5.8), note the weight, place the cleaned slice in the bag, reweigh the bag with its contents, and calculate the FW. Place the sealed bag with the sample identification note in a second bag, seal this bag, and then deep-freeze.

See notes in previous section.

7.2 Determination of dry weight

A clean weighing bottle (5.17), with the ground stopper removed, is put into the drying oven (5.21) (100°C , 2 hours), using a pair of clean pincers. It is important to use the pincers every time the glass is touched, to avoid leaving fingerprints and particles of dirt on the weighing bottle.

The stopper and the bottle are put into the desiccator (5.18) to cool.

The empty bottle and stopper are then carefully weighed on the analytical balance (5.19). The weight obtained is the weight of the dried, empty weighing bottle and stopper. Note the weight.

Remove the stopper and place about 1-2 g of material (7.1.2) in the weighing bottle and replace the stopper.

Determine carefully the weight of the weighing bottle plus stopper. The weight obtained is the FW of the tissue plus the weight of the bottle and stopper.

Place the bottle in the drying oven (100°C), removing the stopper and placing it also in the drying oven.

After 24 hours replace the stopper in the bottle, remove the bottle with stopper from the drying oven and place it in a desiccator to cool.

Weigh the stoppered bottle and note the weight.

Repeat the drying cycle until the difference between subsequent weighings is less than 5 per cent of the total weight; record FW and DW and calculate the FW/DW ratio.

NOTE: The concentrations of elements and substances are usually calculated with reference to fresh weight (FW) and dry weight (DW); and both should, if possible, be determined. FW or live weight is not easily defined, since marine organisms lose water more or less rapidly after being taken from the sea-water. On the other hand, most methods refer to FW because it is easier to compare with volume measurements. DW determinations also present problems, because the requirement that the tissues should be dried until constant weight is reached, cannot always be satisfied, especially when large amounts of lipids are present. Reporting data on ash weight has only slight significance.

The determination of the DW requires that the sample be dried at 100°C until constant weight is obtained (usually after 24 to 48 hours). Repeated weight determinations of the sample are necessary to establish if a constant weight has been reached.

NOTE: Oily fishes (sardine, mullet etc.) cannot be dried to constant weight in an oven at 100°C, because the lipids will liquify and evaporate slowly. These fishes have to be freeze-dried to constant weight following the same procedure but substituting a freeze-drier for the drying oven.

7.3 Mineralization of the biological matrix .

Explosion hazard

If too high amounts of organic materials are placed in closed vessels, (e.g. instead of the amount of material in FW the same amount in dry weight (DW) is used) the vessels may burst.

- Therefore, all digestion procedure must be carried out with the appropriate precautions necessary when working with hot acids. For example, fume hoods must be closed when the vessels are heated. Defective plastic bottles must be discarded and bottles which have been used for a certain length of time must be replaced before there is any risk of bursting.

7.3.1 Cleaning of the digestion vessel before and between digestions.

Clean vessels (5.14), if necessary, and rinse with distilled water (4.1), then run a digestion procedure without adding the sample. Analyse the solution according to the analytical procedure (7.4). If the blank value is high, repeat this "blank digestion".

7.3.2 Predigestion experiments

Determine the minimum amount of concentrated nitric acid (4.3) necessary to destroy completely the organic matter for every new matrix by adding to a 1 g FW sample (7.1.2) increasing amounts of acid from 1 ml to not more than 6 ml HNO_3 , following the digestion procedure (7.3.3).

7.3.3 Digestion procedure

Place a sample of about 1 g FW (7.1.2) in each of the Teflon vessels (5.14), one vessel being charged with a matrix substandard (4.9) containing a known amount of mercury to check the efficiency of each digestion.

Add the predetermined amount (7.3.2) of concentrated HNO_3 , cover the vessels and close them tightly.

Let the samples in the vessels predigest at room temperature for at least an hour.

Place the vessels in a pre-heated oven or on a hot plate (120° - 150°C) (5.15) for at least 6 hours.

Remove the vessels from the oven and let them cool to room temperature and then open. If the solution is not clear or has a yellow-brownish colour, the digestion is not complete and has to be repeated. If the solution is clear, transfer the contents of each vessel into clean 10 ml volumetric flasks (5.16) and bring up to volume with distilled water (4.1). The contents of the volumetric flasks represent the test solution.

