Coordinated Mediterranean Pollution Monitoring and Research Programme

GUIDELINES FOR HEALTH RELATED MONITORING OF COASTAL WATER QUALITY

Published under the joint sponsorship of the United Nations Environment Programme and the World Health Organization

WORLD HEALTH ORGANIZATION
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Report of a Group of Experts
jointly convened by WHO and UNEP
Rovinj, Yugoslavia, 23-25 February 1977

CORRIGENDA

Page 12	"Fig. 4a" should read "Fig. 4b".
Page 16, para. 6.2.2	Below the last reference point measurement parameter "Air temperature", insert new sub-paragraph heading "6.2.3 Multiple-point sampling".
Page 16	Delete last two lines "Parameters in sediment Faecal coliforms/g".
Page 20	Delete lines 1 and 2 starting "Figure 7 gives an example .....".
Page 23, para. 7.3	"Table 3" in last line of first paragraph should read "Table 4 (page 29)".
Page 23, Table 3	Under column "Virus type" the fourth item should read "ECHO virus 1-33".
Page 23, Table 3	Under column "Symptoms of disease", line 3, "echo-strains" should read "ECHO-strains".
Page 23, Table 3	Under column "Symptoms of disease", opposite "Hepatitis virus", delete "hepatitis (acute or chronic)".
Page 24	In line 4 "lustidine" should read "histidine".
Page 24, para. 7.3.2	The last sentence starting "Die-away rates ..." should form a separate paragraph.
Page 25, para. 7.3.5	In line 4, the amount opposite "untreated sewage" should read "10^3/ml". Similarly, in line 5, the amount opposite "secondary treated sewage" should read "10^2/ml".
Page 26, para. 7.3.6	Line 3 should read "symptoms of which are those of enteritis". Delete "with watery diarrhoea".
Page 26, para. 7.3.7	The last sentence of the first sub-paragraph should read "The symptoms of cholera are watery diarrhoea without fever, ..."
Page 26, para. 7.3.7	Delete the second sub-paragraph starting "Non-agglutinable (NAG) vibrios ....
Page 26, para. 7.3.8	Delete "watery" in line 3.
Page 27, para. 7.3.11	In penultimate and last line of third sub-paragraph, "Giaordia and Naegleoria" should read "Giardia and Naegleria".
Page 27, para. 7.3.12	In line 2 "below (9)" should read "in Table 3, page 23 (9)".
Page 27, para. 7.3.12	In last line of third sub-paragraph "1000°C" should read "100°C".
Page 28	In line 1 "echo" should read "ECHO".
Page 28, para. 7.3.13	In line 1 of second sub-paragraph "dioflagellata" should read "dinoflagellata

Page 29, Table 4	Delete asterisk (*) against "M" in column "Sediment" opposite "faecal coliforms".
Page 29, Table 4	Insert "Vibrio" after "NAG" in item 8 in column "Monitoring organism".
Page 33, para. 8.10	In penultimate line of sub-paragraph (2) "(Figs. 9 and 10)" should read "(Figs. 10 and 11)".
Page 35, Fig. 10	In last line of text "20 metres' depth" should read "30 metres' depth".
Page 36, Fig. 11	In penultimate line of text "20 metres' depth" should read "30 metres' depth".
Page 48, para. 9.12	Add asterisk "*" against item 10 "Sterilization liquid" and add footnote at end of tabulation "*: equipment necessary for the minimum programme".
Page 51	In 12th line from bottom of page "1 ml" should read "10 ml".
Page 52	In sub-paragraph (4) in sentence starting "Sample test amount = 10^{-3} g ...." add "etc., as above" after "Petri dish".
Page 60, Table 6	In column "Blackening" opposite "Salmonella" "+" should read "+**".
Page 60, Table 6	In column "Gas production" opposite "Salmonella" "v" should read "+**".
Page 60, Table 6	In column "Blackening" opposite "Citrobacter" "v" should read "+**".
Page 60, Table 6	In column "Lysine" opposite "Faecal coliforms" "+" should read "v".
Page 60, Table 6	In column "Blackening" opposite "Faecal coliforms" "-" should read "-**".
Page 60, Table 6	In column "Gas production" opposite "Yersinia" "-" should read "-**".
Page 60, Table 6	Add under "Notes" a new line "** = few exceptions".
Page 64, Fig. 19	Add "VIBRIO" after "NAG" at end of title.
Page 64, Fig. 19	In second box on right-hand side, opposite "SELECTED SEROLOGICAL AND BIOCHEMICAL TESTING", delete "(TRANSFER YELLOW COLONIES FROM BLOOD AGAR ONLY)".
Page 65, Table 7	In column "Vibrio cholera NAG Vibrio" opposite "Peptone water** Kovac's reagent", "v" should read "+".
Page 65, Table 7	Add two asterisks "**"against "VP-broth" in column 1.
Page 65, Table 7	Under "Notes" at end of table, "** = For VP testing add 2% NaCl" should read "** = Except for V. Cholerae and NAG Vibrio add 2% NaCl".
Page 66, Fig. 20	In first box on right-hand side "TRANSFER OF FLAT, REGULAR, GLOSSY ....." should read "TRANSFER OF LARGE, FLAT, REGULAR, GLOSSY ....."
Page 68, Table 8	In column headings move "SIM medium" to the right so that it includes columns "Indole", "H_2S" and "Motility".
Page 68, Table 8	In columns "H_2S" and "Simmons citrate agar" opposite "Shigella", insert "-".
Page 68, Table 8	In column "H_2S" opposite "Salmonella typhii", "+*" should read "+".
Page 68, Table 8	In column "Simmons citrate agar" opposite "Salmonella typhii", "+" should read "-".
Page 68, Table 8	In column "Gas production" opposite "Salmonella", "v" should read "+*".
Page 68, Table 8	In column "Colour Slant/Butt" opposite "Proteus", "Red/yellow* ..." should read "Red/yellow ....".

Page 68, Table 8 — In column "Colour Slant/Butt" opposite "Citrobacter"and opposite "Yersinia", "Red/yellow*...." should read "Red/yellow ....".

Page 72, Table 9 — In column "Carbohydrates" "Cellolioso" should read "Cellobiose".

Page 72, Table 9 — In column "Cellobiose" opposite "Enterobacter", insert "v".

Page 72, Table 9 — In column "Hugh and Leifson", 10th line, delete "Glucose".

Page 72, Table 9 — In column "Hugh and Leifson" opposite "Alcaligenes" and opposite "Acinetobacter", "-" should read "Gl-".

Page 72, Table 9 — Under "Notes" at end of table, insert new line "Gl = glucose".

Page 73 — In fifth line "Voges-Prosteonuer (VP) ..." should read "Voges-Proskauer (VP).

Page 74, Fig. 23 — In first line of paragraph (1), insert "(see Annex III)" after "(1 : 18)".

Page 76 — In second line of sub-paragraph "Advantages and limitations","be less than" should read "not exceed".

Page 77 — Under "Reagents" line 3 onwards should read as follows:

"1 N NaCl
Al $(OH)_3$ (specially prepared, cf above)
2 M Na_2CO_3
0.025 M $AlCl_3$
0.15 M NaCl
1 N HCl
3% beef extract, pH 7.0 (for elution)".

Page 77 — Under "Reagents", sub-paragraph (b),the formula in line 4 should read "1 N NaCl".

Page 85, para. 11.4.2 — In sub-paragraph (b) "0.2 c" should read "0.2°C".

Page 85, para. 11.4.2 — In last line of sub-paragraph (c) "15/4 c" should read "15°C".

Page 86, para. 11.5.2 — In first line of sub-paragraph (e) "$Na_2S_eO_3$" should read "$Na_2S_2O_3$".

Page 93, para. 11.5.5 — In second line "chlorine" should read "chloride" and "(mg ce^-/litre)" should read "(mg Cl^-/litre).

Page 93, para. 11.5.5 — In fourth line the equation should read "Chlorosity (Cl g/litre) = ..."

Page 93, para. 11.5.5 — In line 6 "chlorine content" should read "chlorosity".

Page 93, para. 11.5.5 — In line 8 "chlorine content" should read "chloride content".

Page 96 — Line 12 should read "i = enumerator, $1 \leqslant i \leqslant n$".

Page 96, para. 13.3 — The first term of equation (3), "s_x^2", should be raised so that the "s" is opposite the "=".

Page 101 — Equation (5) should read "$$t = \frac{(\bar{X} - \bar{Z}) - (\mu_x - \mu_z)}{(s_x^2/n_x + s_z^2/n_z)\ 1/2}$$"

and in next line "X, Z," should read "$\bar{X}$, $\bar{Z}$,"

Page 101 — Equation (7) should read "$$c = \frac{s_x^2/n_x}{(s_x^2/n_x + s_z^2/n_z)}$$

Page 101 — Sixth line from bottom of page should read "n_z = 20 (Table 17)".

Page 101 — Penultimate line should read "Table 18: $t(37)_{95\%}$ = 1.65 (app.)".

Page 103, para. 13.5 — Equation (8) should read "$$\frac{(\bar{X} - \bar{Z}) - (\mu_x - \mu_z)}{(s_x^2/n_x + s_z^2/n_z)^{1/2}} < t(r)_{1-\alpha/2}$$"

Page 103, para. 13.5 — Equation (9) should read "$$\left.\begin{aligned}(\bar{X} - \bar{Z}) - t(r)_{1-\alpha/2} \cdot (s_x^2/n_x + s_z^2/n_z)^{1/2} &< \mu_x - \mu_z \\ (\bar{X} - \bar{Z}) - t(r)_{\alpha/2} \cdot (s_x^2/n_x + s_z^2/n_z)^{1/2} &> \mu_x - \mu_z\end{aligned}\right\}$$"

Page 109, REFERENCES — In third line "EHE/7.6.1" should read "EHE/76.1".

Page 118, para. 4 — In fourth line delete "(to avoid contamination)".

Page 120 — In fourth line from bottom of page "The two tables above" should read "The two tables below".

Page 120 — In third line from bottom of page delete "already".

Page 134 — In paragraph "Cytochrome oxydase reagent*", the third item should read "Tetramethylparaphenyldiamin hydrochloride".

Page 134 — In paragraph "Fluid Yersinia peptone broth (selective)*", line 7, "a final urea concentration of 1.0%" should read "a final urea concentration of 0.4% for sewage and clean water and 0.2% for sludge and sediments".

Page 136 — In paragraph "KF streptococcus agar", insert after item 7,"Bromocresol purple 0.015 g".

Page 138 — In paragraph "Nitrate bouillon*", line 7, "dimethyl -x-naphtylamine" should read "dimethyl-a-naphtylamine".

Page 139 — Title of last paragraph should read "Phenylalamine agar*".

Page 140 — In paragraph "Preuss potassium tetrathionate broth", delete "a a" before "adding" in line 8.

Page 143 — Title of third paragraph should read "Sugar solution for parasitic flotation*".

Page 145 — In line 8 "Red/yellow: glucose, lactose ..." should read "Yellow/yellow: glucose, lactose ... ".

Page 146 — In line 6 title "VP-broth*" should read "Voges-Proskauer broth*".

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Note

This report contains the collective views of an international group of experts and does not necessarily represent the decision or the stated policy of either the World Health Organization or the United Nations Environment Programme.

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CONTENTS

PREFACE

The UNEP-sponsored International Workshop on Marine Pollution in the Mediterranean, convened in Monaco (9-17 September 1974) by the Intergovernmental Oceanographic Commission (IOC) of the United Nations Educational, Scientific and Cultural Organization (UNESCO), the General Fisheries Council for the Mediterranean (GFCM) of the Food and Agriculture Organization of the United Nations (FAO) and the International Commission for the Scientific Exploration of the Mediterranean (ICSEM), defined the pollution of coastal waters as the main environmental problem of the Mediterranean Sea. This pollution was attributed to the general lack of adequate systems for the treatment and disposal of domestic and industrial waste, to the input of pesticides and petroleum hydrocarbons, and to the presence of pathogenic microorganisms.

Based on the recommendations of the Monaco Workshop, the Intergovernmental Meeting on the Protection of the Mediterranean (Barcelona, 28 January - 4 February 1975) approved a Co-ordinated Mediterranean Pollution Monitoring and Research Programme (MED POL) as part of a wider Mediterranean Action Plan.

The MED POL programme consists of seven pilot projects, one of them the WHO/UNEP pilot project on Coastal Water Quality Control (MED VII).

The operational document (1) outlining the framework of MED VII was developed at the WHO/UNEP Expert Consultation in Geneva (15-19 December 1975) taking into account the recommendations of the Monaco Workshop, of the Workshop on Coastal Pollution and other Environmental Health Problems in the Mediterranean (Copenhagen, 16-19 December 1974) and the decisions of the Barcelona Intergovernmental Meeting.

According to the operational document the overall objective of MED VII is to produce statistically significant data, scientific information and technical principles which are required for the assessment of the present level of coastal pollution as it concerns human health. The most important immediate objectives are to design and implement a programme for the sanitary and health surveillance of coastal recreational areas and of shellfish-growing waters in selected coastal areas and to initiate a scientific study on the epidemiological evidence of health effects caused by inadequate sanitary conditions in coastal areas.

The implementation of MED VII is closely coordinated with the implementation of the other MED POL pilot projects. Data obtained through MED POL will be used to produce an overall assessment of the pollution of the Mediterranean Sea and to formulate recommendations to the governments of the Mediterranean States in respect of desirable and possible management decisions which may decrease or eliminate the extent and present level of pollution in the Mediterranean.

Among the activities proposed in the operational document is the development of the present guidelines, so as to select and harmonize monitoring procedures and analytical methods and thus contribute to the better and more efficient surveillance of beaches, marine waters and shellfish waters.

The first draft of the guidelines was prepared by Mr J.Å. Hansen, WHO consultant, with the assistance of Mr K.K. Kristensen, and subsequently amended and modified by a WHO/UNEP Study Group Meeting (Rovinj, 23-25 February 1977) and the participants in the mid-term Review Meeting on the Joint WHO/UNEP Co-ordinated Pilot Project on Coastal Water Quality Control in the Mediterranean - MED VII (Rome, 30 May - 1 June 1977).

The agenda of the Study Group Meeting and the list of participants are included in this report as Annexes VI and VII.

1. Background

The pollution of the coastal waters in the Mediterranean is having an increasing impact on the social and economic wellbeing of the countries bordering it. In addition to the millions of inhabitants living along the coastline of the Mediterranean, millions of tourists spend their holidays on the shores of this sea, and thus there is considerable potential for exchange of pathogenic agents. The present situation constitutes a significant health hazard in many places; salmonellosis, dysentery, viral hepatitis and poliomyelitis have all been endemic in the Mediterranean area, and during recent years there have been a number of cholera outbreaks (Table 1).

The data in Table 1 are incomplete and interpretation is difficult. However, there is little doubt that the figures shown in it underestimate the real situation as to both morbidity and mortality. Many Mediterranean countries are still in a developing phase, where facilities for effective disease control have not yet been established. Consequently there is a distinct need to improve health by introducing measures to control the routes of infection by microbiological agents.

Coastal monitoring may form part of such control programmes and monitoring, combined with selected in-depth epidemiological studies, may demonstrate where control measures could be introduced, and when improvements are likely to appear.

2. Objectives

The general objectives of the WHO/UNEP pilot project on Coastal Water Quality Control (MED VII) carried out as part of the Co-ordinated Mediterranean Pollution Monitoring and Research Programme may be defined as:

(1) the introduction of uniform reference methodologies that will eventually permit comparison of results between laboratories and controlling agencies, nationally and internationally;

(2) establishment of base data for national assessment of the coastal pollution created by land-based pathogenic microbiological discharges;

(3) establishment of strategies for pollution control and initiation of programmes for the efficient implementation;

(4) contribution to a comprehensive assessment of the situation in the Mediterranean.

The present guidelines were drawn up to meet only those needs of participants in MED VII which are relevant to coastal water quality monitoring as expressed in the joint WHO/UNEP Expert Consultation on the subject (1). Many activities in MED VII are far beyond the scope of the monitoring programme proposed here, such as epidemiological studies parallel to coastal monitoring, and there has been no attempt to include methodology for such studies in the present guidelines. As regards microbiological examination procedures, the guidelines will apply more generally.

In case of emergencies, such as an outbreak of an infectious disease, there may be good reasons for extension of on-going monitoring to support the health and food authorities involved or to assist in an epidemiological study. Such emergencies must, however, be handled on an ad hoc individual basis and no general advice is intended in these guidelines.

To help in promoting steadily improving skills and more efficient water quality control the guidelines will propose minimum programmes based on established principles as well as extended and more advanced monitoring programmes.

The guidelines are provisional only, and should be revised after a few years when data and experienced gathered may justify substantial changes in the monitoring parameters, as well as in the proposed field and laboratory procedures.

Table 1

MEDITERRANEAN HEALTH STATISTICS

Disease		1970	1971	1972	1973
Cholera	C	1 023	139	399	280
	D	118	0	12	23
Typhoid and paratyphoid	C	36 752	25 731	27 076	36 646
Typhoid	D	664	653	601	256
Paratyphoid	D	36	33	78	81
Bacillary dysentery	C	45 375	44 307	40 449	44 402
	D	81	56	42	31
Infectious hepatitis	C	85 685	88 978	84 785	112 953
	D	1 529	1 586	1 698	1 224

C = cases D = deaths

Notes: 1. Only 14 of 18 countries reported regularly, and some of them only provisionally.

2. Only a few infectious diseases are included here; for example, diseases caused by vibrios other than Vibrio cholerae are not included.

3. Available statistics may not reflect the actual situation; realistic statistics would probably give a higher annual incidence.

4. Cases exported from the area (tourists) are not included.

Source: World Health Organization: World Health Statistics, Vol.II: 1972, 1976, Geneva (2).

3. Scope of activity

In accordance with the principles laid down in the operational document (1), the monitoring areas will be as follows:

(1) Recreational areas: beaches frequented by the public, and coastal waters used for swimming or other time spent in the water.

(2) Shellfish areas: grounds or areas where sea-food (crustaceans, molluscs, fish, etc.) is harvested either commercially or as a leisure-time activity.

(3) Pollution sources: rivers and effluents discharging in the coastal zone.

To meet adequately the very different national requirements in terms of monitoring and the very different levels of field and laboratory training, it is worth distinguishing between two different approaches (see also Chapters 6 and 7):

(1) Minimum monitoring (MM): involving sampling and analysis of a few indicator organisms. Limited resource allocation and only a basic public health assessment and control would be provided under this approach.

(2) Extended monitoring (EM): involving sampling and analysis of several indicators as well as pathogens. Extended monitoring automatically includes minimum monitoring, and comparisons and assessment on a MM scale is still possible between any two different locations. Extended monitoring is the more resource-demanding and would often involve an element of research, e.g., on survival ratios between certain pathogens and indicators under different environmental circumstances. Ultimately a series of EM programmes may lead to new achievements in terms of pollution control, which may then cause redefinition of the scope of activities, new choice of monitoring organisms, etc.

The activities under MM and EM or any individual programme combining these two approaches appear in Chapters 6 and 7.

4. Area and problem identification

A coastal water quality monitoring programme will be initiated only after careful consideration as to general and specific aims and means pertinent to the region involved. Such aims could be water quality control where criteria already exist; or the background studies necessary to establish criteria and standards; or certain studies of selected pathogens that are epidemiologically significant, or studies that basically have an educational purpose. Available resources must also be taken into account and inevitably influence the extent and degree of sophistication of any studies. Whatever the purpose or the resources available, the provision of adequate maps or nautical charts is an indispensable prerequisite for monitoring programmes.

4.1 Provision of maps, scale modifications

Geodetic and nautical maps of the coastal region to be studied should be provided. The nautical charts will normally be of prime interest, and the local pilot organization, port administration or similar may be of assistance both in acquiring the charts and in using them to best effect.

Figures 1 and 2 show two Mediterranean coastal areas chosen at random, to demonstrate how existing information should be used and that adequate-scale maps are needed for different applications within the same overall coastal monitoring programme. The maps do not represent any actual planning, and no attempt has been made to obtain the supplementary information required in the preparation of any real monitoring programme in both areas.

The two maps are large in scale and have been reproduced photographically, a useful technique for the provision of maps at almost any desired scale, e.g. 1:25 000, for beach surveillance or 1:50 000 for area overviews. The situation and the use of the map will normally define the appropriate scale.

A map of practical size could be the European A3 format (approximately 42 x 60 cm); the two maps shown were originally drawn up in this format, but have been reduced to half size (A4) for convenience of presentation. Many copying machines allow for direct reduction from A3 to A4, allowing for cheap reproduction and presentation of results.

Each map should always be clearly identified by location, coordinates, scale, and orientation. This must be ensured before any copying or reproduction is made.

1. Location: Use the name of a typical town or conspicuous landmark, and always indicate the country.

2. Coordinates: Give approximately longitude and latitude of the location.

3. Scale: Provide a linear measure, e.g., divisions of 100 metres and 1 kilometre. Do not provide a numerical scale because this will change whenever the map is reduced or enlarged.

Fig. 1

EXAMPLE: ALEXANDRIA AREA OVERVIEW

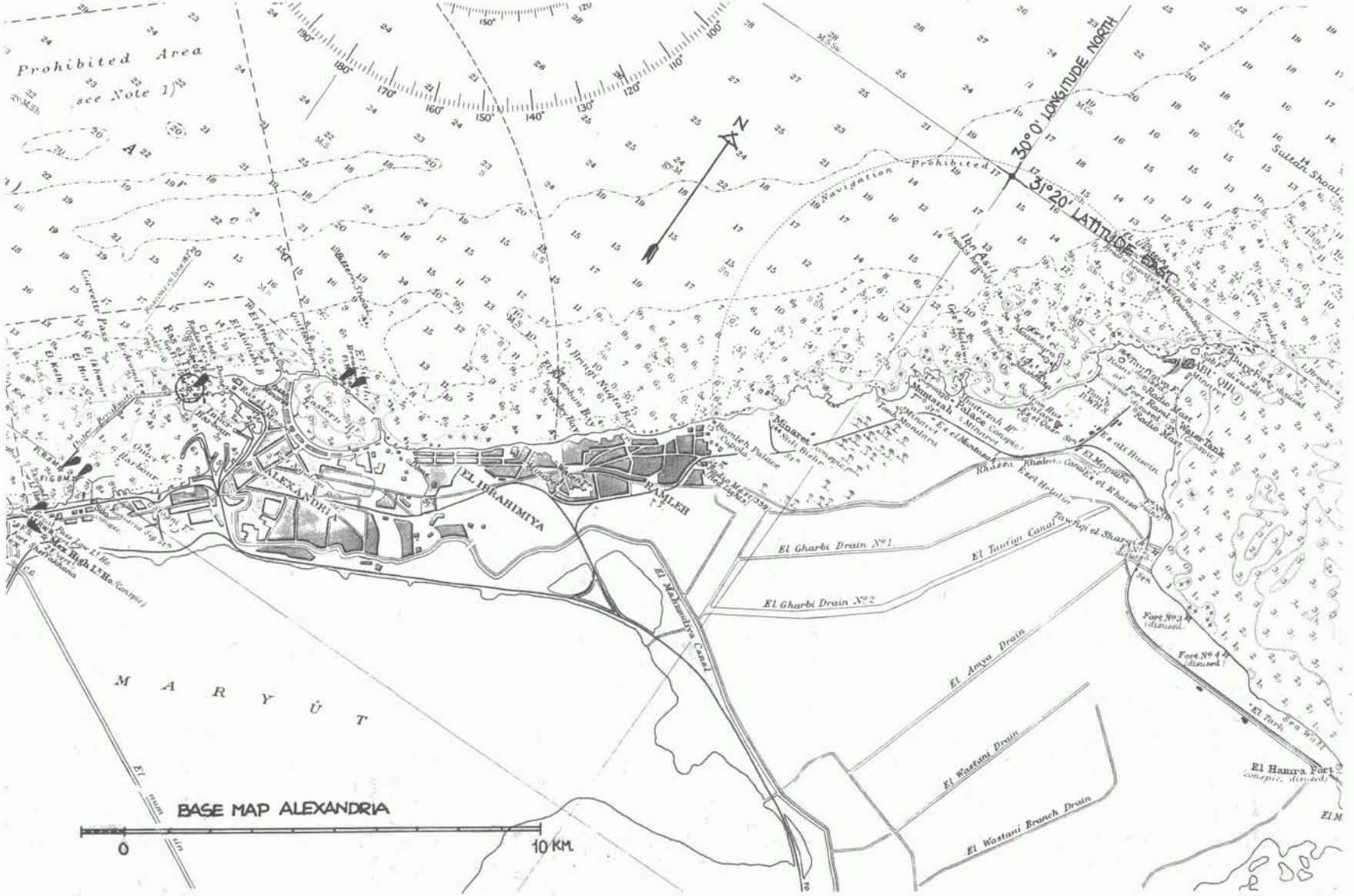
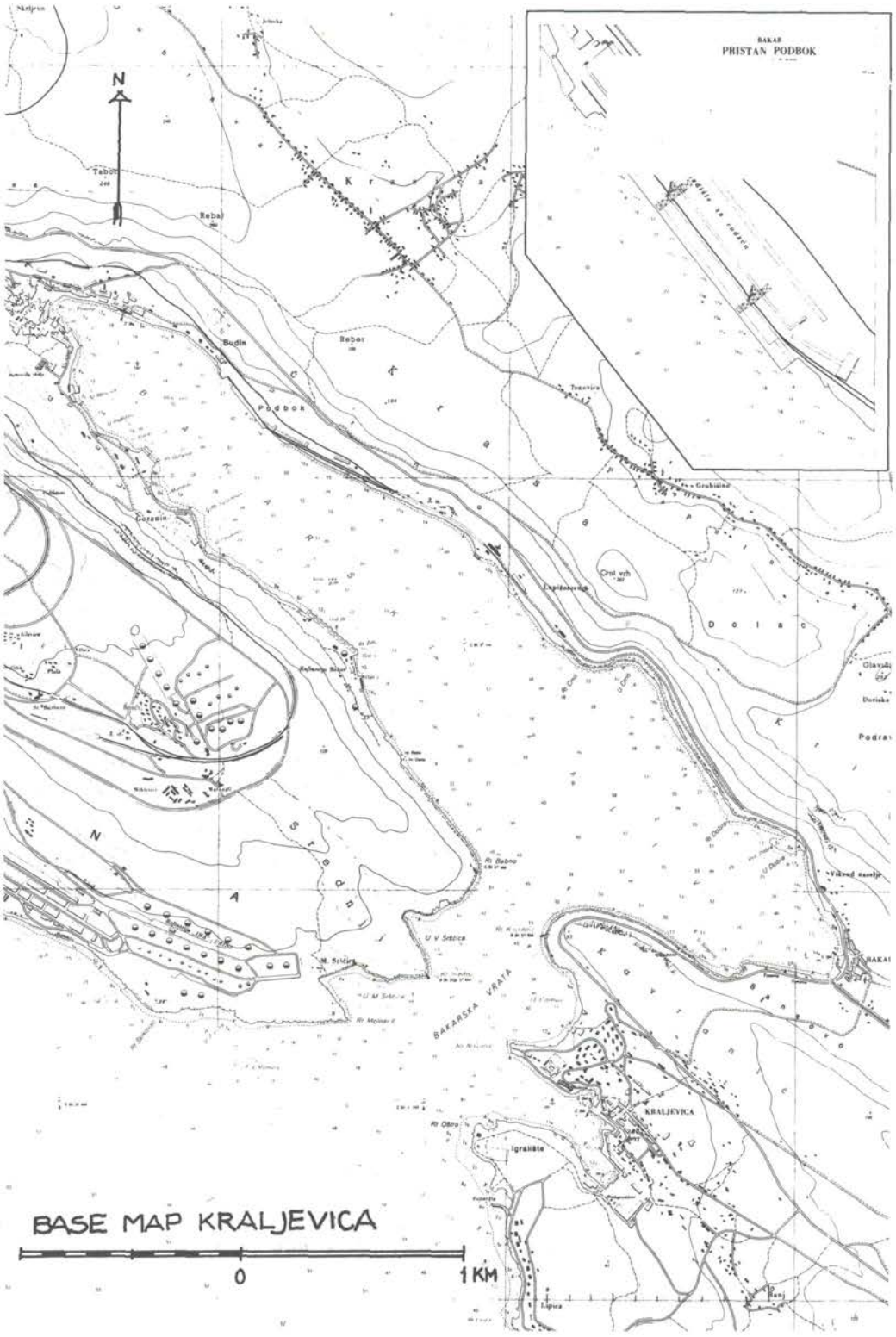


Figure 4.1.2. Area overview, example Kıaljevica.



EXAMPLE: KRALJEVICA AREA OVERVIEW

Fig. 2

4. Orientation: Indicate N for North, or give lines for longitude and latitude of the main location.

The photographic technique for enlarging and/or reducing existing maps is recommended because of its great flexibility and low cost.

4.2 Area assessment, check list

Before commencing any monitoring the situation must be provisionally assessed through facts gathered from existing locally obtained experience and reported facts. Data obtained should be noted either on a fact sheet or on a descriptive map, or both, depending on circumstances, as indicated below.

4.2.1 Landward description

Land use	Distinguish between categories of land use within the area, including use of beaches, as follows: Industrial Residential Mixed Agricultural Forestry Recreational Swimming (beaches and rivers)
Run-off Map and fact sheet	Identify rivers, and streams, location and flow. Estimate individual and total monthly discharge into the sea.
Waste-water discharges and outfalls Map and fact sheet	Identify outfall (beach or offshore) sites and estimate individual and total daily flow into the receiving coastal zone. Distinguish between type: domestic, industrial and mixed. Specify industry.
Treatment Map and fact sheet	Identify location, cubic capacity (m^3) per day and degree of treatment (primary, secondary, tertiary, chlorination).
Dumps Map and fact sheet	Identify dumping sites on land for solid waste or sewage disposal or both. Only dumps close to the beaches should be included (for example less than one kilometre). Indicate capacity/year.
Coastline Map	Indicate type of coastline, viz: Sand Rocks Shallow water Gravel Deep water Cliffs

4.2.2 Seaward description

Shellfish areas Map and fact sheet	Indicate site, catch (tonnage per year), and type of shellfish.
Fishing grounds Map and fact sheet	Indicate type of fish and if possible catch.
Dumping sites Map and fact sheet	Indicate locations, materials dumped and in what quantity per year.
Winds Fact sheet and wind rose	Draw up a wind rose (annual distribution of wind directions) based on statistics from a nearby weather station or the like. Make different wind roses for different seasons.
Precipitation and air temperatures Fact sheet	Establish a table for annual precipitation, for example by monthly averages. Include average monthly air temperatures in the same table.

General currents Map and fact sheet	Certain currents are often typical for longer periods or seasons. Such typical situations should be assessed if possible. Consult local pilots, harbour authorities or fishermen.
Tides Fact sheet	Tidal cycles are normally indicated directly on nautical charts or available from pilots or port authorities or naval stations. Initially only the mean spring flood and ebb and mean neap flood and ebb are required. For more detailed studies, updated tables on daily variations can subsequently be provided.
Salinity levels and temperatures Fact sheet	Provisional information on salinity and temperature variations may be acquired from previous studies or regular routine observations, for example by pilots, studies for hydrographic research institutions, universities, or others. A few typical profiles would be helpful in the first attempt to describe the area.
Depth contours Map	Normally depth contours appear directly from curves on the nautical chart or from depth soundings reported on the chart.
Buoys, navigational aids Map	Buoys and other navigational aids such as lights and conspicuous landmarks should be indicated on the maps. Also indicate important obstacles (wrecks or rocks).

4.3 Earlier coastal pollution studies

A careful examination should be made to see whether any studies have been conducted or reported previously in the coastal region, or studies related to items in the above checklist. Such information should be thoroughly examined and summarized in the area assessment.

4.4 Problem assessment

The search for existing information and new knowledge only becomes meaningful when the aims of, and techniques used in, monitoring are clearly defined. Consequently, a problem assessment is a prerequisite for any monitoring programme and the preparations hereof.

There may be reasons for reassessing already defined aims after a knowledge of the area has been acquired but the principle of having aims established to set adequate limits in the search for data should never be invalidated. For example, extensive meteorological statistics may be readily available but their relevance must be judged not on their availability but rather on their applicability to the public health aspects of coastal water quality monitoring.

4.5 Area and problem assessment, initial report

The report containing an evaluation of the area and problem should be as factual and concise as possible. The report should thus contain at least such items as;

(1) Aims : local assessment of problems and goals.

(2) Maps : as per the indications in the check-list above. Several maps must be drawn.

(3) Fact sheets : Fact sheet indications in the check-list above.

(4) Wind roses : See check-list above.

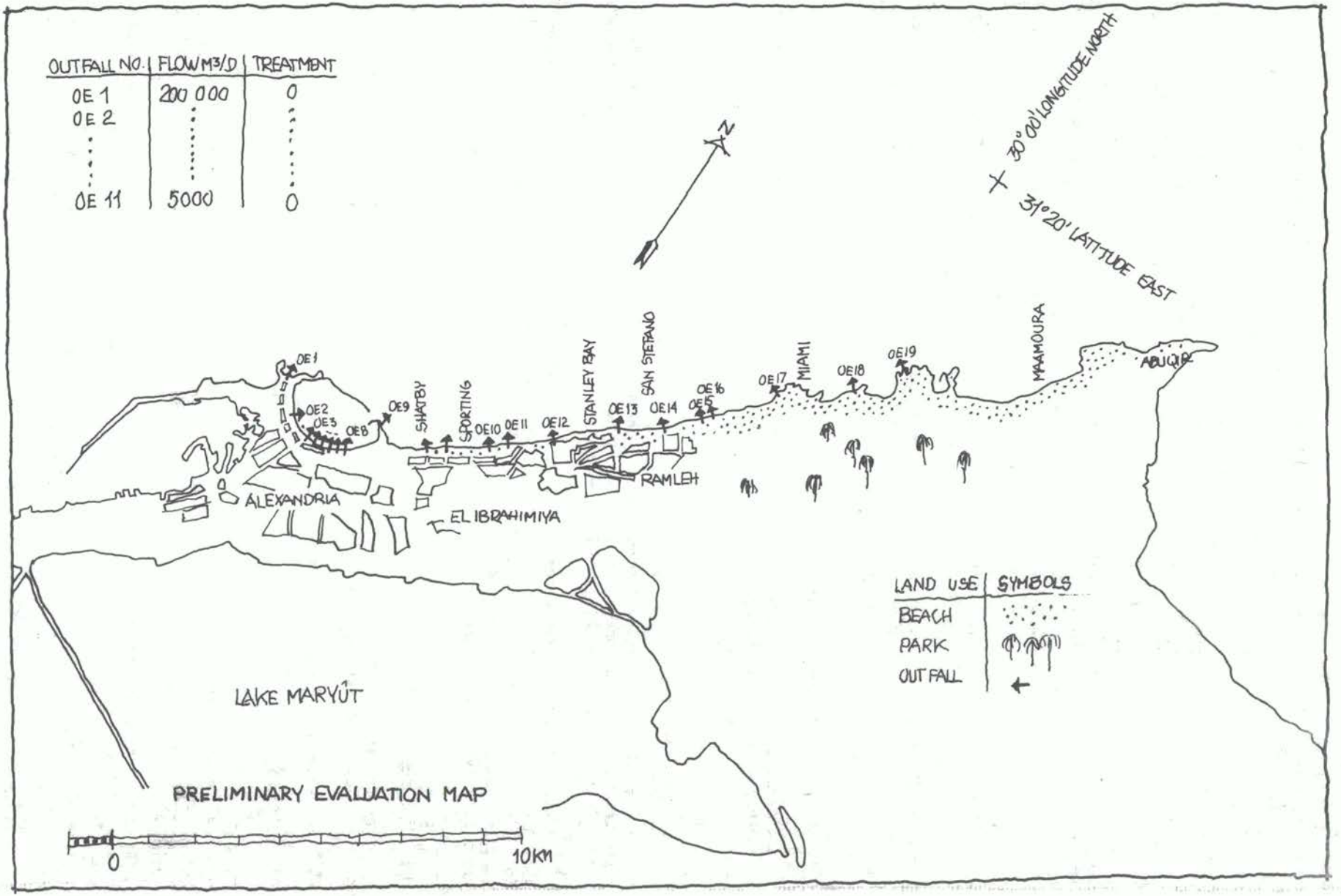
(5) Graphs and tables : See check-lists above; where possible always condense factual information into tables or graphs.

(6) Previous studies : Short summaries supported by maps, graphs, and tables, where obtainable.

Figure 3 shows what kind of maps may be useful in the initial reporting phase; it is related to the situation in Figure 1 (Alexandria); it shows existing outfall sites but is presented in provisional form only; for example, the flows and a more precise indication of beach or sea outfall

Fig. 3

PRELIMINARY EVALUATION STAGE (ALEXANDRIA)



and length of outfall are not given. The figure also shows that maps may always be produced on any scale adequate for a given individual purpose.

5. Beach surveillance

5.1 General

Systematic beach surveillance is a type of pollution control where little experience is available, and few results have been reported. To secure uniform criteria in examining the visual appearance of beaches the classification (25) system may be adopted. In northern England the McKay (26) procedure, a modified version of the Garber classification system (see Table 2) seems to have been successfully employed for several years by different workers, with identical results that have proved useful for improving beach appearance (see Table 2 below).

5.2 Surveillance maps, recording of results

To obtain simple and accurate reporting of observation results, copies of maps should be obtained during surveillance and notes should be made directly onto them. Figures 4 and 5 have been reproduced from the chart given in Figure 1 above, enlarged to a suitable scale, but with the coastline placed at the centre to simplify observation and to allow notes to be made equally easily both to landward and seaward of the central axis.

On Figures 4a-c some observation positions have been marked for demonstration purposes.

5.3 Observation points and frequency

Observation points are where a classified contaminant (Table 2) is observed in a significant quantity.

Contaminants not classified in Table 2 may also be registered by a separate note on the beach surveillance map, but every effort should be made to use an existing class of contaminants rather than establishing a new class.

The map allows observations to be conducted off-shore as well as on the beach. Both types of observations should be entered on the map.

Observations should be conducted once a month in the tourist season, at three equally spaced intervals during the remainder of the year, and should be made on days and at times when the visitor density is low, viz. early mornings or on normal week-days.

The uses that a beach is put to are, however, important for evaluating beach surveillance results. If possible, therefore, peak visitor densities for the beaches surveyed should also be reported, possibly on the maps where dates and times would indicate peak visitor densities relative to regular beach surveillance operations.

6. Minimum monitoring

6.1 General

The present chapter will deal with monitoring as related to recreational waters, shellfish areas and pollution sources such as rivers or sewage effluents. The methodology for work in field and laboratory is described in Chapters 8 - 11 below and in Annexes I - V .

The minimum programme outlined reflects the monitoring that was deemed essential by the WHO/UNEP expert consultation of December 1975; an extended monitoring approach is discussed in Chapter 7 should it become desirable to expand the programme. In field monitoring practice it is worthwhile distinguishing between the following types of studies:

(1) Overall observation of the area, for example local meteorological parameters relevant at the time of observation.

(2) Reference point measurements, for example, local hydrographic parameters at the time of observation.

Table 2

BEACH SURVEILLANCE CODES

Code	Identification	Application, examples
Contaminants		
C	Clear water	Clear, no off-colour water or particulate matter
D	Ocean debris	Driftwood
K	Sewage debris	Match-sticks, hair, sludge floc, some garbage.
HS	Human faecal matter	Intact faeces must be differentiated from animal waste.
HL	" " "	L = Landborne S = Seaborne
ST	Sanitary towels	
CC	Rubber goods	Condoms and rings
R	Refuse, including garbage from beach and land use	Domestic trash such as cartons, cans, boxes, bottles, and garbage from use of beach recreation areas.
TR	Floating trash and garbage from boats and ships	Similar to R above, but judged to originate from boats or ships
S	Seaweed	Any kind of seaweed
B	Dead bird	Any dead marine bird
ML	Dead marine life	Fish or other marine animal
P	Plankton blooms or rafts	Plankton bloom discoloration of water.
SP	Spores	Usually kelp spores that appear as a surface scum or film.
O	Oil	Mineral oil from ships or other sources. Ship bilge pumping, fuel spills, etc.
OS	Mineral oil scum	Mineral oil slicks associated with natural oil seeps.
G	Particulate grease, sewage origin	Grease particles or balls near waste outlet
GS	Grease scum, sewage origin	Slick appearing to originate at a sewage discharge point
T	Tar	Mineral oil tar.
N	Noxious odours, fumes or non-sewage gases	Mercaptans, sulfides, smog odours from industrial activities
NS	Noxious odours, fumes or gases-sewage	Sewage or treated sewage odours present in water or along beach
M	Murky-dirty	Water dirtied by causes other than plankton blooms. M_1 approx. 2 m. Secchi, M_2 approx. 1.5m. Secchi, M_3 approx. 1 m. Secchi.
F	Outflow of water to ocean from land	Usually storm-drain outflow which can affect ocean water condition
Quantity		
1	Small amount	Traces of the coded materials
2	Moderate amount	Some of the coded materials at intervals. Usually not objectionable.
3	Large amount	Enough of coded materials to be objectionable.
Example:		
	H2, P2, O1, GS1, T1	This result should be entered directly on the copy of the map (Figs 4a-4c)

Source:

MacKay: Personal communication (26), based on Garber system (25)

Fig. 4a

BEACH SURVEILLANCE, OBSERVATION SHEET (1)

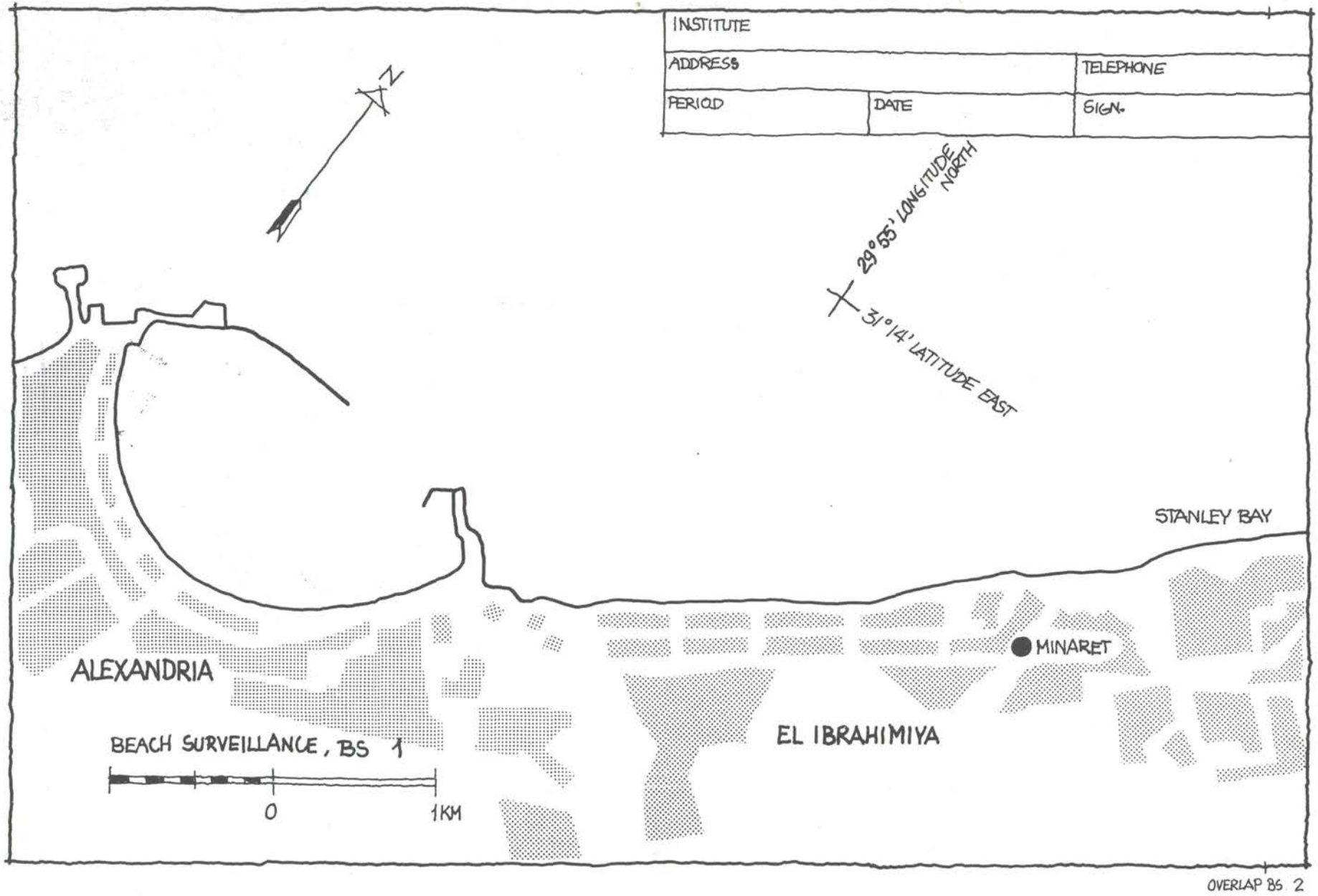


Fig. 4a

BEACH SURVEILLANCE, OBSERVATION SHEET (2)

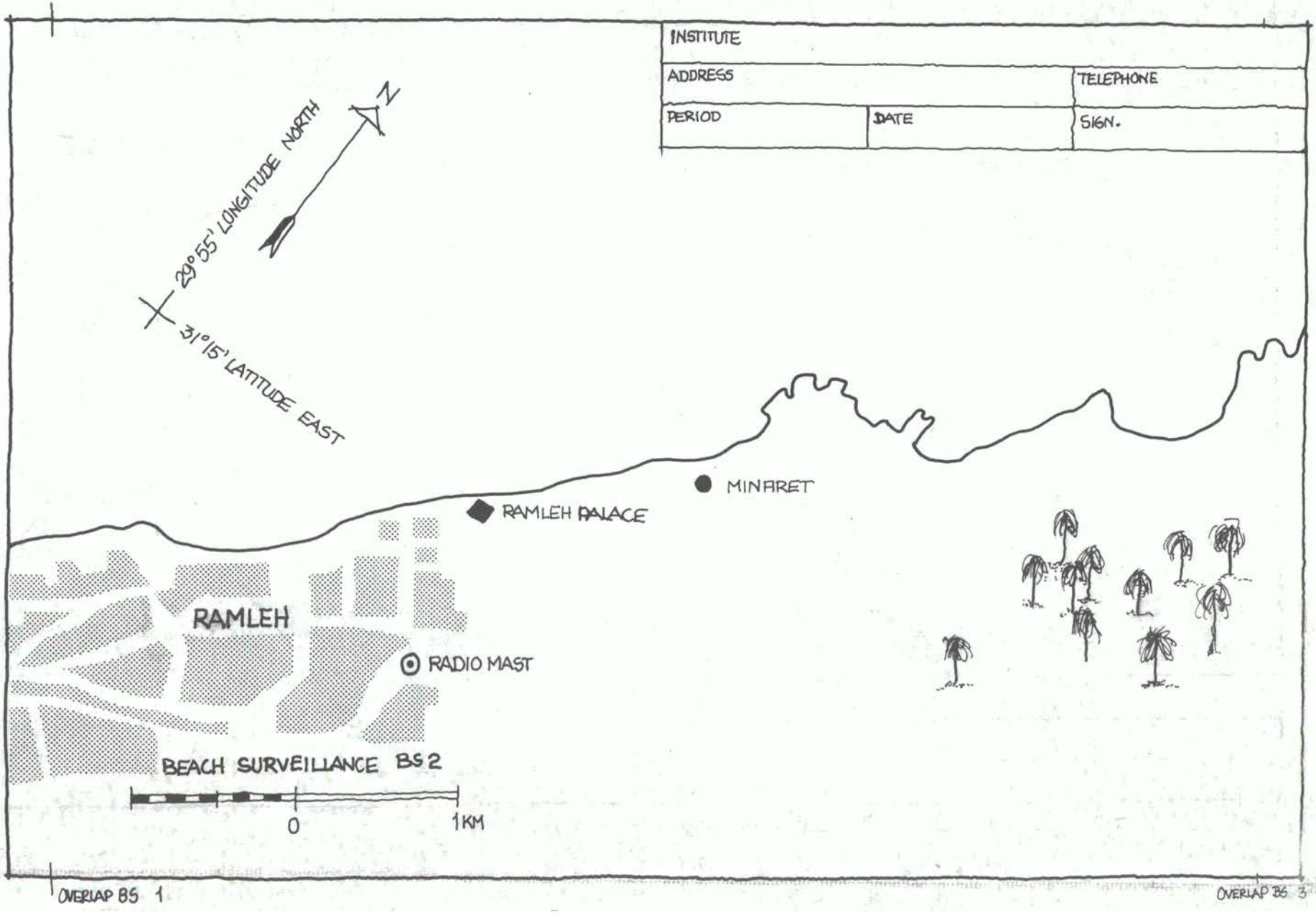


Fig. 4c

BEACH SURVEILLANCE, OBSERVATION SHEET (3)