NOTE: It is important not to exceed the quantities of organic materials recommended. Since excess nitric acid has to be eliminated to avoid interference in the determinations, it is necessary that in preliminary experiments the amount of acid required to destroy the organic matrix is determined for each matrix used in the project.

7.4 Analytic determination of total mercury

7.4.1 Standardization

Before a new matrix is analysed, and at periodic intervals as specified in the quality control procedure (9.3), carry out a digestion procedure with 6 vessels (5.14) all but one charged with the substandard (4.9) in order to standardize (calibrate) the method and the apparatus used.

Prepare an appropriate standard solution (4.8:2) so that 0.1 ml of this standard added to 10 ml of the test solution will result in a Hg concentration approximately equal to the lowest Hg concentration anticipated in the samples to be analysed.

Add about 1 g FW of the substandard (4.9) to five vessels (no substandard is added to the sixth: it is the reagent blank). With a micropipette (5.20), add 0.1 ml of the solution 4.8.2 to vessel no. 2 (no standard is added to the first vessel), 0.2 ml to vessel no. 3, 0.3 ml to vessel no.4, 0.4 to vessel no.5. Then add the predetermined amount (7.3.2) of conc. nitric acid to all six vessels and carry out the digestion procedure (7.3.3) and the aeration and determination (7.4.2).

Construct a standardization working curve (calibration curve).

7.4.2 Aeration and determination

Adjust the controls of the apparatus and the gas flow using an aeration flask (5.24) filled with water (4.1) to the calibration mark. Wait until the apparatus and the gas flow stabilize. Divert the gas flow and replace the aeration flask containing water by one containing 2 ml of the test solution (7.3.3) to which 2 ml stannous chloride solution (4.6) have just been added.

Mix, wait 30 seconds and then restore the gas flow through the aeration flask. The entrainment of the mercury vapour through the measuring cell (5.25) produces a recorder tracing which rapidly attains a maximum. If peak heights are being used as a measure of response, purge the gas circuit and replace the aeration flask with one containing only water as soon as the recorder tracing begins to decrease. If a peak area is being measured, do not interrupt the tracing until the signal has returned to its initial value. After each test, purge the aeration flask.

8. EXPRESSION OF RESULTS

From the height of the peak obtained on the test solution, determine, by reference to the standardization working curve (7.4.1) and making allowance for the blank determination, the concentration of mercury in the test solution. In the case of an apparatus with digital read-out or a maximum response indicator, prepare a graph of mass of mercury against the corresponding instrument read-out. From this value compute the total mercury concentration of the sample, allowing for the volumes of reagents introduced during the treatment of the sample. Express this concentration in ug/kg FW and DW.

9. ESTIMATION OF PRECISION AND ACCURACY

9.1 Precision

Estimate the precision (CV = coefficient of variation) of the entire analytical procedure (7.4) by digesting (7.3.3) 5 different subsamples from one original sample. If the coefficient of variation in the ppm range is greater than 10 per cent, then check the procedure for possible errors and contamination.

9.2 Accuracy

Participate in the intercalibration exercises and, in addition, analyse a known certified standard of a matrix similar to the material under study.

9.3 Quality control

Conduct a quality control programme periodically in order to guarantee the precision and accuracy of your results according to the appropriate quality control method.

10. TEST REPORT

Annex I contains the proper formats and guidelines to be completed by the responsible analyst. In addition to the results obtained, the test report shall include details of the sampling and analytical methods used.

Annex II

PREPARING PLASTIC TWEEZERS

Methylmetacrylate of 4 mm thickness has been found very useful as it will give the right elasticity. The strips from which the tweezers are to be made should be cut according to the strength of the material. The easiest way to heat the plastic and bend it is with a hot air blower used for forming plastics. A drying oven brought to 135^o-140^oC can be used, but it is much more difficult to make tweezers by heating the plastic in an oven since the plastic twists easily.

Material:

- Sheets of acrylic (methylmetacrylate) resin, 4 mm thick (trade names, e.g. Perspex, Plexiglas, Lucite)
- A plastic tube of 40 mm diameter

Equipment:

- A hot air blower (300°-350°C) for plastics (e.g. Karl Leister, Switzerland, W 1850, 400 rpm)
- Or a drying oven at 135°-140°C

Procedure: with a hot air blower

1. Cut with an electric or a hand saw strips about 10 mm in width and 250 mm in length.
2. Heat a part about 60 mm long in the middle of the strip so that it will bend easily, and bend it round the plastic tube carefully in order to make both ends meet. Cool the plastic with cold water.
3. Sharpen the ends with a file and roughen the inside of the tweezers so that they grip well.
4. Wash the tweezers carefully with detergent and rinse them with distilled water.

Procedure : with a drying oven

1. Place the plastic strips on a clean piece of wood in a drying oven (135°-140°C) until the plastic becomes soft.
2. Lift the plastic strip at one end with a pair of tweezers and remove from the oven, letting the other end hang down so that the plastic will not bend.
3. Bend it around the plastic tube without letting the tips meet and cool them immediately by dipping them into a beaker of clean, cold water so that they do not curve.
4. Now bend the tips of the tweezers so that they will meet.
5. Prepare the ends as described earlier.

DETERMINATION OF TOTAL MERCURY IN EDIBLE TISSUE OF
MUSSELS BY FLAMELESS ATOMIC ABSORPTION SPECTROPHOTOMETRY
AFTER LIQUID PRESSURE DECOMPOSITION OF THE ORGANIC MATERIAL

1. SCOPE AND FIELD OF APPLICATION

This reference method describes a flameless atomic absorption spectrophotometric method for the determination of total mercury in biological materials after the organic matter is decomposed by liquid pressure decomposition. Detection limit is 0.05 ug of mercury per kg of fresh weight.

2. REFERENCES

BERNHARD, M. (1976), Manual of methods in aquatic environment research. Part 3. Sampling and analyses of biological material (Guidelines for the FAO(GFCM)/UNEP Joint Co-ordinated Project on Pollution in the Mediterranean) FAO Fish.Tech.Pap.(158): 124 pp.

FAO/UNEP(1975) Report of the FAO(GFCM)/UNEP Expert Consultation on the Joint Co-ordinated Report on Pollution in the Mediterranean, Rome, Italy, 23 June - 4 July 1975, Food and Agriculture Organization, Rome. Access No. H 0975:42 pp.

3. PRINCIPLE

After uncontaminating sampling and sample preparation of mussels an aliquot of the edible tissue is decomposed by oxidation in a pressure container with nitric acid at 120°-150°C. Then the mercuric ion is reduced with an excess of SnCl₂ to elemental mercury. The elemental mercury is then volatilized by aeration and the mercury determined as monoatomic vapour by flameless atomic absorption spectrophotometry (flameless AAS) at a wavelength of 253.7 nm.

4. REAGENTS

For this analysis use only distilled water and reagents of recognized analytical quality, with as low as possible mercury concentrations.

- 4.1 Demineralized distilled water, or water of equivalent quality, free from mercury.
- 4.2 Sulphuric acid ($d_{20^{\circ}\text{C}} = 1.84 \text{ g/ml}$) diluted with an equal volume of water (4.1).

CAUTION - Add the acid to the water slowly and with constant stirring to avoid spattering of concentrated acid.

- 4.3 Nitric acid ($d_{20^{\circ}\text{C}} = 1.4 \text{ g/ml}$, approximately).
- 4.4 Hydrochloric acid ($d_{20^{\circ}\text{C}} = 1.19 \text{ g/ml}$).
- 4.5 Potassium permanganate solution (50 g/l).
- 4.6 Stannous chloride (50g/l $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$)/hydroxylamine sulphate solution. Prepare this solution daily by mixing 10 ml H_2SO_4 (4.2) with about 60 ml H_2O (4.1). After allowing to cool to room temperature, dissolve 3 g NaCl, 3 g hydroxylamine sulphate, and 5 g SnCl_2 in the diluted H_2SO_4 and bring the volume to 100 ml with distilled water (4.1).

NOTE: Instead of hydroxylamine sulphate, an equivalent amount of hydroxylamine chloride can be used.