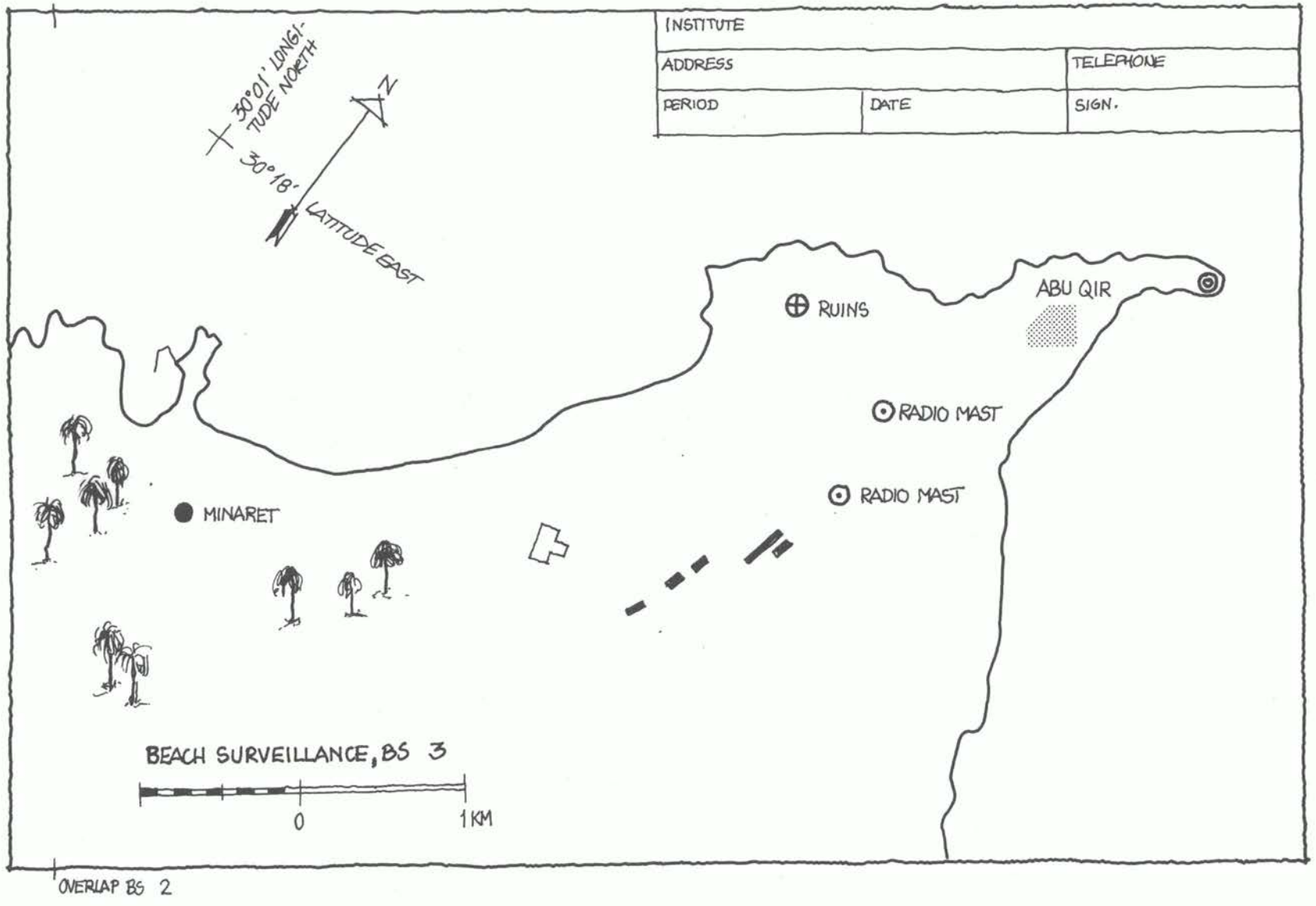


Fig. 5a

MINIMUM RECREATIONAL MONITORING LOCATIONS AND PARAMETERS, ALEXANDRIA (MM1)

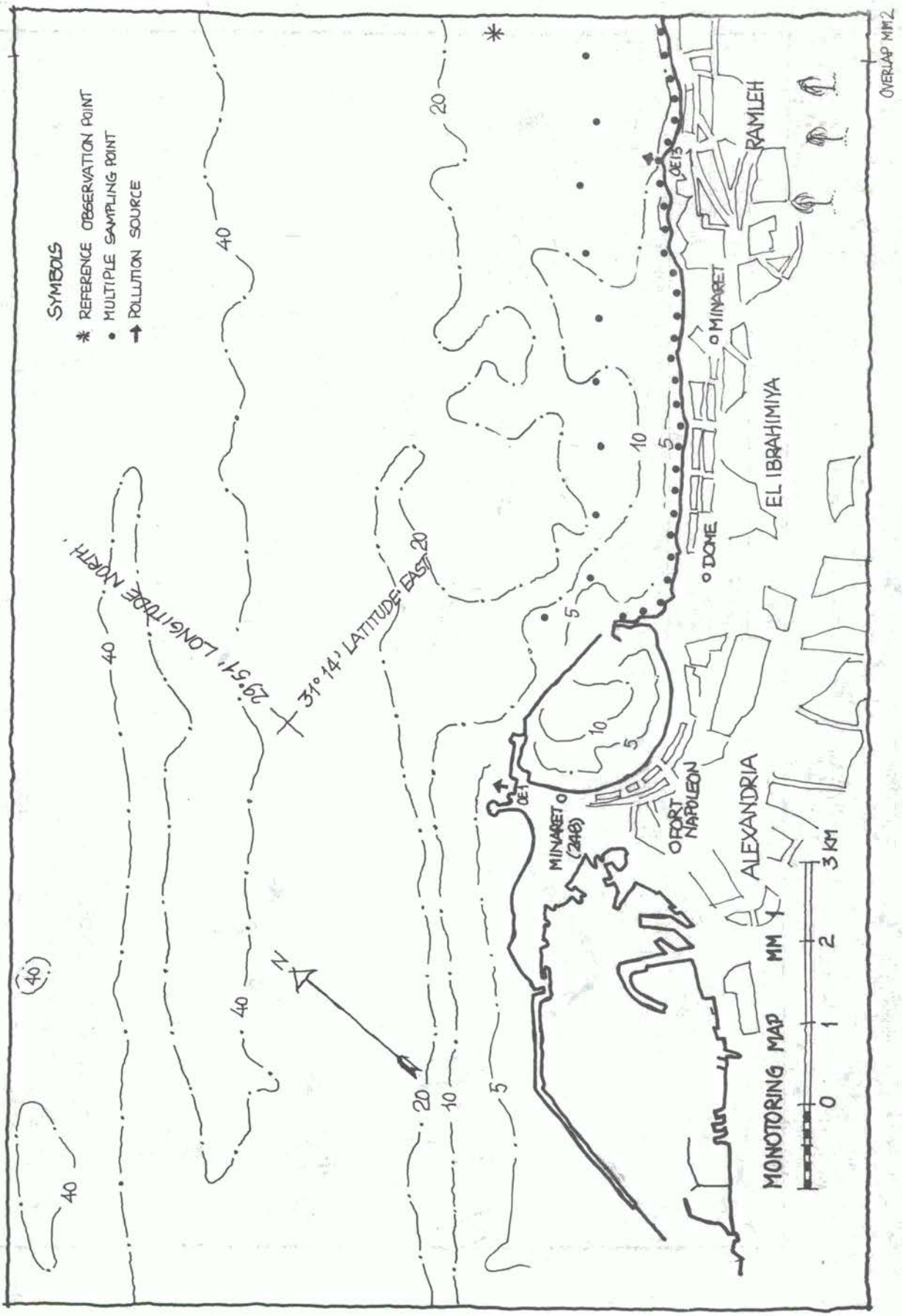
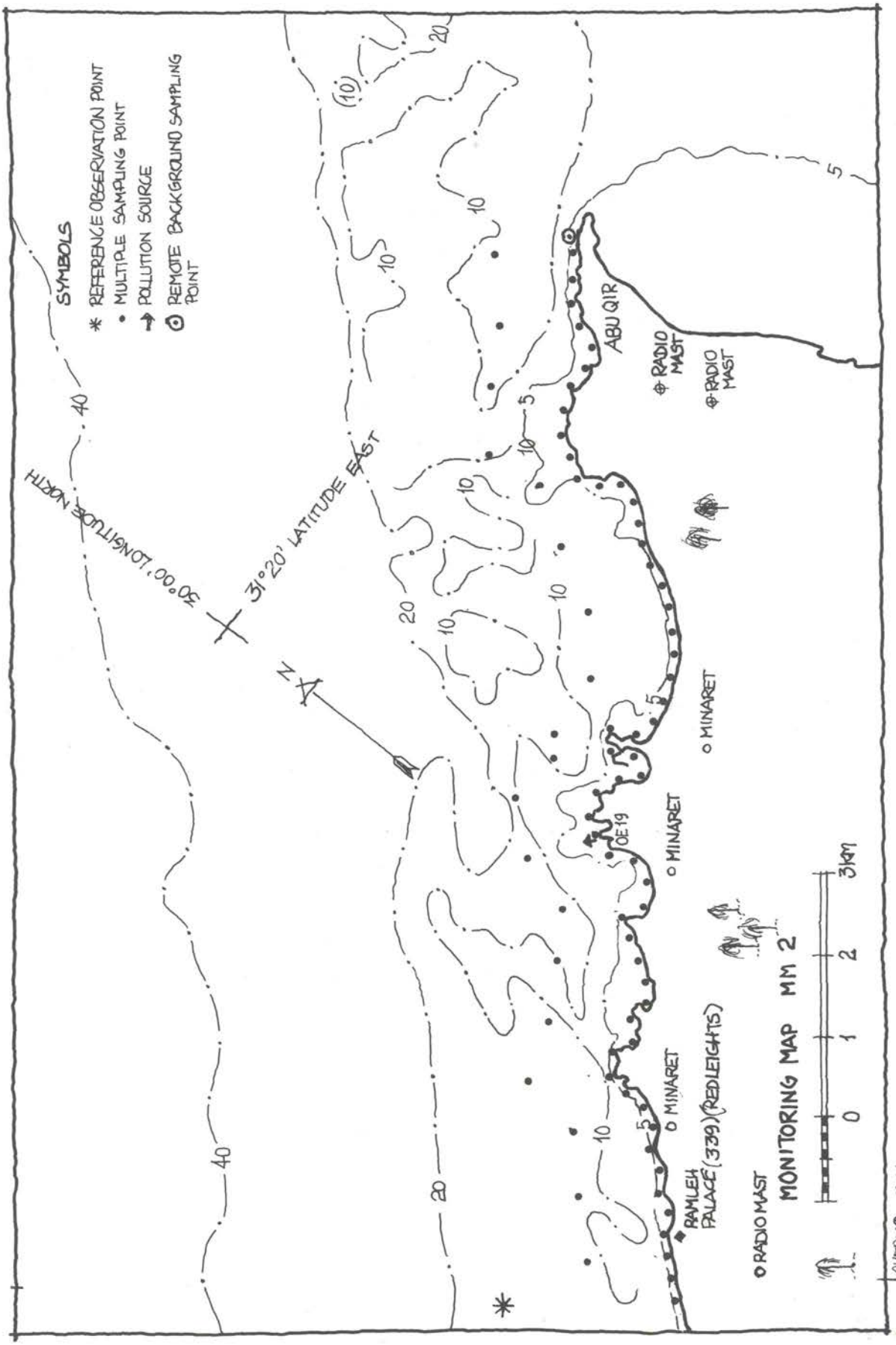


Fig. 5b

MINIMUM RECREATIONAL MONITORING LOCATIONS AND PARAMETERS, ALEXANDRIA (MM2)



(3) Multiple point sampling, for example, bacterial counts, for a particular location, medium, and depth. These should be conducted in the minimum monitoring programme for each type of area.

6.2 Recreational waters

6.2.1 Overall observations

The following parameters should be observed on a continuous basis, preferably at an existing weather station, adequately and regularly sampled:

Wind	Continuous records of direction and velocity should be obtained from a permanent weather station, such as at a nearby airport or harbour.
Tides	Tide tables should be studied and copied for monitoring periods, and for this purpose pilots at the nearest commercial port should be consulted.
Water-level variations	Water-level variation at a nearby port or similar should be registered continuously or at least observed every two hours.

Records of these parameters should be kept as copies of original recorder strips or similar.

6.2.2 Reference point measurements

The following reference point measurement parameters should be observed at the beginning and end of the multiple-point sampling period. The reference point should be fixed, and the location should be as representative as possible for the entire monitoring region in terms of relative parameter changes (see Figure 6). For all these observations use Form 3, Annex IV.

It should be noted that sub-surface metrical current measurements have not been included in the minimum programme since, to provide meaningful results, they require a separate relatively large vessel, lying at anchor in a fixed position. Currents could, however, be estimated by drogue observations conducted at a number of depths at the time surface drift is observed. In this case the same fixed position should be used as reference for speed and direction.

Surface water drift	Observe surface water speed and direction relative to a fixed position for example a buoy.
Waves	Estimate wave height for example every two hours
Salinity and temperature	Observe salinity and temperature at surface, bottom, and in between.
Secchi disc	Observe the disappearance and reappearance depths, in metres.
Oxygen	Observe O_2 at surface, bottom and in between.
Wind	Observe direction and velocity (local winds may register considerable shifts)
Air temperature	Measure air temperature

It was specifically suggested by the WHO/UNEP consultation meeting that multiple-point sampling should take the following form (1):

Parameters in water	Total coliforms/100 ml Faecal coliforms/100 ml Faecal streptococci/100 ml Salinity °/oo (weight/weight)
Parameters in sediment	Faecal coliforms/g

Fig. 6a

EXTENDED RECREATIONAL MONITORING, OPEN SEA, PROVISIONAL PROGRAMME, ALEXANDRIA (MM1)

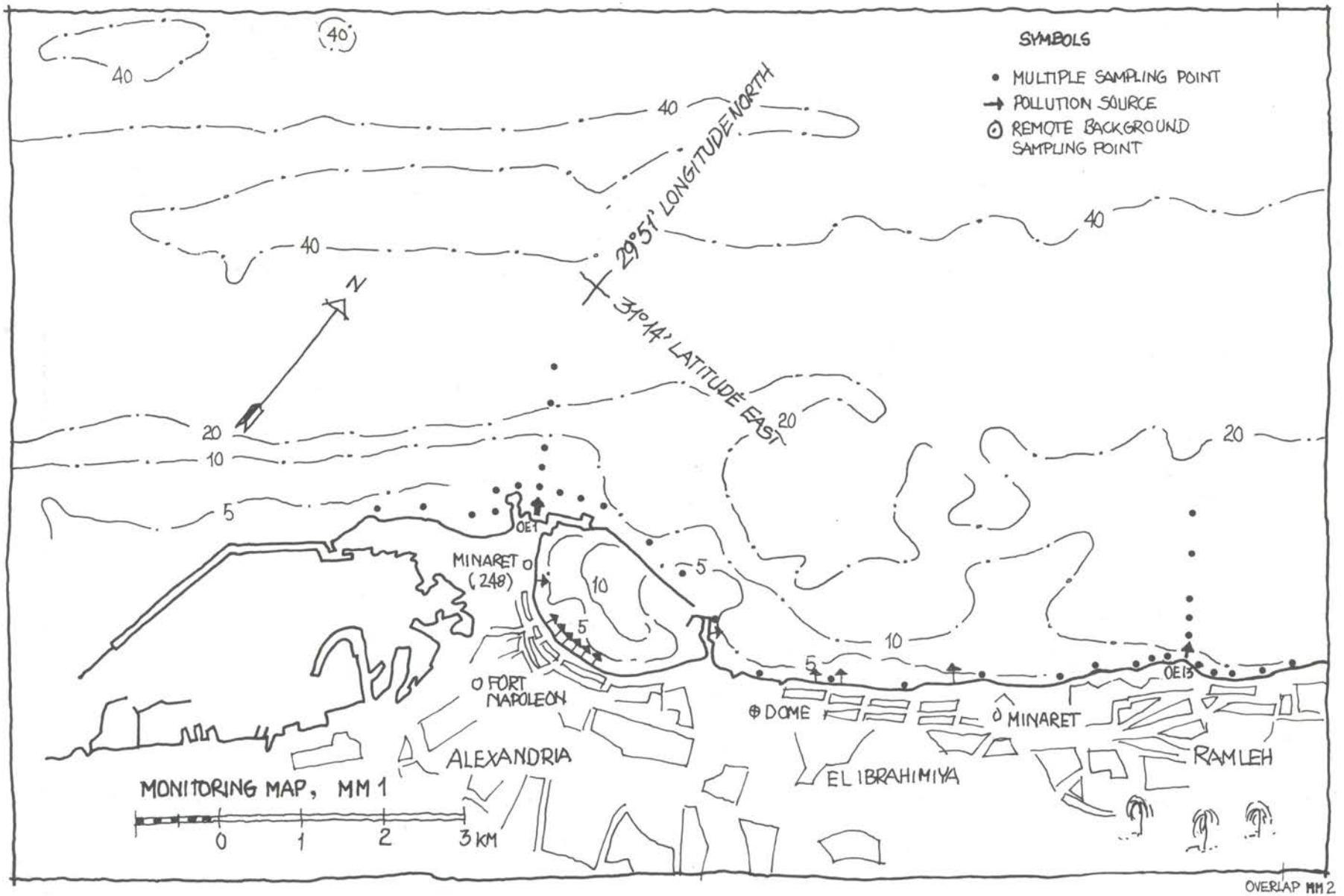
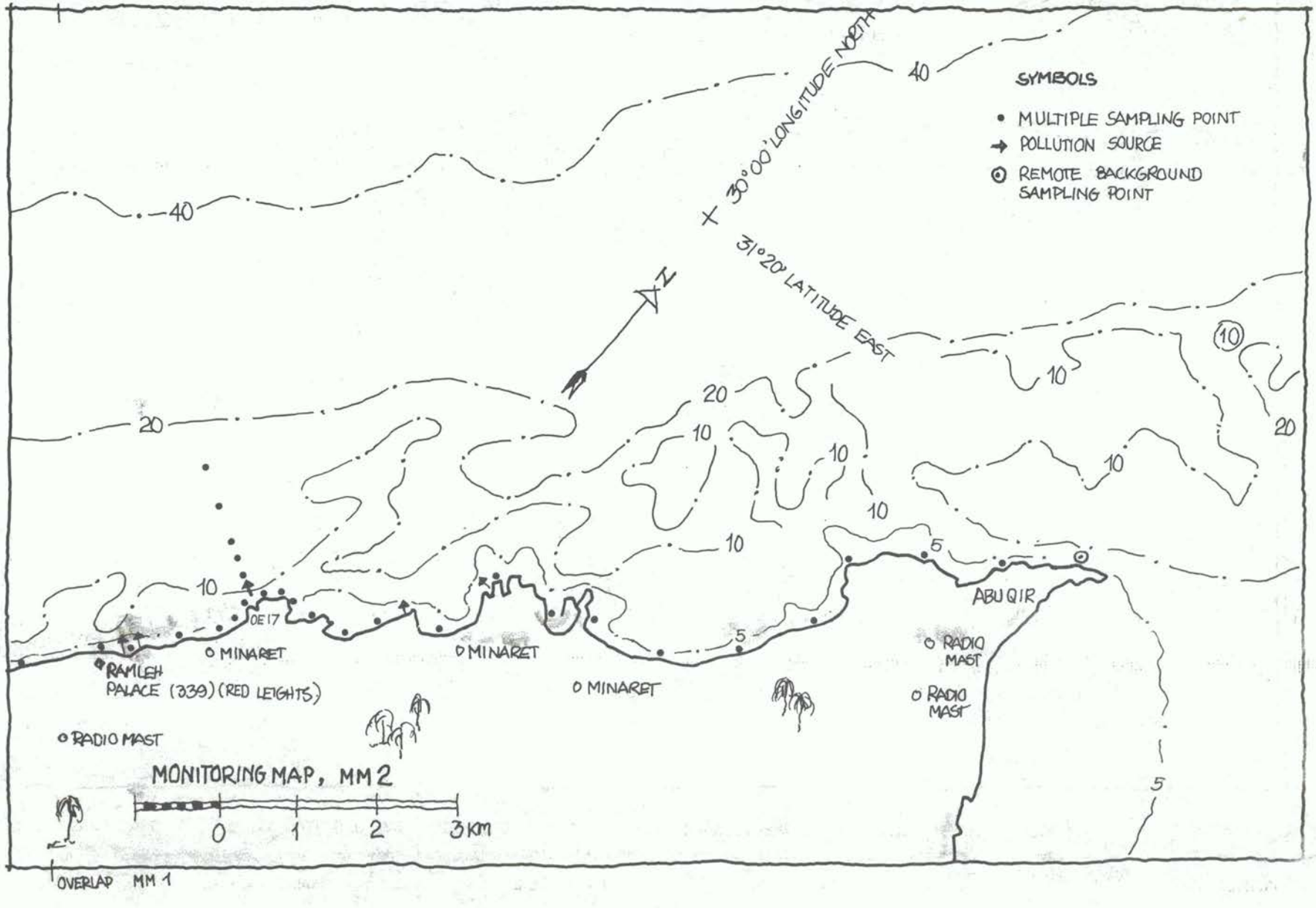


Fig. 6b

EXTENDED RECREATIONAL MONITORING, OPEN SEA, PROVISIONAL PROGRAMME, ALEXANDRIA (MM2)



Sampling point locations	Every 250 metres along the coastline, 10 metres below low-tide mark. Where the water depth exceeds five metres water samples will be taken at the surface, on the bottom and in between. In this latter case a sediment sample should also be taken (not from the rocky bottom, only from sand, silt or mud).
	Multiple-point sampling should include sampling points remote from direct (outfalls) or indirect (rivers, creeks) wastewater discharge points. Results from such points would serve as parameter references for other observations in the area, and would serve the purpose of comparison between areas. The final decision must be made locally, taking into account information gathered throughout the initial sampling cruises.
	A seaward sampling point, for example lying one kilometre off-shore, should also be established for every kilometre of beach being monitored.
Sampling frequency	All points must be sampled at least once a month during the bathing season (normally May - September) and at three equally spaced intervals during the remainder of the year.
	If more than 10% of the samples taken along the coast show a value of faecal E. coli of over 500/100 ml the frequency should be doubled for at least one year.

Form 4 (Annex IV) should be used for recording facts about the sampling. Note that observations of surface pollution as given by the modified Garber classification (25, 27) are also included here.

Figure 6 shows an example of a monitoring programme designed in accordance with these requirements. Obviously the burden of work becomes demanding in terms of laboratory capacity, and depends on the number of sampling sites, depths, media, and organisms to be investigated. A monthly timetable for staged sampling may be necessary in order to obtain an even distribution of work.

Adjustments, such as by increasing or reducing the number of sampling points, may be realistically considered after completing at least one season's monitoring (See Chapters 9 and 15).

6.3 Shellfish areas

A distinction must be made between primary and secondary sources of shellfish pollution. Primary sources, sampling for which is all that space permits to be described here, are those which contaminate shellfish directly by the water route, when shellfish are contaminated before being harvested. Secondary sources of pollution cover all types of potential contamination which occur between harvesting from the shellfish bed and ingestion by the consumer. These must be supervised under sampling programmes (see also Chapter 10 for methods) operated by the local food inspection bureau or equivalent.

The reference point programme is identical to that for recreational waters. The location of the reference point should be close to the shellfish bed, preferably in the direction of the major sources of pollution, and Form 3 should be used for data recording.

The joint WHO/UNEP consultation suggested the following minimum programme for multiple point sampling (1).

Parameters	Total heterotrophic bacteria Total coliforms Faecal coliforms Faecal streptococci
Sampling point location	In the shellfish culture area In the culture area surroundings Close to sources of pollution
Frequency	Minimum, every three months: For large scale (> 500 ton/year)areas, once every month.
	Weekly monitoring in peak consumption period.

Form 4 should be used for data recording.

Figure 7 gives an example of shellfish monitoring design for a hypothetical case of shellfish monitoring according to the above minimum requirements.

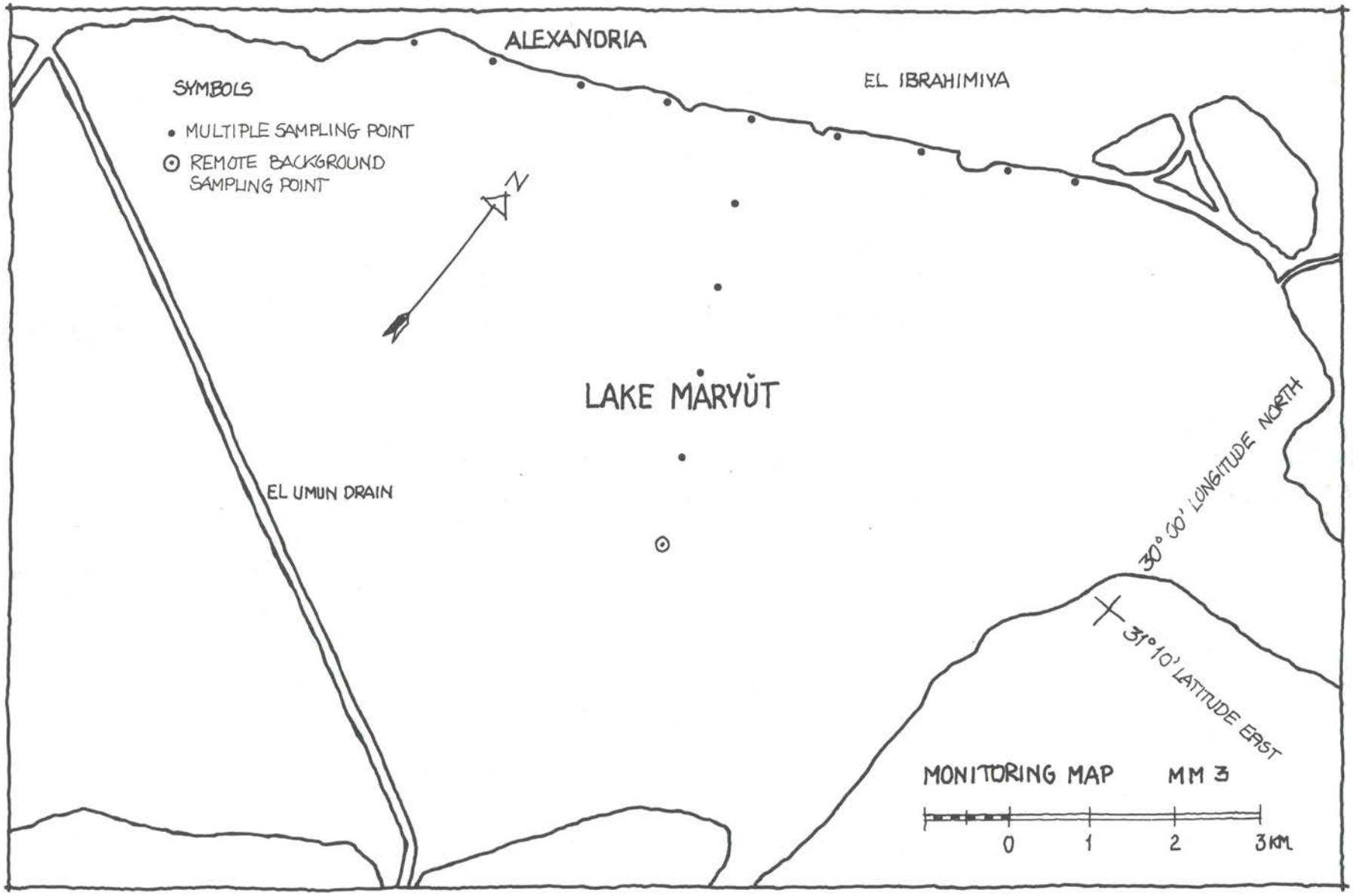
6.4 Pollution sources

Pollution source monitoring is required by the minimum programme only to a limited extent. Form 5 should be used to record results.

Parameters in the effluent	Biochemical oxygen demand (BOD) Settleable solids Flow (estimate only) These three parameters should be determined for recreational as well as for shellfish monitoring. For shellfish monitoring, the following additional parameters should be included: Total coliforms Faecal coliforms Faecal streptococci.
Sampling point location	Sample only major outfalls at points on land as near as possible to outfalls, where easy access allows representative samples to be taken.
Frequency of sampling	Samples are taken on the same days as for recreational or shellfish studies. Grab sampling is used, preferably at the beginning of the monitoring day, rather than at the end.

Fig. 7

EXTENDED RECREATIONAL MONITORING, LAGOON, PROVISIONAL PROGRAMME, ALEXANDRIA (MM3)



7. Extended monitoring

7.1 Pathogens and indicators

Pathogenic microorganisms include bacteria such as Salmonella and Shigella, viruses such as polio and reovirus, and parasites such as hookworms and tapeworms. The presence of pathogens in the environment may constitute a health hazard for humans and animals, for example through human ingestion of fresh seafood harvested from an area where faecal matter is present in high concentrations.

Even though a pathogen might convincingly demonstrate the presence of pollution it does not necessarily follow that it is a good organism for monitoring risks to human health. The following examples will illustrate this.

(1) If shellfish are the monitoring medium the pathogen itself could prove to be the ideal monitoring organism, provided that efficient analytical procedures exist to establish adequate quantitative information on the latter's presence. However, analytical techniques may be insufficient at present or even not feasible, e.g., for certain viruses that cause infectious hepatitis.

(2) If the monitoring medium is offshore water, the pathogen will normally not be the best monitoring agent, as it may be present in small concentrations and only intermittently. Thus, when not monitoring constantly there is a chance of zero-pollution indication, even though the pollution risk may be real, either because shellfish will concentrate the pollutant steadily, or because the recreational area is also used when monitoring is not being conducted.

Evidently, faecal pollution control requires monitoring organisms other than the pathogens themselves and it is for this reason that the concept of the indicator was introduced. Certain criteria have been established (3) according to which the faecal water pollution indicator should ideally:

- always be present and occur in much greater numbers than the pathogens of concern;
- not be able to proliferate in the aquatic environment to any greater extent than the pathogen;
- be more resistant than pathogens to disinfectants and to the aqueous environment; and
- yield characteristic, specific, and rather simple reactions to ensure unambiguous identification.

Very few, if any, faecal microorganisms fulfil all requirements, and an ideal indicator does not exist. However, after careful consideration of environmental factors and pollution problems to be studied, good indicators can be found locally or regionally, and, at least for temperate climates, these include faecal coliforms and Clostridium perfringens. Both types include certain strains that are entero-pathogenic to humans.

Human pathogens are not necessarily of faecal origin but may be widespread in the marine environment. Vibrio parahaemolyticus could serve as an example. A concatenation of circumstances such as peak concentrations in the summer and/or incorrect seafood preparation may cause human enteritis. There are other examples of pathogenic and ubiquitous microorganisms, and only after careful consideration of environmental and epidemiological factors should decisions be reached as to the extent of microbiological monitoring.

7.2 Monitoring media

As outlined already under Scope of Activities, (section 3, page 2) several media must be considered: sewage discharge point, water, sediment, seafood and beaches.

The medium itself may significantly influence the choice of monitoring organism. For instance, in seafood the monitoring organism would often be of the pathogenic type, because accumulation on fish gills or in the oyster mantle cavity provides the enrichment necessary to obtain sufficient analytical accuracy, whereas water sampling would call instead for a more plentiful indicator organism.

When considering the monitoring of sediments, the settling characteristics of particles in sewage rivers, etc. may be of importance if the organisms adhere to or even constitute the settleable particles. The discharge of a river into the coastal zone may significantly influence the marine environment, so that several different monitoring regions must be distinguished, because salinity, temperature, turbidity, etc. vary greatly.

7.3 Characteristics of certain microbiological monitors

In order to assess properly which of the proposed monitoring organisms included in the present guidelines to select for a given monitoring programme, certain facts on the occurrence of the organisms and the etiology of possible diseases will have to be brought out (for examination procedures see Chapter 10). Here, the sequence of the organisms used in Table 3 will be employed.

Table 3

DISEASES[1] WHICH MAY OCCUR AFTER AN INFECTION WITH A HUMAN ENTERIC VIRUS

Virus type	Symptoms of disease[2]
Poliovirus 1-3 Coxsackie virus A 1-24 Coxsackie virus B 1-6 Echo virus 1-33	Fever, headaches, nausea, diarrhoea, muscular pains, meningitis, paralytic poliomyelitis (infrequent with infections other than poliovirus). Some coxsackie- and echo-strains have caused hepatitis. Some enteroviruses cause exanthema.
Adenovirus 1-28	Fever, acute upper and lower respiratory tract infections, inflammations of the conjunctiva, more seldom: symptoms of enteritis or central nervous system involvement.
Reovirus 1-3	Virus has been demonstrated in connexion with common cold and other infections of the upper and lower respiratory tract, with diarrhoea, exanthema, and hepatitis, especially in children.
Rotavirus	Infantile gastroenteritis
Hepatitis virus	Fever, nausea, anorexia, diarrhoea, hepatitis (acute or chronic)
Virus or acute gastroenteritis	Acute gastroenteritis; "winter vomiting disease"

[1] In most cases infections are inapparent even with the highly pathogenic viruses. In addition, the disease symptoms for each virus are variable depending on such host factors as immunity, age and general resistance and on the initial dose of virus.

[2] For each virus type there may be a considerable number of virus strains which may vary in pathogenicity. Routine cultivation procedures for the isolation of viruses provide no information on the pathogenicity of the viruses.

Source: Lund, E. In: Manual on Analysis for Water Pollution Control, Copenhagen, WHO Regional Office for Europe (in preparation) (9)

7.3.1 Total heterotrophic bacteria

The total number of heterotrophic bacteria are determined by a plate count after five days of incubation at 20°C. The significance of the plate count is not universally accepted, although some countries employ a total plate count standard. Molluscs taken from the sea reflect the numbers of bacteria growing naturally in the marine or estuarine environment, and experiments in some areas have shown that the tissues of freshly caught molluscs taken from non-polluted sea areas often yield total plate counts in excess of 10^6 per gram. Thus, control authorities should first establish the range of total plate counts for shellfish from approved areas before submitting them to an arbitrary total plate count standard.

The total heterotrophic count in shellfish depends on a number of factors such as distance from sewage outfalls, algae blooms, alterations in reproduction potential for naturally occurring marine microorganisms, and the filtering activity of the shellfish in the area. Degradation of food and food changes in taste, and more specific occurrences such as decarboxylation of lustidine are possibilities that are enhanced by increased numbers of bacteria, for example, like those reflected in an increased total plate count.

Where molluscs are subjected to subsequent processing or to unsatisfactory storage and transportation conditions, experience has shown that there will be a substantial increase in the total plate count. Thus, the total plate count may be of value in assessing handling and storage conditions etc. It must be emphasized, however, that before such standards can be applied in a given area, an extensive programme of sampling and bacteriological examination is needed.

7.3.2 Total coliforms

The coliform group of bacteria is widely distributed in the soil, surface waters, and also in foodstuffs, and it includes such species as Klebsiella, Enterobacter and Citrobacter.

The coliforms are not specifically linked to the intestines of warm-blooded animals (including man), and major sources of coliforms in the coastal region may come from rivers, soil run-off and certain types of industrial discharges; those such as Klebsiella, for example, may propogate where polycarbohydrates are discharged in major amounts.

The use of total coliforms as indicators of faecal pollution in coastal surveys is by tradition widespread, though hardly justified with regard to the indicator criteria already outlined, and the more specific alternatives now available.

Several bacteria within the coliform group may be pathogenic, but the coliform test would seem least useful for disease control because of its lack of specificity in examination and diagnosis. Die-away rates for total coliforms have often been studied and reported, e.g., by Harremoes (3), who found T-90 (i.e., a 90% reduction in living organisms) values of 2-3 hours in Manila Bay.

7.3.3 Faecal coliforms (thermostable coli)

Faecal coliforms are facultatively anaerobic bacteria belonging to the coliform group. They are often referred to as Escherichia coli although there are several different strains within the group. Faecal coliforms are specific, including human-specific. Certain data on occurrence exist:

Human excreta:	10^9	/g faeces
Non-treated sewage:	$10^6 - 10^8$	/100 ml
Secondary treated sewage:	$10^4 - 10^6$	/100 ml

Faecal coliforms, where found, will normally indicate recent faecal pollution, because they do not propogate in the marine environment. Die-away rates (T-90) of 1-3 hours have been reported depending on salinity, temperature, solar radiation and other factors (3, 23).

Certain faecal E. coli. strains are known to be enteropathogenic, causing diarrhoea among infants as well as adults. The infective dose may approximate 10^8 viable organisms of the pathogenic strains (Bockemuhl: 3).

In many respects E. coli meets the requirements of a good indicator. The major problem is its relatively short survival in sea-water, which may call for the use of supplementary indicators.

7.3.4 Faecal streptococcus

Species of Lancefields group D streptococci occurring in human and animal faeces are likely to be found in polluted waters and include Streptococcus faecalis, S. faecium, S. bovis, and S. equinus.

For different types of polluted waters the ratio of faecal coliforms to faecal streptococci may vary as follows:

Polluted water	Faecal coliforms/ Faecal streptococci
Faecal discharges from wild animals, dogs, cats, cattle	less than 1
Faecal discharge from man	more than 4
Domestic waste-water	4 or more
Storm-water	less than 1

These ratios must be applied carefully. Correlations are most meaningful when developed from bacterial densities in water samples taken at waste outfalls into a stream, and will only be valid during the initial 24-hour travel downstream from point of pollution discharge into the receiving river.

Normally, there is no need for species identification of faecal streptococci in water pollution studies. Density ratios in relation to faecal coliforms are adequate to assign the probable source of water discharge, whether domestic, farm animals, or wild life.

In special circumstances, the use of S. bovis and S. equinus, as a specific indicator group for animal pollution of non-human origin may be useful in studies involving livestock pollution from cattle and dairy farm feed-lots, meat-packing and dairy plant wastes, poultry farms and poultry processing wastes, and pollution from wild bird refuges. These two faecal streptococci strains are the most sensitive indicator organisms within the faecal streptococci group because of their rapid death-rate outside the animal intestinal tract. Laboratory data on the survival of S. bovis and S. equinus in various polluted receiving waters indicate that the two organisms, on discharge into the receiving water, die away faster than other faecal streptococci.

The increasing use of chemical treatment of domestic sewage in some countries may generate more interest in the faecal streptococcus test. Flocculation by lime or other strong bases may lead to pH-values higher than 9 in treated sewage. If the coliform test is used as the only method for estimating the effectiveness of the process in removing faecal organisms, the process will be graded as excellent. Faecal streptococci, however, being able to grow at a pH of 9.6, may not be reduced to the same extent, and may therefore be used as an indicator for pathogenic organisms with similar pH-resistance.

7.3.5 Clostridium perfringens

Clostridium perfringens is an anaerobic sporulating bacterium. It is excreted by man and animals and is found very regularly in stools. Its occurrence is as follows (4, 5):

Human excreta	$10^6 - 10^5$/g
Untreated sewage	10^3
Secondary treated sewage	10^2
Sea sediments without faecal pollution[1]	10/g
Sea sediments with faecal pollution[1]	10^4/g

Certain types of Cl.perfringens are pathogenic; type A may cause slight diarrhoea, and type C may give rise to enteritis necroticans. Infections occur mostly after non-refrigerated storage of food, where the multiplication of organisms may occur, for example, from spores that have germinated after cooking. Ingestion of approximately 10^9 viable cells seems an adequate dose for infection (Bockemuhl: 3).

The use of Cl. perfringens as a monitoring organism is a matter of discussion, as can be seen from the indicator criteria above. However, there are a number of reasons why Cl. perfringens is being used for coastal pollution studies, at least in Scandinavia.

(1) It is discharged in significant numbers with sewage, where it is mainly of human origin.

(2) It does not multiply in sediment.

(3) It survives in the marine environment, as opposed to E. coli, and the two organisms therefore tend to be complementary.

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[1] Applies to Scandinavian waters at least. Reference quantities must be established locally for each survey region.

(4) As there are significant differences between Cl. perfringens counts near to and far from discharge points, interpretation of the counts seems feasible. Although sewage may not be the only source, it is nevertheless often the most significant one.

7.3.6 Salmonellas, including S. typhoid and paratyphoid

Salmonellas are excreted from healthy as well as diseased carriers among animals and humans. Salmonellas may be the most frequent reason for infection of humans by animal-borne pathogens, the symptoms of which are enteritis, with watery diarrhoea.

Infections by typhoid or paratyphoid salmonella bacteria require a human excretor, a carrier whether healthy or diseased, and symptoms include fever and watery diarrhoea (dysentery). Typhoid and paratyphoid are endemic and often epidemic in sub-tropical and tropical areas, but so far seem to be under control in the industrialized countries of Europe and North America, although changing patterns of travel, such as those involved in tourism and trade may require increased attention to be devoted to the infection route leading from animal feeds through animals and food to man. In the marine environment sewage outfalls normally constitute the major source of pollution by salmonella. Salmonellas do not survive for long in water, and infection directly as a result of bathing or other recreational activities is not very likely, due to the infective dose required of 10^5 - 10^6 viable organisms. However, it must be noted that for S. paratyphoid A and B and for S. typhoid the infective dose is considerably lower.

Consumption of seafood is a different problem, because the bacteria are concentrated either by filtering shellfish or on fish gills; in shellfish, the concentration may be 50 times the concentration in water. With consumption of raw seafood such as oysters this route of infection evidently becomes important.

Separate interest must be devoted to typhoid and paratyphoid salmonella types, partly because of the severe consequences of an infection, and partly because of the different route of infection to man. Further isolation of salmonella typhoid requires examination procedures that differ from those of the other salmonellas (see Chapter 10).

7.3.7 Vibrio cholerae and NAG Vibrio

Cholera has been endemic in Southern Asia for centuries and pandemics have spread from here on several occasions, caused firstly by the "classical" and lately (1970 in Europe) by the "El Tor" biotypes of Vibrio cholerae. The symptoms of cholera are diarrhoea without fever, with the patient losing water due to the production of enterotoxins that act on the small intestines and disturb the balance of water and electrolyte movements across the cell walls.

Non-agglutinable (NAG) vibrios will not agglutinate cholera antiserum at a ratio of 0 : 1. NAG vibrios may cause mild, or sometimes severe diarrhoea. The background to NAG vibrio is not yet well known.

In the marine environment discharged wastewater from healthy and diseased carriers is the major V. cholerae source. The most likely routes of infection are by consumption of shellfish that filter large amounts of water and thus accumulate over a period of time the (possibly) intermittently discharged bacteria of the different V. cholerae types.

7.3.8 Vibrio parahaemolyticus

Vibrio parahaemolyticus is native to the marine environment, contrary to previously mentioned bacteria. It is pathogenic to man, causing enteritis, normally of shorter duration, but involving watery diarrhoea, vomiting, and abdominal cramps. The infective dose is greater than 10^6 viable organisms. The most common cause is consumption of shellfish, shrimps or raw fish.

Not all V. parahaemolyticus strains cause diarrhoea; in this respect, it seems relevant to make a distinction between the so-called Kanagawa-positive and Kanagawa-negative strains. However, wound infections seem possible with both K-positive and K-negative strains, also caused by V. alginolyticus.

It appears useful to use shellfish, sediments and sewage as monitoring procedures, because the situation in receiving water, seafood and the population is worth knowing.

7.3.9 Shigellae

The statistics on dysentery in the Mediterranean given in Table 1, and on cases exported from there, call for a more comprehensive assessment of the situation than has hitherto been undertaken.

The shigellas are causative agents for bacillary dysentery, and to assess this part of the problem it is first essential to establish statistics on its occurrence in sewage from major cities. This first primitive approach is based on the fact that shigellas survive only for a short time in the marine environment, and examination procedures are hampered by the absence of cultivation media as selective as those for salmonellas.

7.3.10 Yersinia

Only recently has Yersinia been recognized as an important type of bacteria in terms of epidemiology and zoonoses (20). Interest is attached to Y. pseudotuberculosis and particularly to Y. enterocolitica, and their links with enteritis and arthritis. Pigs and rodents are considered to be natural reservoirs of Yersinia, and it has been demonstrated as being present in fresh water and marine sediments, fish, shellfish, seawater, fresh water and sewage (32).

In this case too, assessment of the situation seems justified, and examination of sewage alone is proposed as an initial step.

7.3.11 Parasites

The human health aspects of parasites such as nematodes (e.g., Trichuris and Ascaris) in the marine environment are not yet well understood and little information is available. However, while an infective dose of bacterial pathogens is often as high as about 10^6 viable organisms, a single parasite egg is sufficient to cause infection. Furthermore, parasite eggs are likely to survive for months, as against the brief survival period for bacteria in the marine environment.

The potential problems created by parasites in the marine environment may be more pertinent to sediments and bottom animals than to water because most parasite eggs will settle after discharge through sewage outfalls. For the same reason, treated sewage is less a problem than is raw sewage. Filtering organisms such as molluscs and oysters should be kept under particularly careful observation if harvested near sewage outfalls.

The route of infection should always be considered, in order to select among numerous possibilities the relatively small number of parasites that require attention when discharging into a marine environment. Schistosomas, for example, should not be included in marine monitoring programmes, because they need intermediate hosts (snails) which live only in fresh water. Particular interest should primarily be devoted to eggs from nematodes and some protozoans such as Endamoeba, Giaordia and Naegleoria.

In order to assess the parasitic problem provisionally, an examination procedure is proposed that will provide basic information on occurrence in sewage, sediments and shellfish (see Chapter 10).

7.3.12 Enteroviruses

A list of diseases which may occur after infection with human enteric virus has been reproduced below (9). Considering the as yet relatively poor information available on pathogenesis and etiology, the studies on virus in the marine environment as related to human health fall within the research type of monitoring and development of techniques to concentrate the very dilute virus suspensions.

It is worth noting that conventional chlorination may kill a number of bacteria, while viruses are not effectively inactivated. Further, there is evidence that viruses survive longer in receiving water than many bacterial indicators and pathogens.

Viral hepatitis type A is the only viral disease for which shellfish have been shown to be a source of infection. Because of the lack of suitable methods for isolating the virus, and because the incubation period is long (usually 20-40 days), the role of shellfish is not fully understood. There is evidence from Sweden and North America that grossly polluted raw or improperly cooked clams and oysters have caused hepatitis. The organism is probably taken up by molluscs during their normal feeding activities, for the virus is probably present in sewage from communities where infectious hepatitis occurs. Shellfish from an area known to be contaminated by sewage derived from a community with cases of viral hepatitis should not be used for human consumption, even after heat treatment, for the virus can withstand a temperature of 1000°C for several minutes (21).

Mussels, oysters, and clams from various parts of the world have been shown to be capable of accumulating enteroviruses from the water in which they feed (21). Members of the poliovirus,

echo virus, coxsackie virus, and reovirus groups have been detected in oysters from the coastal waters of the USA, and in mussels in Italy. Polioviruses have also been detected in oysters exported from Japan. Crabs too have been shown to become contaminated, under experimental conditions, by feeding on enterovirus-contaminated clams. Virological examinations have been suggested for shellfish and for sewage, and the finding of enteric viruses in shellfish should be grounds for their exclusion from commercial markets.

7.3.13 Biotoxin (paralytic shellfish poison)

This biotoxin is included in the microbiological parameter list because the route followed by it is similar to that of many of the microorganisms already mentioned, and sampling for examination is similar to that used with microorganisms (22).

Paralytic shellfish poison (PSP) is produced by some species of dioflagellata, such as Gonyaulax catenella and G. tamarensis. Shellfish store the toxin without harmful effect to themselves, if toxin-producing plankton constitute the chief part of their diet.

Paralytic shellfish poison is a neuromuscular toxin, or possibly several closely related neuromuscular toxins, which under certain conditions cause poisoning following consumption of shellfish. No antidote is known against the poison which has considerable demonstrable signs of toxicity.

Toxicity may vary considerably from species to species under fairly similar conditions. In the cases of poisoning that have occurred in Scandinavia, the common mussel (Mytilus edulis) seems to have been absolutely predominant. Among other species found toxic it is worth mentioning the Californian common mussel (Mytilus californianus), sand mussel species like Saxidomus nutallii, and Saxidomus gigantus, Mya arenaria, and Crassostrea gigas (cultivated oysters).

7.3.14 Other monitoring organisms

Only a few monitoring organisms have been included in these guidelines. Most of the organisms selected were already listed in the report of the joint WHO/UNEP consultation in Geneva in December 1975 (1), with due regard to the characteristics of the Mediterranean in terms of climate, epidemiology etc. A few others have been added, including such organisms as Yersinia, because they are found important in order to assess situations that have not yet been adequately studied, either in the Mediterranean or in many other parts of the world.