- 4.7 Sulphochromic mixture containing 4 g potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) in 1 litre of sulphuric acid (4.2).
- 4.8 Mercury standard solutions:
- 4.8.1 Mercury stock solution: 1 g Hg/l. Weigh 1.354 g mercuric chloride (HgCl_2) to the nearest mg. Transfer to a 1 000 ml volumetric flask. Prepare an approximately 1 % solution of nitric acid (4.3) with distilled water (4.1) and bring the volume to 1 000 ml with this 1 % nitric acid.
- 4.8.2 Mercury standard solution: From the stock solution (4.8.1) prepare, by appropriate dilutions using micropipettes (5.22), a mercury standard solution which in 0.1 ml contains the lowest amount of mercury standard to be used for the standardization (7.4.1). Prepare this standard solution frequently, using 1 ml nitric acid (4.3) diluted with 250 ml distilled water (4.1), for the appropriate dilutions.

NOTE: The concentration of the Hg standard depends on the Hg levels anticipated in the samples to be analysed.

NOTE: All reagents must be checked for Hg contamination by running blanks.

- 4.9 Mussel substandard: prepare a sufficiently large sample of mussel tissue to last for several digestion runs (e.g. 300 g FW) according to 7.1. Homogenize with a stainless steel blender or other homogenizer (5.29).

Test for homogeneity by running 5 mercury determinations following 7.3 and 7.4. If the standard deviation is acceptable the substandard is ready for use. Otherwise homogenize the substandard again or prepare a new substandard.

5. APPARATUS

- 5.1 Plastic buckets or thermoisolated boxes containing plastic grids in order to prevent the mussels from being submerged in the sea-water used to keep them moist. Camping equipment may be adapted for this use.
- 5.2 A plastic bucket or bottle for sea-water used to keep the mussels moist.
- 5.3 A scraper or similar device (see figure 1) or diver's knife of stainless steel (needed only in surveys).
- 5.4 Use of a small rowing-boat (optional in surveys).
- 5.5 Two to four plastic knives made out of high density polyethylene, or similar, of high purity (quartz knives are an alternative).
- 5.6 Pyrex dishes, plastic sheets or similar.
- 5.7 Two to three pairs of plastic tweezers (see annex II).
- 5.8 High density, high quality, polyethylene bags and airtight plastic containers with screw caps for preservation of samples in deep freezer cleaned with detergents and rinsed with distilled water.
- 5.9 High density polyethylene sheets to cover the bench.
- 5.10 Smaller polyethylene sheets to be used as "weighing paper"
- 5.11 Balance (100 - 200 g) with a precision of 0.001 g, or better, for weighing specimens and subsamples; preferably a "top-loading" balance.
- 5.12 Length-measuring device, e.g. a transparent Pyrex dish with a centimeter scale attached underneath.
- 5.13 Plastic washing bottle with glass-distilled water (4.1).
- 5.14 Distilled water or clean sea-water in a plastic dispenser.
- 5.15 Five to nine digestion vessels (Teflon) of at least 25 ml capacity. A digestion block can be used.

NOTE: If smaller digestion vessels are used, the amounts digested in procedure (7.3.3) must be accordingly reduced to avoid explosions.

- 5.16 Oven or hot plate for digestion at 120° - 150°C.
- 5.17 Five to nine 10 ml and two 50 ml volumetric flasks (borosilicate glass).
- 5.18 Weighing bottle with a ground stopper.
- 5.19 Desiccator.
- 5.20 Analytical balance (0.0001 g) for weighing reagents and samples.
- 5.21 Drying oven with thermometer or, better, freeze-drier.
- 5.22 Micropipettes to deliver accurately 0.1 ml.
- 5.23 Atomic absorption spectrophotometer provided with a hollow cathode mercury lamp and background correction; alternatively a mercury vapour analyser may be used.

NOTE: Background correction may not be necessary if it can be shown that the matrix effect is negligible.

- 5.24 A recorder, a maximum signal indicator, or a peak-area integrator.

A tall-form aeration flask with a volume suitable for the sample size and compatible with the rest of the apparatus, bearing a calibration mark corresponding to the optimum filling level, the dead volume of which has been reduced to a minimum and through which the gas current flows under optimum conditions. For this, the aeration tube should be fitted either with a finely drawn-out point, a ball pierced with holes, or a glass frit (porosity between 100 and 250 μm). It is necessary to confirm that a series of different flasks all give the same result. After each use, treat the aeration flask with the sulphochromic solution (4.7) to oxidize any traces of Sn(II) that it may contain.

- 5.26 Measuring cell with windows (e.g. quartz) transparent to UV (253.7 nm), the length and diameter of which are suitable for the spectrophotometer being used.
- 5.27 Equipment which eliminates any condensation of water vapour in the measuring cell. Any appropriate system may be adapted for this: an infra-red lamp, an electrical heating element, a rod heater, by passing some air around the cell, etc.
- 5.28 Equipment to absorb mercury vapour leaving the measuring cell (e.g. 0.25 per cent iodine in 3 per cent potassium iodide solution).

NOTE: Figure 2 shows an example of the way in which the various components are assembled and the principle of operation of the apparatus in the case of a measured system using an open circuit. Any other satisfactory arrangement may be utilized. The entrainment gas may be air, nitrogen or argon, and an absorbing solution other than that given in "J" may be used. It is possible to use an aeration system based on a closed circuit system though this system is less sensitive than the open circuit one. Commercially available kits can also be found.

NOTE: Glassware that is used for the first time in the analytical procedure (7.4) must be thoroughly cleaned e.g. by the following procedure:

- wash with diluted 10 % nitric acid (4.3);
- wash with a mixture of 4 volumes sulphuric acid (4.2) and 1 volume potassium permanganate solution (4.5) prepared in the container just before using it;
- wash with a hydroxylamine sulphate solution to remove all deposits of manganese dioxide;
- finally, wash several times with distilled water (4.1) or leave the glassware in sulpho-chromic mixture (4.7) for several hours, rinse with distilled water (4.1) and leave in about 10 % nitric acid until the glassware is needed. Then rinse with distilled water (4.1).

5.29 Stainless steel homogenizer or other tissue homogenizer made from glass or Teflon.

6. SAMPLING

6.1 Sampling plan

Design a sampling plan in accordance with the objective to be reached. Select the sites so that, besides easy access, enough material will be available from the same site during the entire project without depleting the resource. Clearly identify the sampling sites on a map and describe the general characteristics of each one.

NOTE: If a shipment of mussels is to be analysed, take a representative (random) sample.

NOTE: The minimum number of individual mussels for a composite sample is 10 specimens.

NOTE: If the distribution of mercury in mussels is the main objective of the survey, collect mussels seasonally or monthly. The geographic distribution of sampling areas along the coast should be guided by the presence of major pollution sources such as rivers, towns and industrial complexes which discharge into the sea. Samples should not be taken in the direct vicinity of pollution sources such as pipe-lines from industrial plants, sewage outfalls from towns and villages, nor in ports or in small estuaries of highly polluted rivers, but from areas which are representative of the general conditions of a certain coastline. In addition, highly polluted areas should be sampled in order to assess the existing maximum pollution. Existing or planned marine parks should be used as reference areas, whenever possible.

6.2 Presampling preparations for surveys

Clean scraper or diver's knife (5.3), thermoisolated boxes and buckets (5.1), with detergent and rinse with tap water or clean sea-water from the sampling zone.

6.3 Sampling procedure for surveys

Scrape mussels from their attachments with the scraper or a diver's knife and transfer them into the thermoisolated box (6.2). Take a sea-water sample in a suitable container (5.2) from the collection site to keep the mussels moist if a long transport time is envisaged. Keep mussels moist during transport without submerging them. Report in sampling protocol and label the thermoisolated box (5.1) with station number, sampling area and date.

NOTE: Mussels attached to metal pipes, ships' hulls etc. which may have contaminated the mussels with metals, must not be included in the samples.

NOTE: The sampling of wild mussels may be carried out from the shore, but it is probably more efficient and convenient to sample from a small rowing-boat with a scraper fixed to a long stick. One person is needed to manoeuvre the boat with the oars while another scrapes the mussels from the supporting rocks or substrate. The scraper should be made of stainless steel and the collection net of nylon. One other way would be through the assistance of a skin-diver who collects the mussels by removing them with a good, stainless steel, divers' knife and collects them in a nylon net. If samples are taken from a mussel park, the mussels are to be collected from various depths and from different positions within the culture.