Local circumstances may often justify inclusion of additional parameters and/or the exclusion of some of those proposed in the present guidelines. Pathogenic organisms not included but perhaps of interest include Pseudomonas aeruginosa, Vibrio alginolyticus, Aeromonas Klebsiella, haemolytic staphylococcus, and others that may cause infections through eyes, ears, throat or wounds.

7.3.15 References for other organisms and their examination

To include new monitoring organisms would require a well-trained group of analysts and the use of references that provide information and experience already reported by other investigators. The reference list, included at the end of the present document, may give some guidance in this respect. Often the regional specialized reference laboratory will be of great help in evaluating new parameters and relevant methodology.

7.4 Choice of organisms for extended monitoring

A coastal water quality monitoring programme should normally be designed only after careful consideration of the objectives, and the available resources. With regard to human health protection, several pathogens and biotoxins are of prime interest as monitoring parameters. It is difficult, however, to state exactly which organisms should be included, which media should be sampled, which location used, and at what frequency. Also, with lack of knowledge on pathogenesis and routes of infection there is no clear distinction between monitoring for control purposes, for research, or for combinations thereof.

Table 4 may serve as a guide to extended monitoring programmes, but it applies only to the choice of microbiological parameters.

As with the minimum programme it seems enough to distinguish between recreational, shellfish and pollution source monitoring. The distinction between overall area observations, reference point measurements, and multiple point sampling also seems worth maintaining.

Table 4

EXTENDED MONITORING MICROBIOLOGICAL PARAMETER GUIDE

Monitoring organism	Sea food					Recreation		Sewage
	Water	Sedi-ment	Mollusc	Crust-acean	Fish	Water	Sedi-ment	Source of pollution
Total heterotrophic			M*					
Total coliforms	M*		M*			F*		M
Faecal coliforms	M*	M*	M*			F*	F	M
Faecal streptococci	M*		M*			F*		M
Clostridium perfringens		M					M	M
Salmonella		M	M	M	M	M	M	M
Salmonella typhii			M					
Vibrio cholerae & NAG			M					
Vibrio parahaemolyticus		M	M	M				M
Shigella								M
Yersinia								M
Parasites			M				M	M
Enteric viruses			M					M
Biotoxins (PSP)			M					

Notes: F = Fortnightly sampling; monthly, outside the recreation season
M = Monthly sampling
* = indicates activity that is also part of the minimum programme (see Chapter 6)

7.5 Recreational water

7.5.1 Overall area observations

The following parameters should be observed on a continuous basis, preferably at an existing weather station with regular and reliable attendance.

Wind	See section 6.1.1 (minimum programme)
Tides	See section 6.1.1 (minimum programme)
Water level variation	See section 6.1.1 (minimum programme)
Precipitation	Records of precipitation, amount and distribution over time, should be obtained, particularly when and where effluents (rivers or sewers) are sampled

Records should be kept as copies of original strips or notes or similar, and should cover the period of coastal monitoring, including intervals between actual samplings.

7.5.2 Reference point measurements

Reference point measurements provide background data for interpretation of multiple-point sampling and effluent monitoring. They should be begun well before and carried out continuously during the individual point sampling period, and will therefore require an extra vessel to be anchored at the reference position. Alternatively, the multiple-point sampling vessel could also carry out reference point measurements at the beginning and end of the sampling period although this is less satisfactory regarding subsequent data analysis and time of sample storage before laboratory analysis.

The location of the reference point should be as representative as possible of the entire sampling area. Adjustment may be necessary after experience has been gained on the characteristics of the area. Use Form 3 for data recording.

Currents	Profiles should be established, for example, by current speed and direction measurements for every two metres depth. If current meters are to be used there must be a separate reference point vessel available. Repeat measurements once every 1-2 hours, depending on changes observed.
Surface drift	Observe drift of surface water relative to a fixed position, e.g., the anchored boat. Drift cards may conveniently simulate drift of floattables, sticks and surface and surface trapped sewage. Make observations once every hour.
Waves	Estimate and register wave heights once every hour.
Salinity and temperature	Measure salinity or chlorine content and temperature, for every two metres depth, and in between if changes exceed 0.5% or 0.5°C per increment.
Secchi disc	Observe Secchi disc disappearance and reappearance and register number of metres below surface.
Oxygen	Observe O_2 at surface, bottom and in between, at the beginning and at the end of the sampling period.
Wind	Observe direction and speed once every hour within the sampling period.
Air temperature	Observe air temperature, at the beginning, middle and end of the day.

7.5.3 Multiple point sampling

In principle, extended monitoring automatically includes the minimum monitoring activities. However, as soon as the minimum programme has been working over a long enough period, for example a year, there may be grounds for adjustments in the light of local knowledge and experience (see Chapters 13 and 15).

Such adjustments may involve increasing the number of monitoring organisms, strategically selecting new sampling locations and reducing the number of samples of indicator organisms at locations and in media that show insignificant changes in time or differ insignificantly from observations at neighbouring sampling points.

In general, minimum monitoring should not be allowed to deteriorate into trite routine and mere accumulation of data. Only as long as data are purposefully applied and/or are still meaningful for the education process should the full minimum monitoring be continued without change.

Table 4 and the characteristics of the monitoring organisms as presented in section 7.3 above may assist in planning an extended monitoring programme for a given coastal region, but any new area must be judged on its own merits.

7.6 Pollution-source monitoring

In the extended programme, pollution-source sampling becomes more important, because pathogens may often be included in the monitoring organisms. In itself, sewage examination may thus partially reflect the epidemic and/or endemic situation in the surrounding community. Furthermore, the interpretation of pathogens detected in the receiving water may be meaningful only if the effluent is investigated to assess properly the actual release of these pathogens.

In the extended programme, flow measurement should be carried out more carefully than in the minimum programme. The flow should be determined, if not continuously over the day, then at suitable intervals, such as every two hours, to account for fluctuations. Moreover, the sampling may for various reasons need to be considered on a more strategic basis, for example, to describe the excretion of certain pathogens adequately.

8. Meteorological and hydrographical observations[1]

8.1 General

The present chapter will aim at a description of instrumentation and the methodologies applicable in undertaking the minimum metereological and hydrographical monitoring programme.

It is not possible to list and describe completely the equipment and methodology for extended monitoring because such a programme and instrumentation should be proposed only after careful consideration of local objectives and problems. However, the supplementary equipment necessary to perform extended monitoring will often require instruments and supplies in only slightly greater amounts than for minimum monitoring. To assist readers therefore, somewhat more than is really necessary for the minimum programme is listed and described hereunder. Only the final lists of equipment and supplies (sections 8.15, 9.11 and Annex II, section 3.1) indicate the absolute minimum required in order to commence minimum monitoring.

8.2 Wind and air temperature observations

General wind and local (multiple point of reference) winds must be recorded.

General wind — To assess and evaluate weather conditions properly before and during water quality monitoring, a continuous wind record, i.e. direction and velocity, in metres per second, must be obtained. A nearby weather station should be used, and existing records copied and identified by place, year, date and time.

Local winds — Any commercially available anemometer ± 0.5 m/s is applicable for use aboard the monitoring vessel when not under way. Form 4 should be used for entering data.

Air temperature — The air temperature should be measured on the monitoring vessel, together with wind readings and also entered on Form 4. Air temperatures should also be copied from the weather station together with wind data.

8.3 Precipitation observations

Where possible, precipitation readings should be obtained from an existing weather station, and records of rain periods and total rainfall (in mm) within each period should also be noted.

8.4 Tidal observations

Tidal variations may be important for local currents and transport of pollution. Information on tides may be obtained locally, for example from pilots in the area. Tidal tables are always available in print and should be purchased for any major coastal monitoring. An approximate indication of tidal variation is normally given in any nautical chart (see Fig. 1).

8.5 Observation of water level variations

The water level may fluctuate due to air pressure variations, winds, and tides. Locally, the water level variation is important for the flushing of the coastal receiving water. Water level variations should be registered, preferably by the administration of a nearby port, and copies obtained from the port log-book. Alternatively, an intermediate water level meter may be installed for the monitoring periods, and a separate log-book prepared for that purpose.

8.6 Surface drift observations and dye techniques

Surface water drift (often wind-driven) is an important observation because wastewater in high concentrations tends to float at the sea surface, at least near outfalls.

The drift might be studied by commercially available drift cards (as shown in Fig. 8). Observe drift card movements relative to a fixed position (buoy, anchored vessel, ...).

Surface water movements may be easily studied by use of such dyes as rhodamine (red) or fluorescine (green). Use the same observation technique as for drift cards and drogues. The obvervation period should normally be 15-20 minutes for a release of 0.5 litres of dye solution. Dye solutions must be prepared on land and kept on board in closed jars with quick-release valves for discharge through a hose that reaches down to the sea surface. Use Form 3 to record results.

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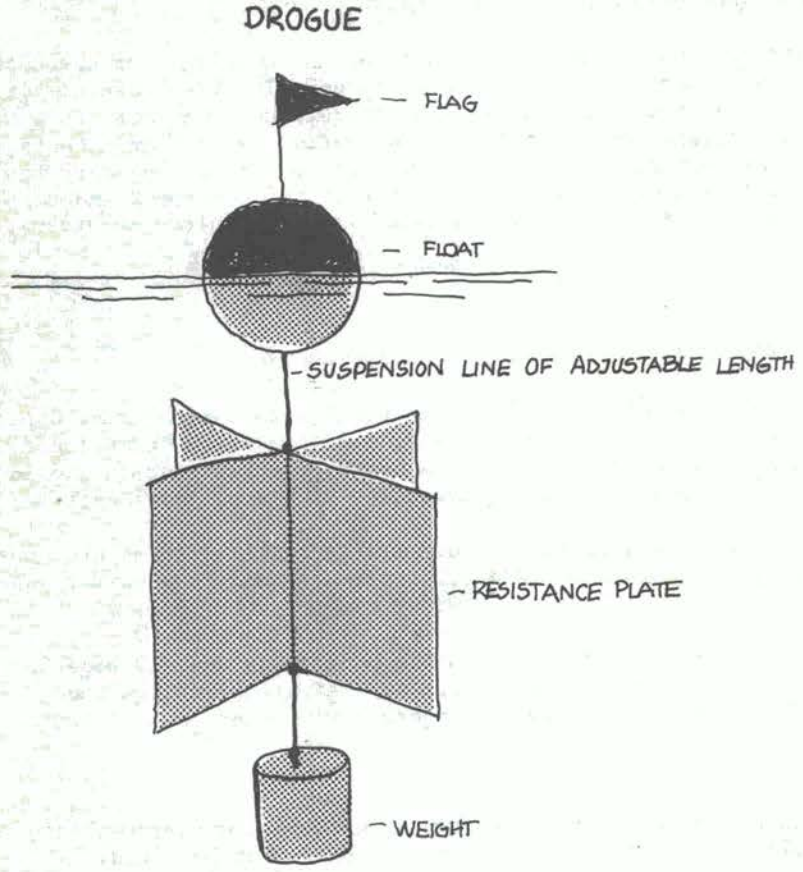
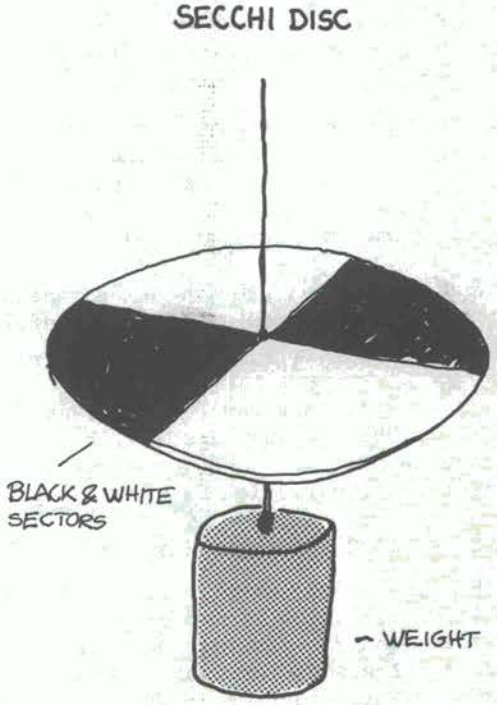
[1] The guidelines in this chapter are intended to provide the minimum indispensable data on hydrographic parameters when these are not available from responsible national services.

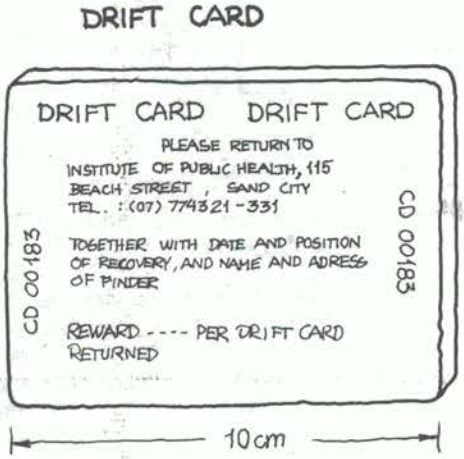
Fig. 8

SECCHI DISC, DROGUE, AND DRIFT CARD

These simple devices should be locally produced and thoroughly field-tested before use to find adequate weights, size of resistance plates, material for the float, etc.







3 mm POLYTHENE
SPEC. GRAV. 0.92

8.7 Observations of currents by drogue

A primitive, though not very accurate, observation of currents may be made by use of drogues (as shown in Fig. 8). The drogue can be locally produced with few materials and little experimentation to find the proper balance and degree of submersion of the floating body. Keep the unimmersed portion of the surface body as small as possible, so as to minimize wind effect.

The drogue movement is observed relative to a fixed position (buoy, anchored boat or the like). By regulating the distance between buoyancy body and resistance plates, currents at different depths can be observed. Enter results on Form 3.

8.8 Observation of currents by current meter

The current meter, preferably a direct reading meter (as shown in Fig. 9) gives the best and most useful current observations. (Required precision ± 3 cm/sec). Manufacturers' instructions for use of the current meter should be followed. Use Form 3 to enter results.

An anchored boat (normally fixed by two anchors) is necessary for accurate observations. In addition, the boat must be relatively large, so as to lie motionless in the sea. The monitoring vessel for multiple-point sampling cannot be used for this type of observation, because it will normally be too small, and because a good sampling speed is required to reduce storage time before laboratory examination of collected samples. As the current meter observations are time-consuming (including time for proper mooring of the boat), no attempt should be made to combine current meter measurements and sampling for microbiological analysis in the laboratory.

Always carry drogues (different depths for resistance plates, see Fig. 8) as substitutes for emergency situations in case of current meter failure.

8.9 Wave observations

Waves should be observed only visually, but at the reference point (Form 3) as well as at the multiple sampling points (Form 4).

8.10 Salinity observations

Salinity must be determined at the reference point (salinity profile and variations with time) and at the multiple sampling points. Two methods are recommended for swift and easy determination:

(1) Salinometer : any commercially available conductivity instrument that gives directly salinity ‰ (± 0.3 ‰) and temperature °C (± 0.1°) at the position of the transducer.

(2) Hydrometer : seawater hydrometers covering the specific density ranges 0.966 - 1.011 and 1.010 - 1.031 are the most suitable. Use the special hydrometer jar for measurements. Make corrections for temperature to obtain salinity by using standard tables such as those given in Standard Methods (10) pp. 100-106 (see also chapter 11). Hydrometers replace the salinometer in case of failure and during repair periods. Water for the hydrometer jar is taken from the water sampler (Figs. 9 and 10). The same sample is used for the microbiological sample bottle as indicated in chapter 9.

Salinity results should be entered on Forms 3 and 4.

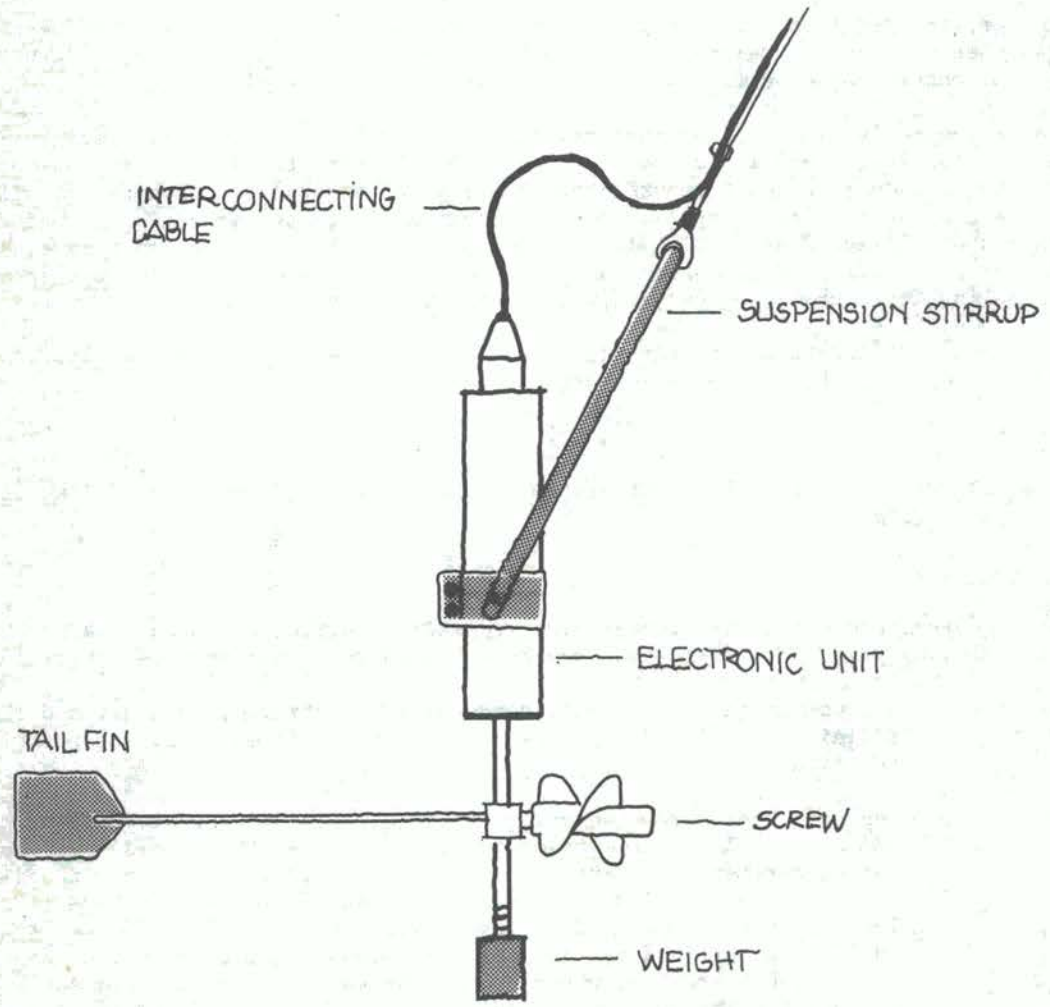
8.11 Temperature observations

Temperature must be determined at the same time as salinity at the reference point, as well as at each of the multiple sampling points.

By using the salinometer the temperature is obtained automatically. A water sampler can be used with the built-in thermometer to give the reading as soon as possible after the sample is brought on deck. The insulating water sample (Fig. 11) should be used when depths greater than 30 metres are sampled. When surface sampling alone is done the temperature scoop (Fig. 12) may be used.

Fig. 9

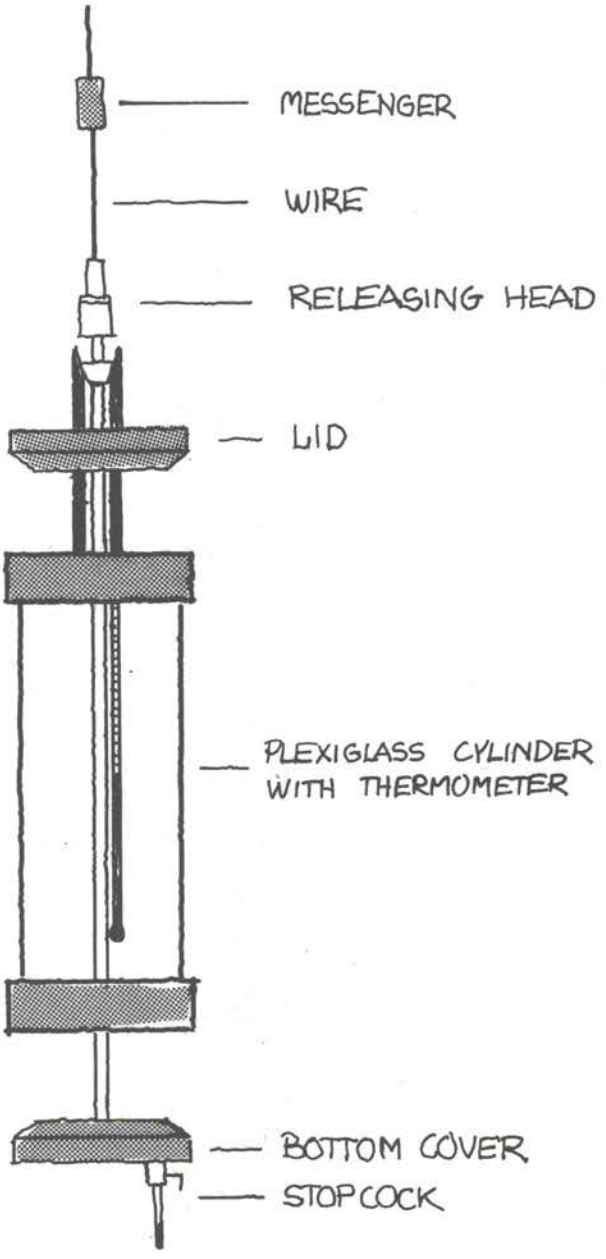
DIRECT READING CURRENT METER



Several brands of such instruments are commercially available. They allow direct reading on board the survey vessel of screw speed (rotations) and direction (tail fin) caused by currents passing the anchored vessel. For low speed currents, e.g., 2-6 cm/s, accuracy and precision may be low because wind and wave action on the survey vessel may cause significant disturbances.

Fig. 10

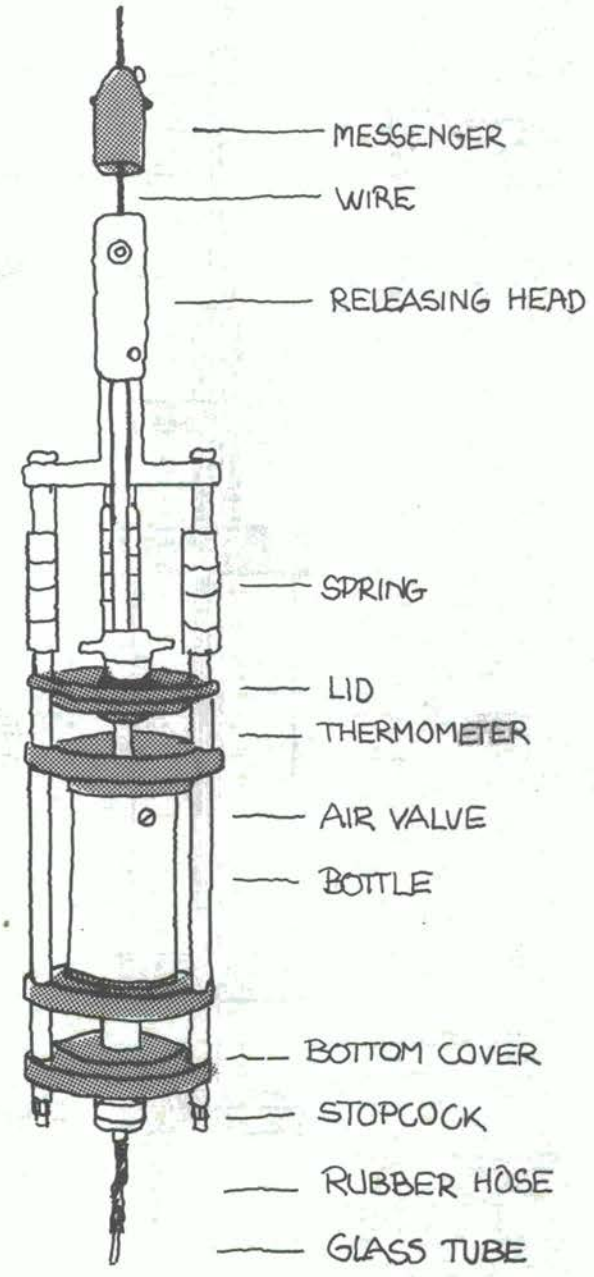
SUBSURFACE WATER SAMPLER



An example of a common, commercially available water sampler. This sampler should be used for subsurface sampling down to approximately 20 metres' depth.

Fig. 11

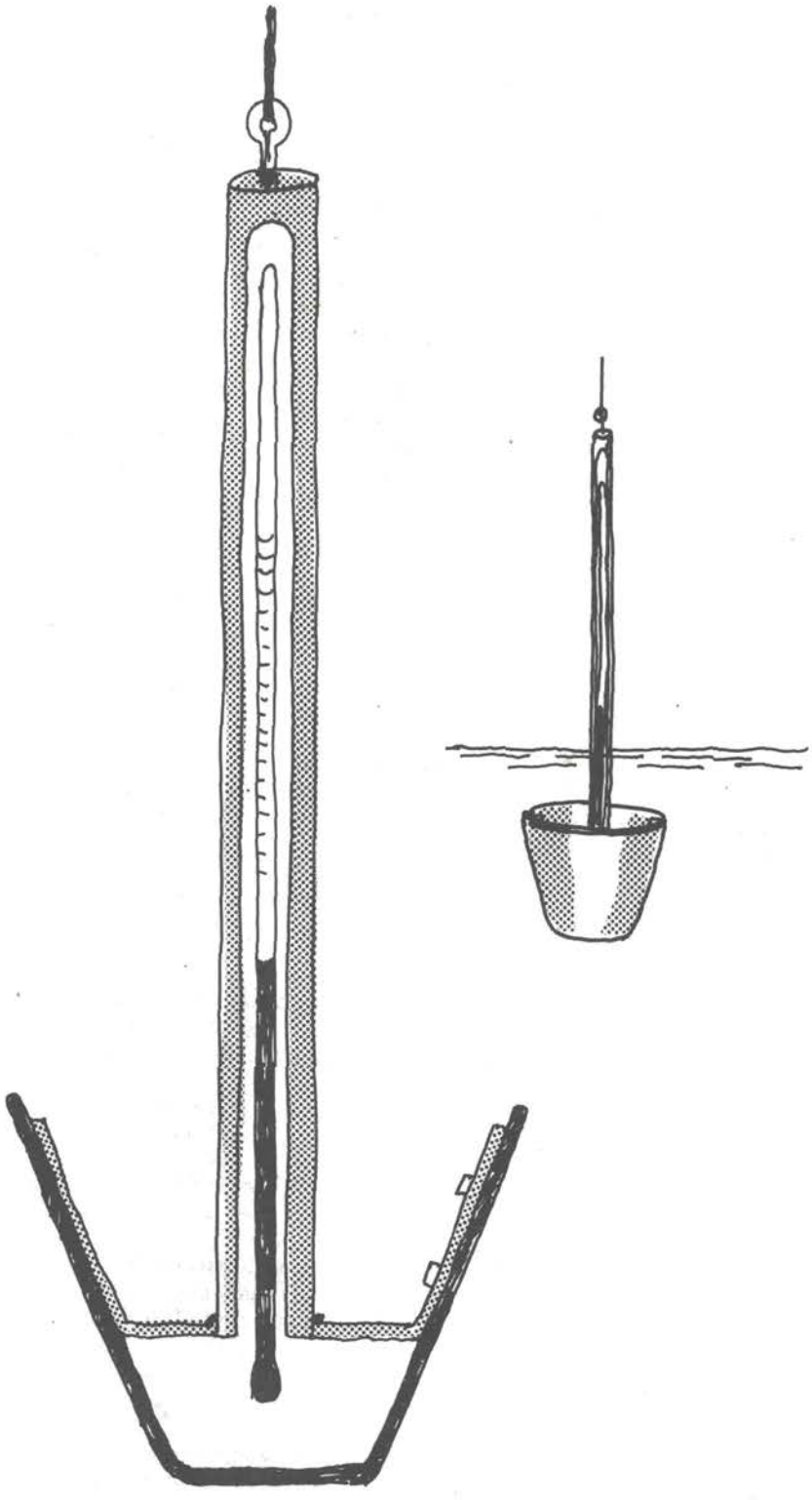
INSULATING SUBSURFACE WATER SAMPLER



For sampling below approximately 20 metres' depth, the insulating water sampler is recommended where temperature accuracy is important.

Fig. 12

SURFACE TEMPERATURE SCOOP



Where surface sampling takes place by direct bottle filling (Figs 13 and 14) the temperature scoop will be an appropriate simple device for measuring temperatures. (Reproduced by courtesy of Dr Castellvi, Spain)

8.12 Transparency

Use the Secchi disc (Fig. 8).

Always lower the disc at the side of the vessel exposed to the sun. The line must have marks at two-metre intervals. Lower rapidly until the disc disappears; read depth of disappearance, then hoist and read depth of reappearance. Enter results on Form 3.

8.13 Oxygen determination

Oxygen may be determined either by chemical methods or by using oxygen-metering apparatus.

Winkler method — The azide modification of the iodometric Winkler method is recommended (10) cf. chapter 11. Record data on Form 3. The sampler used for salinity and bacteriology will also give sufficient water for the 50 ml bottle used for oxygen. A syringe is used to inject 0.4 ml of manganese sulfate and 0.4 ml of alkali iodide azide reagent into the 50 ml bottle before closing it with a grounded stopper.

Oxygen-meter — Any commercially available oxygen-meter with proven reliability may be used. Regular calibration will be necessary, one possible approach being the Winkler method above. The oxygen-meter is convenient where depth profiles are required. Use Form 3 for data recording.

8.14 Identification of samples

All samples for activities such as oxygen determination must be labelled or tagged immediately after sampling and before storage in a dark place in cooling containers. Form 1 can be used, either as a self-adhesive label or attached as a tag. Whatever the circumstances, the responsible institution should have its name and address preprinted on the identification tag or label.

8.15 Navigation and positioning

All navigation, mooring, and signalling should be adequate and meet with local regulations. A nautical chart should always be used for navigational purposes, and the chart should be kept on board the monitoring vessel. Local pilots should be regularly consulted to obtain and update relevant knowledge on navigation in the area.

Hydrographic observations are carried out mainly at the reference point, whose position should be clearly identified by bearings based on adequate landmarks. Ideally, a sextant should be used to determine position from time to time.

If a permanent buoy or the like can be used as a reference point, positioning becomes very easy while carrying out hydrographic measurements. However, it should be remembered that buoys move around on their anchors, as they are fixed only at the sea bed.

Where a number of conspicuous buildings or other landmarks exist, it is preferable for the reference point position to be identified by simple visual cross-bearings.

A sextant should always be kept aboard the vessel for hydrographic observations, for use in confirming already established observation points, or for identifying new points in the area.

There should be short-distance radio equipment available for safety reasons and for communication between the multiple point sampling vessel and the hydrographic reference point observation vessel and between the monitoring vessels and land installations: pilots, harbour or home institute.

8.16 Equipment and supplies

The following list includes instruments and supplies necessary for meteorological and hydrograph observations as given above (sections 8.1 - 8.14, and Annex II). An asterisk (*) indicates that the item is essential for minimum monitoring to meet the criteria laid down by the joint UNEP/WHO consultation (1).

Amounts given in parentheses are totals required for use when there is both a reference point measuring vessel and a multiple point monitoring vessel.

Item	Quantity	Remarks
Anemometer*	1(2)	Two instruments (± 0.5 m/sec) are necessary for extended monitoring. One for the multiple point vessel and one for the reference point vessel.
Driftcards*	50	Any commercially available card, or a locally produced surface drifter whose drift characteristics must be studied (wind/driftcard/sewage drift correlations).
Drogues*	5(7)	The five drogues should be locally produced and tested. Adjust length of wire between float and resistance plates (see Fig. 8). Extended monitoring requires more drogues.
Current meter	1	Any direct reading commercially available instrument (± 2 cm/sec).
Dye		Any dye similar to rhodamine or fluorescine to observe surface water movements.
Salinity meter*	1(2)	Any commercially available conductivity instrument; specifications: approx. ± 0.3 ‰ salinity and ± 0.1°C temperature.
Hydrometers*	4(8)	Specific gravity ranges: 0.966 - 1.011 and 1.010 - 1.031 or equivalent. At least two for each range. For extended monitoring, double the number of instruments.
Thermometers*	3(6)	Spare thermometers for those built into samples and one for air temperature. Normally 0-60 or 100°C range ± 0.1°C for water, ± 1°C for air temperature.
Secchi disc*	2(3)	One for use, and one spare.
Winkler oxygen *	1	Manganese sulfate, e.g., 100 ml.
reagents and	1	Alkali - iodide - azide, e.g., 100 ml.
sample bottles	50	50 ml bottles, with ground glass stopper.
Oxygen-meter	1	Carefully select an instrument with proven reliability suitable for sea water.
Sextant	1(2)	
Tags and labels		For all sample containers
Depth water sampler	1(2)	Approximately one litre content
Insulating depth water sampler	1	Only where sampling depths exceed 30 metres.
Maps, nautical sampling	1(2)	Reference points should be indicated on the nautical map
points	1(2)	as well as on the multiple point map.
Portable radio transceivers	1(2)	For communication ship-to-shore (e.g., harbour or pilot station) and ship-to-ship.

9. Microbiological sampling

9.1 General

A human-health oriented coastal water quality monitoring programme necessarily involves a certain amount of microbiological sampling, since many health risks are directly related to the presence of pathogenic or toxigenic microorganisms.

Microbiological sampling, handling and examination require great care because there are many steps to be made before results (e.g., concentration of organisms) become available, and at each step there is a risk of spoiling the whole procedure, for example, through contamination or by non-controlled growth of microorganisms. Sterility and correct temperature (cooling) are of prime importance in performing microbiological investigations.

In the present guidelines it is assumed that sampling does not include such further sample preparation steps as membrane filtration in the field. Sampling proper is completed once the sample is transferred into the sterile container carried aboard the sampling vessel for that purpose. Even the minimum monitoring programme requires sampling to be conducted from a boat or ship, as beach sampling is both more time-consuming and more inefficient.

The microbiological examination procedures are treated in Chapter 10 and additional reference should be made to Annexes II and V.

9.2 Sterile sample containers

Sterile containers are primarily the responsibility of the microbiological examination laboratory, where all preparations take place, including provision of sterile equipment, sample labels, enumerated sample containers, insulated cooling boxes, and cooling elements to maintain a constant temperature of 4°C throughout sampling and transportation.

9.3 Sterile sampling and handling

The sampling crew should be well-trained in microbiological laboratory practice in order to appreciate the importance of sterile handling of sampling equipment and sample containers. Therefore it is of prime importance when aboard the sampling vessel that there is sufficient working space and a safely fixed position for any equipment (e.g., cooling boxes, sample bottles, water samplers, etc.) so that even in a rough sea everything remains in good order and working routines can be maintained. All storage should be in the shade. Normally a few experimental sea-trials will be necessary to ensure that boat procedures, equipment and crew, are capable of being used for regular monitoring cruises.

The sterile containers should never be touched by hand on surfaces that will be in contact with the sample. After filling a bottle with sample water, discard some 20-30 ml in order to obtain good sample homogenization when the bottle is shaken for testing in the examination laboratory. Place the bottle stopper carefully, without contaminating the sample or the lower part of the stopper.

If water samples are used, there must be facilities for on-board sterilization between two samplings. An inflammable sterilization liquid should be used, for hands as well as samples.

Hands may be made sterile where necessary by the use of cheap sterile gloves.

Normally the bottom samples (for sediments) should be rinsed only in clear sea water between two samplings.

9.4 Storage and transportation

The cooling of samples during on-board storage and subsequent transportation to the laboratory is largely ensured by correct use and handling of the equipment and cooling containers that have been prepared in the laboratory. However, the sampling crew should be careful to do everything possible to maintain low temperatures inside the cooling boxes, by securing shade where the boxes are placed, by keeping lids shut tight, etc. It is important to protect samples against ultraviolet radiation: they should always be kept inside a closed container. In addition, temperatures should be controlled at 3 - 4 hour intervals and any irregularity should be reported, for example, on Form 4.

Transportation from the sampling vessel to the laboratory should take place immediately upon arrival in the port. Normally, 24 hours should not be exceeded from the time sampling begins to the time samples are received in the laboratory. After 30 hours the sample must normally be considered useless for commencing microbiological examination.

9.5 Specifications for the sampling vessel

To ensure the shortest possible sample storage time, the sampling vessel must possess a good speed: for example, 8-10 knots (15-20 km/hour) would in most cases be satisfactory.

Consequently, factors that must be considered in choosing a vessel for microbiological sampling include the working space available, shade and fastening possibilities for cooling containers, speed, and seaworthiness. Further, to perform any sampling at depth, a davit and cable winch or equivalent, capable of being used for repeated lowering and hoisting must be available.

9.6 Water sampling

In general sampling must be commenced at the farthest point of the sampling area, where the pollution should be lowest. This strategy involves the shortest possible storage-time for samples before analysis and means that sampler sterilization can be held at a minimum. Standard Form 3 should by used for records of observations and as a monitoring log-book.

9.6.1 Surface water sampling

Surface sampling will be the commonest sampling procedure for most microbiological water sampling. Wide-mouth 200-300 ml bottles should be used for samples; they should be sterile and be filled directly, either by hand (Fig. 13) or by use of an extension arm (Fig. 14).

9.6.2 Subsurface water sampling

In most microbiological monitoring programmes for subsurface water sampling the simple type of sampler shown in Fig. 10 will suffice, and operations for emptying it and filling the sampling bottle should aim at non-contaminated transfer. Let the first 100 ml of the sampler be discarded before filling the sample bottle, and use normal 250-300 ml bottles.

Where necessary, for instance after a recent sample from a polluted water, the sampler should be thoroughly rinsed in the sterilization fluid which should be available on board. This fact also bears out the need to commence sampling at the farthest sampling point.

If a subsurface sample is taken where the bottom water is thought to be only slightly, or not at all, polluted, but where surface pollution is significant, a more advanced sampling device is required (Fig. 15) (9).

Only very seldom will there be any need to use an insulating subsurface sampler (Fig. 11), because depths will often be modest, and the temperature will not be that critical for a microbiological water quality assessment.

9.7 Sediment sampling

Sediment sampling can normally be carried out only where the bottom consists of relatively soft and homogeneous sands, silts, clays, or muds. A rocky bottom should not be sampled by the equipment described below, and a quantitative interpretation of any such results would be most difficult.

Standard Form 4 should be used for recording observations and as a monitoring log-book.

Core sampling is the only technique advised. The grab samplers commonly used for other biological sampling seem inappropriate as they immediately mix the sediment surface with deeper sediments. The microbiological precipitate normally concentrates in the sediment surface, which is consequently where sampling should take place.

The Albrechtsen sediment sampler (Fig. 16) has been used widely in Scandinavian waters and it is simple to produce as well as use. The van Donsel-Geldreich bottom sampler (Fig. 17) also seems adequate at least for areas where the sea bottom is soft.

In spite of the compatibility of data that can be achieved by using a single sampling device, it should be noted that due to lack of homogeneity in the microbiological density in the sediment, the comparability of results from different samplings will always be questionable. For this reason the use of devices of different types would jeopardize the comparability of results.

The Albrechtsen sediment sampler, where it can be employed, is consequently recommended for common use. Use 200-300 ml wide-mouth sterile plastic bottles for collecting samples.

Fig. 13

DIRECT SURFACE SAMPLING

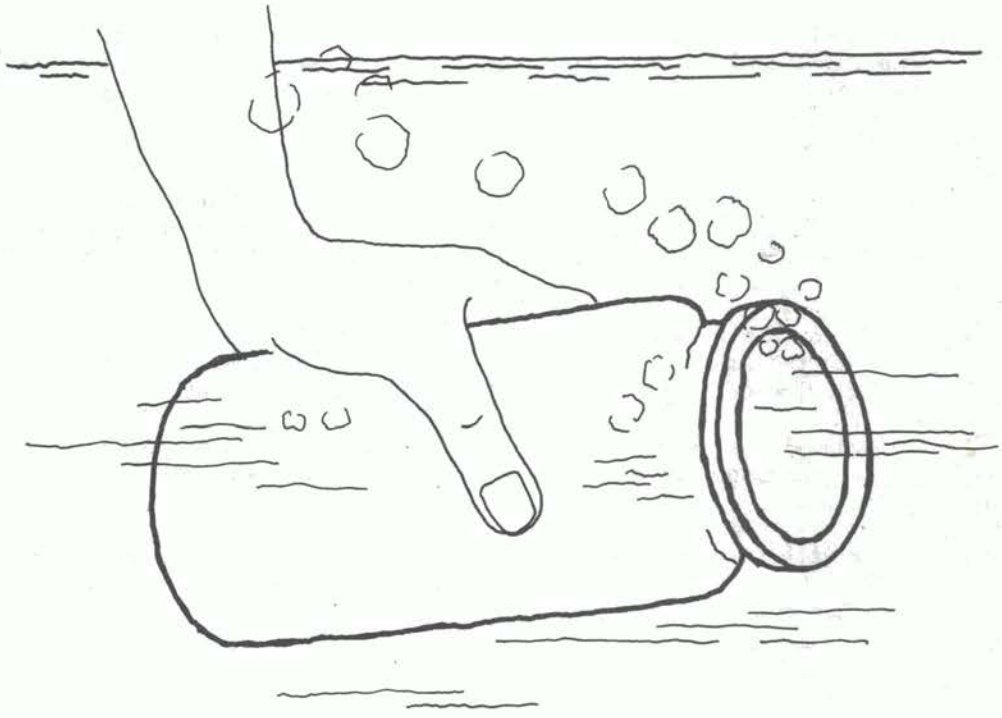
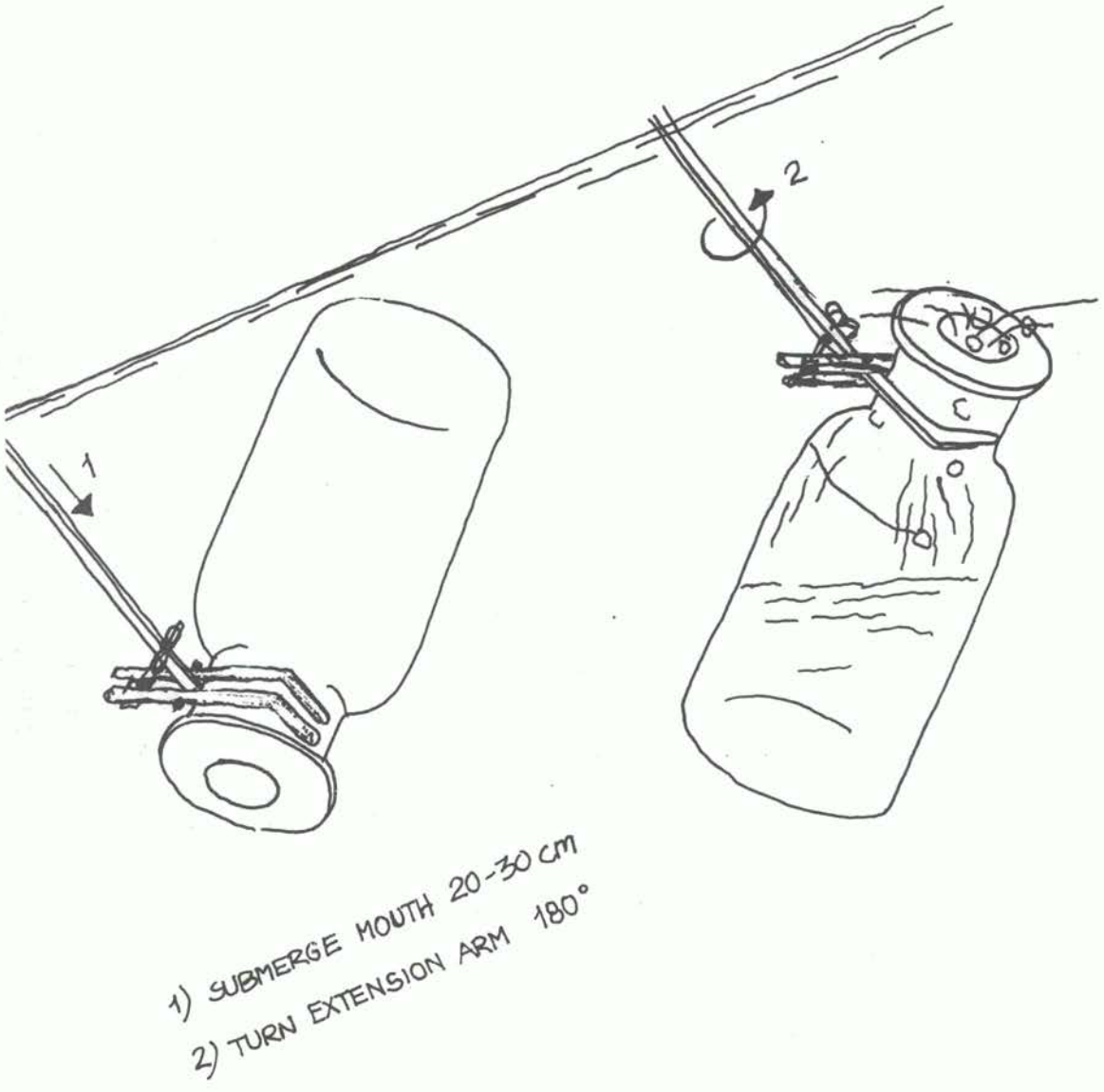


Fig. 14

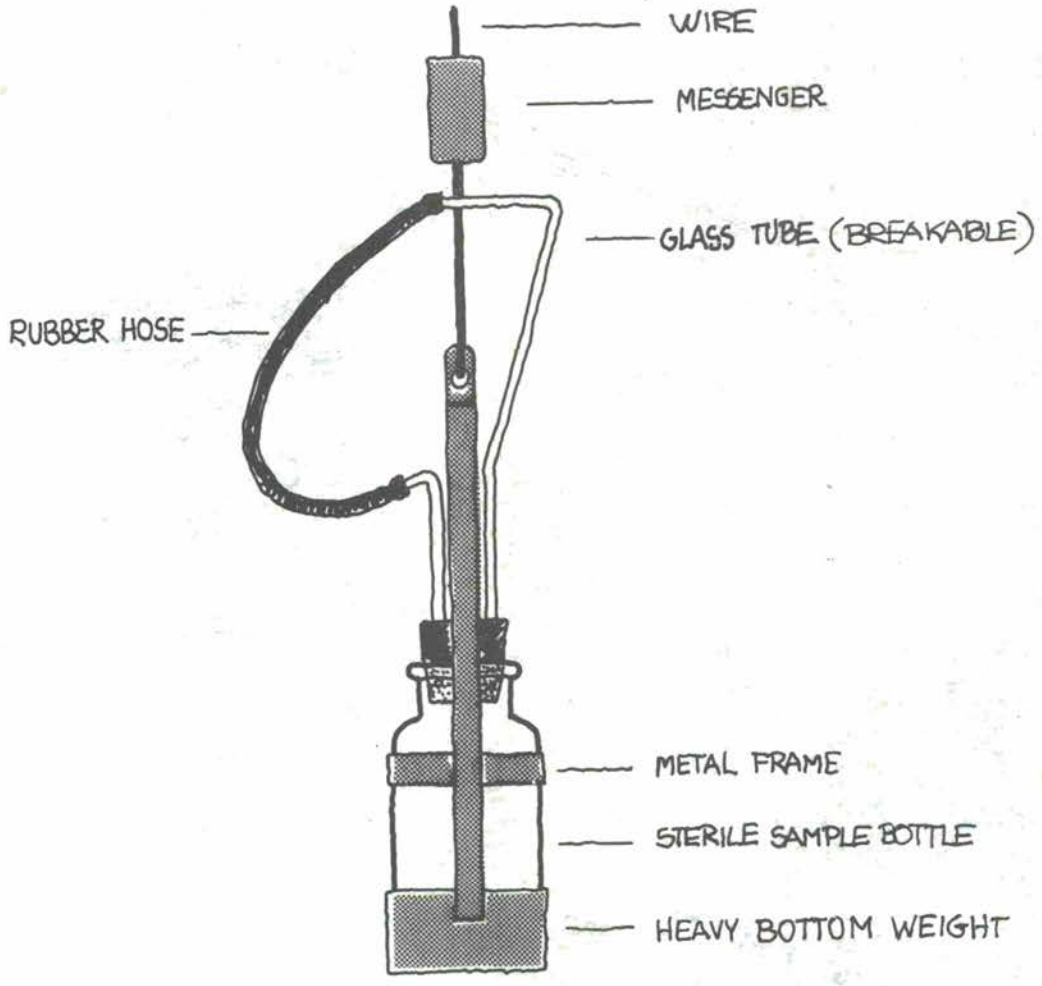
SURFACE SAMPLING EXTENSION ARM



The sterilized bottle is fixed as indicated on a rod and submerged 20-30 cm below the surface from the deck of the sampling vessel.

Fig. 15

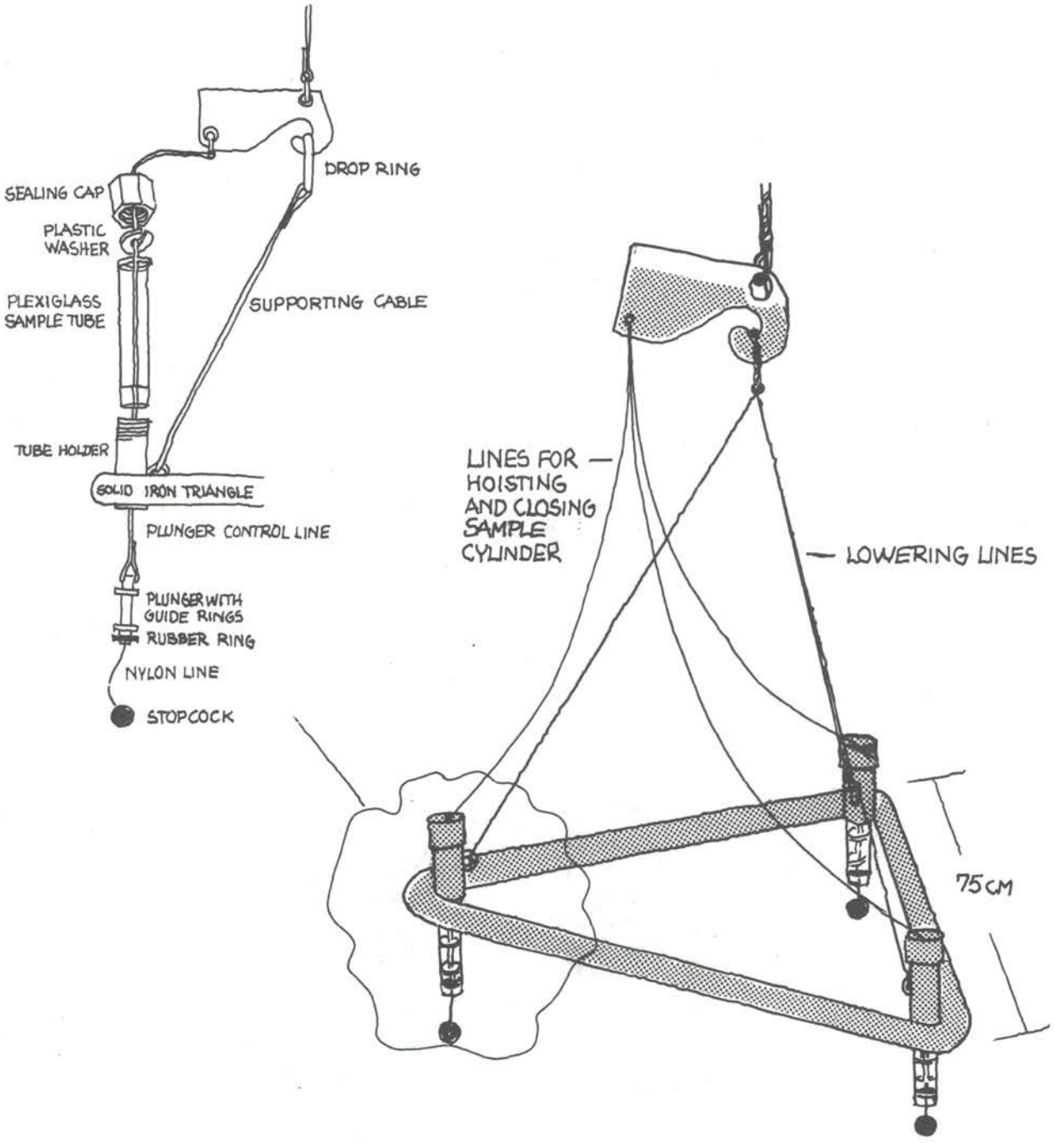
STERILE SUBSURFACE SAMPLING



To perform subsurface sampling where surface pollution is significant, the sterility of the sampling bottle may be secured by use of the devices indicated. After breaking the glass tube the differential pressure (top end of broken glass and suspended rubber hose) will automatically fill the bottle. The risk of contamination when hoisting the bottle is insignificant.

Fig. 16

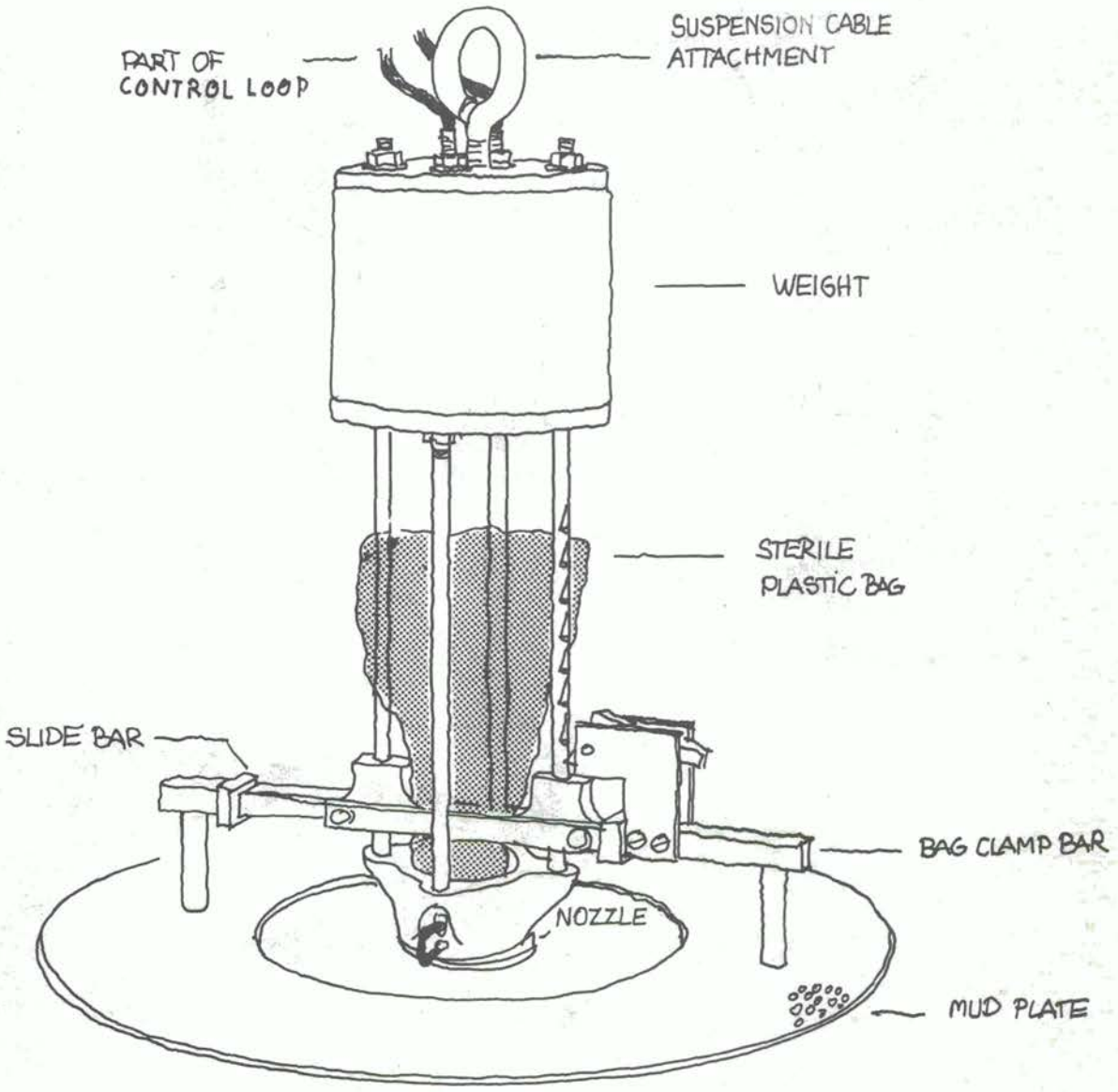
THREE-CORE ALBRECHTSEN BOTTOM SAMPLER



This device is widely used for bottom sampling in marine waters in Denmark. It can be copied, modified and manufactured locally without permission from the inventor. Only some 50-60 g of sediment are sampled in one operation, but the area coverage and the specificity as to layers are obvious benefits of its use. It is designed for use only on relatively soft or sandy bottoms.

Fig. 17

VAN DONSEL-GELDREICH BOTTOM SAMPLER



For description, see Geldreich, E.E. (22)

9.8 Shellfish and fish sampling

Shellfish and fish sampling should mainly be carried out by joining local commercial shellfish producers or fishermen on their boats and in their regular routines. Standard Form 4 should be used as a log-book.

The amount of sample (e.g., five oysters, five molluscs, or two fish) needed to examine for any given microorganism, such as salmonella, is put into a single separate and sterile plastic bag as soon as possible after the catch is hauled aboard the boat. Fish should be handled only by the tail to prevent casual contamination by the sampling crew. The bag is then sealed, tagged and immediately placed in the cool box.

9.9 Flow estimations and effluent sampling

Assessment of the pollution load in coastal waters requires adequate estimates of pollution concentrations and flow of effluents. Of great importance are variations in flow and concentration over periods of time. For rivers, the monthly variation is often of significance, whereas for sewers daily variations must be considered.

Determination of concentrations is normally based on grab sampling according to the specifications given in Chapters 6 and 7. Form 5 should be used as a log. Often the extension arm (Fig. 14) is helpful in sampling.

Flow estimation techniques may vary greatly depending on type of flow and effluent, and the degree of accuracy required may also be crucial.

River flow estimates may be made according to one of the following principles.

(1) Use of existing hydrological data established by other specialized groups.

(2) Establishment of representative cross-section flow-rate profiles in amounts that take adequate account of seasonal flow variations. Plotting speed data (m/s) against area (m^2) will determine the flow directly by simple graphical integration.

Ultimately, this approach implies only an average speed of flow (m/s) and multiplication by the cross-section of the river (m^2). A crude estimate of speed may be established by drogue or float drift time over a known distance.

(3) Tracer methodologies using dilution of known amounts (or flows) of an artificially added tracer. Local aims and circumstances are important in choosing a feasible and efficient strategy. A number of references are available for different tracer applications (33).

It should be recognized that river flow measurement is a comprehensive and resource-demanding exercise that should normally be carried out by people with training in hydrology and hydraulics.

Sewage flow estimates are made differently, depending on type of flow. For pressure lines, two principal methods are recommended.

1. Use of existing calibration curves (e.g., delivery test documents) for already installed pumps and thus utilizing the power consumption of the pumps involved to estimate flow.

2. Use of tracer methodologies (33), the particular choice depending on local aims and means.

For open channels or free flow surface sewers the method would be as follows.

1. Use of existing weirs or flumes; very often this procedure is a questionable one because of poor maintenance of the level meters involved or because of clogging and other disturbances, particularly where raw sewage is involved.

2. Estimates of speed of flow, for example, by a float whose travel time over a known distance (e.g., between two man-holes) is determined. Speed of flow may also be gauged by wet cross-section.

3. Use of tracer methodologies (33).

Generally the work Sewer Flow Measurement - a State of the Art Assessment (33) will be most helpful when planning flow studies, both for pipe and open channel flows.

9.10 Identification of samples

All samples must be identifiable and labelled or tagged immediately after sampling and before storage in cooling or other containers.

Form 1 is recommended for universal use in identification. It should be available both as a self-adhesive label and as a tag to be attached by use of string or rubber band. In any case, the name and address of the responsible sampling institution should be preprinted or prestamped on all identification tags prepared for each monitoring cruise.

9.11 Sample positions and navigation

Local rules of navigation must always be observed. A nautical chart for navigation purposes must always be kept on board the monitoring vessel. Contact and consult local pilots for relevant and updated information.

The number of sampling points may be rather large, and the distance between them relatively short, so that a nautical chart may not adequately identify the sampling points. A large-scale map may then be useful, on which each separate point can be easily distinguished, and often located according to landmarks or local characteristics (off-shore monitoring) that cannot be found on the nautical chart.

9.12 Equipment and supplies

For microbiological sampling there is little difference between equipment and supplies required to perform either the minimum or extended monitoring programmes.

A list of items that must always be stocked on the vessel for microbiological sampling (see also Annex II, section 3) is presented below. If a single vessel is used to perform both hydrographical observations and microbiological sampling, the subsurface water sampler is the same for both parts of the programme.

Item	Number	Remarks
Subsurface water sampler*	1	See Fig. 10
Sterile subsurface water sample*	1	See Fig. 15
Glass bottles 200-300 ml*	D	For subsurface water samples
Wide-mouthed glass bottles 200-300 ml*	D	For surface water sampling
Extension arm*	1	See Fig. 14 for surface water sampling
Sediment sampler*, Albrechtsen type	1	See Fig. 16. Always carry 10 extra tubes for replacement
Plastic bags*	D	For shellfish samples
Plastic bottles 200-300 ml*	D	For sediment samples
Plastic bottles 1-3 litres	D	For effluent sampling
Sterilization liquid	D	For water samples and hands
Cooling containers	D	For storage of samples
Sextant	1	See Chapter 8
Transceiver	1	See Chapter 8
Maps, nautical	1(2)	See Chapters 4, 6 and 7
sampling points	1(2)	
Tags and labels		For all sample bottles and plastic bags

D: the amount must be determined in each case based on local goals and constraints.

10. Microbiological examination procedures

10.1 General

The following information on microbiological examination is simplified to assist the user. As far as possible the procedures described (see Table 5) should be viewed in relation to Annexes I-IV, which provide guidance by means of figures and forms for recording data. The different annexes should be consulted before conducting examinations. Annex I describes general rules for microbiological examination, Annex II provides a minimum inventory of equipment and supplies for laboratory and field use, Annex III lists in alphabetical order the various methods of preparing cultivation substrates and test reagents, Annex IV contains standard forms for recording data, and Annex V deals with microbiology laboratory quality control.

The advanced user may need to refer to more comprehensive manuals for microbiological examination, or use work provided in the list of references at the end of the main text. According to (1), where applicable the membrane filtration method should be preferred to the multiple test tube method. In similar fashion, in the present chapter the first of the different alternative procedures given is normally the method of choice.

There are a number of possible pitfalls in bacteriological examinations, for example, due to non-sterile handling of samples in the field or in the laboratory. Incorrect or unstable temperatures during incubation may seriously affect the results of an examination. Therefore regular use should be made of Annex V (General requirements and quality control for microbiological laboratories) to secure good performance and valid results. Table 5 below lists organisms, sampling media and methods described in this chapter.

Table 5

METHODS FOR INDICATED COMBINATIONS OF MICROORGANISMS AND SAMPLING MEDIA

	Fish	Shellfish	Sediment	Water	Sewage
1. Total heterotrophic bacteria		PP			
2. Total coliforms		MPN,SP		MF,MPN	MF,MPN
3. Faecal coliforms		MPN,SP	PP	MF,MPN	MF,MPN
4. Faecal streptococci		PP		MF	PP
5. *Clostridium perfringens*			ST		ST
6. Salmonellas	1	1	n	n	n
7. *Salmonella typhii*		1			
8. *Vibrio cholerae* & NAG		1			
9. *Vibrio parahaemolytians*		n	n		n
10. *Shigella*					1
11. *Yersinia*					1(n)
12. Parasites		n	n		n
13. Enteric viruses		1		1	1
14. Biotoxin, paralytic shellfish poison		n			

Notes: MF = Membrane filtration (see Annex I.1)
PP = Pour plate (see Annex I.2)
SP = Spread plate (see Annex I.3)
ST = Standing tube (see Annex I.4)
MPN = Multiple test tube (maximum probable no., see Annex I.5)
1 = Qualitative determination of colony presence
n = Quantitative determination of colony density

For 1 and n, applying to pathogens only, the examination procedures are more complicated and often less precise than is the case for the indicator organisms.

10.2 Total heterotrophic bacteria

10.2.1 Definition

Heterotrophic bacteria are all those which will grow on plate count agar at 20°C within five days (five days are chosen in order to include also halophilic bacteria that grow slowly on the non-salt enriched agar).

10.2.2 Procedure

Molluscs, pour plate method. The pour plate method is used (see Annex I, Figs 3 and 4) for the general procedure. The mollusc (oyster) sample consists of a batch of five specimens. Shells should be cleaned cautiously using brush and alcohol and finally be held briefly over a flame using sterile forceps.

Still using sterile forceps, the tissue is transferred to the sterilized laboratory blender vessel. Tissue alone should be examined and transfer of water from within the shells should be avoided. Weigh the vessel before and after transfer of mollusc flesh and add phosphate buffer to obtain a dilution 1 : 10, one unit weight of mollusc to nine units weight of buffer. Homogenize for 1-2 minutes.

The dilution series is now prepared by transferring 1 ml to each test tube as indicated in Annex Figs 3 and 4. Test tubes D1, D2, D3 and D4 will correspond to sample test amounts of respectively 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}g.

From each of the four dilutions, 1 ml is transferred by sterile measuring pipette to four sterile 9-cm Petri dishes. Liquid plate count agar, 12-14 ml, cooled to 40°C, is added to each Petri dish, which is moved to and fro in a circular movement to obtain good mixing and distribution before solidification. Incubation takes place for five days at 20±0.2°C (either water or air incubation). Total heterotrophic count is determined from a plate with a 20-80 colony range according to the formula:

$$\text{Total heterotrophic bacteria/g} = \frac{\text{No. of colonies}}{\text{g sample test amount (STA)}}$$

Readings and sample data are entered on a standard form such as Form 6 (Annex IV).

10.2.3 Equipment and supplies

(1) Apparatus. Bottles, flasks, test tubes, Petri dishes, measuring pipettes
Laboratory blender and accessories
Incubator: 20±0.5°C
Automatic shaker
Bacterial colony counter, autoclave

(2) Substrates and reagents. Phosphate buffer
Plate count agar

10.3 Total coliform bacteria

10.3.1 Definition

Coliform bacteria are aerobic and facultatively anaerobic, Gram-negative, non-sporeforming rods that ferment lactose while producing acid and gas at 36°C.

10.3.2 Procedures

(1) Water sample, membrane filtration method. The membrane filtration technique is described generally in Annex I, Figs 1 and 2. For each sample (approximately 500 ml), the sample test amount should be prepared for filtration according to the degree of pollution of the sample medium. Phosphate buffer is used as diluent.

Sample medium	Recommended dilution series (Annex I, Fig. 1)			
Raw sewage	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Secondary effluent	1	10^{-1}	10^{-2}	10^{-3}
Polluted seawater	10	1	10^{-1}	
Clear seawater	100	10	1	

For sample test amounts less than or equal to 1 ml, it should be emphasized that the pour plate method would seem to be more useful than the membrane filtration method. After filtration the membrane filters are transferred by sterile forceps onto solid M-endo agar in 9-cm Petri dishes. Incubate 24 hours at 36±1°C. Both air and water incubators are acceptable. After incubation, count the coliform colonies on a filter with a 20-80 colony range. The coliform colonies will appear as pink to dark red spots with a metallic sheen, which may vary in size from pinhead to complete colony coverage. The number of colonies on the filter gives the coliform density of the sample:

$$\text{Coliforms/100 ml} = \frac{\text{No. of colonies} \times 100}{\text{ml Sample test amount (STA)}}$$

Readings and sample data are transferred onto a standard form such as Form 6 (Annex IV).

Many non-coliform colonies will not grow on the M-endo agar. Non-coliforms will appear as colourless or light red colonies and should be excluded from the counting. Suspect colonies can be tested separately using the following two procedures:

(a) Cytochrome oxydase test. Streak suspect colonies onto a filter paper moistened with cytochrome oxydase reagent. Coliform colonies will (within 10 sec) yield no reaction; colonies of other bacteria such as Aeromonas and Pseudomonas will turn dark blue within 10 seconds.

(b) MacConkey broth test. The suspect colony is transferred into a tube containing MacConkey broth and incubated at 36±1°C for 24 hours. Coliforms will develop gas (trapped in the Durham tube) after 24 or 48 hours and acid (broth colour turns yellow).

Negative results from a cytochrome oxydase test and positive results from a MacConkey broth test will confirm the presence of a coliform colony.

(2) Multiple test tube method. The multiple test tube (most probable number, MPN) technique is described in Annex I, section 5. For each sample (approximately 500 ml) a dilution series should be prepared according to the degree of pollution of the water. Phosphate buffer is used as diluent.

Sample medium	Proposed dilution series (Annex I, Fig. 10)				
Raw sewage	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Secondary effluent	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Polluted seawater	10	1	10^{-1}	10^{-2}	10^{-3}
Clear seawater	10	1	10^{-1}		

The first test tube series (Annex I, Figs 9 and 10) is prepared by sterile pipette transfer of 1 ml from each tube in the dilution series to the quintuple test tubes each already holding 10 ml of single strength sterile MacConkey broth. For sample test amounts of 1 ml, the test tubes should hold 10 ml of sterile double strength MacConkey broth. The test tubes should be positioned in a suitable test tube rack (Annex I, Fig. 10) and placed in an air or water incubator at 36±1°C for 18-24 hours before the first reading of gas and acid formation. Gas is observed in the inverted vial (Durham tube). Slight tapping on the test tube wall by a fingernail may release gas bubbles and speed up the readings. Acid production is indicated by a yellow colour in the broth. To provide positive readings, the test tubes must register both gas and acid production, and the results should be registered on a standard form such as Form 7. For tubes where there is neither gas nor acid formation after 24 hours, incubation is continued for another 24 hours (total incubation time 48±3 hours) to double-check for acid and gas formation. Results are also recorded on Form 7.

The total coliform density in the water sample is now estimated, using the maximum probable no. index from Annex I, Table 1. Results are recorded on Form 7 together with relevant sampling data.

(3) Molluscs, multiple test tube method. The multiple test tube technique, to obtain the most probable number, is shown graphically in Annex I, Figs 9 and 10, and should be performed as follows.

Five molluscs must be used for each sample (random choice among several molluscs from the same sample station). Before opening the shells, they should be carefully cleaned using brush and alcohol. Finally the shells are held in sterile forceps over a flame to dry for a short while. Sterile tools are also used to remove tissue from the shells and to transfer it into a sterile and preweighed laboratory blender vessel. Transfer of water from the shells must be avoided. Add phosphate buffer to obtain 10 : 1 dilution (9 units of buffer to 1 unit of animal water). Approximately 6 g flesh is sufficient. Homogenize for 1-2 minutes.

The contents of the blender vessel, which holds 60 ml, now consist of dilution D1. One ml is transferred from D1 to make up a new dilution, D2, to which is added 9 ml of phosphate buffer. Incubation tubes (quintuple test tube series 1) are now prepared:

Sample test amount = 1 g by transfer of 5 × 10 ml from D1 to one test quintuple, each single tube containing 10 ml of sterilized, double strength MacConkey broth.

Sample test amount = 0.1 g by transfer of 5 × 1 ml from D1 to another test quintuple, each single tube containing 10 ml of sterilized single strength MacConkey broth.

Sample test amount = 0.01 g by transfer of 5 × 1 ml from D2 to a third test quintuple, each single tube containing 10 ml of sterilized single strength MacConkey broth.

Incubation is carried out at 36±1°C in either a water or air incubator, as described in the above-mentioned multiple test tube methods, for obtaining a total coliform count in water samples. The resultant total number of coliforms/100 g molluscs is obtained by using the maximum probable no. index given in Annex I, section 5, Tables 1 and 2, as 100 g corresponds to the 100 ml used in the index. Results should be entered on a standard form such as Form 7 (or 8 if both total and faecal coliforms are being determined).

(4) Molluscs, spread plate method. The spread plate method is described in Annex I, section 3. Five molluscs are used as one batch sample. A dilution series (D1 and D2) is prepared in exactly the same way as in the multiple test tube method described above. In addition, a dilution D3 is prepared by pipetting 1 ml from D2 into D3, as shown in the figures in Annex I. The spread plate series for incubation is now prepared in the following way:

Sample test amount = 10^{-2}g by sterile pipette transfer of 0.1 ml from the laboratory blender dilution (D1), to a 9-cm Petri dish containing solidified M-endo agar. The inoculate is evenly distributed by use of a sterile inoculation spatula (Drigalski).

Sample test amount = 10^{-3}g by sterile pipette transfer of 0.1 ml from dilution D2 to a second Petri dish.

Sample test amount = 10^{-1}g by sterile pipette transfer of 0.1 ml from dilution D3 to a third Petri dish. Incubation of the three Petri dishes is done at 36±1°C for 24 hours in either a water or air incubator.

Total coliform colonies will appear as pink to dark red spots with a metallic sheen. Make a plate count in the 20-80 colony range. The total coliform density of the sample is now determined as follows.

$$\text{Total coliform/100 g} = \frac{\text{Number of colonies} \times 100}{\text{Sample test amount}}$$

Results should be recorded on a standard form such as Form 7 (or 8 if faecal coliforms are being determined too).

10.3.3 Equipment and supplies

(see below, section 10.4.3)

10.4 Faecal coliforms (heat tolerant)

10.4.1 Definition

Faecal coliforms are Gram-negative, aerobic and facultatively anaerobic, non-sporeforming rods capable of fermenting lactose, producing acid and gas at both 36°C and 44°C in less than 24 hours. They produce indole in peptone water containing tryptophan at 44°C.

10.4.2 Procedures

(1) Water sample, membrane filtration method. The membrane filtration method is described in Annex I, section 1. To obtain adequate sample test amounts, the following dilution series are recommended, with phosphate buffer to be used as dilution liquid.

Sample medium	Recommended dilution series				
Raw sewage	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Secondary effluent	1	10^{-1}	10^{-2}	10^{-3}	
Polluted seawater	10	1	10^{-1}		
Clear seawater	100	10	1		

For sample test amounts less than or equal to 1 ml, the pour plate method may often be more appropriate than membrane filtration. After filtration the membrane filters are transferred by sterile forceps onto solid M-FC agar in Petri dishes. Incubation is carried out by immersing the Petri dishes, sealed with lids and inverted, inside plastic bags in a water bath at 44±0.2°C for 24 hours. After incubation, the faecal coliform count is made on a filter with a 20-80 colony range. Faecal coliforms appear as blue colonies. Occasionally colonies with grey to cream colouring may be observed on M-FC agar. Only blue colonies should be included in the count and the faecal coliform density should be determined according to the following formula:

$$\text{Faecal coliforms/100 ml} = \frac{\text{Total no. of blue colonies} \times 100}{\text{Sample test amount in ml}}$$

Enter results on a standard form such as Form 6. Where there is some doubt about the nature of given colonies, one optional verification procedure consists in streaking off the colony from the filter and transferring it into a MacConkey broth for incubation at 44±.2°C in 24 hours, followed by indole testing of positive colonies incubated in peptone water (see procedure for faecal coliform confirmation for water samples below by the multiple test tube method).

(2) Water sample, multiple test tube method. The principles of the multiple test tube method are described in Annex I, section 5 and shown graphically in Figs 9 and 10 and Tables 1 and 2 of the same Annex. For each sample, a dilution series should be prepared according to the degree of pollution of the water, with phosphate buffer being used for dilution.

Sample medium	Recommended dilution series (Annex I, Fig. 5)				
Raw sewage	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Secondary effluent	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Polluted seawater	10	1	10^{-1}	10^{-2}	10^{-3}
Clear seawater	10	1	10^{-1}		

Test tubes are arranged in quintuple series before inoculation and incubation. Approximately 10 ml single strength MacConkey broth is added to each test tube before any 1 ml inoculation. For 10 ml inoculations, 10 ml double strength MacConkey broth should be used. A Durham tube should be placed in each test tube.

The first quintuple series is now incubated in a water bath at 36±1°C for 18-24 hours before the first reading of acid and gas formation. Gas is observed in the Durham tube. Slight tapping on the tube wall may help release gas bubbles that are produced. Acid is indicated by the broth colour turning yellow. To yield a positive reading, tubes must display both gas and acid production. Positive readings should be entered on a standard form such as Form 8. For tubes not showing gas

formation after 24 hours, the reading must be repeated after another 24 hour period (the total incubation time at 36°C is then 48±3 hours), and checked again for acid and gas production. These results should also be entered on Form 8. All positive readings from test tubes at the 36°C incubation must be confirmed in new tubes incubated at 44°C, to demonstrate whether faecal coliforms are present. Thus, from each test tube yielding a positive reading from the 36°C incubation, one drop is transferred by Pasteur pipette (not wire loop) into a test tube in the same position in the second quintuple test series, where each tube contains approximately 10 ml single strength MacConkey broth, and is incubated at 44±.2°C for 24 hours. Faecal coliforms will generate acid (yellow broth) and gas (Durham tube). Test tubes giving a negative reaction in the first (36°C) incubation should be omitted; the empty space in the test tube rack is considered a negative reaction in the 44°C incubation too. Results should be entered on Form 8.

A third quintuple test tube series is prepared at the same time as the second series by sterile transfer, using the Pasteur pipette, of another single drop from the first series (36°C incubation) to the identically positioned tube in the third quintuple test series, where each of the small tubes contains approximately 5 ml peptone water. Incubation is done at 44±.2°C for 24 hours. Positive indication of faecal coliforms is shown by red surface colouring after adding Kovac's reagent and shaking the tube. A tube in the first incubation series (36°C) which gives a negative reaction should automatically leave an identical empty position in the rack containing the third incubation series; the empty position is read as a negative reaction. Results should be entered on Form 8.

Criteria for positive identification of faecal coliforms are now:

(a) generation of both acid and gas after 48 hours at 36°C;

(b) generation of both acid and gas after 24 hours at 44°C;

(c) positive indole reaction after 24 hours at 44°C, i.e., red colour in reaction to addition of Kovac's reagent.

To obtain the faecal coliform density of the sample using the multiple test tube method, count the tube positions that positively match all three criteria and enter results directly on Form 8, where the readings obtained by using the most probable number index accompanying the multiple test tube method (Annex I, Tables 1 and 2) may also be recorded.

It should be noted that, in using the above procedure for faecal coliform determination, the total coliform density is obtained automatically by using the positive reactions yielded from the first incubation series (generation of both acid and gas after 48 hours at 36°C), and by taking results from the most probable number index given in Annex I, Tables 1 and 2. If the multiple test tube method is used, Form 8, when completed, will consequently always show total coliforms as well as faecal coliforms.

(3) Sediment, pour plate method. The pour plate method is described in Annex I, Figs 3 and 4. For each sediment sample (minimum 50 g) a dilution series should be prepared according to the expected degree of pollution. Phosphate buffer is used for dilution.

Sediment pollution	Proposed dilution series (Fig. 3)
Slight	1 10^{-1} 10^{-2} 10^{-3}
Heavy	10^{-1} 10^{-2} 10^{-3} 10^{-4}

Vigorous shaking of the sample for approximately 30 minutes is required before preparing the dilutions The inoculation is done by sterile pipette transfer of 1 ml from each of the prepared dilutions onto sterile Petri dishes. Then pour 12-14 ml of liquefied M-FC agar onto each dish.

Incubation of inverted Petri dishes is done at 44±.2°C for 24 hours, preferably in a water bath incubator.

A count is best made on a plate with a 20-80 colony range. Count only blue colonies. The faecal coliform density is determined as follows:

$$\text{Faecal coliforms/g} = \frac{\text{Number of blue colonies}}{\text{g sample test amount}}$$

Results should be entered on a standard form such as Form 6.

(4) Molluscs, spread plate method. The spread plate method is described generally in Annex I, section 3. Five molluscs are used to prepare a sample. Preparation of dilutions and sample test amounts is described in full in the section dealing with the spread plate method for producing total coliform counts in molluscs (section 10.3 above).

The 0.1 ml inoculate is transferred onto Petri dishes containing solidified M-FC agar, which are inverted and incubated for 24 hours in a water bath at 44±0.2°C.

Faecal coliform colonies will appear as blue colonies and may be counted on a plate in the 20-80 colony range. The faecal coliform density of the sample may be determined as follows:

$$\text{Faecal coliforms/100 g} = \frac{\text{Number of blue colonies} \times 100}{\text{g sample test amount}}$$

Results should be entered on a standard form such as Form 6.

Where low faecal coliform concentrations are expected it may be advantageous to use the pour plate method, which will allow a 10-fold increase in sensitivity over the spread plate technique (transfer of 1 ml instead of 0.1 ml).

(5) Molluscs, multiple test tube method. The multiple test tube or most probable number method is described in Annex I, section 5, which indicates three test tube series and coherent incubations (Annex I, Figs 9 and 10). As always, the first step in this procedure is identical for total and for faecal coliform counts (see section 10.3 above on investigation of total coliforms in molluscs by this method). Results from this initial incubation should be entered on Form 8.

The second test tube series is prepared by sterile pipette transfer of one drop from each test tube that provides a positive reading during the first 24 hours' incubation at 36°C to test tubes in identical positions on the tube rack used for the second incubation. Each of these tubes contains approximately 10 ml single strength MacConkey broth. Incubation is now carried out for 24 hours at 44±0.2°C. Durham tubes are used inside the test tubes to detect gas production. Empty rack positions indicating negative yields at the 36°C incubation are also considered as being negative for the purposes of the second incubation. Positive yields after incubation are indicated by tubes displaying generation of acid (yellow broth) and gas (Durham tube). Results should be entered on Form 8.

The third incubation series is prepared at the same time as the second, by sterile pipette transfer of one drop from the contents of each test tube providing positive yields after incubation at 36°C, to small test tubes in identical positions on the third test tube rack. Each tube contains approximately 5 ml of peptone water. Incubation is carried out at 44±0.2°C for 24 hours. Positive yields are indicated by those tubes which, after incubation, addition of approximately 1 ml of Kovac's reagent, and shaking, display a red surface colour. Results should be entered on Form 8.

All intermediate results have been recorded on Form 8 and it only remains to obtain the maximum probable number of faecal coliform in the sample by reading off the number of positive yields (both second and third incubation, cf. Form 8) from the index of most probable numbers given in Annex I, section 5, Tables 1 and 2. The resulting number is also recorded on Form 8.

10.4.3 Equipment and supplies

(1) Apparatus. Flasks, test tubes, vials, spatulas
Measuring pipettes, Pasteur pipettes, wire loops, spatulas, plastic bags
Laboratory blender and accessories
Filter pump (vacuum), membrane filters (0.45µ) and membrane filter apparatus
Incubator (36±0.5°C, 44±0.2°C)
Automatic shaker, bacterial colony counter, autoclave

(2) Substrates and reagents. Phosphate buffer
MacConkey broth (single strength)
MacConkey broth (double strength)
M-FC agar
Peptone water
Kovac's indole reagent

10.5 Faecal streptococci

10.5.1 Definition

All Gram-positive, oblong bacteria capable of multiplying as visible dark red to pink colonies within 48±3 hours at 44±0.2°C in the prescribed KF-streptococcus agar are considered to be faecal streptococci.

10.5.2 Procedures

(1) Water sample, membrane filtration method. The membrane filtration method is described generally in Annex I, section 1. For each sample (approximately 500 ml), the sample test amount for filtration should be selected according to the degree of pollution of the sample, in accordance with the following table:

Sample medium	Recommended dilution series			
Raw sewage	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Secondary effluent	1	10^{-1}	10^{-2}	10^{-3}
Polluted seawater	10	1	10^{-1}	
Clear seawater	100	10	1	

Phosphate buffer is used as diluent and rinsing agent. Note that for sample test amounts less than or equal to 1 ml, the pour plate method will often be preferable to membrane filtration. After filtration, the membrane filters are transferred by sterile forceps onto solidified KF-streptococcus agar in 9-cm Petri dishes. Note that the KF-agar needs special preparation (add triphenyl tetazolium chloride and adjust pH, see Annex III).

Incubate the inverted Petri dishes at 44±.2°C for 48±3 hours in either air or water incubator. Red colonies or colonies with a red centre are considered faecal streptococci. Take a plate count for a plate with a 20-80 colony range.

The faecal streptococci density of the sample is determined according to the formula:

$$\text{Faecal streptococci/100 ml} = \frac{\text{Number of red or red centred colonies} \times 1000}{\text{ml sample test amount}}$$

Results should be entered on a standard form such as Form 6.

(2) Water sample, pour plate method. The pour plate method is described generally in Annex I, section 2. It may be advantageous to use the pour plate method instead of the membrane filtration method, if the water is very turbid or contains suspended material such as raw sewage. In general, the pour plate method should be preferred to membrane filtration for sample test amounts less than or equal to 1 ml.

Recommended dilutions appear from the table shown for membrane filtration (see (1) above). The sample should be shaken efficiently for good homogenization before dilutions are prepared by sterile measuring pipette. The sterile 9-cm Petri dishes are inoculated by sterile transfer of 1 ml from each of the prepared dilutions. Pour 12-14 ml of liquefied KF-streptococcus agar over the dish and secure even distribution and good mixing. Note the special KF-agar preparation (Annex III).

Incubation of inverted Petri dishes is done at 44±0.2°C for 48±3 hours. Red colonies or colonies with red centres are considered to be made up of faecal streptococci. Make a count for a plate in the 20-80 colony range (preferably) and then use the formula:

$$\text{Faecal streptococci/100 ml} = \frac{\text{Number of red or red centred colonies} \times 100}{\text{ml sample test amount}}$$

Enter the results on a standard form such as Form 6.

(3) Molluscs, pour plate method. The pour plate method is described in Annex I, section 1. The batch sample requires five molluscs, which are prepared and homogenized in the laboratory blender as described in section 10.3 above for the multiple test tube method of obtaining total coliform counts for molluscs (use only tissue and avoid transfer of water). Approximately 5 g flesh will suffice for the 10^{-1} dilution.

The dilution series prepared should correspond to sample test amounts of 10^{-1}, 10^{-2} and 10^{-3} g by transfer of 1 ml from the dilution series to the Petri dishes (Annex I, Figs 3 and 4). Transfer 1 ml from each dilution by sterile pipette to corresponding 9-cm Petri dishes. Pour 12-14 ml of liquefied KF-streptococcus agar over each dish and secure even distribution by mixing. Note the special features of KF-agar preparation (Annex III). Incubate the inverted dishes at 44±0.0.2°C for 48±3 hours.

Counting should be carried out on a plate with a 20-80 colony range to establish the number of red and red centred colonies, using the formula:

$$\text{Faecal streptococci/100 g} = \frac{\text{Number of red or red centred colonies} \times 100}{\text{g sample test amount}}$$

Enter the results on a standard form such as Form 6.

10.5.3 Equipment and supplies

(1) Apparatus. Flasks, test tubes, vials, spatulas, measuring pipettes
Laboratory blender and accessories
Vacuum filter pump, membrane filter apparatus and 0.45µ filters
Incubator (44±0.2°C)
Automatic shaker, bacterial colony counter, autoclaves

(2) Substrates and reagents. Phosphate buffer
KF-streptococcus agar

10.6 Clostridium perfringens

10.6.1 Definition

Obligatory, anaerobic, sporeforming, Gram-positive rods that reduce sulfite to sulfide within 24 hours at 48°C.

10.6.2 Procedures

(1) Sediment, standing tube method. The standing tube method is briefly outlined in Annex I, section 4. The following dilution series are recommended (Annex I, Figs 7 and 8) where there has been no previous experience of using the method.

Sediment pollution	Proposed dilution series (Annex I, Fig. 7)
Heavy	10^{-1} 10^{-2} 10^{-3} 10^{-4}
Slight	1 10^{-1} 10^{-2} 10^{-3}

Sterile phosphate buffer is used for dilution. Shake each tube vigorously before any transfer into other tubes, to obtain even distribution of sediment and organisms.

Approximately 15 ml of liquefied tryptone sulfite neomycin agar is used for each of the quintuple test tubes. The tubes may be heated to liquefy the agar and expel air. After cooling to 45°C, each tube is inoculated with 1 ml from the tube in the appropriate dilution series. After the agar in the tubes has solidified, some 2 ml of liquid TSN agar is added to each tube to maintain anaerobic conditions.

Incubation is carried out at 48±.2°C for 24 hours in a water bath. Development of black colonies will indicate the presence of Cl. perfringens. After incubation, counting is carried out on a single test tube quintuple, preferably one where the black colonies are separated sufficiently and of a size ⩾ 1 mm. From the single quintuple selected for counting, the colony density of the original sample is obtained by means of the following formula:

$$\text{Cl. perfringens/g} = \frac{\text{Total 5-tube count (one quintuple)}}{\text{g sample test amount} \times 5}$$

It should be remembered that a high number of colonies counted gives greater precision in the determination, i.e., it is worth selecting a test tube quintuple containing 100 rather than 25 colonies, which may increase precision from ±20% to ±10%.

This procedure will yield a total Cl. perfringens count (spores and vegetative cells). To obtain spores alone, a sample may be preheated to 80°C for five minutes, which would kill just the vegetative cells. Incubation, by the method described above, will now give the number of spores, and hence the number of vegetative cells.

(2) Sewage, standing tube method. The procedure for examination of Cl. perfringens in sewage is exactly as described above for sediments, with only two modifications, affecting the dilution series and determination of colony density. The dilution series should be as follows:

Sewage	Proposed dilution series (Annex I, Fig. 7)
Raw sewage	10^{-1} 10^{-2} 10^{-3} 10^{-4}
Secondary treatment	1 10^{-1} 10^{-2} 10^{-3}

The colony density for sewage should be expressed as a number per 100 ml, such as other liquid concentrations:

$$\text{Cl. perfringens/100 ml} = \frac{\text{Total 5-tube count (one quintuple)} \times 100}{\text{ml sample test amount} \times 5}$$

10.6.3 Equipment and supplies

(1) Apparatus. Test tubes (large), flasks, bottles, measuring pipettes
Incubator (48±.2°C)
Automatic shaker, autoclave

(2) Substrates and reagents. Phosphate buffer
Tryptone sulfite neomycin (TSN)
Agar

10.7 Salmonellas

10.7.1 Definition

Salmonellas belong to the Enterobacteriaceae and comprise motile (apart from a few exceptions) oxidase-negative, catalase-positive, aerobic and facultatively anaerobic, Gram-negative rods. Salmonellas attack sugars by fermentation with gas production (with one important exception), reduce sulfite to sulfide, and decarboxylize lysine (with a few exceptions).

10.7.2 Procedures

(1) Water, total salmonellas

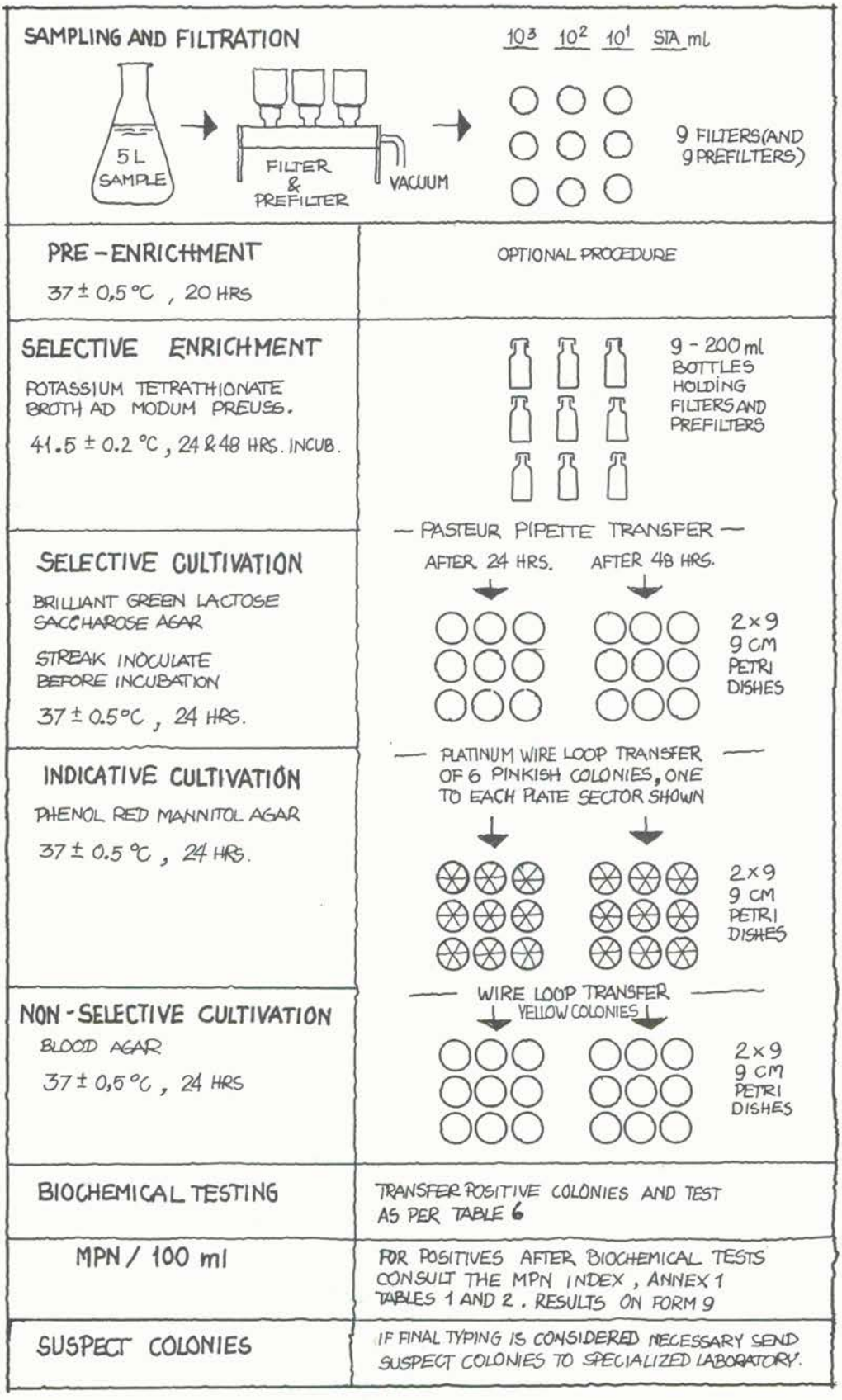
(a) Quantitative. The membrane filtration method is used initially (Fig. 18 below and Annex I, Figs 1 and 2). The amount of water to be filtered will depend on the degree of pollution of the water. The following filtration quantities are recommended:

Sample medium	Recommended sample test amounts		
Raw sewage	10	1	10^{-1}
Secondary effluent	100	10	1
Polluted seawater	100	10	1
Clear seawater	1000	100	10

Prefiltration (1.25µ) may be necessary and is to be recommended whenever the water is turbid. After filtration (.45µ), both prefilters and filters are transferred by sterile forceps to 200 ml flasks containing pre-enrichment medium and incubated for 18-20 hours at 37±0.5°C.

Selective enrichment is carried out at 41.5±0.2°C on potassium tetrathionate broth ad modum Preuss in 200 ml flasks (Fig. 18). Twenty-four hours after the start of enrichment, 9 (3 × 3) brilliant green lactose saccharose agar plates (selective enrichment medium) are inoculated by sterile

Fig. 18


SAMPLING AND FILTRATION
10³ 10² 10¹ STA ml
5 L SAMPLE
FILTER & PREFILTER
VACUUM
9 FILTERS (AND 9 PREFILTERS)
PRE-ENRICHMENT
37 ± 0,5 °C , 20 HRS
OPTIONAL PROCEDURE
SELECTIVE ENRICHMENT
POTASSIUM TETRATHIONATE BROTH AD MODUM PREUSS.
41.5 ± 0.2 °C , 24 & 48 HRS. INCUB.
9 - 200 ml BOTTLES HOLDING FILTERS AND PREFILTERS
— PASTEUR PIPETTE TRANSFER —
AFTER 24 HRS.
AFTER 48 HRS.
2×9 9 CM PETRI DISHES
SELECTIVE CULTIVATION
BRILLIANT GREEN LACTOSE SACCHAROSE AGAR
STREAK INOCULATE BEFORE INCUBATION
37 ± 0.5°C , 24 HRS.
INDICATIVE CULTIVATION
PHENOL RED MANNITOL AGAR
37 ± 0.5 °C , 24 HRS.
— PLATINUM WIRE LOOP TRANSFER OF 6 PINKISH COLONIES, ONE TO EACH PLATE SECTOR SHOWN —
2×9 9 CM PETRI DISHES
NON-SELECTIVE CULTIVATION
BLOOD AGAR
37 ± 0,5 °C , 24 HRS
— WIRE LOOP TRANSFER —
YELLOW COLONIES
2×9 9 CM PETRI DISHES
BIOCHEMICAL TESTING
TRANSFER POSITIVE COLONIES AND TEST AS PER TABLE 6
MPN / 100 ml
FOR POSITIVES AFTER BIOCHEMICAL TESTS CONSULT THE MPN INDEX , ANNEX 1 TABLES 1 AND 2 . RESULTS ON FORM 9
SUSPECT COLONIES
IF FINAL TYPING IS CONSIDERED NECESSARY SEND SUSPECT COLONIES TO SPECIALIZED LABORATORY.