NOTE: The transport of mussels collected near the laboratory will not present any special transport and storage problems. Mussels, especially when gathered from the intertidal zone, will be able to survive aerial exposure for more than one day. Mussels submerged in a bucket will open their valves and start pumping water and excreting waste products, while during aerial exposure their metabolic rate is greatly reduced. It is, therefore, advisable to keep the mussels exposed to the air and moisten them with sea-water collected from the sampling area so that they do not excrete their waste products into the water and contaminate other mussels. A grid on the bottom of the bucket or box may help to prevent the mussels from being submerged. If longer transport is necessary the mussels are to be stored in plastic buckets or bags placed in thermoisolated boxes. Great care is again necessary to avoid accumulation of sea-water in the bottom of the containers.

7. PROCEDURE

7.1 Sampling preparation

7.1.1 Pre-operational preparations

Clean knives (5.5), dishes (5.6), tweezers (5.7), length-measuring device (5.12) and polyethylene sheets (5.10) with detergent, rinse with distilled water (4.1). Cover the working area with pre-cleaned plastic sheets (5.9).

7.1.2 Sample preparation procedure

Clean hands carefully with detergent and rinse them with water.

Scrape off all foreign materials still attached to the outer shell surface with a plastic knife (5.5) used only for this purpose, handling the mussels as little as possible.

Rinse each mussel with distilled water (4.1) (or clean sea-water) and let the water drain off.

Pull out the byssus which extrudes from between the closed shells on the concave side of the shells.

Weigh (5.11) the whole mussel to the nearest 0.1 g and note the weight.

Insert a second clean plastic knife (5.5) into the opening from which the byssus extruded and cut the posterior adductor muscle by turning the knife as indicated in figure 3, then cut in the other direction and open the mussel. Do not try to break the mussel open with the knife: if the two muscles are cut the mussel will open easily. Check if the byssus has been eliminated completely; if not, remove the remainder with clean tweezers (5.7).

Rinse the soft part of the mussel in the shell with distilled water (4.1) or clean sea-water.

Loosen all tissue with the second knife and remove the soft tissue from the shell with a pair of clean plastic tweezers (5.7), without touching the outer part of the shell, and let all the water drain off.

Composite sample: weigh a cleaned empty plastic container (5.8) on the balance and note the weight. Fill it with at least 10 soft parts of mussels prepared as described above. Reweigh the plastic container in which the 10 or more specimens are placed. Note the weight and calculate the fresh weight. Homogenize the specimens in a stainless steel blender (5.29), replace the homogenate in the plastic container. Note the total weight and the calculated weight of the specimens in the protocol. Label the plastic container.

Place containers in clean plastic bags, include an identification note, seal the bags and deep-freeze.

Determine the length of one shell by placing it with the inner part facing the cm scale (5.12). Note length together with the total weight of the mussel and the weight of its soft parts.

NOTE: If the sample will only be used for mercury analysis the composite sample can be homogenized with a clean stainless steel blender. If the sample will be used to determine also other metals the use of a tissue homogenizer made from glass or Teflon should be considered.

7.2 Determination of dry weight

A clean weighing bottle (5.18), with the ground stopper removed, is put into the drying oven (5.21) (110°C, 2 hours), using a pair of clean pincers. It is important to use the pincers every time the glass is touched, to avoid leaving fingerprints and particles of dirt on the weighing bottle.

The stopper and the bottle are put into the desiccator (5.19) to cool.

The empty bottle and stopper are then carefully weighed on the analytical balance (5.20). The weight obtained is the weight of the dried, empty weighing bottle and stopper. Note the weight.

Remove the stopper and place about 1-2 g of material (7.1.2) in the weighing bottle and replace the stopper.

Determine carefully the weight of the weighing bottle plus stopper. The weight obtained is the FW of the tissue plus the weight of the bottle and stopper.

Place the bottle in the drying oven (100°C), removing the stopper and placing it also in the drying oven.

After 24 hours replace the stopper in the weighing bottle, remove the bottle with stopper from the drying oven and place it in a desiccator to cool.