Pasteur pipette transfer. Another transfer and inoculation of the other 9 plates may be done after 48 hours. Use 9-cm Petri dishes and incubate. Alternatively 15 plates (3 × 5) may be used throughout the examination.

Continue, using Fig. 18 as a model, and employing phenol red mannitol agar as indicative cultivation medium. Before biochemical testing, the suspect colonies (yellow) are transferred to pure culture blood agar and incubated for 24 hours at 37°C.

Biochemical tests are now conducted on at least two positive colonies from each plate (see Table 6). Only one of the two plates in identical positions on the two parallel sets of tubes need be positive, i.e., +/+, -/+, +/-, are considered positive for that rack position, and only -/- indicates negative reaction, where neither of the two parallel plates show a single yellow colony. Suspect colonies are isolated by wire loop transfer and placed on pure culture blood agar for incubation for 24 hours at 37°C before biochemical testing.

For determination of the Salmonella concentration of the sample, the most probable number index in Annex I, section 5, Table 1, must be consulted. Results should be entered on a standard form such as Form 9.

Table 6

BIOCHEMICAL SCREENING FOR SALMONELLA

Test media / Bacteria	Lysine	Indole	Urea	Sulfite agar	Reactions on TSN-agar (tilted tubes)		
					Colour Slant/butt	Blackening	Gas production
Salmonella	+	-	-	+	Red/yellow	+	v
Citrobacter	-	v	v	-*	Red/yellow or yellow/yellow	v	+
Proteus Rettgeri	-	+	+	-	Red/yellow	-	-
Shigella	-	v	-	-	Red/yellow	-	-
Faecal coliforms	+	+	-	-	Yellow/yellow	-	+
Hafnia	+	-	-	-	Red/yellow or yellow/yellow	-	+
Yersinia	-	v	+	-	Red/yellow or yellow/yellow	-	-
Pseudomonas	v	-	v	-	Red/red	-	-

Notes: All reactions are given in Annex III.

v = various reactions

+ = positive reaction

- = negative reaction

* = slight blackening may be visible

(b) Serological testing. More precise identification, e.g., of paratyphoid A and B may be of interest, in which case the assistance of a specialized central laboratory is necessary, and a sample must be prepared for the purpose, as given below.

Provisional serological testing may be carried out locally by studying salmonella strain agglutination in Salmonella-O, antisera A-I (serotypes 0.1 - 0.16), and other antisera reacting with somatic antigens higher than 0.16. Although local serotyping may be useful, the reference laboratory should always be consulted for ultimate identification of different strains if this is considered necessary.

(c) Preparing shipping for final identification. Colonies for ultimate identification are taken after the pure culture blood agar incubation (Fig. 18). The colonies are stabbed into approximately 5 ml of solid sugar-free agar in small tubes. The inoculated tubes are incubated for 18 hours at 37±.5°C before being corked and sealed by liquefied paraffin. The tubes must be accompanied by proper identification, i.e.:

(i) sender institution (name and address);

(ii) sampling time and data;

(iii) sampling station;

(iv) sampling medium;

(v) time of salmonella identification;

(vi) time of dispatch;

(vii) remarks as to unusual reactions (biochemically or using antisera).

(2) Sediment, total salmonellas - quantitative. A total sample amount of approximately 100 g will normally suffice. The examination procedure to be applied is only initially different from the salmonella/water method described above. The sample test amounts should be introduced directly to the enrichment broth (alternatively first in the pre-enrichment broth) according to the following general recommendations:

Growth ingredients	Enrichment flask content three flasks for each amount		
Sediment (amount in g)	30	3	0.3
Enrichment broth (ml)	300	50	50

Enrichment is carried out as per Fig. 18.

All further procedures are exactly as indicated in the method given for salmonella/water above and in Fig. 18 together with Table 6 and the most probable number indices (Annex I, section 5, Tables 1 and 2). Remember to correct the most probable no. per 100 ml according to actual sample test amounts, in this case 30, 3, and 0.3, i.e., to divide by 3 when finding the most probable no. index that applies to 10, 1 and 0.1 respectively.

(3) Shellfish and fish, salmonella - qualitative. Procedures for fish and shellfish will be qualitative only, because for fish a qualitative (gill scraping) sampling is inherent from the beginning, and for shellfish the criteria will often be of the presence/absence type. Further, the qualitative procedure requires less resources and may permit more samples to be taken.

The fish sample examination is based on gill scraping of the two fish needed to make up a sample. Use sterile cotton swabs to remove slime from the gills, two swabs per gill, i.e., a total of eight swabs per fish sample. All eight swabs are put into a single 200 ml pre-enrichment flask. Use only the sterile part of the swab pin and discard the part touched by the fingers. The two fish making up a sample should be of the same type, i.e., either bottom fish or pelagic, in order to ease the subsequent interpretation of results later on.

The shellfish sample (batch of five molluscs for example) should yield some 25 g of flesh. Avoiding transfer of water from the shells, they should be brushed with alcohol and slightly flamed before opening. The flesh is put all at once into the pre-enrichment broth in a single 200 ml flask. After preparing the pre-enrichment flask, the remainder of the examination procedure is the same as for a single enrichment flask, as given in Fig. 18. Use the quantitative procedure described in testing for the presence of salmonellas in water.

The result, after biochemical testing, indicates the presence or absence of salmonella in the sample. Numbers are not indicated. Further serological and biochemical testing is possible and should be done according to the recommendations given above. Suspect colonies can still be ultimately identified at a specialized laboratory.

10.7.3 Equipment and supplies

(see below, section 10.8.3)

10.8 Salmonella typhii

10.8.1 Definition

Unlike the other salmonellas, S. typhii, however, do not produce gas when fermenting sugars.

10.8.2 Procedure

Shellfish, qualitative. The qualitative approach is the only method available (selective substrates are insufficiently developed), and for the shellfish investigation this approach seems justified. Preparation of the shellfish and the general procedure for examination is the same as for total salmonella counts. However, some of the substrates used must be changed (see also Fig. 18

Pre-enrichment:	Same (pre-enrichment medium)
Enrichment:	Selenite cystine enrichment broth
Selective medium 1:	Bismuth sulfite agar
Selective medium 2:	Same, phenol mannitol agar
Biochemical tests:	Same as for total salmonella counts

However, reaction to triple sugar iron agar should not include gas. Also for S. typhii, the final typing must essentially be done by a specialized central laboratory.

10.8.3 Equipment and supplies

(1) Apparatus. Flasks, test tubes, bottles, Petri dishes
Measuring pipettes, Pasteur pipettes, wire loops, spatulas
Laboratory blender and accessories
Filter pump (vacuum), membrane filter apparatus, filters (0.45µ) and pre-filters (1.25µ)
Incubator (37±0.5°C and 41.5±0.2°C), autoclave

(2) Substrates and reagents. Phosphate buffer
Potassium tetrathionate broth ad modum Preuss
Selenite cystine enrichment broth
Brilliant green lactose saccharose agar
Bismuth sulfite agar
Phenol red mannitol agar
Pre-enrichment medium
Urea broth
Lysine decarboxylation base medium
Peptone water (indole test)
Kovac's indole reagent
Sulfite agar
Triple sugar iron (TSI agar)
Sugar-free agar (for shipment of samples)
Antisera, e.g., O-antiserum A-I (optional)

10.9 Vibrio cholerae and non-agglutinable (NAG) vibrios

10.9.1 Definition

Cholera bacteria are facultatively anaerobic, motile, catalase-positive, oxidase-positive, Gram-negative, often curved, rods which have a single polar flagellum. V. cholerae do not hydrolyse arginine, but decarbolyze lysine and ornithine. Sugars are decomposed fermentatively without gas production.

NAG vibrios are vibrios which morphologically and biochemically resemble V. cholerae, but usuall must be distinguished serologically.

10.9.2 Procedure

Shellfish, qualitative. Only a qualitative procedure is described here, because the result should initially be used only for determining the absence/presence of the V. cholerae and NAG vibrio strains in shellfish. The sample should consist of approximately 25 g of flesh from the shellfish. For crustaceans, use the front part, i.e., mouth and abdomen, and avoid transfer of water when placin

the flesh sample in the cultivation broth. For molluscs, the shells should be brushed, rinsed and flamed cautiously by alcohol before being opened, and the flesh transferred into the broth. Normally, five molluscs or crustaceans should be used for one sample.

The general examination procedure consists of:

(a) cultivation in selective fluid enrichment broth;

(b) plating from selective fluid growth medium onto selective primary indicator plates;

(c) isolation of pure cultures from suspect colonies using non-selective substrates;

(d) screening of suspect colonies by means of serological tests and selected biochemical tests;

(e) mailing of suspected cultures to specialized laboratory for final biochemical-serological typing and phage-typing to distinguish between classical cholera, El Tor biotypes and NAG vibrios.

The details of the examination procedure are given in Fig. 19 and Table 7. If biochemical screening does not eliminate the possibility of V. cholerae or NAG vibrio, the pure culture should be prepared for ultimate typing at a specialized central laboratory. Use sugar-free agar and proceed as described for salmonellas, with adequate labelling and information.

10.9.3 Equipment and supplies

(1) Apparatus. Flasks, bottles, test tubes, Petri dishes
Measuring pipettes, Pasteur pipettes, wire loops, spatulas
Incubator (37±0.5°C), automatic shaker
Microscrope and accessories, autoclave

(2) Substrates and reagents. Phosphate buffer
Taurocholate - tellurite - pepton substrate
Thiosulfate - citrate - bole - salt - sucrose (TCBS) agar
Blood agar, cytochrome oxydase reagent
Hugh and Leifson medium, arabinose broth, sucrose broth
Starch salt agar
Inositole broth
Lysine decarboxylation base medium
Ornithine decarboxylation medium
Argenine broth
Tryptone 1% broth (plus variable NaCl)
Peptone water (indole test)
Kovac's reagent
VP broth
Nitrate bouillon
Pteridine (antibioticum)

10.10 Vibrio parahaemolyticus

10.10.1 Definition

V. parahaemolyticus is a facultatively anaerobic halophilic, curved, Gram-negative rod, which possesses a single polar flagellum. The bacteria are indole-positive (few exceptions), catalase-positive and oxidase-positive. V. parahaemolyticus attack sugars by fermentation, but without gas production. They do not hydrolize arginine but they decarboxylate lysine.

10.10.2 Procedures

(1) General. Only quantitative procedures based on the maximum probable number principles will be described below. Note that for V. parahaemolyticus, 2% brine is always used as diluent instead of phosphate buffer. The general strategy for the V. parahaemolyticus examination reads as follows:

(a) selective cultivation in fluid media;

(b) plating from the selective growth medium onto selective primary indicator plates;

Fig. 19

QUALITATIVE EXAMINATION OF SHELLFISH FOR VIBRIO CHOLERA AND NAG

SAMPLE PREPARATION

CF. TEXT. PLACE SHELLFISH FLESH DIRECTLY IN ENRICHMENT BROTH.

SELECTIVE ENRICHMENT

TAUROCHOLATE TELLURITE PEPTONE WATER

37 ± 0.5 °C

SELECTIVE CULTIVATION

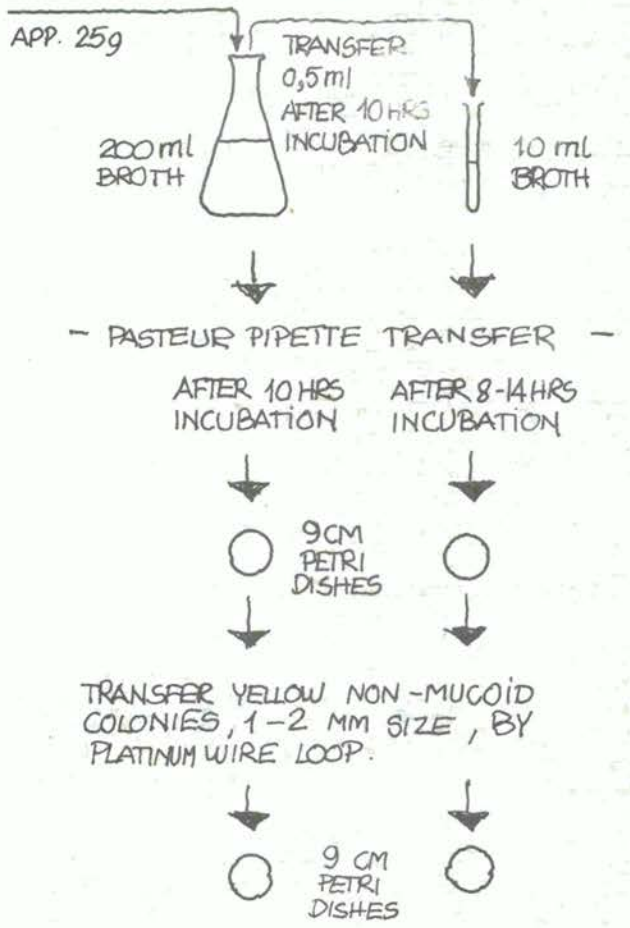
TCBS AGAR

37 ± 0.5 °C

NON-SELECTIVE CULTIVATION

BLOOD AGAR

37 ± 0,5 °C, 24 HRS.



SELECTED SEROLOGICAL AND BIOCHEMICAL TESTING

COMPLETE THE SERIES OF TESTS IN TABLE 7 (TRANSFER YELLOW COLONIES FROM BLOOD AGAR ONLY) FOR THOSE COLONIES THAT ARE GRAM-NEGATIVE, MOBILE, AND CYTOCROME-OXYDASE POSITIVE

SUSPECT COLONIES TO SPECIALIZED LABORATORY

COLONIES THAT PASS ALL BIOCHEMICAL TESTS MUST BE SENT TO A SPECIALIZED LABORATORY FOR FINAL IDENTIFICATION ENTER RESULTS ON FORM 10

Table 7

BIOCHEMICAL SCREENING FOR VIBRIOS

Organism: / Test:	Vibrio parahaemolyticus	Vibrio alginolyticus	Vibrio cholera NAG Vibrio	Vibrio Proteus	Vibrio anguillarum
Gram staining	gr -	gr -	gr -	gr -	gr -
Motility	+	+	+	+	+
Haemolysis (24 hours) calf blood	-	-	v	+	+
Cytochrome oxydase	+	+	+	v	+
Hugh & Leifson**	F	F	F	F	F
Arabinose**	+	-	-	-	v
Sucrose**	-*	+	+	+	+
Starch salt agar	+	+	-	+	v
Inositol**	-	-	-	-	-
Lysine**	+	+	+	-	-
Ornithine**	+*	+	+	-	-
Arginine**	-	-	-	-	+
Tryptone 1%	-	-	+	-	-
Tryptone 1% NaCl 4%	+	+	+	+	+
Tryptone 1% NaCl 8%	+	+	-	(+)	-
Tryptone 1% NaCl 10%	-	+	-	-	-
Peptone water** Kovac's reagent	+*	+	v	v	+*
VP-broth	-	+	v	v	v
Antibiotica test Pteridine	sensitive	sensitive	sensitive	sensitive	sensitive
42° Tryptone 1%**	+	+	v	-	-
Nitrate reduced**	+	+	+	-	+

Notes: For each test medium reactions are described in Annex III.

F = Fermentative

v = various reactions

* = Few exceptions

** = For VP testing add 2% NaCl

(+) = Weak reaction

(c) preparation on non-selective plates of pure cultures of suspect colonies from the primary plates;

(d) screening for suspect strains by means of serological and selected biochemical tests;

(e) derivation of most probable number from the positive plate combination found as a result of screening;

(f) referral of suspect colonies to a specialized central laboratory for final identification.

(2) Sewage: Quantitative. The procedure for examining V. parahaemolyticus in sewage is outlined in detail in Fig. 20. The minimum sufficient amount of sample is 100 ml. It should be stirred before the 3 × 3 sample test amounts (respectively 20, 2, and 0.2 ml) are inoculated directly into

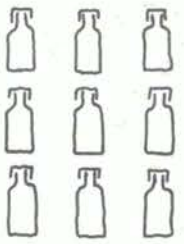
Fig. 20

QUANTITATIVE EXAMINATION OF SEWAGE FOR V. PARAHAEMOLYTICUS

SAMPLE PREPARATION

DIRECT INOCULATION INTO BROTH OF SAMPLE SIZE INDICATED

20 2 0.2 STA (ml)



9 - 200 ml BOTTLES.
200 ml BROTHS FOR 20 ml INOCULATE.
50 ml BROTH FOR LESS.

SELECTIVE ENRICHMENT

V.P. SALT MEAT BROTH

41 ± 0.5 °C, 18 HRS

— PASTEUR PIPETTE TRANSFER —

SELECTIVE PLATE CULTIVATION

TCBS AGAR

37 ± 0.5 °C, 48 HRS.

READ AFTER 24 AND 48 HRS.

9 - 9 CM PETRI DISHES

TRANSFER OF FLAT, REGULAR, GLOSSY, AND GREEN COLONIES

NON-SELECTIVE CULTIVATION

BLOOD AGAR

37 ± 0.5 °C, 24 HRS.

9 - 9 CM PETRI DISHES

BIOCHEMICAL AND SEROLOGICAL COLONY TESTING

TRANSFER NON-HAEMOLYTIC (BEFORE 18-24 HRS) GRAM-NEGATIVE, CYTOCHROME OXYDASE POSITIVE COLONIES FROM BLOOD AGAR FOR COMPLETE BIOCHEMICAL TESTING, CF. TABLE 7

SUSPECT COLONIES

SEND SUSPECT COLONIES FOR FINAL TYPING AT SPECIALIZED LABORATORY

MPN / 100 ml

TAKE THE POSITIVE COMBINATIONS OF PLATES. READ OFF THE MPN FROM ANNEX 1 TABLE 1 OR 2. ENTER MPN ON FORM 9

the nine selective enrichment bottles. The most probable number index of Annex I, section 5, Tables 1 and 2, is used to determine colony densities. Remember corrections as shown in Fig. 20 for actual sample test amounts (e.g., 20, 2, and 0.2 instead of 10, 1 and 0.1 in the index). Enter readings and results on a standard form such as Form 9. Results must be sent for final typing to a specialized laboratory. Strains that biochemical and provisional serological testing may identify as V. parahaemolyticus should be sent to a specialized central laboratory for final identification, including determinations for such phenomena as positive and negative reactions to the Kanagawa test (see procedures for testing salmonellas).

(3) Sediment: Quantitative. The sediment samples for testing should be approximately 100 g. The 3 × 3 sample test amounts (respectively 20, 2, and 0.2 g) should be inoculated directly into the nine flasks containing fluid enrichment broth (200 ml for 20 g, 50 ml for smaller sample amounts). Procedures and media used are the same as for the sewage sample described above and in Fig. 20, but the most probable number is expressed in grams instead of 100 ml, i.e., figures determined from the most probable no. index should be divided by 100. Moreover, corrections should also be made for actual sample test amounts.

(4) Shellfish: Quantitative. The sample should consist of approximately 40 g of flesh to obtain 3 × 3 sample test amounts of 10 g, 1 g and 0.1 g respectively. Avoid transfer of water when preparing the sample. In taking samples from crustaceans, use the mouth and abdomen. Molluscs should be brushed, rinsed, and cautiously flamed with alcohol before opening the shells. The sample test amounts can be inoculated as shown in Fig. 20. Thereafter, the maximum probable no. is derived and the density of V. parahaemolyticus is expressed as most probable no./100 g tissue. Use a standard form such as Form 9 to enter readings for positives and resultant most probable nos. Final identification of the vibrio strains should be done at a specialized laboratory.

The spread plate method may be used instead of the most probable no. method, in which case a synthetic brine (2%) should be used as diluent when preparing sample test amounts, such as 10^{-1}, 10^{-2}, and 10^{-3} g, to be inoculated directly on the surface of the selective-plate TCBS solidified agar (Fig. 20). In this case the amount of tissue sample need be only 5 g, and mixed in the blending vessel with 45 g (ml) of the 2% brine (= 10^{-1} g). Further cultivation and identification is as described for the most probable number method given above. However, the colony density is obtained by using a plate with a positive V. parahaemolyticus colony count preferably in the 20-80 range:

$$\text{V. parahaemolyticus/100 g} = \frac{\text{Total no. of substrate coloured and green centred colonies} \times 100}{\text{g sample test amount}}$$

10.10.3 Equipment and supplies

(1) Apparatus.
Flasks, bottles, test tubes, Petri dishes
Measuring pipettes, Pasteur pipettes, wire loops, spatulas
Laboratory blender and accessories
Incubator (41.5±0.2°C and 37±0.5°C), automatic shaker
Microscope and accessories
Bacterial colony counter, autoclave

(2) Substrates and reagents.
2% brine
VP selective salt meat broth (+NaCl)
Thiosulfate citrate bile salt sucrose
TCBS agar
Blood agar
Gram reagents
Cytochrome oxydase reagent
Hugh & Leifson +2% NaCl (VP) broth
Arabinose broth +2% NaCl
Sucrose broth +2% NaCl
Starch salt agar
Inositol broth +2% NaCl
Lysine decarboxylation medium +2% NaCl
Ornithine decarboxylation medium +2% NaCl
Arginine broth +2% NaCl
Tryptone 1% broth
Tryptone 1% +2% NaCl
Tryptone 1% +4% NaCl
Tryptone 1% +8% NaCl
Tryptone 1% +10% NaCl
Peptone water +2% NaCl
Kovac's reagent
Pteridine (antibioticum)

10.11 Shigella

10.11.1 Definition

Shigellas belong to the Enterobacteriaceae. They are non-motile, oxydase-negative, usually catalase-positive, Gram-negative rods. They are aerobic and facultatively anaerobic and attack sugars by fermentation, but without gas production.

10.11.2 Procedures

(1) General. This involves the following stages:

(a) cultivation in selective fluid growth media;

(b) streaking from selective fluid substrates and directly from the sample onto selective primary indicator plates;

(c) streaking onto non-selective pure culture media;

(d) biochemical and serological testing;

(e) despatch of suspect colonies to specialized laboratory for final identification of strains.

(2) Sewage, shigella. Only a qualitative procedure will be described for the examination of shigellas. A quantitative method is feasible along the lines of the salmonella examination, but due to the comprehensive testing done for the qualitative examination, a quantitative assessment would normally require special justification. A sample size of 200 ml is sufficient. Stir the sample before diluting, in line with Fig. 21, which also gives details of further examination procedures. After non-selective cultivation on blood agar, biochemical tests are carried out (Table 8) but only for Gram-negative, cytochrome oxydase-negative, and non-haemolytic colonies. Colonies that provide positive reactions to all tests must be sent to the specialized laboratory for final typing and identification. Use the lagging procedure as recommended for referral of salmonella samples.

Table 8

BIOCHEMICAL SCREENING FOR SHIGELLA

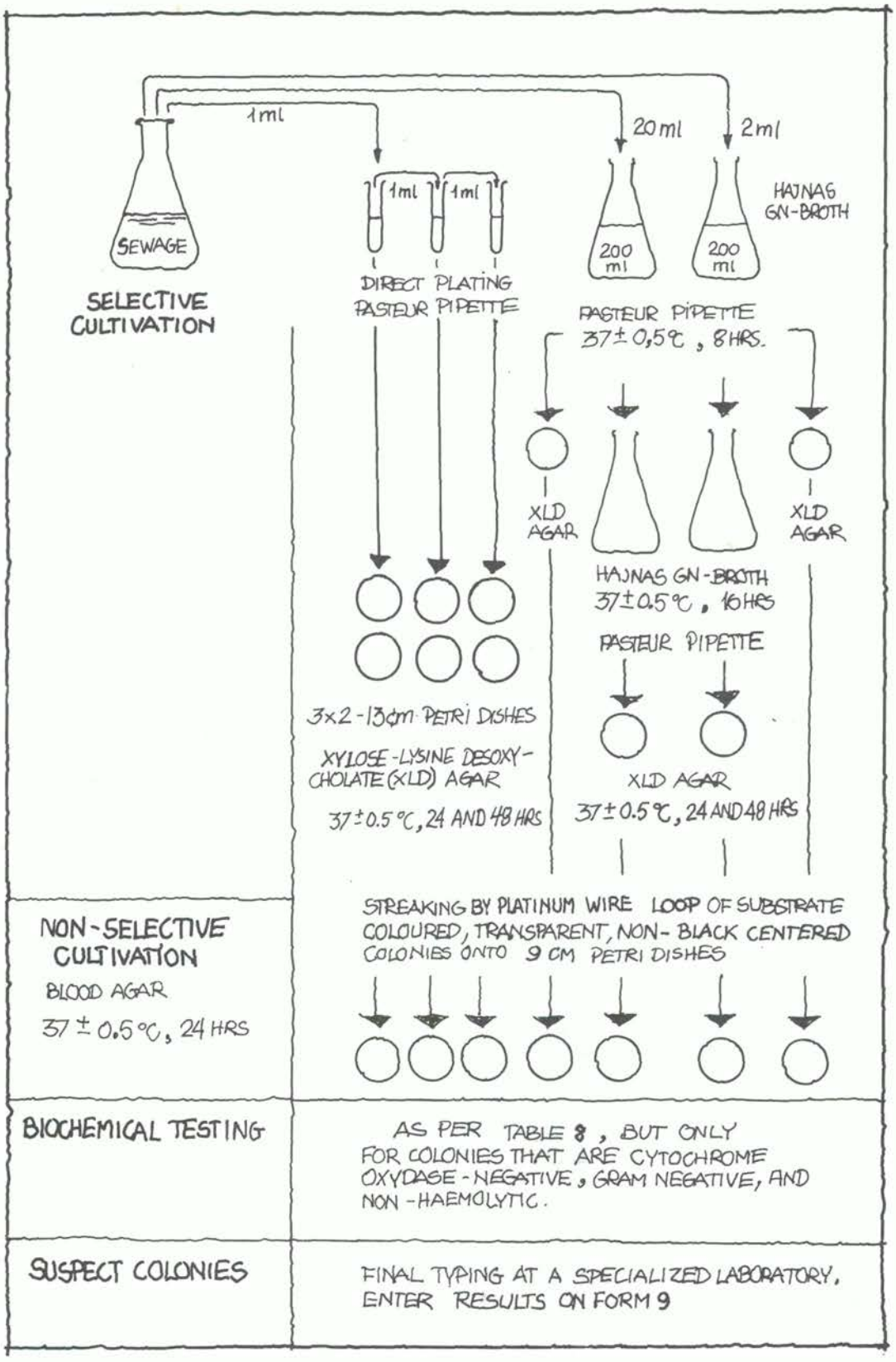
Test medium / Bacterium	SIM medium							Reactions on TSI agar		
	Urea	Lysine	Arginine	Indole	H_2S	Motility	Simmons citrate agar	Colour Slant/Butt	Blackening	Gas production
Shigella	-	-	v	v		-		Red/yellow	-	-
Salmonella typhii	-	+	v	-	+*	+*	+	Red/yellow	v	-
Salmonella	-	+*	v	-	+*	+*	+*	Red/yellow	+	v
Faecal coliforms	-	v	v	+	-	v	-	Yellow/Yellow*	-	+
Proteus	+	-	-	v	v	+	v	Red/yellow* or yellow/yellow	v	+*
Providencia	-	-	-	+	-	+	+	Red/yellow or yellow/yellow	-	+*
Edwardsiella	-	+	-	+	+	+	-	Red/yellow	+	+
Citrobacter	v	-	v	v	v	+	+	Red/yellow* or yellow/yellow	v	+
Yersinia	+	-	-	v	-	+**	v	Red/yellow* or yellow/yellow	-	-
Pseudomonas	v	v	v	-	v	+	v	Red/red	-	-

Notes: For each test medium the reactions are given in Annex III.

* = Few exceptions; ** = Only at 22°C; v = various reactions.

Fig. 21

QUALITATIVE EXAMINATION OF SEWAGE FOR SHIGELLA



10.11.3 Equipment and supplies

(1) Apparatus. Flasks, bottles, test tubes, Petri dishes
Measuring pipettes, Pasteur pipettes, wire loops, spatulas
Incubator (37±0.5°C), automatic shaker
Microscope and accessories
Bacterial colony counter, autoclave

(2) Substrates and reagents. Phosphate buffer
Hajnas GN broth
Xylose lysine desoxycholate (XLD) agar
Blood agar
Gram reagents
Cytochrome oxydase reagent
Urea broth
Lysine decarboxycholate medium
Arginine broth
SIM substrate
Kovac's reagent
Simmons' citrate agar
Triple sugar iron (TSI) agar

10.12 Yersinia

10.12.1 Definition

Yersinia bacteria are Gram-negative, motile, facultatively anaerobic, oxydase-negative rods. With few exceptions, they ferment sugars without gas production and hydrolize urea.

10.12.2 Procedures

(1) General. This involves the following stages:

(a) cultivation in fluid selective growth media at low termperature and anaerobic conditions;

(b) streaking from fluid media to primary selective plates for incubation;

(c) streaking from primary selective plates onto pure culture non-selective media;

(d) biochemical tests; and

(e) referral of suspect colonies to specialized laboratory for final identification.

(2) Sewage; Qualitative. In making preparations for the first inoculation of selective fluid growth medium, it must be ensured that incubation is anaerobic (see Fig. 22). The cooling (sterilization) of the substrate should be done with paraffin already added, so that inoculation may be made beneath the paraffin after the substrate is cooled to 30°C.

Further cultivation procedures and substrates are shown in Fig. 22. Biochemical testing should be carried out in line with Table 9 for colonies on the pure culture medium (blood agar) that are cytochrome oxydase-negative, small and non-haemolytic. Colonies that provide positive reactions to all tests (Table 9) must be shipped to a specialized laboratory for final typing and identification.

(3) Sewage; Quantitative. One method for performing quantitative examination for Yersinia is by combining the procedure given in Fig. 22 with the multiple test tube (most probable number) method described in detail under the quantitative examination for salmonellas, demonstrated in relation to water samples (see section 10.7 above and Fig. 18).

10.12.3 Equipment and supplies

(1) Apparatus. Flasks, bottles, large Petri dishes, test tubes
Measuring pipettes, Pasteur pipettes, wire loops, spatulas
Incubators (4±0.5°C and 2±0.5°C), automatic shaker
Microscope and accessories
Bacterial colony counter, autoclave

Fig. 22

QUALITATIVE EXAMINATION OF SEWAGE FOR YERSINIA

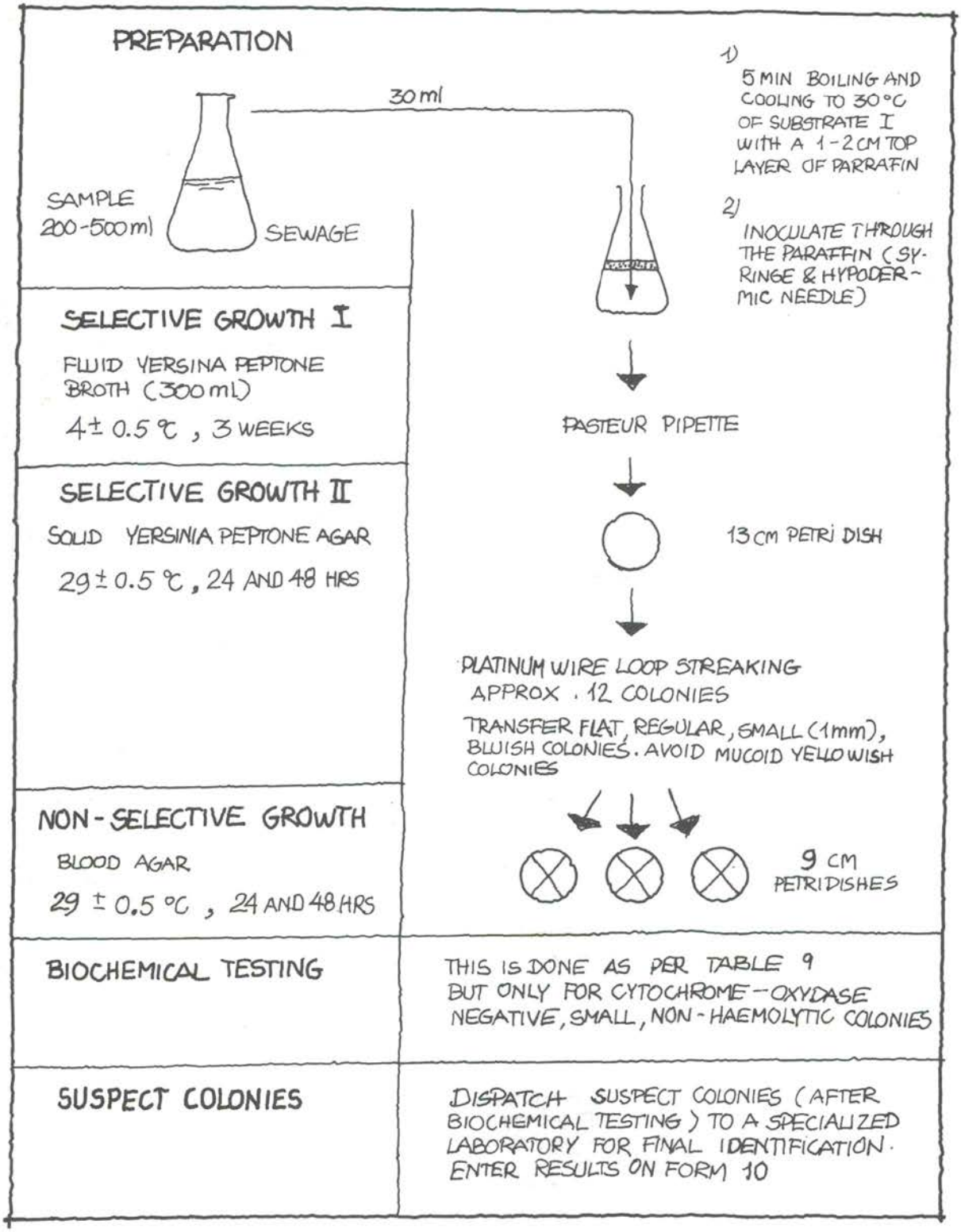


Table 9

BIOCHEMICAL SCREENING FOR YERSINIA

Test / Bacterium	Presumptive tests			Carbohydrates						Amino-acids		SIM medium 22°C			SIM medium 37°C						
	Urea	Phenylalanine	Arginine	Hugh and Leifson	Arabinose	Sucrose	Xylose	Cellobiose	Rhamnose	Lysine	Ornithine	H_2S	Motility	Indole	H_2S	Motility	Indole	Simmons' 22°C	Simmons' 37°C	VP broth 22°C	VP broth 37°C
Yersinia enterocolitica	+	-	-	F	+	+	v	+	v	-	+	-	+	v	-	-	v	v	-	+	-
Yersinia pseudotub.	+	-	-	F	+	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-
Klebsiella	v	-	-	F	+	+	+	v	+	v	-	-	-	-	-	-	-	v	v	v	v
Enterobacter	v	-	v	F	+	+	+		+	v	+	-	+	-	-	+	-	+	+	+	+
Pseudomonas	v	v	v	Ox		v	v		v	v	-	v	+	-	v	v	-	v	v		
Vibrio anguillarum	-	-	+	F	v	+	-	+	-	-	-	-	+	v	-	-	v	v	-	+	v
Aeromonas	-	-	+	F	v	v	-	v	v	-	-	v	+	v	v	+	v	v	v	v	v
Serratia	v	-	-	F	v	+	v	v	-	v	v	-	+	-	-	+	-	+	+	+	+
Faecal coliforms	-	-	v	F	+	v	v		v	+	v	-	v	+	-	v	+	-	-	-	-
				Glucose																	
Alcaligenes	v			-	-	-	-	-	-			-	+	-	-	+	-	+	+		
Acinetobacter	v	-	-	-		v							-	-		-	-	v	v		
				or Ox																	
Proteus	+	+	-	F	-	v	v	v	-	-	v	v	+	v	v	+	v	v	v	v	-
Providencia	-	+	-	F	-	v	-	v	-	-	-	-	+	+	-	+	+	+	+	-	-
Citrobacter	v	-	v	F	+	v	+	v	+	-	v	v	+	v	v	+	v	+	+	-	-

Notes: For each test medium, reactions are given in Annex III.

v = various reactions Ox = oxydative reaction + = positive reaction

F = fermentive blank = ambiguous response - = negative reaction

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(2) Substrates and reagents.

Fluid Yersinia peptone broth (selective)
Solid Yersinia peptone agar (selective)
Blood agar
Gram reagents
Cytochrome oxydase reagent
Urea broth
Phenylalanine agar
Arginine broth
Hugh and Leifson medium
Arabinose broth
Sucrose broth
Xylose broth
Cellobiose broth
Rhamnose broth

Lysine decarboxylation medium
Ornithine decarboxylation medium
SIM substrate
Simmons' citrate agar
Voges-Prosteonuer (VP) broth (Clark and Lub)

10.13 Parasites

10.13.1 Definition

For present purposes, parasites are defined as protozoans and parasites whose eggs are isolated through the procedure outlined below. The eggs of nematodes (such as Ascaris, Ancylostoma, Trichuris) are of particular interest, because no intermediate host is required for human infection, and they are able to survive in the marine environment for considerable periods of time. For reasons of space, the methodology described here will exclude the eggs of trematodes, as these require fresh water intermediate hosts, unable to survive in a marine environment.

10.13.2 Procedures

(1) General. The methodology applied leads to a quantitative assessment of the parasitic pollution of the medium studied. The final identification and enumeration of the isolated eggs must be left to the specialized laboratory. However, the preparation of slide specimens for microscopy must be the responsibility of the local laboratory. Generally speaking, centrifuging in liquids of different densities (heavier and lighter than the eggs to be studied) is used for isolation.

(2) Molluscs; Quantitative. A sample of 10 molluscs (or oysters) is used for the examination. The specific procedure for preparing the examination is described in Figs 23 and 24. The results received from the specialized laboratory should be expressed in terms of:

(a) total number of parasite eggs and protozoans/10 animals;

(b) specific numbers of parasite eggs and protozoans of different species/10 animals; and

(c) number of germinating eggs, for certain species/10 animals.

(3) Sediment; Quantitative. 20 g of sediment (settled, wet weight) should be suspended in 360 ml of saccharose solution and mixed carefully in a mortar. The further steps are as described under the general procedure given in Fig. 24. Results should be expressed per 100 g of sediment (wet weight).

(4) Sewage; Quantitative. Centrifuge the sewage sample to obtain approximately 10 g of precipitate (residue after removal of supernatant by syringe). Choose the sample size so that 10 g of precipitate (wet weight after settling) are obtained. Grab sampling or continuous sampling combined with any primitive settling technique may also be used. The further procedure is as described in Fig. 23.

The volume of sewage used to produce 10 g of precipitate must be recorded so that the final enumeration be correctly related to the volume of sewage: concentration is expressed in number of parasite eggs/100 ml of sewage.

10.13.3 Equipment and supplies

(1) Apparatus. Mortars (500 ml minimum), flasks, 20 ml syringes with hypodermic needles
Filter holders (5μ filters) and syringe adaptation
Filter sieves (250μ) and holders
Centrifuge (min. 4000 r/min., 2900 g)
Centrifuge tubes (polyester, 100 ml)
Microscope (100 × min) and accessories

(2) Substrates. Sugar solution (specific gravity 1.28)

Fig. 23

GENERAL PROCEDURE FOR PARASITE EXAMINATION

(1) The prepared saccharose diluted sample (1 : 18) is distributed in an adequate number of centrifuge tubes. Rotate at 2500-3000 r/min (1100-1600 g) for 10 minutes.

(2) Transfer the upper 10 ml of flotate by syringe into other centrifuge tubes.

(3) Restir the residue from the centrifuged tubes and repeat centrifugation as for (1). Transfer the flotate to centrifuge tubes, in addition to those mentioned under (2).

(4) All centrifuge tubes (cf. 2 and 3) are topped up with distilled water and centrifuged at 3500-4000 r/min (2200-2900 g) for 15 minutes. Remove supernatant by syringe without disturbing the precipitate and press through a 5μ filter placed in a filter holder with syringe adaptation.

(5) Remove precipitate from centrifuge tubes by syringe after sufficient dilution with distilled water to liquefy the precipitate. The liquefied precipitate is pressed through a 5μ filter placed in a filter holder with syringe adaptation. Apply only the amount of precipitate to each filter that will allow transparency of the filter in the microscope, i.e., use several filters if necessary. Rinse centrifuge tubes by distilled water, and let rinse water pass a filter to secure good recovery of eggs and protozoans.

(6) Dry filters overnight, but keep them flat in order to facilitate microscopy (use two micro slides, for example).

(7) Prepare filters for microscopy using microslide, micro cover glass and immersion oil. Use immersion oil as the filter becomes clearly transparent. Make the oil film uniform under the cover glass, and line with Canada Balsam. Allow to dry, and the microscope specimen is ready, possibly in a number of copies for one original sample.

(8) Shipping to the specialized laboratory must include adequate information on the sample prepared:

(a) sender institution, name and address;

(b) sampling time and date;

(c) sampling station;

(d) sampling medium;

(e) time of sample preparation;

(f) time of dispatch; and

(g) remarks as to unusual observations during sample preparation, any accidents or suspicious circumstances, etc.

Fig. 24

INITIAL PROCEDURE FOR EXAMINATION OF SHELLFISH FOR PARASITES

(Sample preparation and parasite separation)

10 oysters make 1 sample. Separate water and flesh in two mortars.

The water is centrifuged at 4500 r/min (3600 g) for 10 minutes.

Transfer supernatant into other tubes and centrifuge again.

Discard supernatant and retain precipitate.

Mix precipitates = 1 part with 18 parts of saccharose solution (weight basis).

The flesh is ground by knife and poured over a filter sieve, 250µ mesh.

Rinse by 400 ml, 0.1 ‰ Tween 80 at pH = 11 (NaOH adjusted) through the filter sieve.

Repeat rinsing process. Discard filter material.

Centrifuge filtrate 4500 r/min (3600 g) for 10 minutes.

Remove supernatant for repeated centrifugation.

Retain and conjugate the two precipitates; discard supernatant.

Mix precipitates = 1 part with 18 parts of saccharose solution.

Combine the two saccharose dilutions of isolated eggs and protozoans.

Continue as indicated in Fig. 23.

10.14 Enteric viruses

10.14.1 Definition

Enteric viruses are those listed and described in Table 3 (section 7.3.12).

10.14.2 Procedures

(1) General. Virological examinations at an introductory stage of monitoring are encouraged only for shellfish (molluscs and oysters) and sewage. The virus examination should be undertaken only by a specialized laboratory, because advanced and resource-demanding techniques are involved, and because quantitative examination procedures are still in the development stage.

Locally, however, there is a need for good quality sampling by trained microbiologists. The present guidelines deal only with the sampling and concentration procedures that are necessary before dispatch to a specialized laboratory.

(2) Shellfish. The batch of shellfish is sampled and packed (sealed plastic bag) at the monitoring site. A batch of five molluscs or oysters or the equivalent weight of crustaceans is adequate for each location. There is no further sample preparation before dispatch to the specialized laboratory.

(3) Sewage. The three virus concentration methods that follow (Lund, 9) all apply to sewage at different degrees of treatment, but their characteristics vary according to the specific virus type being isolated.

Al $(OH)_3$ concentration - this method concentrates enteroviruses, reoviruses and adenoviruses by adsorption and elution.

Protamine sulfate concentration - reoviruses and adenoviruses are concentrated more efficiently by this method.

Two-phase polymer concentration - enteroviruses and adenoviruses have been recovered by this separation method.

(a) Aluminium hydroxide concentration of virus in water. When added to sewage or other waters at pH 6, aluminium hydroxide adsorbs virus, which may then be recovered by filtration or centrifuging. The viruses are eluted from the complex with beef extract or some other suitable elutant.

Scope and application

The quantity of virus adsorbed by aluminium hydroxide bears a direct relationship to the quantity of salt employed. However, ease of recovery of a salt-virus complex diminishes with increasing volumes of salt, so a compromise must be reached between adsorption efficiency and technical feasibility. The method is best suited to raw sewage, highly polluted water, or partially treated effluents, because with such samples a relatively small sample of a few litres or less suffices.

Advantages and limitations

The method requires only equipment commonly found in a water or microbiological laboratory. The sample size should be less than two litres. The desorption procedure may not elute all adsorbed virus.

Preparation

To prevent adsorption of virus to the discs, pretreat filter discs as follows: stack three or four prefilter discs in a filter holder and use negative or positive pressure to pass through them a 0.1% solution of Tween 80 in sufficient quantity to completely wet the discs (about 200 ml), followed by distilled water for thorough rinsing (about 2 litres). Sterilize treated discs by autoclaving.

Prepare the insolube aluminium hydroxide salt in accordance with the method of Wallis and Melnick (11), but make the stock suspension twice as concentrated. Add 3 ml of 2 M Na_2CO_3 to 100 ml of 25 mM $AlCl_3$ and place flask on a magnetic stirrer at room temperature for 15 minutes. Centrifuge the suspension at 2000 r/min (700 g) for 15 minutes and discard the supernatant fluid. Wash the sediment once with 0.15 M NaCl, resuspend it on 0.15 M NaCl, and autoclave it at 120°C for 15 minutes. Cool the suspension, sediment the precipitate by centrifuging, and resuspend in 50 ml of sterile 0.15 M NaCl. Store the stock suspension at 4°C.

Prefiltration

To remove gross particulate matter, filter sample through two layers of Tween 80-treated prefilters of 124 mm diameter. Adjust pH of sample to 6.0 with 1 N HCl. Add stock aluminium hydroxide suspension to give a 1 : 100 dilution of the stock suspension in the sample. Place the flask of reactants on a magnetic stirrer for two hours at room temperature. Collect salt-virus complex either by filtration or centrifuging:

By filtration: Filter the sample through a prefilter disc (e.g., Millipore AP20) treated with Tween 80. Often, only a few hundred ml can be filtered through a 47 mm diameter disc before it cloggs. The clogged disc may be set aside and replaced with a fresh disc. Alternatively, a larger diameter filter disc may be used. After filtration is completed, remove the disc(s) to a Petri dish and cut it (them) into pieces suitable for transfer to a wide-mouthed screw-capped test tube. Add elutant (3% beef extract, pH 7) in sufficient quantity to cover the pieces of prefilter, and either strap the tube to a mechanical shaker for 5-15 minutes or place it in a vortex mixer for several minutes and mix vigorously.

By centrifuging: Centrifuge the sample treated with aluminium hydroxide at 2500 r/min (1100 g) for 15-20 minutes. Discard the supernatant and add 5-10 ml or other selected colume of elutant (3% beef extract, pH 7.0) to the sediment. Mix thoroughly for several minutes. Centrifuge the

mixture at 2500 r/min (1100 g) for 30 minutes to precipitate the aluminium hydroxide and bacteria. The supernatant is then ready for inoculation after decontamination.

Equipment

Prefilters (e.g., Millipore AP20:47 or 90, and 129 mm diameter)
Filter flasks
Rubber or plastic tubing for filter equipment
pH meter or alternative method for pH determination
Magnetic stirrer (or other means of mixing reagents)
Low-speed centrifuge
Mechanical shaker
Mixer, or alternative method for mixing reagents

Reagents

De-ionized or distilled water
Tween 80^{R}, 0.1% (v/v) in water
1 N NaCl, Al $(OH)_3$ (specially prepared, cf above)
2 M Na_2CO_3, 0.025 M $AlCl_3$
0.15 M NaCl
3% beef extract, pH 7.0 (for elution)

(b) Sewage, protamine sulfate concentration of enteric viruses. Negatively charged proteins can be precipitated by positively charged protamine sulfate in aqueous solution. If protamine sulfate is used to treat protein-supplemented samples of raw sewage or partially treated effluents, certain viruses are taken up into the precipitate, which can be dissolved in 1 M NaCl.

Scope and application

Reoviruses and adenoviruses are concentrated and recovered more efficiently in this way than by many other methods. Some enteroviruses are precipitated and others are not, but the explanation for this remains unknown.

Advantages and limitations

Reoviruses and adenoviruses are more efficiently concentrated and recovered than by many other methods. If the precipitate that contains the virus is dissolved, it can be completely recovered. The procedure requires only equipment generally found in a water microbiology laboratory. The method is not ideal for concentrating enteroviruses; some enteroviruses are concentrated, others are not. To obtain more complete recovery it may therefore be preferable to combine it with the aluminium hydroxide method.

The procedure cannot be used with highly treated effluents or natural waters, because (a) the sample size must be limited to no more than a few litres, and (b) proportions of reagents would have to be altered, because the protamine reaction is greatly influenced by the ion strength of the sample, as well as by pH and protein content. Different batches of protamine sulfate may vary in reactivity.

Preparations

To prevent adsorption of virus to the discs pretreat filter discs as follows: stack three or four discs in a filter holder and pass through them about 200 ml of 0.1% Tween 80 by negative or positive pressure. Follow this with about two litres of de-ionized or distilled water to rinse the discs thoroughly. Sterilize the treated discs by autoclaving them for 15 minutes at 120°C.