Weigh the stoppered bottle and note the weight.

Repeat the drying cycle until the difference between subsequent weighings is less than 5 per cent of the total weight; record FW and DW, and calculate the FW/DW ratio.

NOTE: Freeze-drying will give better results than oven drying.

NOTE: The concentrations of elements and substances are usually calculated with reference to fresh weight (FW) and dry weight (DW), and both should, if possible, be determined. FW or live weight is not easily defined, since marine organisms lose water more or less rapidly after being taken from the sea-water. On the other hand, most methods refer to FW because it is easier to compare with volume measurements. DW determinations also present problems, because the requirement that the tissues should be dried until constant weight is reached, cannot always be satisfied, especially when large amounts of lipids are present. Reporting data on ash weight has only slight significance.

The determination of the DW requires that the sample be dried at 100°C until constant weight is obtained (usually after 48 hours). Repeated weight determinations of the sample are necessary to establish if a constant weight has been reached.

NOTE: The fresh weight of mussels is difficult to determine because of the continuous water loss during handling. The fresh weight determined can therefore give only a rough indication. For comparison it is suggested that the FW should be derived by dividing the exactly determined DW by a factor of 5.5.

7.3 Mineralization of the biological matrix

Explosion hazard:

If too high amounts of organic materials are placed in closed vessels, (e.g. instead of the amount of material in FW the same amount in dry weight (DW) is used) the vessels may burst. Therefore, all digestion procedure must be carried out with the appropriate precautions necessary when working with hot acids. For example, fume hoods must be closed when the vessels are heated.

Defective plastic bottles must be discarded and bottles which have been used for a certain length of time must be replaced before there is any risk of bursting.

7.3.1 Cleaning of the digestion vessel before and between digestions

Clean vessels (5.15), if necessary, and rinse with distilled water (4.1), then run a digestion procedure without adding the sample. Analyse the solution according to the analytical procedure (7.4). If the blank value is high, repeat this "blank digestion".

7.3.2 Predigestion experiments

Determine the minimum amount of concentrated nitric acid (4.3) necessary to destroy completely the organic matter for every new matrix by adding to a 1 g FW sample (7.1.2) increasing amounts of acid from .2 ml to not more than 6 ml HNO_3 , following the digestion procedure (7.3.3).

7.3.3 Digestion procedure

Place a sample of about 1 g FW (7.1.2) in each of the Teflon vessels (4.9), one vessel being charged with a substandard containing a known amount of mercury to check the efficiency of each digestion.

Add the predetermined amount (7.2.2) of concentrated HNO_3 , cover the vessels and close them tightly.

Let the samples in the vessels predigest at room temperature for at least one hour.

Place the vessels in a pre-heated oven or on a hot plate (120° - 150°C . (5.15) for at least 6 hours.

Remove the vessels from the oven and let them cool to room temperature and then open. If the solution is not clear, or has a yellow-brownish colour the digestion is not complete and has to be repeated. If the solution is clear, transfer the contents of each vessel into clean 10 ml volumetric flasks (5.17) and bring up to volume with distilled water (4.1). The contents of the volumetric flasks represents the test solution.

NOTE: It is important not to exceed the quantities of organic materials recommended. Since excess nitric acid has to be eliminated to avoid interference in the determinations, it is

necessary that in preliminary experiments the amount of acid required to destroy the organic matrix is determined for each matrix used in the project.

7.4 Analytical determination of total mercury

7.4.1 Standardization

Before a new matrix is analysed, and at periodic intervals as specified in the quality control procedure (9.3), carry out a digestion procedure with 6 vessels (5.14) all but one charged with the substandard (4.9) in order to standardize (calibrate) the method and the apparatus used.

Prepare an appropriate standard solution (4.8.2) so that 0.1 ml of this standard added to 10 ml of the test solution will result in a Hg concentration approximately equal to the lowest Hg concentration anticipated in the samples to be analysed.