Prefiltration

To remove gross particulate matter, the sample should be filtered through two layers of Tween 80-treated prefilters of 124 mm diameter. Supplement the sample with beef albumin to a final concentration of 0.25%. Adjust pH to 7.5 - 7.8 with 1 N HCl or 1 N NaOH. Add protamine sulfate (stock 1% solution) to optimum concentration, in the 0.025% or 0.05% range (to be determined for each batch by prior titration with a known quantity of virus, so as to determine recovery efficiency). Place flask of reactants on a magnetic stirrer for 30 minutes at room temperature. The precipitate may not be grossly apparent. Collect the precipitate by passing a vacuum filtration of sample through a prefilter disc (47 mm diameter), pretreated with Tween 80, to prevent viral adsorption. If the filter disc clogs, replace with a fresh disc, putting the clogged disc on one side but

retaining it for later use. Then stack the used discs in the filter holder before passing 1 N NaCl through them to dissolve the precipitate.

After vacuum filtration of the sample, turn off the vacuum and supply a small sterile vessel or test tube (e.g., Millipore stainless-steel hydrosol filter with 15 ml conical glass tube) to collect the concentrated viral filtrate.

Dissolve the precipitate on the upper surface of the prefilter discs(s) by adding, with vacuum off, a small volume (e.g., 0.5 ml) of 1 N NaCl, ensuring that it completely covers the disc surface. Wait five minutes and then use the vacuum to extract the fluid into the receiving vessel. To the upper surface of the prefilter disc, add water, six times the volume of the 1 N NaCl, and extract this as well, rinsing the disc and effecting an isotonic filtrate.

Add foetal beef serum (approximately 10% by volume) to the filtrate to stabilize the virus.

An alternative method exists, for collecting and dissolving the precipitate. After the 30 minute mixing period, centrifuge the sample at 2500 r/min (1700 g) for 15 minutes. Discard the supernatant fraction. Add 0.5 ml of 1 N NaCl to the sediment and mix well. Add 3.0 ml of water to effect an isotonic solution, and 0.5 ml of foetal beef serum to stabilize the virus.

Equipment

Filter holders for prefiltration and for collecting precipitates
Prefilters (such as Millipore AP20), 47 and 124 mm diameter
Side-arm filter flasks
Rubber or plastic tubing for filter equipment
pH meter or alternative method for determining pH
Magnetic stirrer or other means of mixing reagents
Centrifuge

Reagents

De-ionized or distilled water
Tween 80, 0.1% (v/v) in water
Beef albumin, fraction V.5% (w/v) in water
Foetal beef serum, inactivated for 30 minutes at 56°C
1 N HCl and 1 N NaOH, for adjustment of sample pH

(The three latter items may be sterilized by filtration and stored at 4°C.)

(c) Sewage, two-phase polymer concentration; enteric virus. Viruses can be concentrated by an aqueous polymer two-phase separation. Polyethylene glycol and dextran are added to the sample under proper physical-chemical conditions, so that the water separates in two phases. The polyethylene glycol will form an upper phase consisting of about 99% of the water and containing most of the impurities in the sample. The lower phase will contain about 1% of the water, the dextran and the virus. The maximum concentration factor is 100. This method is best suited for samples of high virus densities because of the limited sample volume that can be handled.

Scope and application

The aqueous polymer two-phase system was first developed as a method for the concentration and purification of virus suspensions (12). Because of its simplicity and economy, it has been employed for the detection of virus in raw sewage and treated effluents. Only if the ion strength and pH are correct for the specific virus will the separation and concentration be successful. Enteroviruses and adenoviruses (13, 14) have been recovered with this technique.

Advantages and limitations

The method offers the advantages of simplicity and economy because minimal equipment and labour are needed, and it is practicable for field use. The method may recover large numbers of a large variety of viruses from naturally polluted samples.

Only limited volumes can be processed. Although it is possible to add additional two-phase separation steps, this becomes rather complicated because of the required adjustments of ion conditions. For field samples, the method is best used in a one-step procedure and consequently a maximum concentration factor of 100. The sample volume may be in the 200 ml - 10 l range, but the 200 ml - 1 l range is the most convenient. The method is therefore best suited for moderately to grossly polluted waters.

Virological assay by plaque forming units (PFU) is not recommended because the samples to be inoculated may be cytotoxic or contain particulate matter. The method is unsuitable for sludges and other particulate samples because it yields erratic results (15).

In some reports virus recovery from sewage samples was good (14) when employing LD_{50} assays in tissue culture, but in other reports (16) the recovery rate was poor when PFU assays were used.

Only a limited number of viruses have been tested, and it has not been established that viruses of naturally polluted waters behave like those contained in artificially contaminated samples. It is possible that the physical and chemical conditions for optimum virus recovery vary among the enteric viruses; because of this, the optimum conditions for maximum virus recovery have not been established.

It has been reported that coxsackie virus B_2 and echovirus type 6 become rapidly inactivated by dextran sulfate (17) and that substituting the dextran sulfate 2000 for dextran T-500 minimizes virus losses (18).

Concentration

A sample of 200 ml is placed in a 500 ml bottle, to which is added 20 g of 5 N NaCl, 58 g of 30% polyethylene glycol and 2.7 g of 20% dextran sulfate. For raw urban sewage, treated effluent and brackish waters, the sample's ion strength has been found to be so low that it may be disregarded when determining the total ion strength, which then depends essentially on the added NaCl.

It is essential that the pH be adjusted to 7.20±0.05. For this purpose 0.01 M phosphate buffer is employed. If the buffer volume required is liable to increase the sample volume significantly, adjust with 1 N HCl or 1 N NaOH.

Shake the sample vigorously for about one hour; with less efficient shaking the concentration of the virus in the dextran sulfate bottom phase may become less efficient. After the shaking, the sample is placed in a separatory funnel of suitable size.

The funnel is stored at 2-6° for 24 hours, and the fluid should then be separated in two phases consisting of dextran sulfate phase (about 2.0 - 2.5 ml) and the remainder. In a number of cases, especially raw sewage, a great deal of particulate matter will be found to collect at the interphase. It has been found very useful to make a separate test of the first bottom sample, i.e., the dextran sulfate phase, as well as the "interphase" (i.e., the next 2 ml sample) (14). Different viruses and mixtures of viruses may be found in the two sub-samples (13).

No further treatment, apart from decontamination, is required. To ensure maximum recovery, the concentrated samples should be tested on tube cultures, making blind passages.

Equipment

Sample bottles with caps or rubber stoppers, such as 1 litre glass bottles with plastic screw caps
pH meter
Magnetic stirrer or shaking machine, although shaking by hand can also be used
A simple conductivity meter, essential to measure the ion strength of some types of water
Pear-shaped separatory funnels (Funnels with burette-shaped bottoms are very useful, but may have to be specifically made.)

Reagents

Sodium dextran sulfate 2000 in a 20% solution by weight
Polyethylene glycol
Carbonwax 6000, in a 30% solution by weight
Phosphate buffer (0.01 M), pH 7.2
Sodium chloride solution (5M)

Remark on dextran: Two enteric viruses have been reported (17) to lose infectivity through the effects of dextran sulfate 2000. Dextran sulfate 2000 and 500 may be employed for the separation, but dextran T-500 may also be used (18). Dextran of lower molecular weights and DEAE-dextran are ineffective.

10.14.3 Shipping of concentrated samples

Dispatch of samples of shellfish as well as sewage to the specialized laboratory must include the basic information, as specified for parasites (Fig. 23).

10.15 Biotoxins, Paralytic shellfish poison

10.15.1 Definition

Paralytic shellfish poison (PSP) is a single neuromuscular toxin or possibly several closely related neuromuscular toxins which under certain conditions cause poisoning following consumption of shellfish. The definition of paralytic shellfish poison is at present closely linked to the method of demonstrating toxicity of the shellfish.

10.15.2 Procedure

(1) Principle biotoxin. The method described hereunder (30) is based on the injection into mice of an acid extract of shellfish meat for identification and titration of the toxin. When the dilution degree of the extract is known, together with the weight of the injected mice, and the respective time of death (time from injection till death sets in), after a few injections the toxicity can be read from Sommer's tables in mice units (ME) per weight unit mussel flesh. By means of PSP produced in pure form, the mouse strain's tolerance to the toxin can be determined. PSP for reference is obtainable from standards departments such as Shellfish Sanitation Section, Washington 25, D.C.

(2) ME unit of toxicity. For the detection and titration of the toxin, mice are generally used. As unit of measurement the mouse unit (ME) is applied. One ME is defined as the smallest amount of toxin which in 15 min kills a 20 g mouse when 1 ml of toxin is intraperitoneally injected as extract. One ME is equivalent to 0.183 µg ± 0.022 µg PSP.

(3) Sampling. For each test 25 mussels or more are sampled, depending on their size.

(4) Preparation of sample.

(a) Common mussels or the like, sand mussels, oysters.

(i) The mussels are washed on the outside under running water, and opened by cutting the adductor muscle.

(ii) The contents of a suitable number of mussels are removed with least possible crushing of tissue, rinsed under running water, and collected in beaker to an amount of 100-150 g.

(iii) The mussel meat is transferred as soon as possible to the wire sieve, and is drained for 5 min.

(iv) The meat is homogenized in blender.

(v) Exactly 100 g homogenized mussel flesh is mixed with 100 ml 0.1 N HCl, and boiled cautiously for 5 min. with constant stirring.

(vi) The mixture is cooled, and pH is adjusted to between 2 and 4 by adding 5 N HCl or 0.1 N NaOH. The volume is adjusted to exactly 200 ml with distilled water, and the extract is thoroughly mixed.

(vii) The extract is put to stand for sedimentation, and is then centrifuged for 5 min. at 3000 r/min (2900 g). The preparation of the clear supernatant is now ready for testing on experimental animals.

(b) Scallops.

(i) As for common mussel, etc.

(ii) The edible adductor muscle is separated off, and collected in an amount of 100-150 g. The test is carried out on this portion alone.

(iii) Continue, as directed for common mussel, etc.

(c) Tinned mussels.

(i), (ii), (iii) and (iv). Transfer the whole contents of the tin to a blender, and homogenize. In the case of large tins, the contents of solid particles and liquid are measured separately, and a smaller portion is reconstructed from the found ratio of mixture.

(v) Continue, as directed for common mussel, etc.

(5) Titration biotoxin. The titration is based on accurate measurement of the "time of death" and weight of mice. The exact toxicity is then read from tables. The method gives highest accuracy at a weight of mice of 19-21 g and a time of death of 5-7 min.

Injection is made into 1-3 mice each with 1 ml extract i.p. as an orienting titration.

The time of death, estimated from the moment of injecting to the last yawning pant movement, is measured with a stop watch at an accuracy of 5 sec. If the time of death at the orienting titration is <5 min., the extract is diluted until the time of death is increased to 5-7 min. If a high dilution is required, pH must be readjusted to 2-4 by adding 1 N HCl or 0.1 N NaOH.

When a time of death of >7 min. is obtained from the orienting titration, or a time of death of 5-7 min. has been obtained from such a titration, if necessary after dilution, the final titration is carried out by injecting 3 mice in all.

The average time of death is calculated for the 3 mice in a final titration (including any surviving), and the corresponding toxin content in ME/ml is determined from Sommer's table I (Table 10) (the time of death for surviving is estimated at >60 min. or equivalent to <0.875 ME). This value is multiplied by dilution degree, if any, and by 200 which represents the total amount of extract. The result represents the toxin content of the sample in ME/100 g mussel meat.

If mice weighing 19-21 g (weighed before injection) are used, no weight correction is made. In weight differences exceeding these values the corresponding toxin concentration is read for each mouse. These concentration values are multiplied by each separate corresponding concentration factor, determined from the weight of mouse and Sommer's table II (Table 10).

The average toxicity for the group is then calculated. The result is multiplied by degree of dilution, if any, and by 200 as above.

(6) Accuracy. The method has a lower limit of measurement of 200 ME/100 g (or about 38.2 µg/100 g when using mice weighing 20 g).

10.15.3 Equipment and supplies.

(1) Apparatus.
- Beaker, tared, 250 ml
- Beaker, 1000 ml for storing and inspection of injected mice
- Blender
- Brush with bristles
- Household-wire sieve
- Indicator paper, pH 2-5
- Injection syringes, sterile, 1 ml
- Hypodermic needles, sterile, 17-19 gauge
- Knife, sharp, thin-bladed with convex cutting edge
- Volumetric flasks, 100 ml
- Graduated cylinder, 200 ml
- Pipettes
- Centrifuge, 3000 r/min with glass
- Stop-watches, 3 items

(2) Reagents.
- Sodium hydroxide, 0.1 N
- Hydrochloric acid, 0.1 N, 5 N
- Water, distilled

(3) Experimental animals. White mice weighing about 20 g and not over 23 g are the most practical for use.

Table 10

SOMMER'S TABLES

(Sommer's table I)				(Sommer's table II)	
				Correction factor for weight of mouse	
Time of death min/sec	Number of ME	Time of death min/sec	Number of ME	Weight of mouse	Number of ME
1.00	100	5.00	1.92	10	0.50
10	66.2	05	1.89	10 1/2	0.53
15	38.3	10	1.86	11	0.56
20	26.4	15	1.83	11 1/2	0.59
25	20.7	20	1.80	12	0.62
30	16.5	30	1.74	12 1/2	0.65
35	13.9	40	1.69	13	0.675
40	11.9	45	1.67	13 1/2	0.70
45	10.4	50	1.64	14	0.73
50	9.33	6.00	1.60	14 1/2	0.76
55	8.42	15	1.54	15	0.785
2.00	7.67	30	1.48	15 1/2	0.81
05	7.04	45	1.43	16	0.84
10	6.52	7.00	1.39	16 1/2	0.86
15	6.06	15	1.35	17	0.88
20	5.66	30	1.31	17 1/2	0.905
25	5.32	45	1.28	18	0.93
30	5.00	8.00	1.25	18 1/2	0.95
35	4.73	15	1.22	19	0.97
40	4.48	30	1.20	19 1/2	0.985
45	4.26	45	1.18		
50	4.06	9.00	1.16	20	1.000
55	3.88	30	1.13		
3.00	3.70	10.00	1.11	20 1/2	1.015
05	3.57	30	1.09	21	1.03
10	3.43	11.00	1.075	21 1/2	1.04
15	3.31	30	1.06	22	1.05
20	3.19	12.00	1.05	22 1/2	1.06
25	3.08	13	1.03	23	1.07
30	2.98	14	1.015		
35	2.88	15	1.000		
40	2.79	16	0.99		
45	2.71	17	0.98		
50	2.63	18	0.972		
55	2.56	19	0.965		
4.00	2.50	20	0.96		
05	2.44	21	0.954		
10	2.38	22	0.948		
15	2.32	23	0.942		
20	2.26	24	0.937		
25	2.21	25	0.934		
30	2.16	30	0.917		
35	2.12	40	0.898		
40	2.08	60	0.875		
45	2.04				
50	2.00				
55	1.96				

Table 11

BIOTOXIN DILUTION DIAGRAM

Amount of extract in ml for dilution	2	2	2	2	2	2	2	2
Amount of distilled water in ml for dilution	0	1	2	3	4	5	6	7
Degree of dilution	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
1 ml extract of the above dilutions - if it has lethal effect on 20 g mice - corresponds at least to the following number of ME/ml undiluted extract	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
or at least to the following number of ME/100 g sample	200	300	400	500	600	700	800	900

By using this type of dilution diagram and Sommer's table I, the operator will, after orienting titration, with some experience, be able quickly to decide what dilution degree to apply to produce the time of death at 5-7 min.

Table 12

DEGREE OF BIOTOXIN DILUTION

Degree of dilution determined by orienting titration = F = 4.0 Final titration

Mouse No.	Weight of mouse before injection	Time of death (DT)	Number of ME corresp. to DT
1	19.9 g	5 min. 20 sec.	
2	19.5 g	4 min. 55 sec.	
3	19.8 g	5 min. 25 sec.	
		Σ DT = 15 min. 40 sec.	
		DT 1,2,3 = 5 min. 15 sec.	1.83 ME
Number of ME/ml = ME·F = 1.83 · 4.0 = 7.32 ME/ml Number of ME/100 g of mussel flesh = ME·F·200 = 7.32 · 200 = 1464 ME/100 g			

11. Chemical and other examination procedures

11.1 General

In addition to the pathogens and indicator organisms a certain number of other parameters must be monitored, even though they are only indirectly related to human health aspects of coastal water quality. Examples of such supporting parameters are salinity, oxygen, and transparency; and in effluents, biological oxygen demand and settleable matter may be of importance. These parameters may change the survival conditions of microorganisms in coastal water, in sediments or in shellfish and may significantly influence the microbiological counts and assessment of human health risks. In a direct way, several parameters such as odour and turbidity also influence at least the recreational value of coastal waters. Where supplementary parameters have been introduced, methods for their determination exist.

Parameters related more specifically to eutrophication, such as nutrients, phytoplankton production, and biomass will not be included here. In any case they are of prime interest in another context, and there is a whole programme designed for their study (MED V). Secondly, the complexity of the nutritional balance and the primary production in a coastal region is such that neither the sampli strategy already proposed nor the analytical capacity and the personnel requested would justify attempts to describe the eutrophication mechanisms as well. Only if plankton production becomes a nuisance must it be registered in the context of the present monitoring programmes.

Below, find analytical procedures for the following parameters:

Effluents: biochemical oxygen demand (BOD)
settleable matter (volumetric)

Seawater: salinity and chloride (hydrometric)
oxygen (Winkler method)

Except for oxygen the methods are reproduced from Standard Methods (10), pp. 54 et ff., which should be consulted for full references.

11.2 Biochemical oxygen demand, BOD5

11.2.1 Methodology

The samples are prepared in several different dilutions with oxygenated water, and stored in a waterbath at 20°C in darkness for five days. The oxygen content in the incubation bottles is determined before and after the five days, and the oxygen-depletion is calculated. From this amount the BOD5 can be calculated.

11.2.2 Reagents

Dilution water,
oxygenated distilled water or deionized water with addition of mineral salts,
cf. Standard Methods (10) which also give reagents for dissolved oxygen.

11.2.3 Procedure

Seeding is not required if the sample already contains microorganisms, which is normally the case with domestic wastewater, nonchlorinated effluents and surface waters. If few microorganisms are present, settled domestic wastewater should be used for seeding.

The BOD5 of the sample must be estimated prior to analysis. If no experience exists (e.g. from previous examinations) the following formula may give an adequate order of magnitude:

$$a = /3500/COD \text{ ml}$$

where: a = amount of sample, ml

/3500 = empirical number applicable to effluents with a medium to high BOD

COD = chemical oxygen demand, mg O_2/l, cf. Standard Methods p. 550.

Samples of 2a, a, and .5a ml/l dilution should be prepared to secure a BOD5 calculation based on 40-70% oxygen depletion in one of the incubation bottles. Two bottles are prepared for each solution

one is incubated for five days at 20±0.5°C in darkness and residual oxygen determined; the other bottle is analysed immediately for oxygen content. For any test series, a blank containing dilution water (a = 0) is also analysed.

11.2.4 BOD5 assessment

The BOD5 is assessed by use of the following formula:

$$BOD5 = (DO1-DO2)\ \frac{1000}{a} - (I1-I2)\ \frac{1000-a}{a}$$

where: BOD5 = 5 day biochemical oxygen demand, mg O_2/l

DO1 = mg O_2/l, dissolved oxygen in the sample dilution before incubation

DO2 = mg O_2/l, dissolved oxygen in the sample dilution after incubation

a = amount of sample, ml, per litre of sample dilution

I1 = mg O_2/l, dissolved oxygen in dilution water before incubation

I2 = mg O_2/l dissolved oxygen in dilution water after incubation.

Enter results on Form 5.

11.3 Settleable matter, volumetric test

Only a volumetric test is proposed. The sole equipment necessary for the determination in ml/litre is an Imhoff cone.

11.3.1 Procedure

Fill the Imhoff cone to the litre mark with a thoroughly mixed sample. Settle for 45 minutes, gently stir the sides of the cone with a rod or by spinning, settle 15 minutes longer, and record the volume of settleable matter in the cone as ml/lt. The practical lower limit is about 1 ml/litre/hr. Where a separation of settleable and floating materials occurs, do not estimate the floating material.

11.3.2 Result

The result is read directly after 45 minutes as ml/litre. Use Form 5 for recording.

11.4 Salinity, hydrometric method

The hydrometric method for salinity determination is reproduced here to give an easy alternative in cases where the salinity meter is not available or where operational problems have been encountered.

11.4.1 Principle

Salinity is determined by measuring specific gravity with a hydrometer, correcting for temperature, and converting specific gravity to salinity by means of salinity density tables.

11.4.2 Apparatus

(a) Hydrometer jar: use a special 400 mm tall jar with 45 mm inner diameter, or a rubber-stoppered transparent plastic tube with the same dimensions, or a 500 ml graduated cylinder.

(b) Thermometer, graduated in 0.2 c divisions.

(c) Hydrometer, seawater. For usual work a set of two, with specific gravity ranges 0.966-1.011, and 1.010-1.031 is needed. Hydrometer divisions should be 0.002. A set should be calibrated for specific gravity of NaCl solutions at 15/4 c.

11.4.3 Procedure

(a) Fill hydrometer jar two-thirds full of sample.

(b) While holding the jar vertically, place the thermometer and hydrometer in the jar.

(c) Read and record the temperature.

(d) Read and record the specific gravity. Estimate the fourth decimal place.

(e) Make temperature corrections for the specific gravity reading from the factors listed in Table 13.

11.4.4 Calculation

Determine salinity from Table 14. Locate corrected density and read salinity from opposite column. Report salinity as parts per thousand (‰).

11.5 Dissolved oxygen (Winkler, azide modification)

11.5.1 General discussion

A Winkler method (azide modification) is presented here so as to have an alternative method when the oxygen meter cannot be used, and as a calibration method to be used parallel to the oxygen meter, for occasional checking and adjustment. It should be noted that a more refined and elaborate methodology is prescribed in Standard Methods (10). In principle, there is no disagreement between the two methods, but for most applications the method below is adequate.

11.5.2 Reagents

(a) Manganese sulfate solution: dissolve 364 g Mn SO_4, 1 H_2O in distilled water, filter and dilute to 1 litre.

(b) Alkali-iodide-azide reagent: dissolve 500 g NaOH and 135 g Na I in distilled water and dilute to 1 litre. Then add 10 g sodium azide (NaN_3) dissolved in 40 ml distilled water.

(c) Conc. sulfuric acid, H_2SO_4.

(d) Starch solution: 5-6 g potato starch are emulsified in a mortar with a small quantity of distilled water. The emulsion is poured into 1 litre of boiling water, allowed to boil for a few minutes, and left to settle overnight. The clear supernatant is used.

(e) Sodium thiosulfate stock solution, 0.10 N: dissolved 24.82 g $Na_2S_2O_3$, 5 H_2O in boiled and cooled distilled water and dilute to 1 litre. Preserve by adding 5 ml chloroform or 1 g NaOH/litre.

(f) Standard sodium thiosulfate titrant, 0.005 N: dilute 50 ml of the above of stock solution to 1 litre.

11.5.3 Procedure

The 50 ml bottle received from the field contains a precipitate which is dissolved by adding 0.4 ml concentrated H_2SO_4. Then titrate 20 ml of the sample with the above titrant (f above) using the starch solution (d above) as the indicator, until the blue colour disappears. Amount of titrant = X ml. Back titration, if necessary, can be done by adding a measured extra volume of sample.

11.5.4 Calculation

The oxygen content of the sample, mg/litre, is obtained by means of the following formula:

$$\text{Dissolved oxygen mg/litre} = \frac{X.0.005.f.1000}{20} \cdot \frac{32}{4}$$

Where the first fraction indicates equivalents of titrant and the second fraction equivalent weight of oxygen, f is a correction factor for addition of reagents (0.4 ml) in the field, i.e., f is normally 50/49.2 = 1.016.

Using the numbers and volumes indicated means 2.03 mg oxygen per litre for 1 ml of titrant.

Enter results on Form 3.

Table 13

DIFFERENCES TO CONVERT HYDROMETER READINGS AT ANY TEMPERATURE TO DENSITY AT 15°C
(The table reading is in 0.0001 units of the observed reading.)

Observed Reading	Temperature of Water in Jar, C												
	−2.0	−1.0	0.0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
0.9960													
0.9970													
0.9980													
0.9990	−1	−2	−3	−4	−5	−5	−6	−6	−6	−6	−6	−5	−5
1.0000	−2	−3	−4	−5	−5	−6	−6	−6	−6	−6	−6	−5	−5
1.0010	−3	−4	−4	−5	−6	−6	−6	−7	−7	−6	−6	−6	−5
1.0020	−3	−4	−5	−6	−6	−7	−7	−7	−7	−7	−6	−6	−5
1.0030	−4	−5	−6	−6	−7	−7	−7	−7	−7	−7	−6	−6	−5
1.0040	−4	−5	−6	−7	−7	−7	−8	−8	−7	−7	−7	−6	−6
1.0050	−5	−6	−6	−7	−8	−8	−8	−8	−8	−7	−7	−6	−6
1.0060	−6	−6	−7	−8	−8	−8	−8	−8	−8	−8	−7	−6	−6
1.0070	−6	−7	−8	−8	−8	−8	−8	−8	−8	−8	−7	−7	−6
1.0080	−7	−8	−8	−9	−9	−9	−9	−9	−8	−8	−7	−7	−6
1.0090	−7	−8	−9	−9	−9	−9	−9	−9	−9	−8	−8	−7	−6
1.0100	−8	−9	−9	−10	−10	−10	−10	−9	−9	−8	−8	−7	−6
1.0110	−9	−9	−10	−10	−10	−10	−10	−10	−9	−9	−8	−7	−6
1.0120	−9	−10	−10	−10	−10	−10	−10	−10	−10	−9	−8	−7	−7
1.0130	−10	−10	−11	−11	−11	−11	−11	−10	−10	−9	−8	−8	−7
1.0140	−10	−11	−11	−11	−11	−11	−11	−11	−10	−10	−9	−8	−7
1.0150	−11	−11	−12	−12	−12	−12	−11	−11	−10	−10	−9	−8	−7
1.0160	−12	−12	−12	−12	−12	−12	−12	−11	−11	−10	−9	−8	−7
1.0170	−12	−12	−12	−13	−13	−12	−12	−12	−11	−10	−9	−8	−7
1.0180	−13	−13	−13	−13	−13	−13	−12	−12	−11	−10	−9	−8	−7
1.0190	−13	−13	−14	−14	−13	−13	−13	−12	−12	−11	−10	−9	−8
1.0200	−14	−14	−14	−14	−14	−13	−13	−12	−12	−11	−10	−9	−8
1.0210	−14	−14	−14	−14	−14	−14	−13	−13	−12	−11	−10	−9	−8
1.0220	−15	−15	−15	−15	−15	−14	−14	−13	−12	−11	−10	−9	−8
1.0230	−15	−15	−15	−15	−15	−15	−14	−13	−12	−12	−10	−9	−8
1.0240	−16	−16	−16	−16	−15	−15	−14	−14	−13	−12	−11	−10	−8
1.0250	−16	−16	−16	−16	−16	−15	−15	−14	−13	−12	−11	−10	−8
1.0260	−17	−17	−17	−16	−16	−16	−15	−14	−13	−12	−11	−10	−8
1.0270	−18	−17	−17	−17	−17	−16	−15	−14	−14	−12	−11	−10	−9
1.0280	−18	−18	−18	−17	−17	−16	−16	−15	−14	−13	−11	−10	−9
1.0290	−19	−18	−18	−18	−17	−17	−16	−15	−14	−13	−12	−10	−9
1.0300	−19	−19	−19	−18	−18	−17	−16	−15	−14	−13	−12	−10	−9
1.0310	−20	−19	−19	−19	−18	−17	−16	−16	−15	−13	−12	−10	−9

Table 13 (contd)

Observed Reading	Temperature of Water in Jar, *C*											
	11.0	12.0	13.0	14.0	15.0	16.0	17.0	18.0	18.5	19.0	19.5	20.0
0.9960												
0.9970												
0.9980							3	4	5	6	7	8
0.9990	—4	—3	—2	—1	0	1	3	4	5	6	7	8
1.0000	—4	—3	—2	—1	0	1	3	4	5	6	7	8
1.0010	—4	—3	—2	—1	0	1	3	4	5	6	7	8
1.0020	—4	—3	—2	—1	0	1	3	4	5	6	7	8
1.0030	—4	—3	—2	—1	0	1	3	4	5	6	7	8
1.0040	—5	—4	—3	—1	0	2	3	5	6	6	7	8
1.0050	—5	—4	—3	—1	0	2	3	5	6	7	8	9
1.0060	—5	—4	—3	—1	0	2	3	5	6	7	8	9
1.0070	—5	—4	—3	—2	0	2	3	5	6	7	8	9
1.0080	—5	—4	—3	—2	0	2	3	5	6	7	8	9
1.0090	—5	—4	—3	—2	0	2	3	5	6	7	8	9
1.0100	—5	—4	—3	—2	0	2	3	5	6	7	8	9
1.0110	—5	—4	—3	—2	0	2	3	5	6	7	8	9
1.0120	—6	—4	—3	—2	0	2	3	5	6	7	8	9
1.0130	—6	—4	—3	—2	0	2	4	5	6	7	8	10
1.0140	—6	—4	—3	—2	0	2	4	5	6	8	9	10
1.0150	—6	—4	—3	—2	0	2	4	5	6	8	9	10
1.0160	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0170	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0180	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0190	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0200	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0210	—6	—5	—3	—2	0	2	4	6	7	8	9	10
1.0220	—7	—5	—3	—2	0	2	4	6	7	8	9	11
1.0230	—7	—5	—4	—2	0	2	4	6	7	8	9	11
1.0240	—7	—5	—4	—2	0	2	4	6	7	8	10	11
1.0250	—7	—5	—4	—2	0	2	4	6	7	8	10	11
1.0260	—7	—5	—4	—2	0	2	4	6	7	9	10	11
1.0270	—7	—5	—4	—2	0	2	4	6	7	9	10	11
1.0280	—7	—6	—4	—2	0	2	4	6	8	9	10	11
1.0290	—7	—6	—4	—2	0	2	4	6	8	9	10	11
1.0300	—7	—6	—4	—2	0	2	4	6	8	9	10	12
1.0310	—8	—6	—4	—2	0	2	4					

Table 13 (contd)

Observed Reading	Temperature of Water in Jar, C												
	20.5	21.0	21.5	22.0	22.5	23.0	23.5	24.0	24.5	25.0	25.5	26.0	26.5
0.9960											19	20	21
0.9970			10	11	12	14	15	16	17	18	19	20	22
0.9980	9	10	11	12	13	14	15	16	17	18	19	21	22
0.9990	9	10	11	12	13	14	15	16	17	18	20	21	22
1.0000	9	10	11	12	13	14	15	16	17	19	20	21	22
1.0010	9	10	11	12	13	14	15	17	18	19	20	21	23
1.0020	9	10	11	12	13	14	16	17	18	19	20	22	23
1.0030	9	10	11	12	13	15	16	17	18	19	21	22	23
1.0040	9	10	11	12	14	15	16	17	18	20	21	22	23
1.0050	10	11	12	13	14	15	16	17	19	20	21	22	24
1.0060	10	11	12	13	14	15	16	18	19	20	21	23	24
1.0070	10	11	12	13	14	15	17	18	19	20	21	23	24
1.0080	10	11	12	13	14	16	17	18	19	20	22	23	24
1.0090	10	11	12	13	15	16	17	18	19	21	22	23	25
1.0100	10	11	12	14	15	16	17	18	20	21	22	24	25
1.0110	10	12	13	14	15	16	17	19	20	21	22	24	25
1.0120	10	12	13	14	15	16	18	19	20	21	23	24	25
1.0130	11	12	13	14	15	16	18	19	20	22	23	24	26
1.0140	11	12	13	14	15	17	18	19	20	22	23	24	26
1.0150	11	12	13	14	16	17	18	20	21	22	23	25	26
1.0160	11	12	13	14	16	17	18	20	21	22	24	25	26
1.0170	11	12	13	15	16	17	18	20	21	22	24	25	27
1.0180	11	12	14	15	16	17	19	20	21	23	24	25	27
1.0190	11	12	14	15	16	18	19	20	21	23	24	26	27
1.0200	11	13	14	15	16	18	19	20	22	23	24	26	27
1.0210	12	13	14	15	17	18	19	21	22	23	25	26	27
1.0220	12	13	14	15	17	18	19	21	22	23	25	26	28
1.0230	12	13	14	16	17	18	20	21	22	24	25	26	28
1.0240	12	13	14	16	17	18	20	21	22	24	25	27	28
1.0250	12	13	15	16	17	18	20	21	23	24	25	27	28
1.0260	12	13	15	16	17	19	20	22	23	24	26	27	29
1.0270	12	14	15	16	17	19	20	22	23	24	26	27	29
1.0280	12	14	15	16	18	19	20	22	23	25	26	28	29
1.0290	13	14	15	16	18	19	21	22	23				
1.0300	13	14	15	16	18								
1.0310													

Table 13 (contd)

Observed Reading	Temperature of Water in Jar, C												
	27.0	27.5	28.0	28.5	29.0	29.5	30.0	30.5	31.0	31.5	32.0	32.5	33.0
0.9960	23	24	25	27	28	29	31	32	34	35	37	38	40
0.9970	23	24	26	27	28	30	31	33	34	36	37	39	40
0.9980	23	25	26	27	29	30	31	33	34	36	38	39	41
0.9990	24	25	26	28	29	30	32	33	35	36	38	39	41
1.0000	24	25	26	28	29	31	32	34	35	37	38	40	41
1.0010	24	25	27	28	30	31	32	34	35	37	39	40	42
1.0020	24	26	27	28	30	31	33	34	36	37	39	41	42
1.0030	25	26	27	29	30	32	33	35	36	38	39	41	42
1.0040	25	26	28	29	30	32	33	35	36	38	40	41	43
1.0050	25	26	28	29	31	32	34	35	37	38	40	42	43
1.0060	25	27	28	30	31	32	34	36	37	39	40	42	44
1.0070	26	27	28	30	31	33	34	36	38	39	41	42	44
1.0080	26	27	29	30	32	33	35	36	38	39	41	43	44
1.0090	26	28	29	30	32	33	35	36	38	40	41	43	45
1.0100	26	28	29	31	32	34	35	37	38	40	42	43	45
1.0110	27	28	30	31	32	34	36	37	39	40	42	44	45
1.0120	27	28	30	31	33	34	36	37	39	41	42	44	46
1.0130	27	29	30	32	33	35	36	38	39	41	43	44	46
1.0140	27	29	30	32	33	35	36	38	40	41	43	45	46
1.0150	28	29	31	32	34	35	37	38	40	42	43	45	47
1.0160	28	29	31	32	34	35	37	39	40	42	44	45	47
1.0170	28	30	31	33	34	36	37	39	40	42	44	46	47
1.0180	28	30	31	33	34	36	38	39	41	42	44	46	48
1.0190	29	30	32	33	35	36	38	39	41	43	44	46	48
1.0200	29	30	32	33	35	37	38	40	41	43	45	47	48
1.0210	29	31	32	34	35	37	38	40	42	43	45	47	49
1.0220	29	31	32	34	36	37	39	40	42	44	45	47	49
1.0230	30	31	33	34	36	37	39	41	42	44	46	47	49
1.0240	30	31	33	34	36	37	39	41	42	44	46	48	49
1.0250	30	31	33	35	36	38	39	41	43	44	46	48	50
1.0260	30	32	33	35	37	38	40	41	43	45	46	48	50
1.0270	30	32	34	35	37	38	40						
1.0280	31	32											
1.0290													
1.0300													
1.0310													

Table 14

CORRESPONDING DENSITIES AND SALINITIES

Density	Salinity	Density	Salinity	Density	Salinity	Density	Salinity
0.9991	0.0	1.0036	5.8	1.0081	11.6	1.0126	17.5
0.9992	0.0	1.0037	5.9	1.0082	11.8	1.0127	17.7
0.9993	0.2	1.0038	6.0	1.0083	11.9	1.0128	17.8
0.9994	0.3	1.0039	6.2	1.0084	12.0	1.0129	17.9
0.9995	0.4	1.0040	6.3	1.0085	12.2	1.0130	18.0
0.9996	0.6	1.0041	6.4	1.0086	12.3	1.0131	18.2
0.9997	0.7	1.0042	6.6	1.0087	12.4	1.0132	18.3
0.9998	0.8	1.0043	6.7	1.0088	12.6	1.0133	18.4
0.9999	0.9	1.0044	6.8	1.0089	12.7	1.0134	18.6
1.0000	1.1	1.0045	6.9	1.0090	12.8	1.0135	18.7
1.0001	1.2	1.0046	7.1	1.0091	12.9	1.0136	18.8
1.0002	1.3	1.0047	7.2	1.0092	13.1	1.0137	19.0
1.0003	1.5	1.0048	7.3	1.0093	13.2	1.0138	19.1
1.0004	1.6	1.0049	7.5	1.0094	13.3	1.0139	19.2
1.0005	1.7	1.0050	7.6	1.0095	13.5	1.0140	19.3
1.0006	1.9	1.0051	7.7	1.0096	13.6	1.0141	19.5
1.0007	2.0	1.0052	7.9	1.0097	13.7	1.0142	19.6
1.0008	2.1	1.0053	8.0	1.0098	13.9	1.0143	19.7
1.0009	2.2	1.0054	8.1	1.0099	14.0	1.0144	19.9
1.0010	2.4	1.0055	8.2	1.0100	14.1	1.0145	20.0
1.0011	2.5	1.0056	8.4	1.0101	14.2	1.0146	20.1
1.0012	2.6	1.0057	8.5	1.0102	14.4	1.0147	20.3
1.0013	2.8	1.0058	8.6	1.0103	14.5	1.0148	20.4
1.0014	2.9	1.0059	8.8	1.0104	14.6	1.0149	20.5
1.0015	3.0	1.0060	8.9	1.0105	14.8	1.0150	20.6
1.0016	3.2	1.0061	9.0	1.0106	14.9	1.0151	20.8
1.0017	3.3	1.0062	9.2	1.0107	15.0	1.0152	20.9
1.0018	3.4	1.0063	9.3	1.0108	15.2	1.0153	21.0
1.0019	3.5	1.0064	9.4	1.0109	15.3	1.0154	21.2
1.0020	3.7	1.0065	9.6	1.0110	15.4	1.0155	21.3
1.0021	3.8	1.0066	9.7	1.0111	15.6	1.0156	21.4
1.0022	3.9	1.0067	9.8	1.0112	15.7	1.0157	21.6
1.0023	4.1	1.0068	9.9	1.0113	15.8	1.0158	21.7
1.0024	4.2	1.0069	10.1	1.0114	16.0	1.0159	21.8
1.0025	4.3	1.0070	10.2	1.0115	16.1	1.0160	22.0
1.0026	4.5	1.0071	10.3	1.0116	16.2	1.0161	22.1
1.0027	4.6	1.0072	10.5	1.0117	16.3	1.0162	22.2
1.0028	4.7	1.0073	10.6	1.0118	16.5	1.0163	22.4
1.0029	4.8	1.0074	10.7	1.0119	16.6	1.0164	22.5
1.0030	5.0	1.0075	10.8	1.0120	16.7	1.0165	22.6
1.0031	5.1	1.0076	11.0	1.0121	16.9	1.0166	22.7
1.0032	5.2	1.0077	11.1	1.0122	17.0	1.0167	22.9
1.0033	5.4	1.0078	11.2	1.0123	17.1	1.0168	23.0
1.0034	5.5	1.0079	11.4	1.0124	17.3	1.0169	23.1
1.0035	5.6	1.0080	11.5	1.0125	17.4	1.0170	23.3

Table 14 (contd)

Density	Salinity	Density	Salinity	Density	Salinity	Density	Salinity
1.0171	23.4	1.0211	28.6	1.0251	33.8	1.0291	39.0
1.0172	23.5	1.0212	28.8	1.0252	34.0	1.0292	39.2
1.0173	23.7	1.0213	28.9	1.0253	34.1	1.0293	39.3
1.0174	23.8	1.0214	29.0	1.0254	34.2	1.0294	39.4
1.0175	23.9	1.0215	29.1	1.0255	34.4	1.0295	39.6
1.0176	24.1	1.0216	29.3	1.0256	34.5	1.0296	39.7
1.0177	24.2	1.0217	29.4	1.0257	34.6	1.0297	39.8
1.0178	24.3	1.0218	29.5	1.0258	34.8	1.0298	39.9
1.0179	24.4	1.0219	29.7	1.0259	34.9	1.0299	40.1
1.0180	24.6	1.0220	29.8	1.0260	35.0	1.0300	40.2
1.0181	24.7	1.0221	29.9	1.0261	35.1	1.0301	40.3
1.0182	24.8	1.0222	30.1	1.0262	35.3	1.0302	40.4
1.0183	25.0	1.0223	30.2	1.0263	35.4	1.0303	40.6
1.0184	25.1	1.0224	30.3	1.0264	35.5	1.0304	40.7
1.0185	25.2	1.0225	30.4	1.0265	35.7	1.0305	40.8
1.0186	25.4	1.0226	30.6	1.0266	35.8	1.0306	41.0
1.0187	25.5	1.0227	30.7	1.0267	35.9	1.0307	41.1
1.0188	25.6	1.0228	30.8	1.0268	36.0	1.0308	41.2
1.0189	25.8	1.0229	31.0	1.0269	36.2	1.0309	41.4
1.0190	25.9	1.0230	31.1	1.0270	36.3	1.0310	41.5
1.0191	26.0	1.0231	31.2	1.0271	36.4	1.0311	41.6
1.0192	26.1	1.0232	31.4	1.0272	36.6	1.0312	41.7
1.0193	26.3	1.0233	31.5	1.0273	36.7	1.0313	41.9
1.0194	26.4	1.0234	31.6	1.0274	36.8	1.0314	42.0
1.0195	26.5	1.0235	31.8	1.0275	37.0	1.0315	42.1
1.0196	26.7	1.0236	31.9	1.0276	37.1	1.0316	42.3
1.0197	26.8	1.0237	32.0	1.0277	37.2	1.0317	42.4
1.0198	26.9	1.0238	32.1	1.0278	37.3	1.0318	42.5
1.0199	27.1	1.0239	32.3	1.0279	37.5	1.0319	42.7
1.0200	27.2	1.0240	32.4	1.0280	37.6	1.0320	42.8
1.0201	27.3	1.0241	32.5	1.0281	37.7		
1.0202	27.5	1.0242	32.7	1.0282	37.9		
1.0203	27.6	1.0243	32.8	1.0283	38.0		
1.0204	27.7	1.0244	32.9	1.0284	38.1		
1.0205	27.8	1.0245	33.1	1.0285	38.2		
1.0206	28.0	1.0246	33.2	1.0286	38.4		
1.0207	28.1	1.0247	33.3	1.0287	38.5		
1.0208	28.2	1.0248	33.5	1.0288	38.6		
1.0209	28.4	1.0249	33.6	1.0289	38.8		
1.0210	28.5	1.0250	33.7	1.0290	38.9		

11.5.5 Saturation percentage

Use the tables below to obtain oxygen saturation in percent at 760 millibars atmospheric pressure.

A correction for chlorine content (mg ce^{-}/litre) is necessary and may be done approximately by using the salinity already established for the same sampling position and depth:

$$\text{Cl g/litre} = \text{salinity } ‰ \frac{\text{density kg/litre}}{1.807}$$

where salinity as well as density are known from section 11.3 and Table 14.

Tables for conversion from salinity to chlorine content (g/litre) are given in Standard Methods (10) (pp. 109-120) and may substitute the above equation.

Oxygen saturation is determined from the tables below when chlorine content and sample temperature are used as entries:

$$\% \text{ oxygen saturation} = \frac{\text{DO mg/l x 100}}{\text{DO (from Table 15)}}$$

Enter results on Form 3.

12. Recording of data

Essentially, data is recorded by using standard forms such as those presented in Annex IV.

The overall aim is to assist in easy and controlled working procedures during field monitoring or laboratory analysis. Particular emphasis is on reliable identification of each sample or observation. The forms are not used directly for subsequent data analyses.

Table 16 has been produced to illustrate the kind of reorganization that may be useful before statistical or other analysis of data, and is based on information extracted from standard forms such as Forms 3 and 8. It should be noted that observation dates have been excluded from the table, even though they are available and actually used to combine data on wind, currents and faecal coliform observations. The example given in the table relates to a situation in the Sound (the straits lying between Denmark and Sweden) where monitoring has been carried out for many years (5), and is used here for the sake of convenience. The monitoring points reported are situated close to the beaches north of Copenhagen (6).

Once data have been recorded and the basic standard forms have been filed for easy retrieval, the manner of additional processing for them depends on the application to which they will be put and the needs of the particular problem for analysis. Without carefully defined applications, further processing of data should be discouraged. However, any monitoring programme should be carefully and regularly reassessed and where necessary redesigned (see Chapter 15).

13. Statistical evaluation

13.1 General

Monitoring usually creates huge amounts of data that must be handled in orderly fashion and often by using advanced methods of condensing data and presentation of results. Statistical methods can to a certain extent assist in the summarizing process, and may also help to sort out facts and assign significant probabilities.

A few statistical methods of wide applicability will be presented below. It is, however, advisable to seek information from existing literature as to general applicability of the methods suggested below, and as to other methods not included.

It should be emphasized that some statistical evaluation will be essential given the comprehensiveness of the monitoring programmes already envisaged.

Table 15

SOLUBILITY OF OXYGEN IN WATER EXPOSED TO WATER-SATURATED AIR

Temperature	Chloride Concentration in Water *mg/l*					Difference /100 mg
C	0	5,000	10,000	15,000	20,000	Chloride
	Dissolved Oxygen *mg/l*					
0	14.6	13.8	13.0	12.1	11.3	0.017
1	14.2	13.4	12.6	11.8	11.0	0.016
2	13.8	13.1	12.3	11.5	10.8	0.015
3	13.5	12.7	12.0	11.2	10.5	0.015
4	13.1	12.4	11.7	11.0	10.3	0.014
5	12.8	12.1	11.4	10.7	10.0	0.014
6	12.5	11.8	11.1	10.5	9.8	0.014
7	12.2	11.5	10.9	10.2	9.6	0.013
8	11.9	11.2	10.6	10.0	9.4	0.013
9	11.6	11.0	10.4	9.8	9.2	0.012
10	11.3	10.7	10.1	9.6	9.0	0.012
11	11.1	10.5	9.9	9.4	8.8	0.011
12	10.8	10.3	9.7	9.2	8.6	0.011
13	10.6	10.1	9.5	9.0	8.5	0.011
14	10.4	9.9	9.3	8.8	8.3	0.010
15	10.2	9.7	9.1	8.6	8.1	0.010
16	10.0	9.5	9.0	8.5	8.0	0.010
17	9.7	9.3	8.8	8.3	7.8	0.010
18	9.5	9.1	8.6	8.2	7.7	0.009
19	9.4	8.9	8.5	8.0	7.6	0.009
20	9.2	8.7	8.3	7.9	7.4	0.009
21	9.0	8.6	8.1	7.7	7.3	0.009
22	8.8	8.4	8.0	7.6	7.1	0.008
23	8.7	8.3	7.9	7.4	7.0	0.008
24	8.5	8.1	7.7	7.3	6.9	0.008
25	8.4	8.0	7.6	7.2	6.7	0.008
26	8.2	7.8	7.4	7.0	6.6	0.008
27	8.1	7.7	7.3	6.9	6.5	0.008
28	7.9	7.5	7.1	6.8	6.4	0.008
29	7.8	7.4	7.0	6.6	6.3	0.008
30	7.6	7.3	6.9	6.5	6.1	0.008

Table 16

SUMMARIZED DATA ON MONITORING OF FAECAL COLIFORMS (FROM TWO DIFFERENT SAMPLING POINTS AND TWO DIFFERENT PERIODS AT THE SAME POINT)

Point 142, 1967			Point 142, 1971			Point 141, 1967		
Faecal coliforms MPN/100 ml	Current direction (from)	Wind direction (from)	Faecal coliforms MPN/100 ml	Current direction (from)	Wind direction (from)	Faecal coliforms MPN/100 ml	Current direction (from)	Wind direction (from)
79	N	W	13	S	S	130	N	W
540	S	W	17	S	SS	1400	S	W
1100	S	S	33	N	E	540	S	S
350	S	E	2	S	S	350	S	E
8	S	SW	49	N	NW	23	S	SW
33	SE	NE	13	N	NW	220	SE	NE
33	S	S	8	S	SW	5	S	S
14	S	S	350	N	W	13	S	S
113	S	W	240	N	W	9	S	W
170	N	W	49	N	W	330	N	W
280	S	S	11	N	W	240	S	S
33	S	W	1600	N	W	17	S	W
170	S	E	46	N	E	27	S	E
130	S	W	13	S	SE	49	S	W
430	S	S	46	N	NW	260	S	S
33	N	W	350	S	N	23	N	W
220	S	S	22	S	SW	240	S	S
33	S	S	17	S	W	22	S	S
110	N	N	700	N	N	130	N	N
350	S	S	11	N	W	350	S	S

13.2 Distributions, probability paper

Application of statistical tools to existing observation data is greatly enhanced if a known statistical distribution can be used. The possibility of fitting data, either directly or by transformation, to the normal distribution should always be examined carefully.