Add about 1 g FW of the substandard (4.9) to five vessels (no substandard is added to the sixth: it is the reagent blank). With a micropipette (5.22), add 0.1 ml of the solution 4.8.2 to vessel no. 2 (no standard is added to the first vessel), 0.2 ml to vessel no. 3, 0.3 ml to vessel no. 4, 0.4 ml to vessel no. 4. Then add the predetermined amount (7.3.2) of conc. nitric acid to all six vessels and carry out the digestion procedure (7.3.3) and the aeration and determination (7.4.2).

Construct a standardization working curve (calibration curve).

7.4.2 Aeration and determination

Adjust the controls of the apparatus and the gas flow using an aeration flask (5.25) filled with water (4.1) to the calibration mark. Wait until the apparatus and the gas flow stabilize. Divert the gas flow and replace the aeration flask containing water by one containing 2 ml of the test solution (7.3.3) to which has just been added 2 ml stannous chloride solution (4.6).

Mix, wait 30 seconds and then restore the gas flow through the aeration flask. The entrainment of the mercury vapour through the measuring cell (5.26) produces a recorder tracing which rapidly attains a maximum. If peak heights are being used as a measure of response, purge the gas circuit and replace the aeration flask with one containing only water as soon as the recorder tracing begins to decrease. If a peak area is being measured, do not interrupt the tracing until the signal has returned to its initial value. After each test, purge the aeration flask.

8. EXPRESSION OF RESULTS

From the height of the peak obtained on the test solution, determine, by reference to the standardization working curve (7.4.1) and making allowance for the blank determination, the concentration of mercury in the test solution. In the case of an apparatus with digital read-out or a maximum response indicator, prepare a graph of mass of mercury against the corresponding instrument read-out. From this value compute the total mercury concentration of the sample, allowing for the volumes of reagents introduced during the treatment of the sample. Express this concentration in ug/kg FW and DW.

9. ESTIMATION OF PRECISION AND ACCURACY

9.1 Precision

Estimate the precision (CV = coefficient of variation) of the entire analytical procedure (7.4) by digesting (7.3.3) 5 different subsamples from one original sample. If the coefficient of variation in the ppm range is greater than 10 per cent, then check the procedure for possible errors and contamination.

9.2 Accuracy

Participate in the intercalibration exercises and, in addition, analyse a known certified standard of a matrix similar to the material under study.

9.3 Quality control

Conduct a quality control programme periodically in order to guarantee the precision and accuracy of your results according to the appropriate quality control method.

10. TEST REPORT

Annex I contains the proper formats and guidelines to be completed by the responsible analyst. In addition to the results obtained, the test report shall include details of the sampling and analytical methods used.

Annex II

PREPARING PLASTIC TWEEZERS

Methylmetacrylate of 4 mm thickness has been found very useful as it will give the right elasticity. The strips from which the tweezers are to be made should be cut according to the strength of the material. The easiest

way to heat the plastic and bend it is with a hot air blower used for forming plastics. A drying oven brought to 135°-140°C can be used, but it is much more difficult to make tweezers by heating the plastic in an oven since the plastic twists easily.

Material:

- Sheets of acrylic (methylmetacrylate) resin, 4 mm thick (trade names, e.g. Perspex, Plexiglas, Lucite)
- A plastic tube of 40 mm diameter

Equipment:

- A hot air blower (300°-350°C) for plastics (e.g. Karl Leister, Switzerland, W 1850, 400 rpm)
- Or a drying oven at 135°-140°C

Procedure: with a hot air blower:

1. Cut with an electric or a hand saw strips about 10 mm in width and 250 mm in length.
2. Heat a part about 60 mm long in the middle of the strip so that it will bend easily, and bend it round the plastic tube carefully in order to make both ends meet. Cool the plastic with cold water.
3. Sharpen the ends with a file and roughen the inside of the tweezers so that they grip well.
4. Wash the tweezers carefully with detergent and rinse them with distilled water.

Procedure: with a drying oven

1. Place the plastic strips on a clean piece of wood in a drying oven (135°-140°C) until the plastic becomes soft.
2. Lift the plastic strip at one end with a pair of tweezers and remove from the oven, letting the other end hang down so that the plastic will not bend.
3. Bend it around the plastic tube without letting the tips meet and cool them immediately by dipping them into a beaker of clean, cold water so that they do not curve.
4. Now bend the tips of the tweezers so that they will meet.
5. Prepare the ends as described earlier.