One way of estimating the applicability of the normal distribution is by use of probability paper which may be done, for example, by using arithmetical, as well as logarithmic abscissas versus probability ordinates (cf. Figs 25-26). If a truly normal distribution is plotted on probability paper it will always show a single straight line; if a straight line results from the plotting of a certain set of data on the probability paper the hypothesis of this data belonging to a normal distribution is possible (but not proven).

In Figures 25-27, data from Table 16 have been plotted on logarithmic probability paper. It should be noted that plotting has taken place after rearranging the original observations according to values (concentrations of faecal coliforms) and applying the Hazen formula (Fig. 25).

$$P_i = \frac{i - 0.5}{n} \cdot 100\% \qquad (1)$$

where: n = number of observations

i = enumerator, $1 \leq i \leq m$

P_i = probability % to be plotted versus observations X_i, Y_i, and Z_i or transformations hereof.

Also, after several trials, the logarithmic transformations turned out to yield the best linear fits, and the logarithms have therefore been listed in Table 17.

In cases where the linear fit seems poor there may be reasons to try different data transformations. If a linear fit is still not obtained, other methods for characterizing the data should be attempted, and statistical handbooks should be consulted.

Here it is assumed that the data of Table 17 adequately represent three normal distributions, where log values are used. For convenience X, Y and Z will mean logarithms of X, Y, and Z, unless otherwise indicated.

13.3 Average and empirical variance

The average and the empirical variance may be calculated as follows:

$$\text{Average} \quad \bar{X} = \sum_1^n X_i/n \qquad (2)$$

$$\text{Empirical variance} \quad s_x^2 = \sum_1^n (X_i - \bar{X})^2/(n-1) \qquad (3)$$

Averages and empirical variances are always calculated from a limited number of observations, i.e., these observations represent the real situation only to a certain extent, the poorer the lower the number of observations.

If the real situation were monitored continuously, the mean μ_x and the variance σ^2 would appear immediately (true average and variance). The normal distribution is completely defined by these two parameters, and hence the real situation is known completely and described by utilizing existing tables on normal distribution. For example, probabilities of certain concentrations of faecal coli could be explicitly expressed by using the tabulated normal distribution.

However, the assessment of a situation must normally be based on samples, not on continuous monitoring results. Consequently $\bar{X}$ and s_x^2 are only obtained, and they must be used to estimate μ_x and σ^2; luckily enough $\bar{X}$ and s_x^2 are good estimates of μ_x and σ_x^2, the higher the number of observations the better - if the distribution is normal.

Averages and empirical variations may be adequate parameters for summarizing a number of observations, for example, for a whole recreational season, if the distribution is fairly normal.

13.4 Testing significance of deviating averages

It is often of interest to test whether two different observation sites differ significantly from each other in terms of water quality. Or it may be of interest to know whether significant changes may be seen over time, for example, as a consequence of improved techniques for wastewater disposal.

Fig. 25

FAECAL COLIFORM DISTRIBUTION ON A BEACH NORTH OF COPENHAGEN, 1967

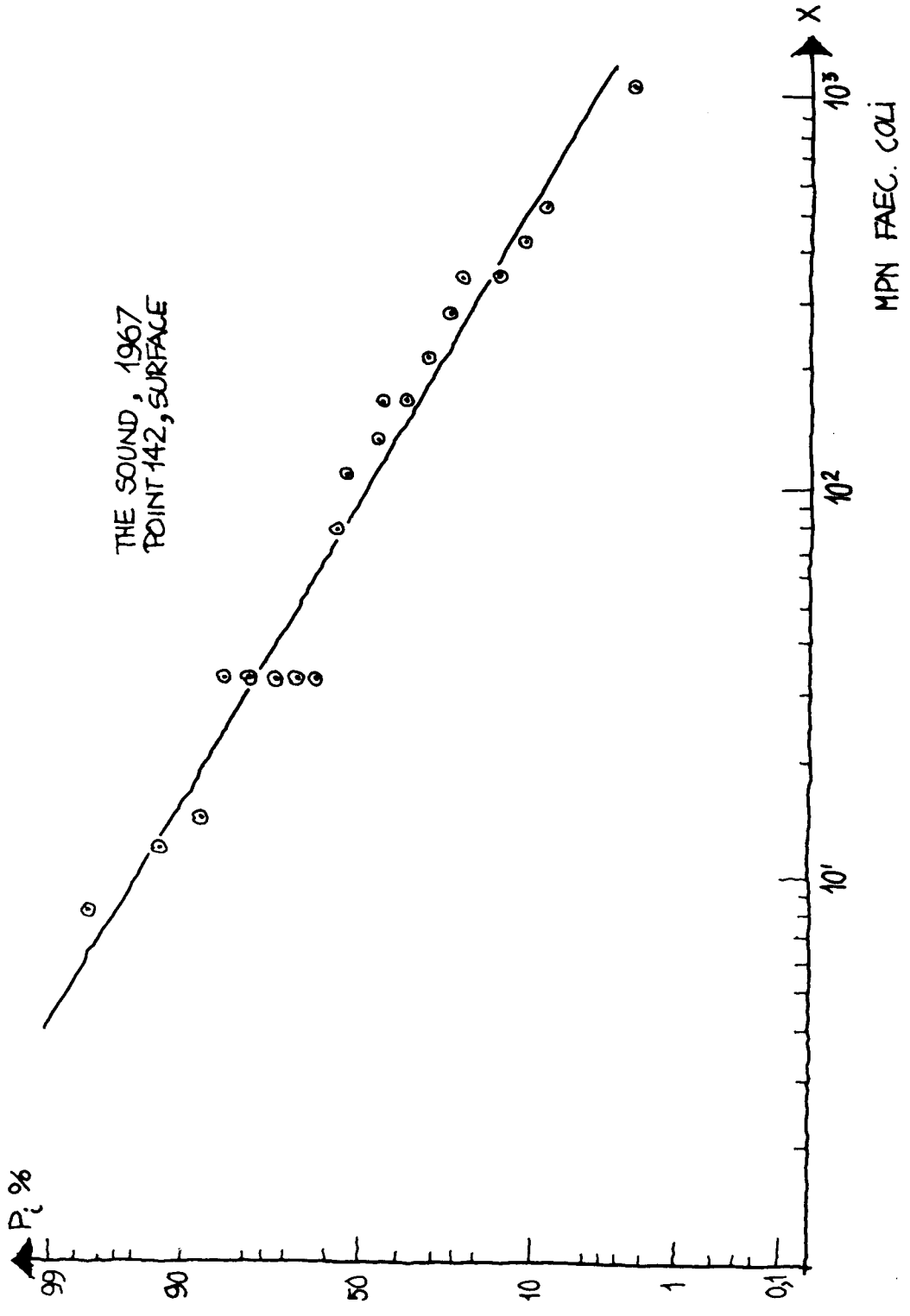


Fig. 26

FAECAL COLIFORM DISTRIBUTION ON A BEACH NORTH OF COPENHAGEN, 1971

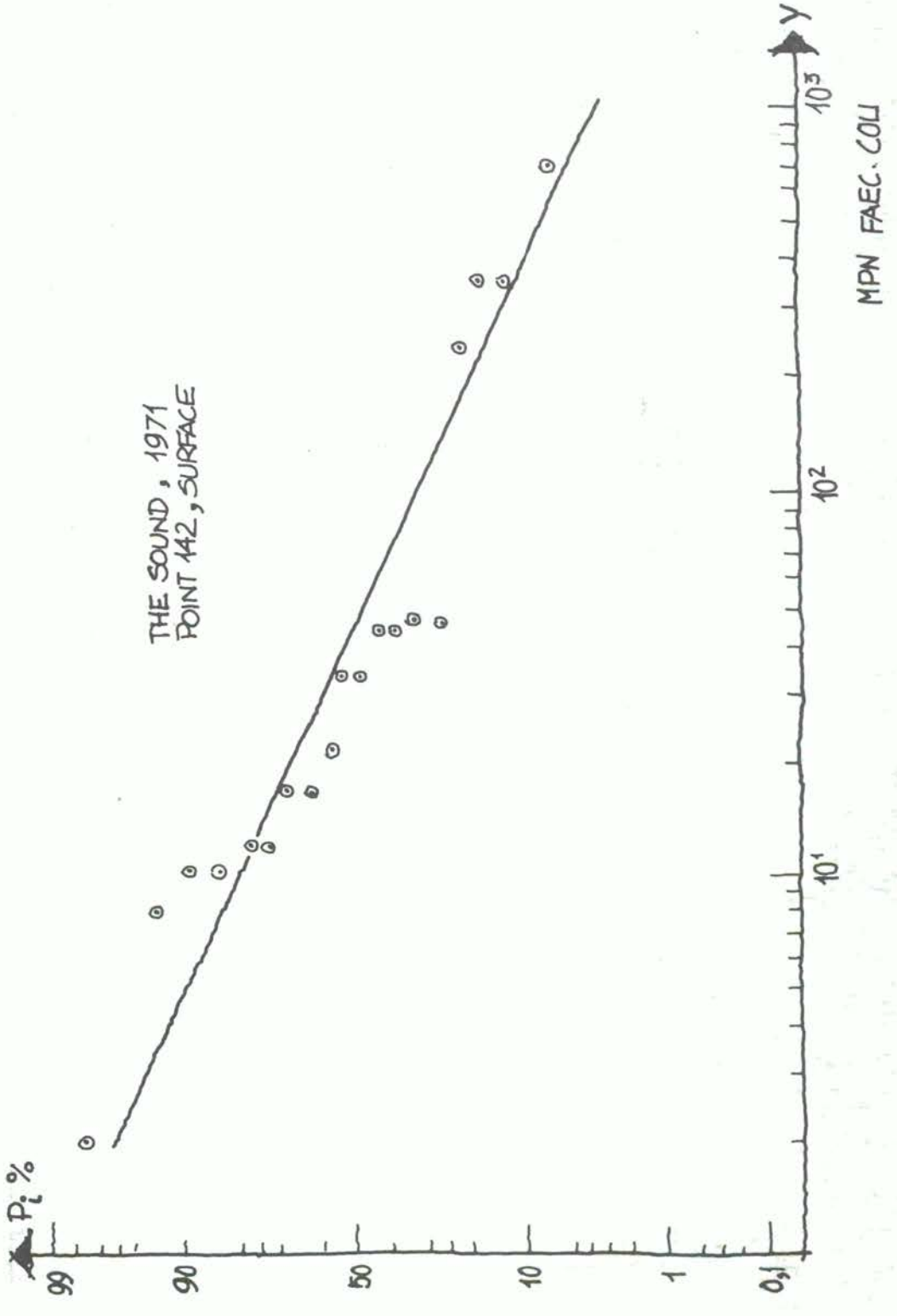


Fig. 27

FAECAL COLIFORM DISTRIBUTION ON A SECOND BEACH NORTH OF COPENHAGEN, 1967

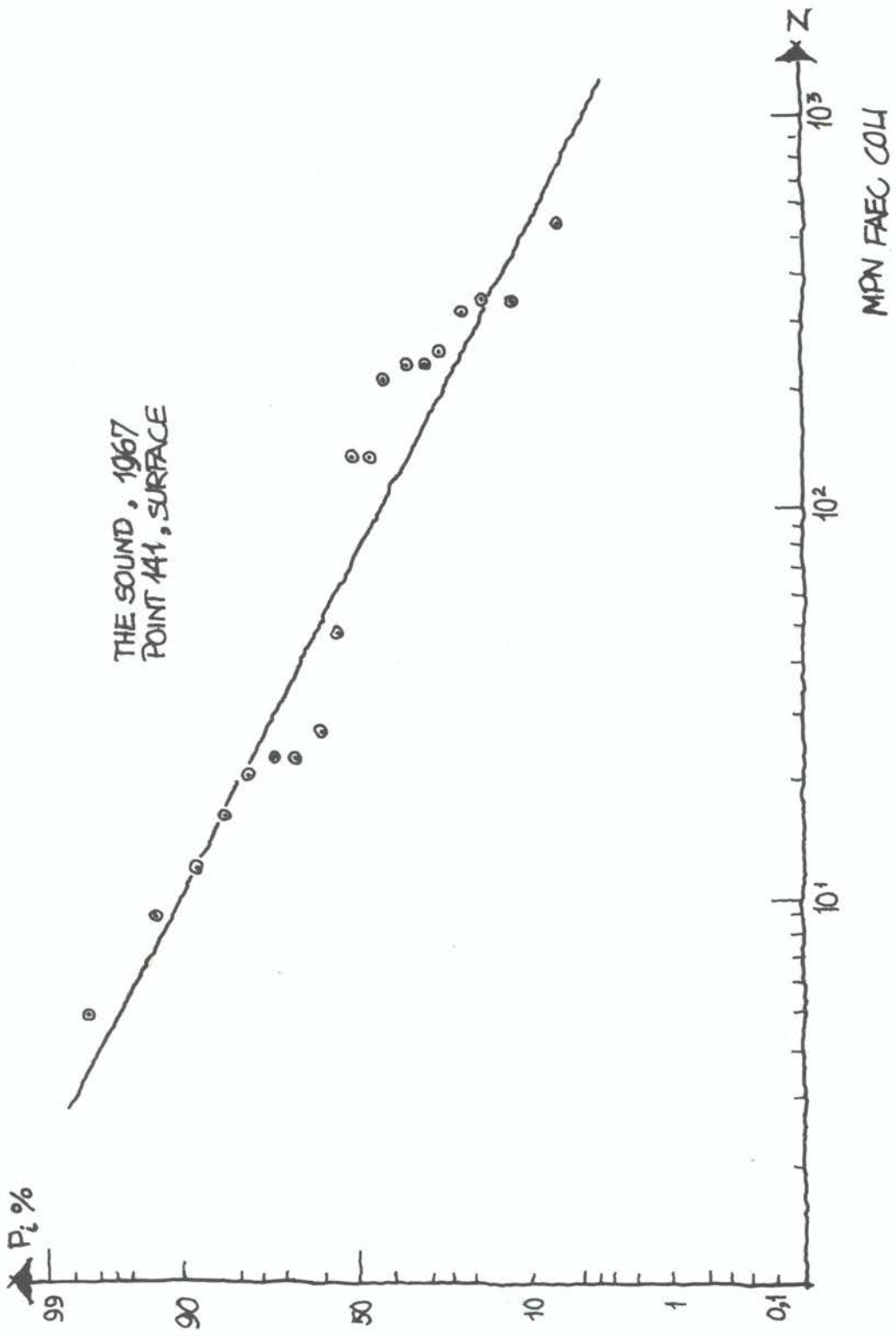


Table 17

TRANSFORMATION OF DATA FROM TABLE 16 FOR PLOTTING ON PROBABILITY PAPER

i	P_i%	X_i*	log X_i*	Y_i*	log Y_i**	Z_i*	log Z_i**
20	97.5	8	0.903	2	0.301	5	0.699
19	92.5	13	1.114	8	0.903	9	0.954
18	87.5	14	1.146	11	1.041	13	1.114
17	82.5	33	1.519	11	1.041	17	1.230
16	77.5	33	1.519	13	1.114	22	1.342
15	72.5	33	1.519	13	1.114	23	1.362
14	67.5	33	1.519	17	1.230	23	1.362
13	62.5	33	1.519	17	1.230	27	1.431
12	57.5	79	1.898	22	1.342	49	1.690
11	52.5	110	2.041	33	1.519	130	2.114
10	47.5	130	2.114	33	1.519	130	2.114
9	42.5	170	2.230	46	1.663	220	2.342
8	37.5	170	2.230	46	1.663	240	2.380
7	32.5	220	2.342	49	1.690	240	2.380
6	27.5	280	2.447	49	1.690	260	2.415
5	22.5	350	2.544	240	2.380	330	2.519
4	17.5	350	2.544	350	2.544	350	2.544
3	12.5	430	2.633	350	2.544	350	2.544
2	7.5	540	2.732	700	2.845	540	2.732
1	2.5	1100	3.041	1600	3.204	1400	3.146
Average (log)			1.978		1.629		1.921
s^2 (log)			0.364		0.534		0.472
s (log)			0.604		0.731		0.687

Notes: * X, Y and Z = Faecal coli MPN/100 ml

** log means $\log_{10}$ (base 10)

To distinguish the different sets of observations, the terms X, Y and Z have been adopted (X = point 142, 1967, Y = point 142, 1971, and Z = point 141, 1967, cf. Table 16).

Based on the data of Table 17 (log transformations of original observations, Table 16) such tests of significance may be demonstrated. The so-called t-test will be applied, but it should be stressed that statistical text-books should be consulted to understand better the applicability and limitations of this test procedure; t is tabulated and a simplified version of such a table is reproduced in Table 18.

General t-test procedure and formulae

1. Test hypothesis H_o: the two series X and Z of samples represent the same situation, $\mu_x = \mu_z$.

2. Critical area of test parameter t(r), i.e., the region where H_o is rejected, is defined through the following inequality:

$$t(r)_{1-\alpha/2} < t < t(r)_{\alpha/2} \qquad (4)$$

where:
$$t = \frac{(X - Z) - (\mu_x - \mu_z)}{s_x^2/m_x + s_z^2/n_z} \qquad (5)$$

X, Z, s_x^2, s_z^2, n_x, and n_z are

defined above, cf. (1), (2) and (3).

$$r^{-1} = c^2/(n_x - 1) + (1 - c)^2/(n_z - 1) \qquad (6)$$

$$c = s_x^2/n_x(s_x^2/n_x + s_z^2/m_z \qquad (7)$$

α = level of significance, e.g. 10%, i.e., the hypothesis H_o is rejected erroneously in not more than 10% of cases where it ought to be accepted.

It should be noted that X and Z applied in the formulae have been chosen arbitrarily; they could represent observations of any two populations (assumed normal), which are going to be tested.

Examples of t-test application

The t-test could be used to test the hypothesis (H_o) that observations X and Z (points 142 and 141, year 1967, Table 16) do not significantly deviate, say at the α = 10% level. Further parameters and variables in the t-test are now as follows:

$X = 1.978$ (Table 17)

$Z = 1.921$ (Table 17)

$s_x^2 = 0.364$ (Table 17)

$s_z^2 = 0.472$ (Table 17)

$n_x = 20$ (Table 17)

$n_2 = 20$ (Table 17)

$\mu_x = \mu_z$ cf. H_o

Hence: Formula (5): $t = 0.279$

Formula (6): $r = 37.4 = 37$ (app., degrees of freedom)

Table 18: $t\ (37)_{5\%} = 1.65$ (app.)

Formula (4): $-1.65 < 0.279 < 1.65$

Table 18

SIMPLIFIED TABULATION OF t-DISTRIBUTION

Degrees of freedom n	Probability of a deviation greater than t						Probability of a deviation greater than t					
	.005	.01	025	.05	.1	.15	.2	.25	.3	.35	.4	.45
1	63.657	31.821	12.706	6.314	3.078	1.963	1.376	1.000	.727	.510	.325	.158
2	9.925	6.965	4.303	2.920	1.886	1.386	1.061	.816	.617	.445	.289	.142
3	5.841	4.541	3.182	2.353	1.638	1.250	.978	.765	.584	.424	.277	.137
4	4.604	3.747	2.776	2.132	1.533	1.190	.941	.741	.569	.414	.271	.134
5	4.032	3.365	2.571	2.015	1.476	1.156	.920	.727	.559	.408	.267	.132
6	3.707	3.143	2.447	1.943	1.440	1.134	.906	.718	.553	.404	.265	.131
7	3.499	2.998	2.365	1.895	1.415	1.119	.896	.711	.549	.402	.263	.130
8	3.355	2.896	2.306	1.860	1.397	1.108	.889	.706	.546	.399	.262	.130
9	3.250	2.821	2.262	1.833	1.383	1.100	.883	.703	.543	.398	.261	.129
10	3.169	2.764	2.228	1.812	1.372	1.093	.879	.700	.542	.397	.260	.129
11	3.106	2.718	2.201	1.796	1.363	1.088	.876	.697	.540	.396	.260	.129
12	3.055	2.681	2.179	1.782	1.356	1.083	.873	.695	.539	.395	.259	.128
13	3.012	2.650	2.160	1.771	1.350	1.079	.870	.694	.538	.394	.259	.128
14	2.977	2.624	2.145	1.761	1.345	1.076	.868	.692	.537	.393	.258	.128
15	2.947	2.602	2.131	1.753	1.341	1.074	.866	.691	.536	.393	.258	.128
16	2.921	2.583	2.120	1.746	1.337	1.071	.865	.690	.535	.392	.258	.128
17	2.898	2.567	2.110	1.740	1.333	1.069	.863	.689	.534	.392	.257	.128
18	2.878	2.552	2.101	1.734	1.330	1.067	.862	.688	.534	.392	.257	.127
19	2.861	2.539	2.093	1.729	1.328	1.066	.861	.688	.533	.391	.257	.127
20	2.845	2.528	2.086	1.725	1.325	1.064	.860	.687	.533	.391	.257	.127
21	2.831	2.518	2.080	1.721	1.323	1.063	.859	.686	.532	.391	.257	.127
22	2.819	2.508	2.074	1.717	1.321	1.061	.858	.686	.532	.390	.256	.127
23	2.807	2.500	2.069	1.714	1.319	1.060	.858	.685	.532	.390	.256	.127
24	2.797	2.492	2.064	1.711	1.318	1.059	.857	.685	.531	.390	.256	.127
25	2.787	2.485	2.060	1.708	1.316	1.058	.856	.684	.531	.390	.256	.127
26	2.779	2.479	2.056	1.706	1.315	1.058	.856	.684	.531	.390	.256	.127
27	2.771	2.473	2.052	1.703	1.314	1.057	.855	.684	.531	.389	.256	.127
28	2.763	2.467	2.048	1.701	1.313	1.056	.855	.683	.530	.389	.256	.127
29	2.756	2.462	2.045	1.699	1.311	1.055	.854	.683	.530	.389	.256	.127
30	2.750	2.457	2.042	1.697	1.310	1.055	.854	.683	.530	.389	.256	.127
∞	2.576	2.326	1.960	1.645	1.282	1.036	.842	.674	.524	.385	.253	.126

The probability of a deviation *numerically* greater than t is twice the probability given at the head of the table.

Source: Hoel, P.G. A first course in the theory of modern statistical methods, New York, Wiley, 1963 (28)

* This table is reproduced from *Statistical Methods for Research Workers*, with the generous permission of the author, Professor R. A. Fisher, and the publishers, Messrs. Oliver and Boyd.

Consequently the H_o hypothesis is satisfactory, and the two distributions could be regarded as equal. Even if the criterion for errors is lowered, e.g. to α = 70%, the H_o hypothesis would be maintained.

The observation series X and Y (observation point 142 in two different years, 1967 and 1971, cf. Table 16) could be tested the same way assuming, H_o, that there be no significant deviation with time. In this case the t-value is found to be 1.647, and according to formula (4) we get:

$$-1.65 < 1.647 < 1.65$$

Again, it must be concluded that the hypothesis about equal distributions should not be rejected.

The t-test should be considered a simple tool for handling comparative evaluations of the kind demonstrated above.

13.5 Confidence intervals and limits

Estimates of intervals of variation for parameters such as sample averages can conveniently be established after performing a t-test (and also other tests).

Formulae (4) and (5) can be combined as follows:

$$\frac{(\bar{X} - \bar{Z}) - (\mu_X - \mu_Z)}{(s_x^2/n_x + s_z^2/n_z)^{1/2}} < t(r)_{-\alpha/2} \qquad (8)$$

where $\bar{X}$, $\bar{Z}$, s_x, s_z, n_x, n_z, r, and α are all known from the t-test calculations, and α is chosen arbitrarily, e.g., = 5%, to obtain 95% confidence intervals.

Rewriting (8) then leads to the following confidence interval for the difference (unknown) between the true means μ_x and μ_z:

$$\left.\begin{aligned} (\bar{X} - \bar{Z}) - t(r)_{1-\alpha/z} \cdot (s_x^2/n_x + s_z^2/n_z) &< \mu_x - \mu_z \\ (\bar{X} - \bar{Z}) - t(r)_{\alpha/z} \cdot (s_x^2/n_x + s_z^2/n_z) &> \mu_x - \mu_z \end{aligned}\right\} \qquad (9)$$

The interval defined by (9) is called the confidence interval for $\mu_x - \mu_z$, and the two end points of this interval called the confidence limits. The use of the term confidence interval implies that the probability is $1 - \alpha$ that this interval contains $\mu_x - \mu_z$ in its interior.

Equation (9) will be useful in cases where the t-test has indicated that μ_x and μ_z do differ significantly (which was not the case in the example above, cf. 13.4).

If the t-test has indicated that there is no significant deviation between X and Z, all the individual X and Z observations may as well be pooled and a single combined average and empirical variance calculated according to (2) and (3) for normally distributed observations.

13.6 Other statistical methods

Many other statistical methods and tools (27, 28) are available when evaluating monitoring data. Analysis of variance (for studying significance of different factors such as wind and currents acting on coastal water quality), and regression analysis are obvious possibilities. Reference is made to existing literature (27, 28).

14. Periodical reports

Periodical reports must be prepared for any monitoring programme in order to evaluate and assess results obtained in the period with respect to the goals defined before commencing the field and laboratory work. These goals will in general define the contents and the scope of the periodic report. A few practical hints can be given as to the preparation of the report.

14.1 The period reported

Often, the seasonal variations of recreational activities or shellfish harvesting will clearly define distinct periods of interest; for example, May to September is the period of interest so far as

recreational activities in the Mediterranean are concerned. Where major improvements of sanitary waste disposal are introduced, this may also clearly define distinct periods of interest for the assessment of effects in the receiving water.

14.2 Use of tables and graphs

The results from the monitoring activities are recorded initially in a number of standard forms (Annex IV) designed to support practical procedures in field and laboratory. For presentation of results in a periodic report these results must be condensed further and summarized. The use of tables and graphs is strongly recommended.

Annex IV includes a standard form, Form 11, which may adequately serve the purpose of presenting a summary of microbiological sampling results for a certain period. As an example, consider the results of Table 16, where the presentation is inadequate, because important information such as date, depth, temperature, salinity etc., is not included. A presentation including the information requested on Form 11 would be much more satisfactory, and such information will always be available directly from the other standard monitoring forms already recommended.

To support understanding of tables, graphs should be prepared. Figure 28 has been established to demonstrate graphically the contents of Table 16 at point 142, 1967. In the figure the dates have also been included to obtain a time scale on the abscissa axis. A criterion of 1000 faecal E. coli/100 ml has been shown to give a reference for a first crude assessment of the situation. Graphs of this type may often be helpful where routine control, e.g., by a public health authority is desirable.

Figure 29 has been included to demonstrate the difference in confidence intervals, when applying two different modifications of the maximum probable number technique for faecal E. coli examination.

The three-tube modification, as shown in Annex I, Table 2, gives a wider 95% confidence interval than the five-tube version. Which version to choose must be decided locally, and aims and resources must be considered in reaching a conclusion. A graph such as Figure 29 may be more illustrative than Table 1 in Annex I, and may be of help in deciding which version of the most probable number technique to choose.

Figure 30 shows typhoid cases in Alexandria according to months of the year. The graph illustrates an epidemiological situation much more clearly than tabular form. This figure demonstrates moreover, how there may be solid grounds for an extended monitoring programme that would particularly provide data on coastal water quality in areas selected for epidemiological studies and in major outfalls that, depending on hydrographic conditions, contribute to pollution in these areas.

14.3 Evaluation and assessment

It is recommended that all results from a monitoring period should be presented in tables and graphs and that all calculations and statistics based thereon should be thoroughly done before commencing the written report. If this is done, the periodical report will be as brief, concise, and well-documented as possible.

15. Adjusting the monitoring programme

There may be several reasons for periodic adjustments of any monitoring programme:

1. The original objectives have been fulfilled; a programme was established to acquire knowledge and experience on methodologies which have now become safe routines in field and laboratory; or the relation between certain discharges and contaminated beaches has been established to the extent that is deemed necessary to initiate adequate remedial action; etc.

2. New objectives have been established and will require either supplementary monitoring parameters, or substitution of existing activities, or combinations thereof. For example, the number of indicator organisms may be reduced and more effort spent on the study of certain pathogens in the effluents, in the receiving water, in the sediments, or in certain kinds of seafood.

3. The frequency of existing sampling and the number of sampling points may be deemed irrelevant after due consideration of results already obtained. Such reevaluation would often result from a statistical analysis as indicated in Chapter 13. For example, the insignificant difference between two sampling points may lead to a decision to drop one of them in the future, or the insignificant variation from period to period may allow the sampling frequency to be lowered

Fig. 28

EXAMPLE OF GRAPHIC PRESENTATION OF FAECAL E. COLI DATA

Five-tube most probable number confidence range

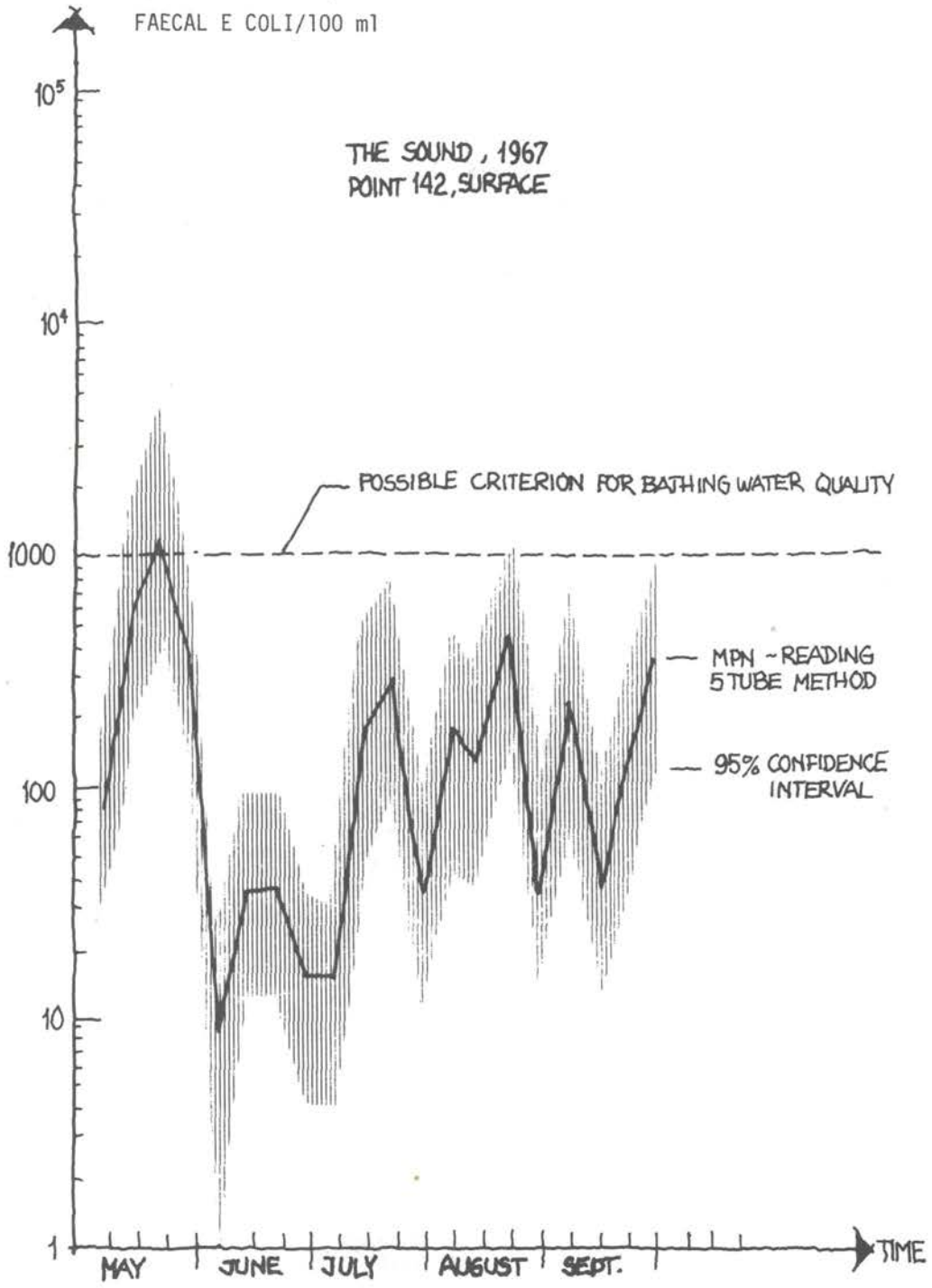


Fig. 29

CONFIDENCE RANGE FOR THREE-TUBE MOST PROBABLE NUMBER FOR FAECAL E. COLI

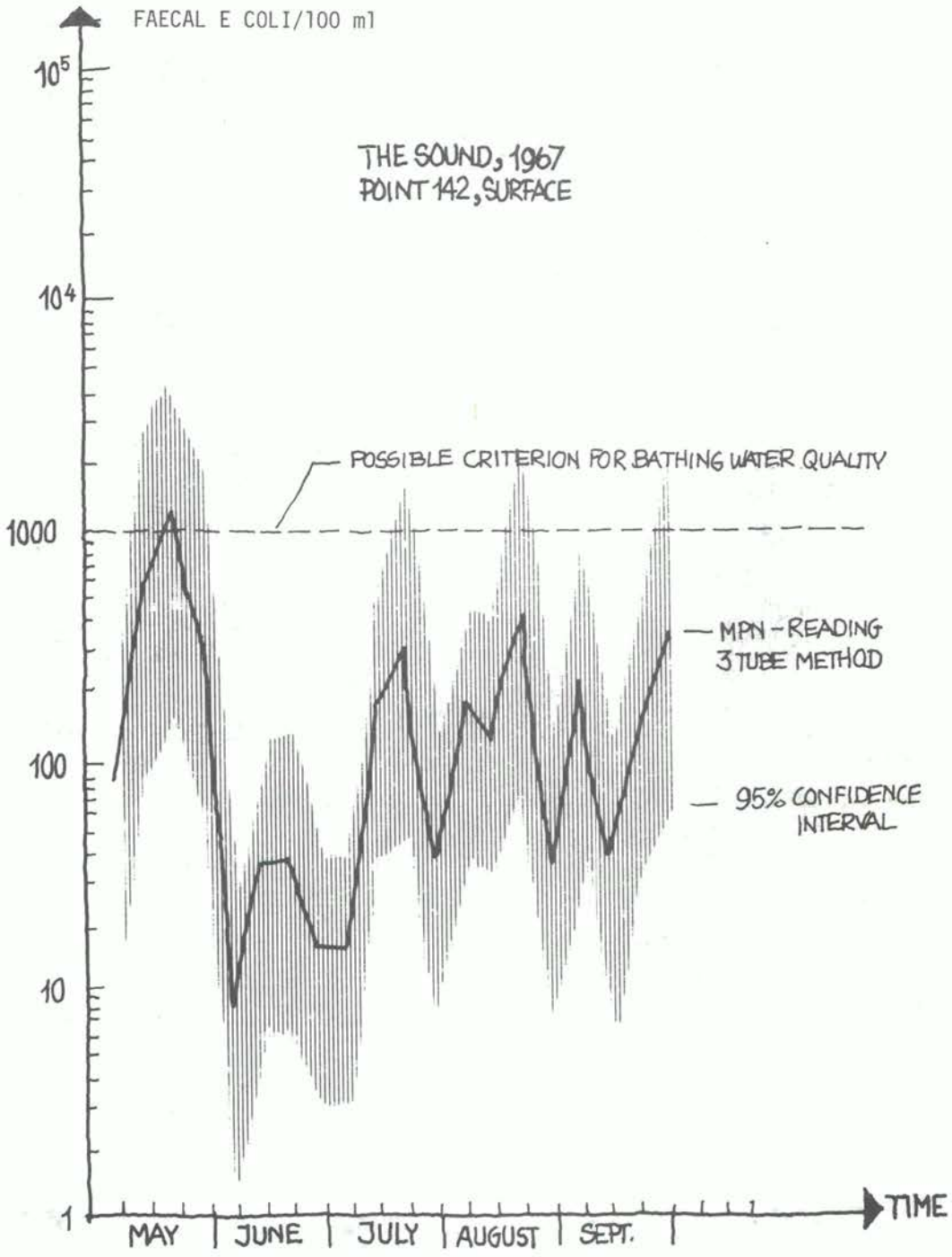
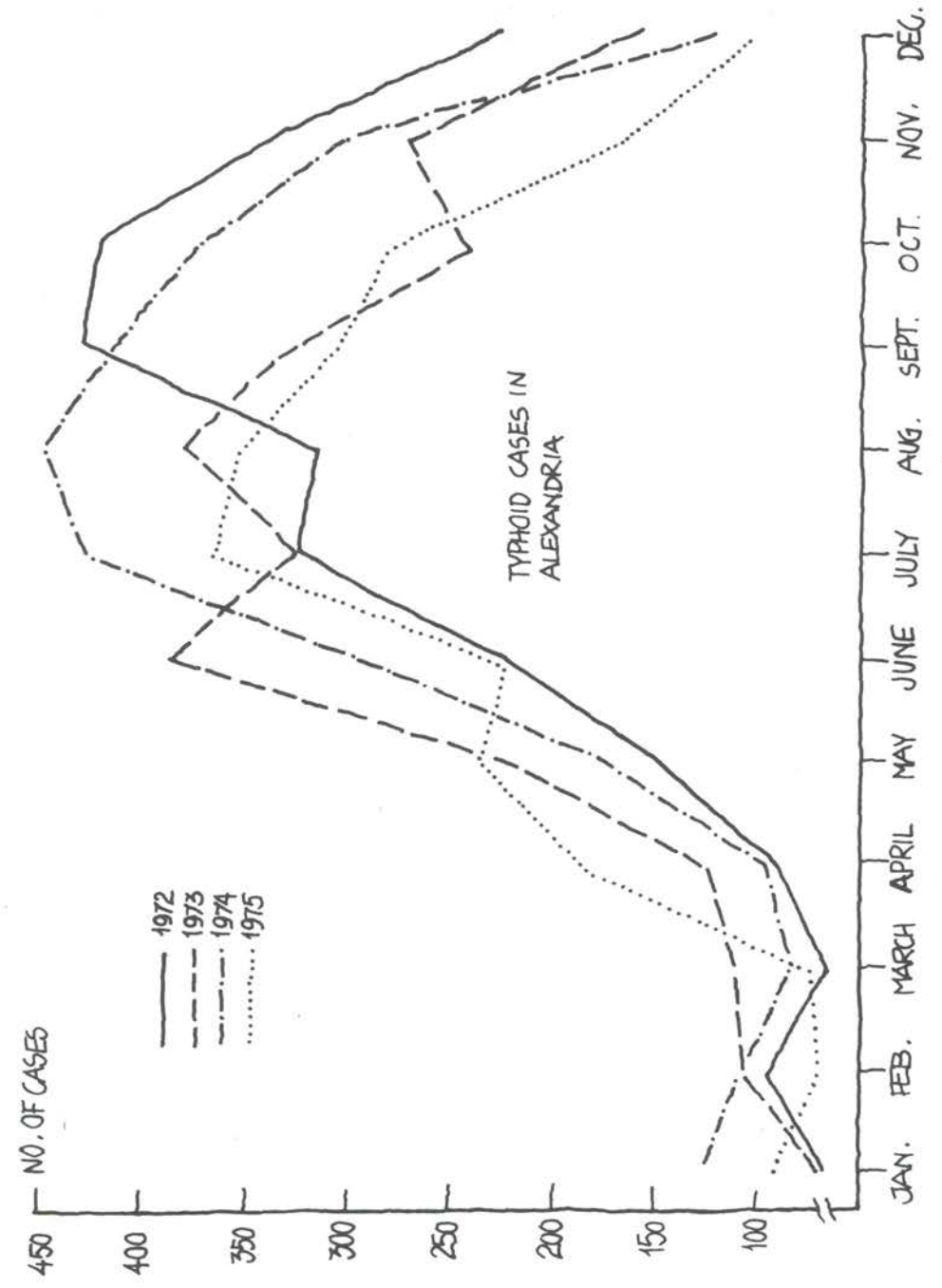


Fig. 30

TYPHOID CASES IN ALEXANDRIA 1972-75



Source: Sharkawi, F. (personal communication)

considerably. It should be noted that more advanced statistical methods exist for the design of a sampling frequency according to the desired precision and the probability of a correct decision with respect to a particular water quality criterion (27) and other textbooks on statistical sampling theory.

Relevant adjustments of ongoing monitoring programmes can be defined locally, but it should be a requirement of any periodical report to include a critical assessment of needs and reasons for making adjustments to existing programmes.

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ANNEX I

SOME GENERAL METHODS FOR MICROBIOLOGICAL EXAMINATION

Only to a certain degree can microbiological examination procedures be described generally, because in practice different microorganisms are identified by their specific response to individual test procedures.

Initial procedures for examining certain microorganisms, however, often belong to one of the following standard routines, employed to test for the presence of the various monitoring organisms mentioned in Chapter 10. Although more comprehensive and sophisticated manuals are available (9, 10), the following procedures together with those set out in Chapter 10 will normally suffice for straightforward work in minimum monitoring.

1. Membrane filtration method

The membrane filtration method consists of a direct count of organisms in a given sample test volume. It is typical of this method that larger volumes of water can easily be filtered on the same membrane, i.e., relatively few organisms in a dilute suspension can be detected. The method is illustrated in Figs 1 and 2.

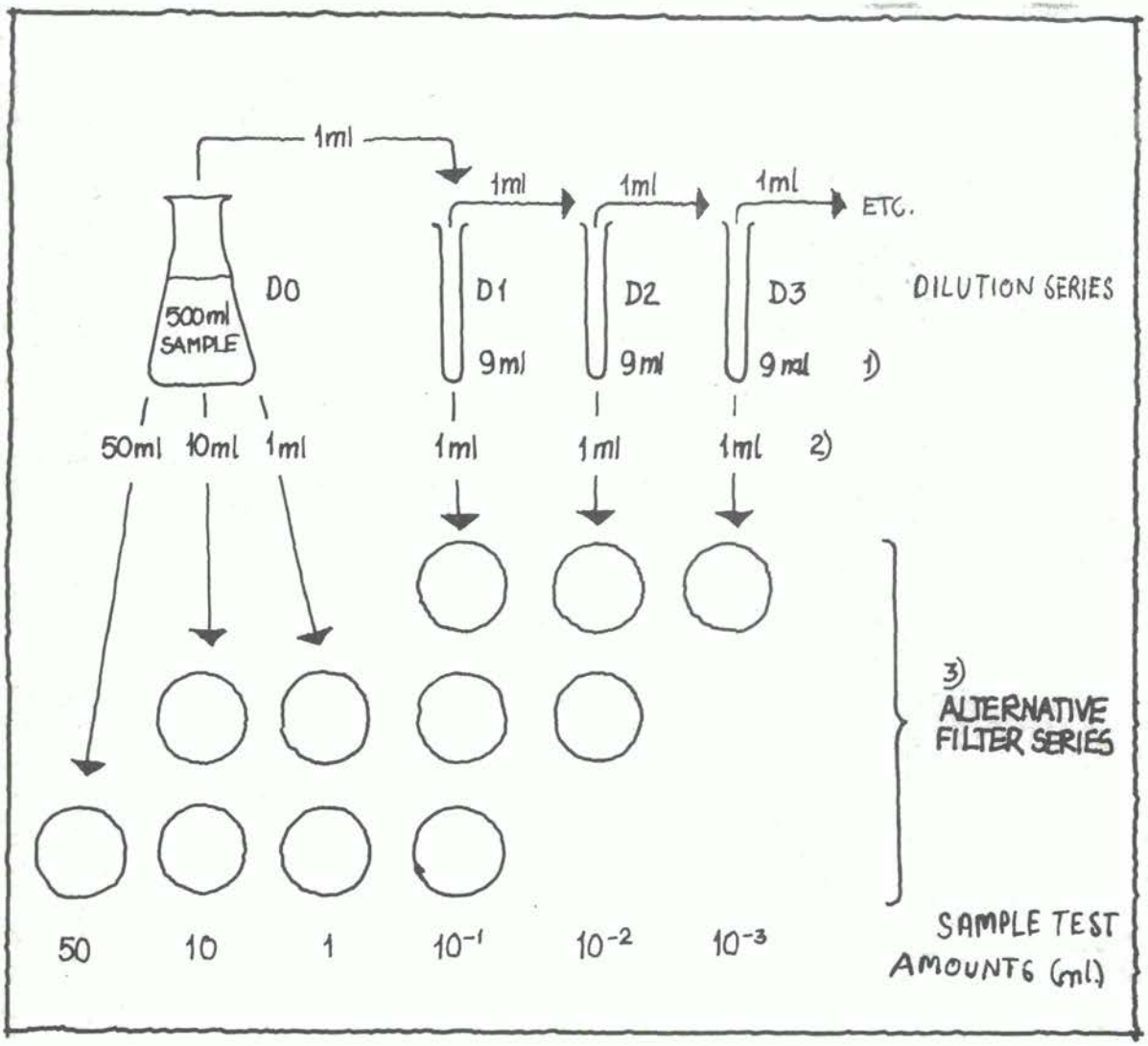
One advantage of the method is that it avoids false positives resulting from growth of anaerobic bacteria such as Clostridium perfringens. However, unlike the multiple test tube method, gas production will not be detected. When examining sample test amounts less than or equal to 1 ml the membrane filtration method should not be used. Instead, with amounts equal to 1 ml, use the pour plate method; with amounts less than 1 ml, the spread plate method is to be preferred.

The general description given in Figs 1 and 2 should be used only in conjunction with the specific procedure outlined in Chapter 10 for the organism and medium to be examined.

In addition to the Petri dishes used for incubation of one series of the filters shown in Fig. 1, an extra dish should be prepared for incubation using identical solid growth medium but not a membrane filter, so as to check the sterility of the medium, the equipment and the handling procedures (see Annex V).

Fig. 1

MEMBRANE FILTER TEST, PRINCIPLES



Notes:

1. 9 ml of dilution liquid, as per Chapter 10.

2. For volumes smaller than 10 ml, approximately 10 ml sterile phosphate buffer is added, to mix and distribute the sample adequately across the filter.

3. Circles indicate membrane filters, 0.45 µ. Only one of the series is used for the incubation.

Rinse the filter funnel before removing the filter by applying 2 × 30 ml phosphate buffer.

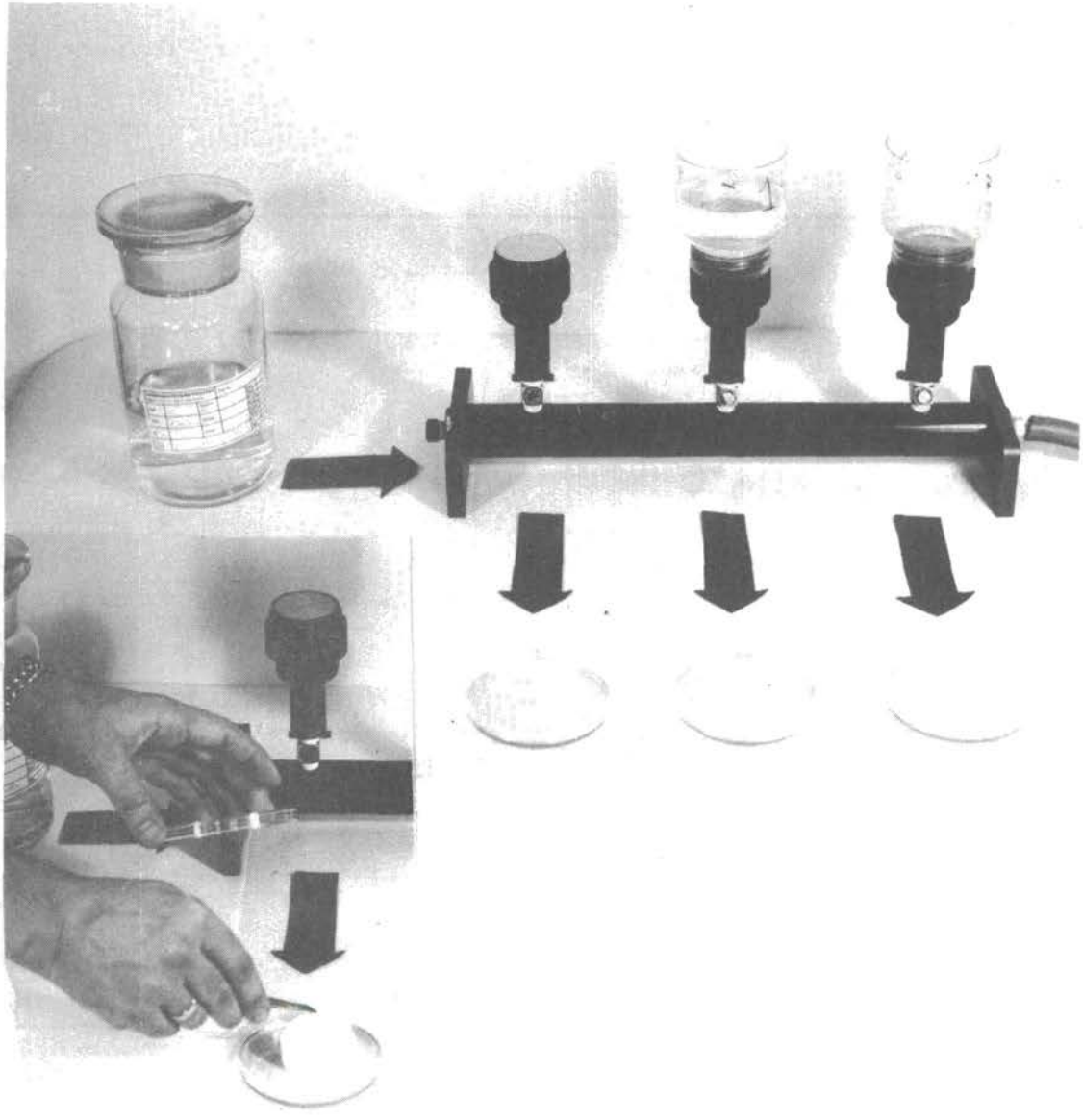
After filtration each filter is transferred by sterile forceps onto solid growth media in a 9 cm Petri dish. If necessary reroll filters to remove air bubbles between filter and agar. Bubbles are recognized as colourless or lighter spots on the membrane.

After incubation the test organism density of the sample can be established from the count of the filter with a 20-80 colony range, according to the following formula:

$$\text{Organisms/100 ml} = \frac{\text{No. of colonies on 1 filter} \times 100}{\text{ml sample test amount}}$$

Fig. 2

MEMBRANE FILTER TEST, ILLUSTRATION



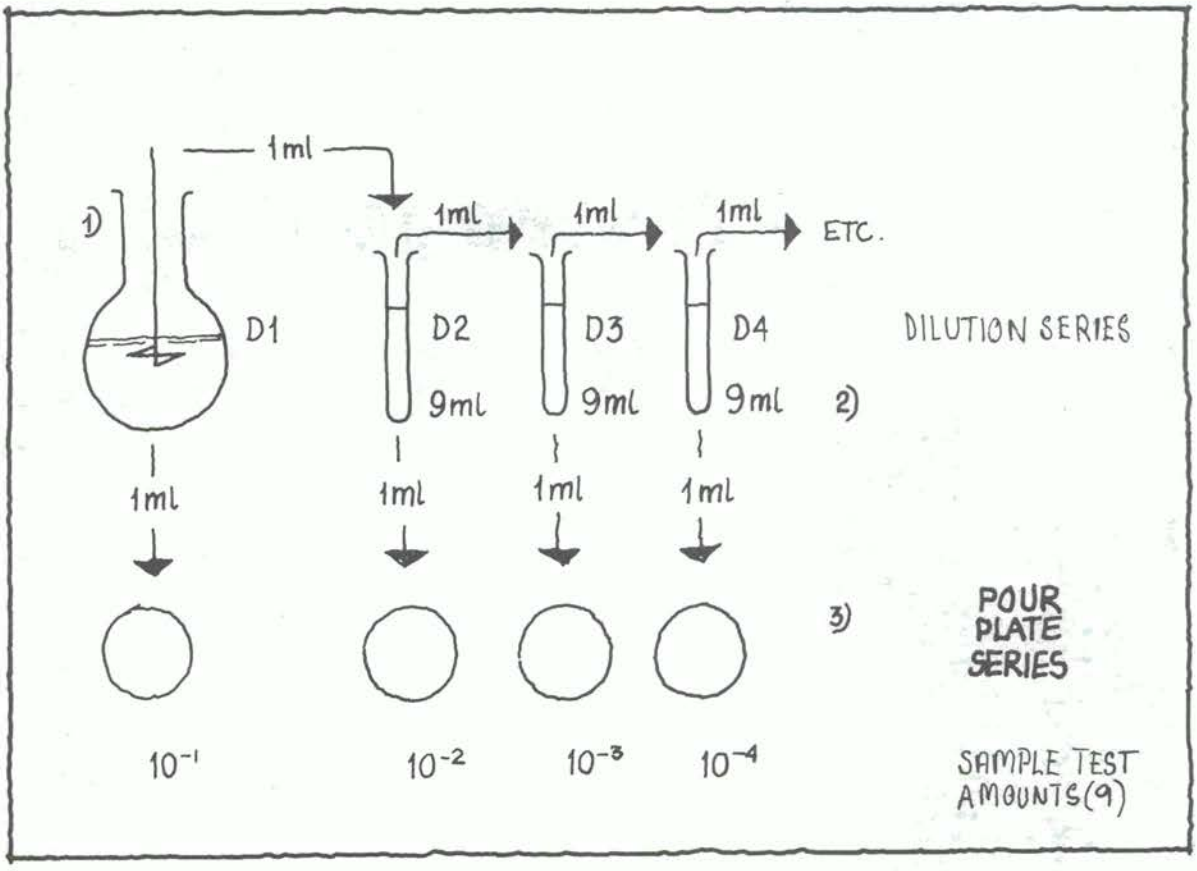
2. Pour plate method

The pour plate method is a colony count procedure like the membrane filtration method, but it has an upper limit of 1 ml to the amount of sample that can be used, because the growth medium should be able to absorb the transferred sample and still solidify without difficulty. For sample test amounts less than or equal to 1 ml, the pour plate method should normally be preferred to membrane filtration. The method is illustrated in Figs 3 and 4.

Sterility of the growth medium and sterile handling and operation should always be checked through incubation of a control (non-inoculated) plate.

Fig. 3

POUR PLATE METHOD, PRINCIPLES



Notes:

1. The sterilized laboratory blender is used to grind down the shellfish flesh effectively (but not their shells and water). Dilution liquid is added on a weight for weight basis, e.g., 1 : 10 = 9 units of dilution liquid and 1 unit of shellfish flesh, before homogenization. Transfer by pipette is then possible.

2. The 9 ml in the dilution tubes consist of phosphate buffer diluent. Stir vigorously without contaminating the solution in the tube.

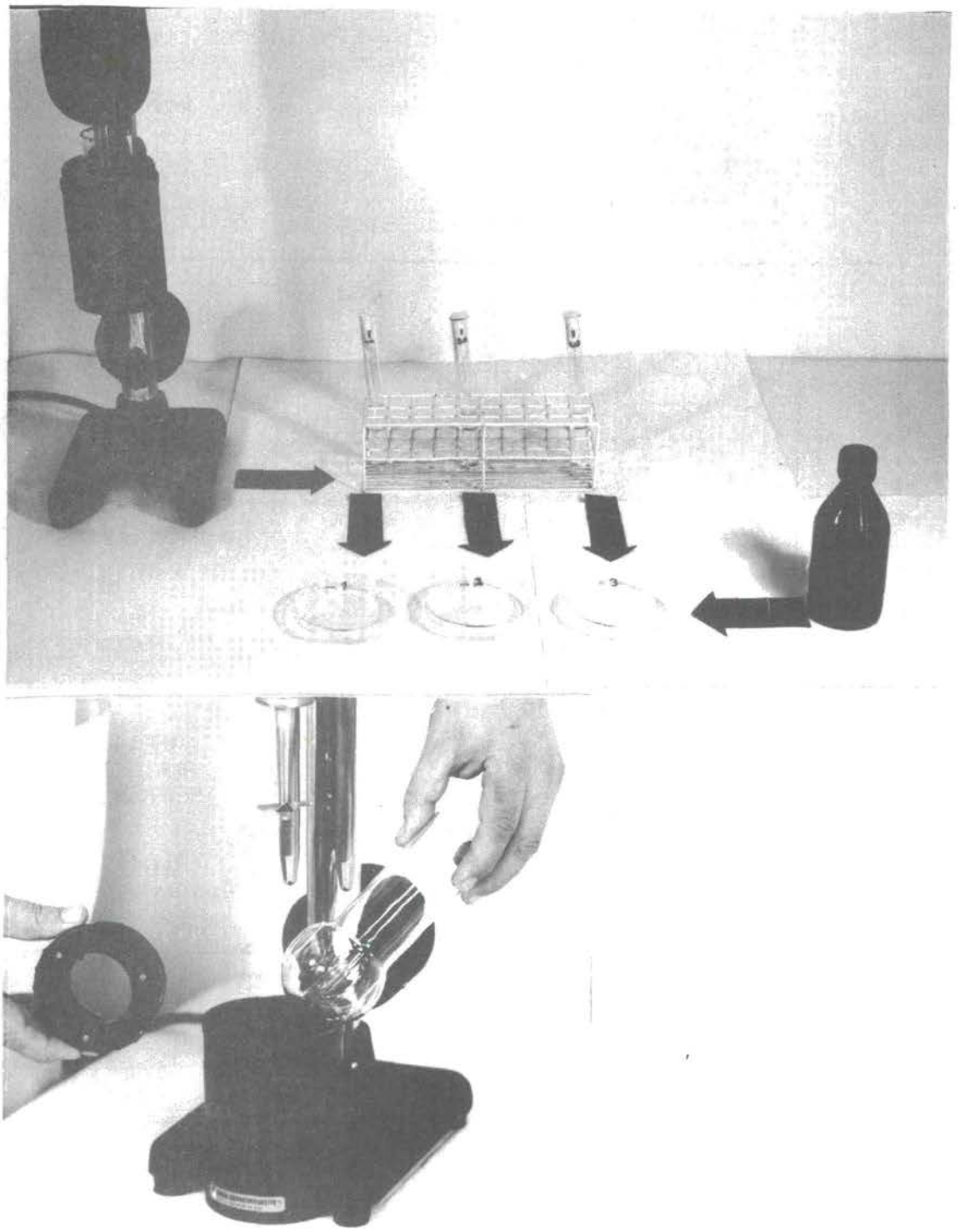
3. Pour plates are sterile 9-cm Petri dishes. Some 12-14 ml of liquefied incubation substrate is added after inoculation of the 1 ml from the dilution series. Use circular to and fro movements of the Petri dish to have the inoculate mixed and the agar evenly distributed across the dish.

After the prescribed incubation period, counting is done on a plate with a 20-80 colony range. The concentration of organisms in the original sample is found from the following formula (as applied to shellfish):

$$\text{Organisms/100 g} = \frac{\text{No. of colonies on 1 plate} \times 100}{\text{g sample test amount}}$$

Fig. 4

POUR PLATE METHOD, ILLUSTRATION



3. Spread plate method

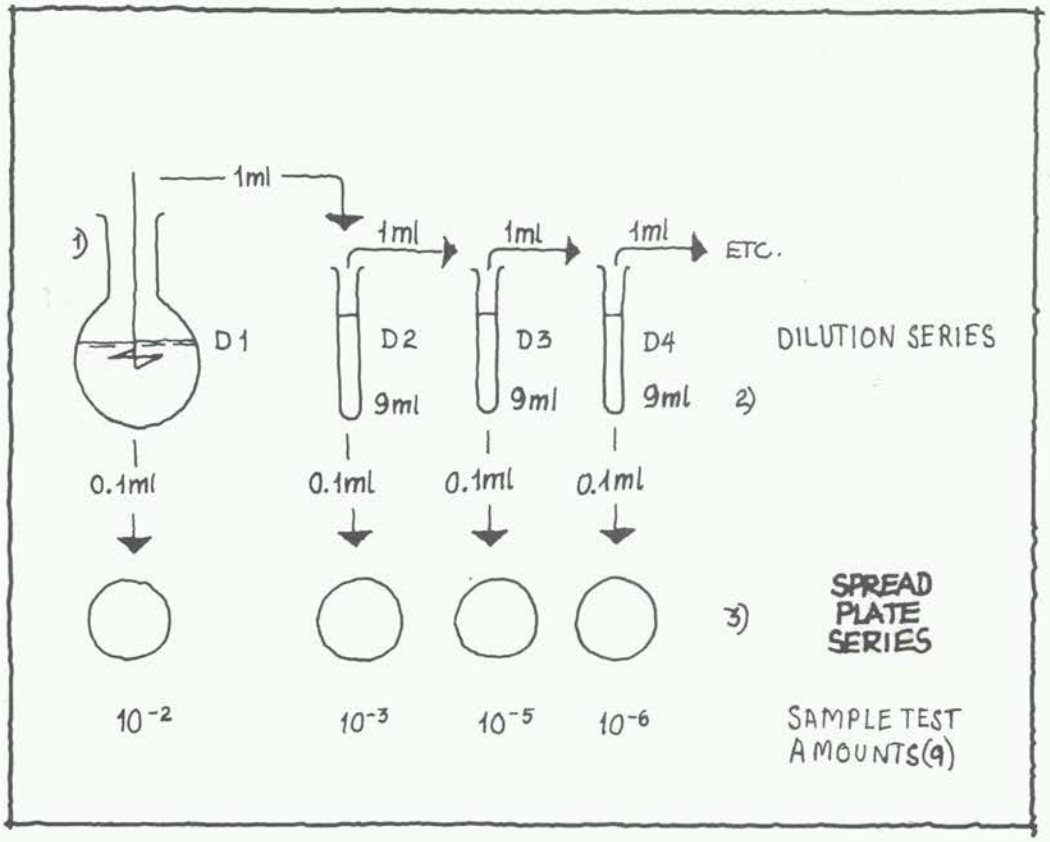
The spread plate method is a third colony count procedure, in which a special solidified agar (e.g., M-endo agar MF) is used. Only 0.1 ml of liquid is transferred from the dilution series to the surface of the agar in the 9-cm Petri dishes. The transferred 0.1 ml is spread over the agar surface by use of a sterile inoculation spatula (Drigalski).

The spread plate method resembles the pour plate method in its principles and procedures. Due to the small inoculation volume there is a lower limit to the density of organisms that can be detected by the spread plate method. The method is illustrated in Figs 5 and 6.

Sterility of growth media and handling procedures should always be checked by passing a control (non-inoculated plate) through incubation and counting.

Fig. 5

SPREAD PLATE METHOD, PRINCIPLES



Notes:

1. The sterilized laboratory blender is used for grinding down the shellfish flesh effectively (but not their shells and water). Dilution liquid is added on a weight for weight basis, e.g., 1 : 10 = 9 units of dilution liquid and 1 unit of shellfish flesh, before homogenization. Transfer by pipette is then possible.

2. The 9 ml in the dilution tubes consist of phosphate buffer diluent. Stir vigorously without contaminating the solution in the dilution tube.

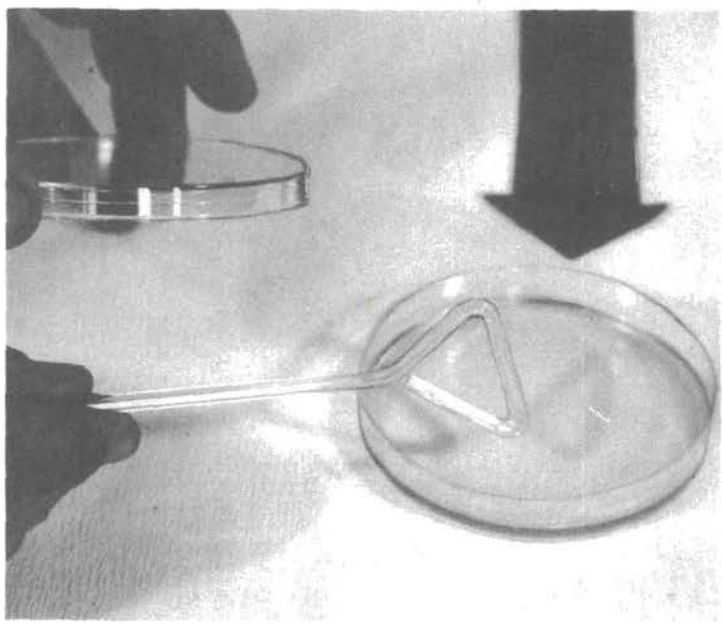
3. Spread plates are pre-prepared solid medium dishes inoculated by transfer of only 0.1 ml from the dilution series. Distribution evenly over the substrate surface is done by an inoculation spatula (Drigalski). The number of dishes and the dilutions are indicated separately in Chapter 10 for each organism and medium.

After the prescribed incubation period, counting is done on a plate with a 20-80 colony range. The concentration of organisms in the original sample is found from the following formula (as applied to shellfish samples). The specific formula to be used is indicated separately for each organism, method, and medium in Chapter 10:

$$\text{Organisms/100 g} = \frac{\text{No. of colonies on 1 plate} \times 100}{\text{g sample test amount}}$$

Fig. 6

SPREAD PLATE METHOD, ILLUSTRATION



4. Standing tube method

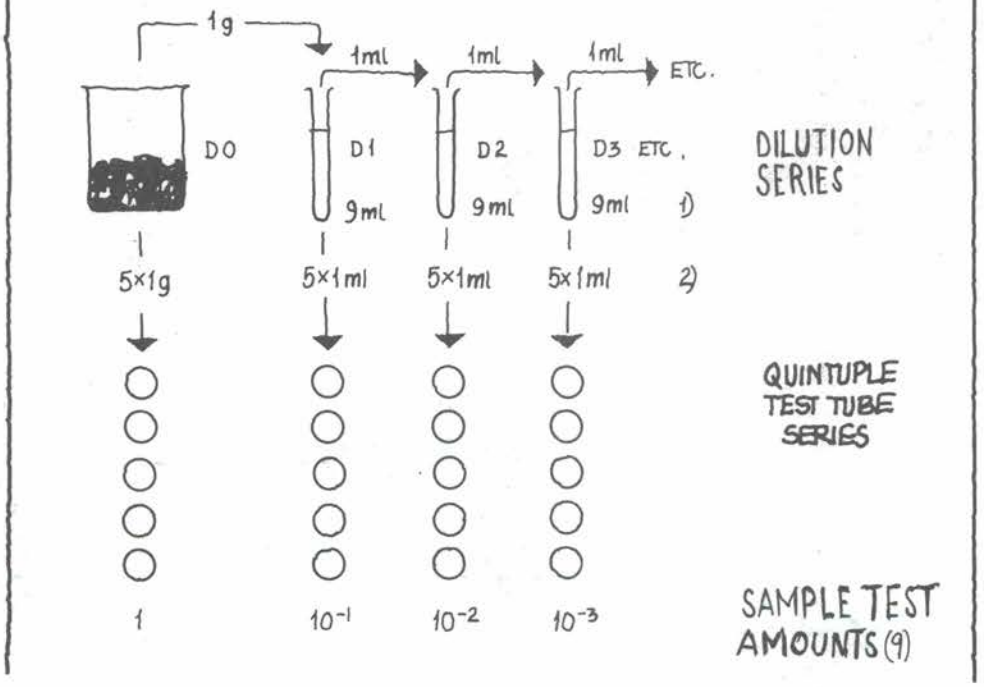
The standing tube method is another direct colony count method. It resembles the pour plate technique in its principles, but instead of one plate per dilution, a number of test tubes are used (normally five) for inoculation into a liquefied growth medium. The inoculate is distributed in the growth agar by inverting the test tubes (to avoid contamination), so that the bacterial colonies become distributed vertically in the test tubes before the agar solidifies. By increasing the number of test tubes the amount of sample can be increased proportionately, but the increase is not comparable to what can be achieved for fluid samples if a membrane filter is used.

The standing tube method is illustrated in Figs 7 and 8 but it should be used only in conjunction with the specific modifications indicated in Chapter 10.

Growth medium sterility and sterile handling and operation should be checked by incubation of controls (non-inoculated test tubes).

Fig. 7

STANDING TUBE METHOD, PRINCIPLES



Notes:

1. Before transfer of 1 ml each dilution tube holds exactly 9 ml of phosphate buffer diluent.

2. Before inoculation, each test tube holds approximately 10 ml of liquefied growth substrate. Stir the tubes to obtain efficient mixing of substrate and inoculate. For anaerobic cultures use 2 cm liquefied agar to top the already solidified substrate, thus securing anaerobic conditions during cultivation.

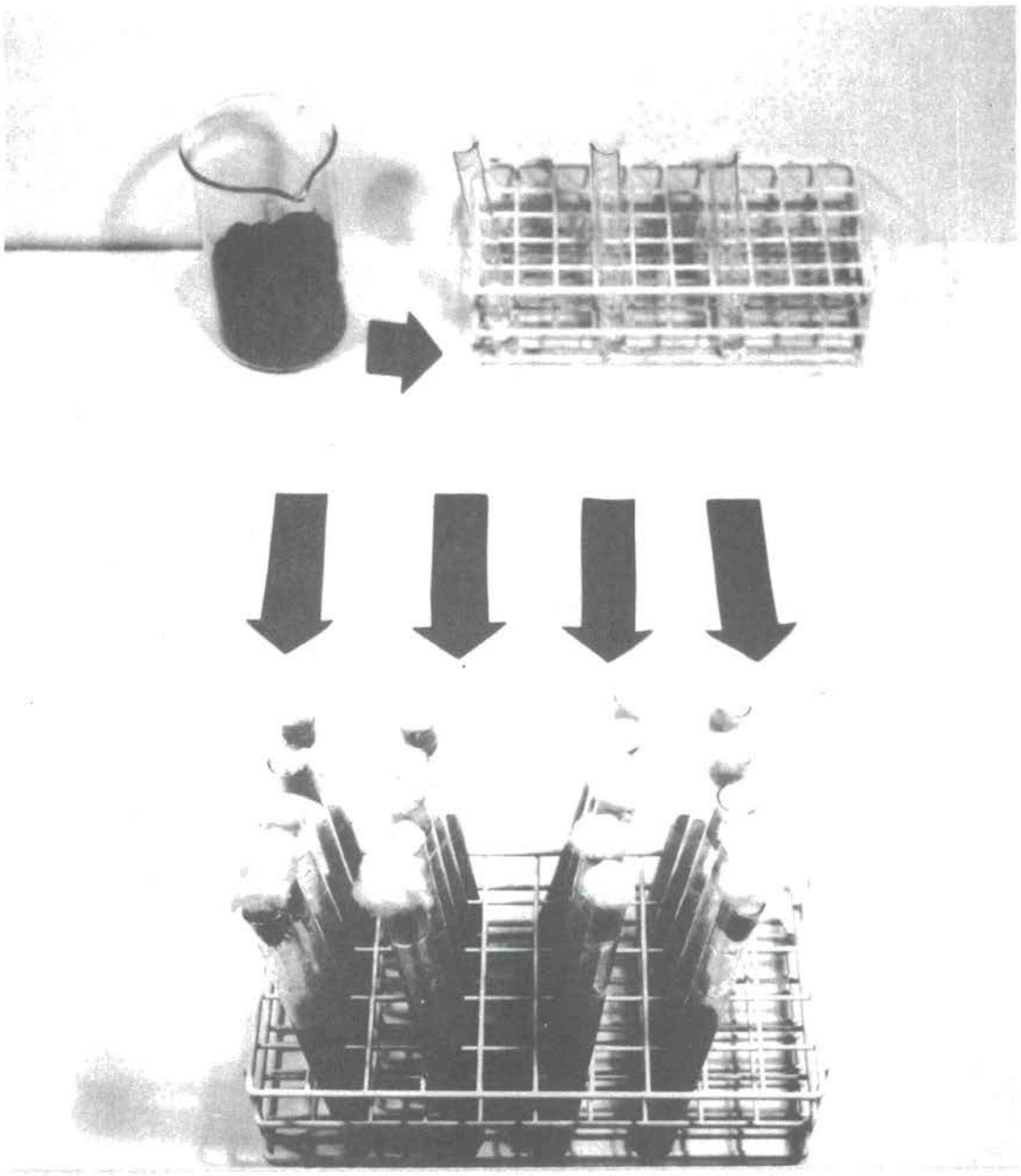
Colony counts are read from only one set of five test tubes, preferably where colonies are separated and of adequate size.

The colony density is determined from the following type of formula (here sediments: cf. Chapter 10 for correct formula for each particular organism/medium):

$$\text{Organisms/g} = \frac{\text{Number of colonies in five tubes (one quintuple)}}{\text{g sample test amount} \times 5}$$

Fig. 8

STANDING TUBE METHOD, ILLUSTRATION



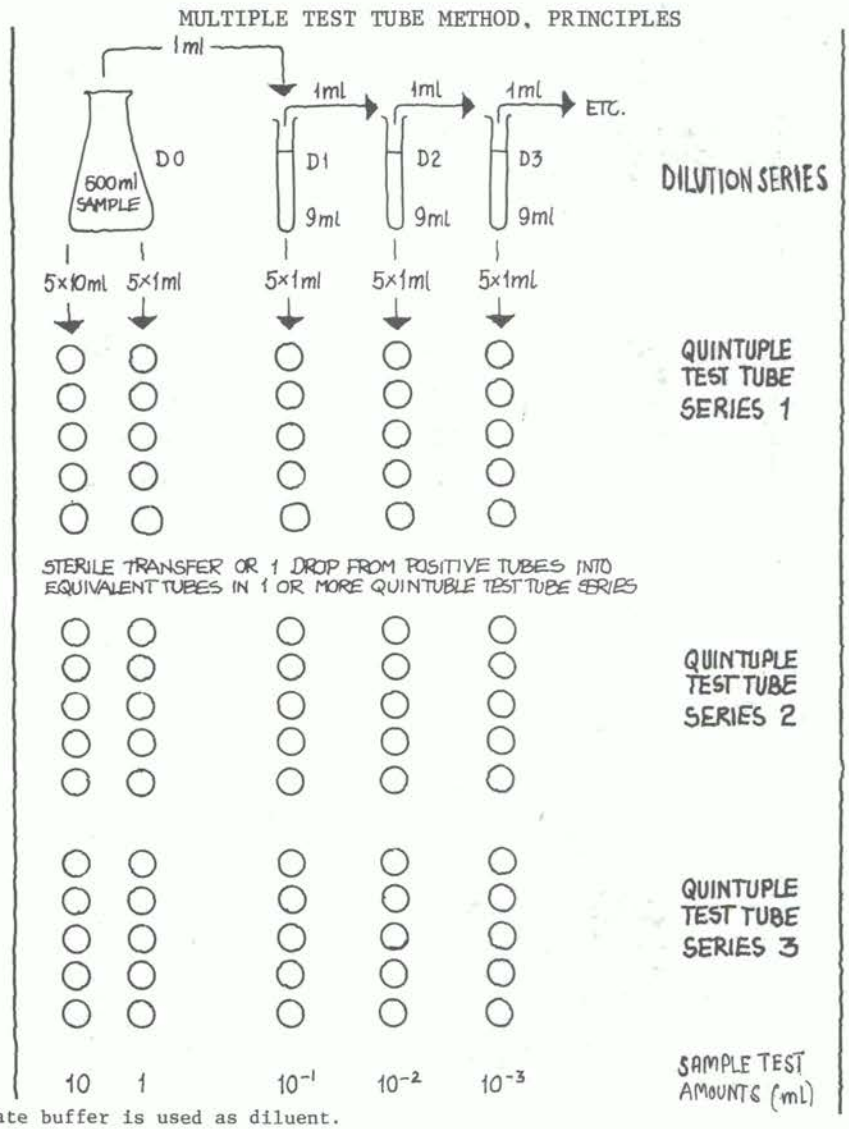
5. Multiple test tube method

This method, providing the most probable number, is an indirect count technique relying on statistical interpretation of growth/no growth observations in the inoculated tubes. These may be numerous, depending on the number of identification tests; cf. Figs 9 and 10, which illustrate the basic principles and operations of the method.

As can be seen from Fig. 9, several quintuple test tube series must be used, corresponding to at least three, and preferably five, dilutions of the sample in order to obtain positive readings (between five or three and one) from at least three consecutive quintuples.

Tables 1 and 2 are those necessary to determine the most probable number of the density of organisms in the sample. A few examples of the use of the tables are given with the table, for both triplet and quintuple test tube series.

Fig. 9



Notes:

1. Phosphate buffer is used as diluent.

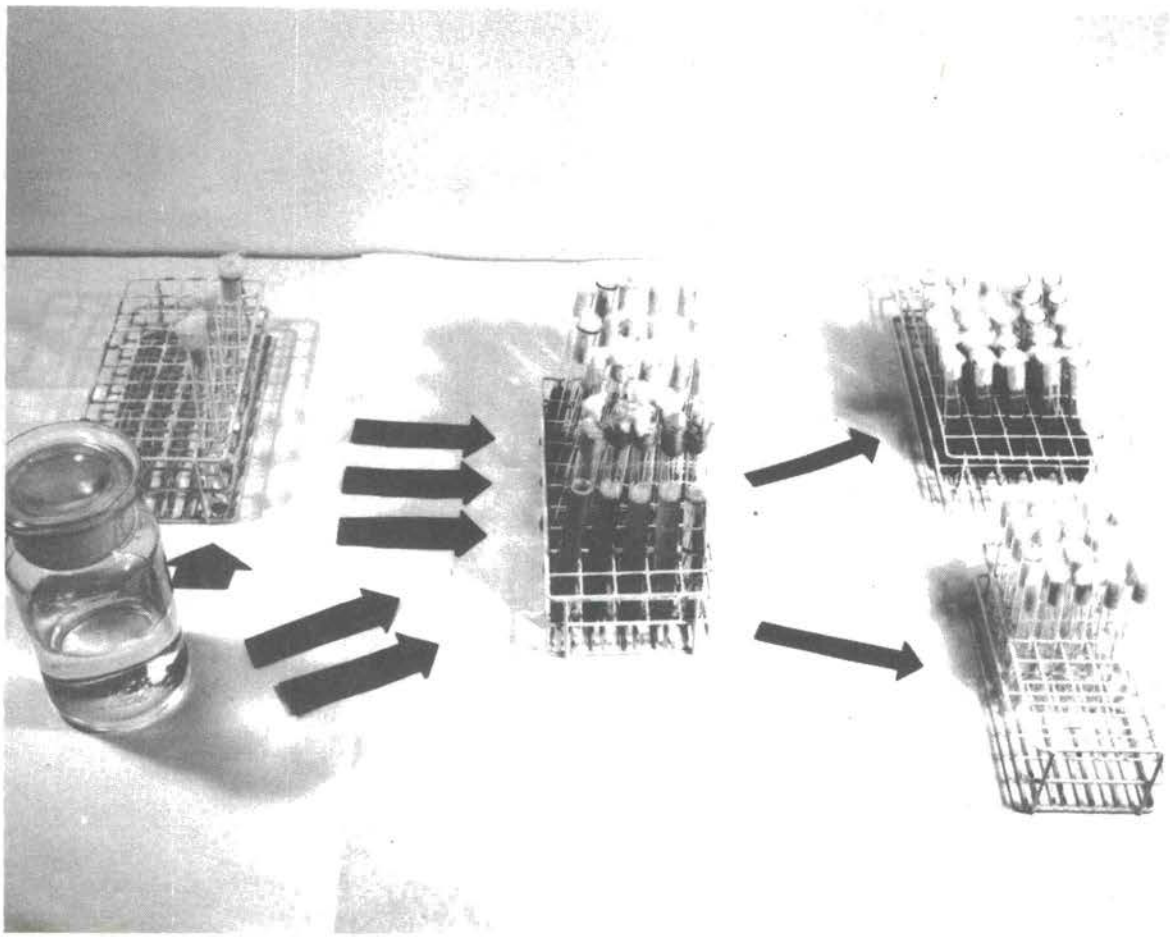
2. Before inoculation, each test tube holds approximately 10 ml of prescribed substrate.

Scaling

The two tables above apply to sample test amounts in the 10, 1 and 0.1 ml dilution series. As already indicated in the examples, the maximum probable number per 100 ml must be adjusted according to actual sample test amount combinations; sample test amounts = 10^{-2}, 10^{-3}, and 10^{-4} ml require multiplication by 1000 ($10/10^{-2}$) etc.

Fig. 10

MULTIPLE TEST TUBE METHOD, ILLUSTRATION



Quintuple reading

From the final quintuple test tube series, normally used for certain specified confirmatory tests in relation to Chapter 10, positive readings from the test tubes are made, to identify the most probable number colony density of the sample according to the following index:

Table 1

No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits		No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits	
5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper	5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper
0	0	0	<2								
0	0	1	2	<0.5	7	4	2	1	26	9	78
0	1	0	2	<0.5	7	4	3	0	27	9	80
0	2	0	4	<0.5	11	4	3	1	33	11	93
						4	4	0	34	12	93
1	0	0	2	<0.5	7						
1	0	1	4	<0.5	11	5	0	0	23	7	70
1	1	0	4	<0.5	11	5	0	1	31	11	89
1	1	1	6	<0.5	15	5	0	2	43	15	110
1	2	0	6	<0.5	15	5	1	0	33	11	93
						5	1	1	46	16	120
2	0	0	5	<0.5	13	5	1	2	63	21	150
2	0	1	7	1	17						
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
						5	3	1	110	31	250
3	0	0	8	1	19	5	3	2	140	37	340
3	0	1	11	2	25	5	3	3	180	44	500
3	1	0	11	2	25	5	4	0	130	35	300
3	1	1	14	4	34	5	4	1	170	43	490
3	2	0	14	4	34	5	4	2	220	57	700
3	2	1	17	5	46	5	4	3	280	90	850
3	3	0	17	5	46	5	4	4	350	120	1,000
4	0	0	13	3	31	5	5	0	240	68	750
4	0	1	17	5	46	5	5	1	350	120	1,000
4	1	0	17	5	46	5	5	2	540	180	1,400
4	1	1	21	7	63	5	5	3	920	300	3,200
4	1	2	26	9	78	5	5	4	1600	640	5,800
4	2	0	22	7	67	5	5	5	≥2400		

Source: APHA-AWWA-WPCF. Standard methods for the examination of water and wastewater, Thirteenth edition, Washington, D.C., 1971

Quintuple examples

Number of positive tubes for sample test amount as indicated:							Resulting most probable number per 100 ml
10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
	5	2	0	0			490
	5	4	2	0			2 200
		0	1	0	0		200
				3	2	1	170 000
	5	5	5	3	1		110 000
		5	3	2	0		14 000
		5	3	1	1		14 000

Generally use three numbers to obtain a most probable number. The first number is preferably a 5 from the smallest possible sample test amount and two other numbers from the succeeding two smaller sample test amounts (higher degrees of dilution).

Triplet reading

From the final triplet test tube series the combination of positive test tubes is used to assess the most probable number according to the following index. The table applies to sample test amounts 10, 1 and 0.1 ml respectively, cf. Table 1 above.

Table 2

Combination of positives	Most probable number Index /100 ml	95% Confidence Limits Lower	Upper
0-0-0	<3		
0-0-1	3	<0.5	9
0-1-0	3	<0.5	13
0-2-0			
1-0-0	4	<0.5	20
1-0-1	7	1	21
1-1-0	7	1	23
1-1-1	11	3	36
1-2-0	11	3	36
2-0-0	9	1	36
2-0-1	14	3	37
2-1-0	15	3	44
2-1-1	20	7	89
2-2-0	21	4	47
2-2-1	28	10	150
2-3-0			
3-0-0	23	4	120
3-0-1	39	7	130
3-0-2	64	15	380
3-1-0	43	7	210
3-1-1	75	14	230
3-1-2	120	30	380
3-2-0	93	15	380
3-2-1	150	30	440
3-2-2	210	35	470
3-3-0	240	36	1 300
3-3-1	460	71	2 400
3-3-2	1 100	150	4 800
3-3-3	≥2 400		

Source: APHA-AWWA-WPCF. Standard methods for the examination of water and wastewater, Fourteenth edition, Washington, D.C., 1975

Triplet examples

Number of positive tubes for sample test amount as indicated: 10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Resulting most probable number per 100 ml
	3	1	1	0		750
3	1	1	0			75

ANNEX II

MINIMUM INVENTORY OF EQUIPMENT AND SUPPLIES FOR USE IN LABORATORY AND FIELD

1. Equipment

The following lists of equipment and supplies will serve to specify certain essentials for the performance of a minimum coastal water quality control programme related to human health; (cf. Chapters 3 and 6).

Well-established laboratories may find the lists of only limited value, but for others the inventory may be helpful in assessing initially available essential supplies, including field and laboratory instrumentation and supplies such as substrates, media, and reagents necessary for the minimum monitoring and examination procedures.

Any microbiological laboratory should have good water supply and disposal facilities. Electricity and gas must be available, and working and storage rooms should be air-conditioned. Each analyst there should be provided with at least 2 metres of laboratory bench. There must be good toilet facilities and separate rooms for lunch breaks, etc.

1.1 Major items of equipment	Number required
pH meter, 0.1 pH unit	1
Analytical balance, 0.200 g, ± 0.3 mg	1
Balance, 0 -1000 g, ± 50 mg	1
Shakers for flasks	1
Blender and accessories:	1
for homogenization of shellfish flesh; must be possible to sterilize mixer blades and vessel (or flask).	
Centrifuge, min. 5000 5.p.m.	1
Microscope, magnification var.: 100-1000	1
Bacterial colony counter	1
Vacuum pump and accessories:	1
Manifold, 3 filters	3
Filter funnels	30
Accessories, tubes, pipes, etc.	
Autoclave, approx. 100 litres	1
Instrument containers	2
Dry air sterilizer: 50-200°C, minimum 250 litres	1
Low temperature incubator 0-50°C, ± 0.5°C,100-200 litres	1
Air incubator 20-70°C, ± 0.5°C, 300-400 litres	1
Water-bath, approximately 40 x 100 x 15 cm and plastic balls for preventing evaporation	1
Water-bath temperature control immersion type with circulation pump: 20-70°C, ± 0.2°C	

Note: Both for air and water incubation below 30° it is necessary to have air-conditioned work spaces or else to have cooling equipment for the incubator.

Major items of equipment	Number required
Casserole autoclave, pressure cooker, for preparing approximately 10 litres	1
Refrigerator, 5-800 litres total (preferably of several units)	1-4
1.2 Glassware and similar	
Bottles with screw caps, e.g. bakelite:	
250-300 ml: clear glass	100
250-300 ml: amber glass	200
200-300 ml: wide mouth	100
Bottles, 250-300 ml ground stopper	50
flared mouth, for biological oxygen demand	30
Flasks, Ehrlenmeyer, 300 ml	30
ordinary, 200-300 ml	30
Funnels, plastic, 12 cm diameter	5
Imhoff cone, 1 litre	2
Test tubes, different sizes:	
Large, e.g. 180 x 18 mm, 5 (most probable number)	500
Medium, e.g. 160 x 16 mm, 50 (most probable number)	5000
Small, e.g. 130 x 13 mm, 30 (most probable number)	3000
Durham, e.g. 35 x 8 mm, 50 (most probable number)	5000
Preferably use non-reuseable tubes	
Mortars, porcelain:	
500 ml	5
300 ml	10
Pestles, porcelain	5
Graduated pitchers, plastic, 1 litre	2
Graduated cylinders, 100 ml, glass	5
Volumetric flasks, 100 ml, glass	10
Beakers, graduated, 100 ml polypropylene	10
Pipettes, measuring 100 ± 0.1 ml	5
measuring 25 ± 0.05 ml	10
measuring 10	100
measuring 5	20
measuring 2	10
measuring 1	1000
Pasteur, serological	3000
Filling device (Peleus ball)	5
Dishes, Petri 9 cm	1500

	Number required
Spatulas, inoculation, Drigalski:	100
ordinary	2
Centrifuge tubes, polyethylene, 100 ml	50
Inoculation loops (platinum wire)	5
Microscope cover glasses:	
24 × 36 mm, 0.13-0.17 mm	300
Microscope slides, cut-off edges:	
76 × 26 mm	600
Syringes, plastic, non-reuseable, 20 ml:	
(for parasite examination, must fit filter holder for 5 µ filter)	100
Bottles, brown, ground glass stopper and flared mouth (for oxygen)	50
1.3 Miscellaneous equipment and supplies	
Bunsen burner (decide on gas type)	8
Gas cooker	1
Asbestos mittens, pairs	4
Casserole, 8-10 litres (for media preparation if necessary)	1
Pipette washer system	1
Test tube racks (supports), plastic coated wire:	
20 × 20 mm mask, 5 × 10 tubes, 2 (most probable number)	40
16 × 16 mm mask, 5 × 10 tubes, 1 (most probable number)	20
Pair of scissors	1
Crucible tongs, 20 cm	2
Forceps:	
for slides	2
for filters	2
Filters:	
0.45 µ (membrane filtration method, adjusted to filter manifold)	1000
1.25 µ (prefilters)	500
0.22 µ (sterilization)	100
5 µ (for parasitic examinations)	100
Preferably buy single-packed sterilized filters.	
Filter-holders must be suitable for the filters available.	
Thermometers, 0.2°C increments, 0-100°C	5

	Number required
Wire baskets (e.g. 12 × 12 × 8 cm) for biochemical testing	25
Tripods and iron wire-gauze with asbestos	2
Biological waste bins for inoculation pipettes	2
Corks for shipment of samples in test tubes (small)	1000
Cotton, water repelling (tube plugs)	10
Stopwatch, with timing alarm device	1
Aluminium foil	30
Paraffin oil	1 litre
Paraffin (pure refined wax) for sealing test tubes for shipment	1 kg
Sterile plastic bags for fish and shellfish samples for incubation of Petri dishes, etc.	500
Sterilizable plastic bottles, 200-300 ml:	100
preferably with wide mouths, approx. 1 litre	50
approx. 3 litres	25
Cooling boxes, 10-15 litres	10
Cooling elements (for cooling boxes)	30

2. Minimum laboratory cultivation media, reagents, and other chemicals

The following inventory lists apply only to the minimum programme (see Chapters 3 and 6). For extended monitoring there is no obvious limit as to the extent of the programme or equipment and supplies.

2.1 Cultivation media and biochemical test reagents

Media and reagents not commercially available are marked with an asterisk. They must be prepared locally, using the methods suggested in Annex III, and the chemicals listed below. Always follow the manufacturer's instructions to the letter:

	Approx. amount to be stocked
Bismuth sulfite agar	1 kg
Blood agar base medium	1 kg
Brilliant green lactose saccharose agar	1 kg
Cytochrome oxydase reagent*	
Gram reagents*	
KF-streptococcus agar	1 kg
Kovac's indole reagent	
Lysine decarboxylation base medium	2 × 100 g
M-endo agar-MF	1 kg
M-FC agar	1 kg
MacConkey broth	3 kg
Peptone water*	

Phenol red mannitol agar	200 g
Phosphate buffer*	
Plate count agar	1 kg
Potassium tetrathionate broth, ad modum Preuss	1 kg
Pre-enrichment medium*	
Selenite cystine enrichment broth	500 g
Sugar-free agar*	
Sugar solution*	
Sulfite agar*	
Triple sugar iron agar	500 g
Tryptone sulfite neomycin agar	1 kg
Urea broth	200 g

2.2 Other chemicals and base compounds for microbiological examination

Agar	2 × 250 g
Alcohol, 96%	1 litre
Ascorbic acid	200 g
Blood, cattle or sheep	cf. A.3
DL-phenylalamine	100 g
Ferricitrate	200 g
Iodine (I)	200 g
KI	200
KH_2PO_4	1 kg
$K_2H\ PO_4$	1 kg
L-Lysine	100 g
Meat extract	500 g
Methyl violet	100 g
NaCl	2 kg
NaOH	2 kg
$Na_2HPO_4,\ 12H_2O$	1 kg
Neutral red (stain only)	100 g
Peptone, dehydrated	200 g
Saccharose	1 kg
Sodium citrate	200 g
Sodium oxylate	200 g
Sodium sulfite	200 g
Tetramethyl paraphenyl diamine hydrochloride	200 g
Tryptone (dehydrated)	200 g
Xylose	50 g
Yeast extract	500 g

2.3 Chemicals and reagents for biological oxygen demand and analyses

$CaCl_2$	H_2SO_4, conc.
$FeCl_3,\ 6H_2O$	$M\ SO_4,\ 1H_2O$

KH_2PO_4	NaI
K_2HPO_4	NaN_3
$MgSO_4$, $7H_2O$	NaOH
Na_2HPO_4, $7H_2O$	$Na_2S_2O_3$, $5H_2O$
Na_2SO_3	Potato starch
NH_4Cl	

For all these chemicals, approximately 1 kg or 1 litre should be stocked.

3. Minimum field requirements

3.1 Monitoring vessels

At least one boat or ship is required, even for the simplest monitoring programme. With only one vessel the main task is to take samples from individual sampling points, particularly for studies of microbiological water quality.

If two vessels are available one should be assigned to microbiological sampling at individual points and the other to hydrographical observations at the reference point. The multiple-point sampling vessel, in addition to being properly seaworthy, must possess:

(1) ample space for:

- work with sampling equipment,
- work with samples,
- work with cooling containers;

(2) ample space and safe fastening for cooling containers;

(3) safe fastening of all equipment, even in rough seas;

(4) shade for storing containers;

(5) davits or similar and winch for lowering and hoistening sampling equipment (water and sediment);

(6) reasonably high speed, e.g. above 8 knots (15-20 km/h) to decrease sampling and storage time.

The reference point hydrographic vessel must possess:

(1) adequate size (not less than 10 metres in length) and shape to keep the vessel relatively stable in slightly choppy seas and some wind;

(2) good mooring equipment, not less than two anchors and associated winding apparatus;

(3) adequate space for working with sampling equipment, direct recording instruments such as salinity-meters, oxygen-meters, anemometers and current meters; and

(4) davits and fittings for lowering and hoisting hydrographic equipment.

Before payment is made or contracting out, any monitoring vessel should be subjected to intensive trials.

3.2 Instrumentation

The list applies to the minimum monitoring programme only. Quantities indicated in parentheses refer to cases where two vessels are used. The use of an asterisk indicates dependence on the circumstances and extent of the monitoring programme. In all cases, quantities stocked must be estimated locally.

Item	No. of items	Remarks
Anemometer	1(2)	
Drift cards	50	
Dye	50 kg	
Drogues	5(7)	
Salinity-meter	1(2)	
Hydrometers	2(4) 2(4)	0.966-1.011 sp. grov. 1.010-1.031 sp. grov.
Thermometers	3(6)	0-60 or 0-100°C
Secchi discs	2(3)	
Oxygen-meter	1	
Sextant	1(2)	
Subsurface water sampler	1(2)	Approx. 1 litre
Insulating sampler	1	
Bottom sampler	1	e.g. Albrechtsen type
Extension arm	1	Surface water sampling
Local radio transceiver	2(3)	

3.3 Sample containers

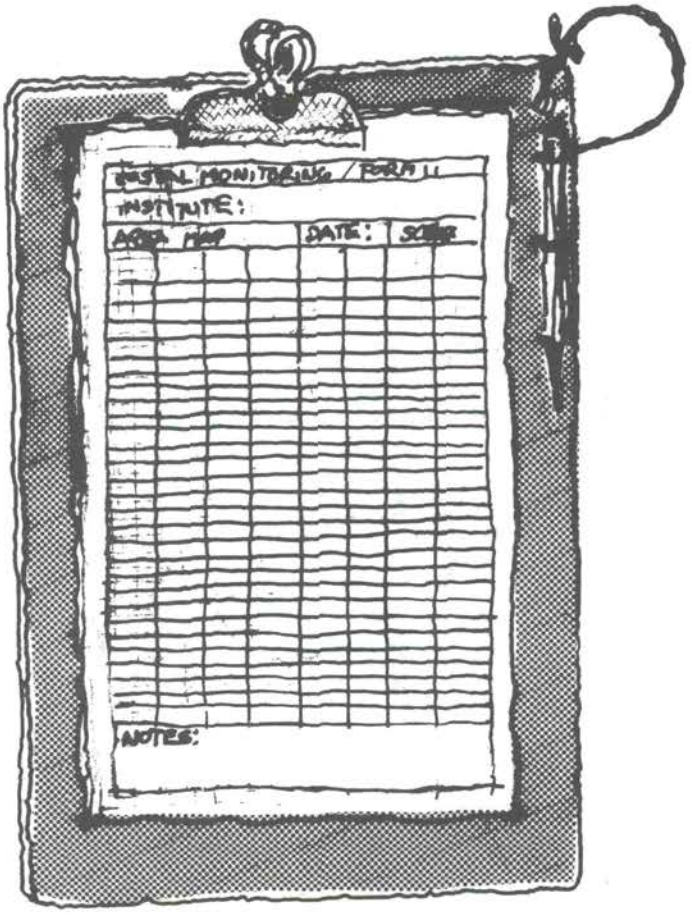
50 ml bottles, glass	*	Oxygen
200-300 ml bottles, glass	*	Microbiology, water
200-300 ml bottles, plastic	*	Microbiology, sediment
Plastic bags	*	Microbiology shellfish
Plastic bottles and jars	*	Effluent sampling

3.4 Miscellaneous

Tags and labels	*	
Maps, nautical	1(2)	
Maps, sampling points	1(2)	
Cooling containers	*	
Sterilization liquid	*	
Winkler reagents for oxygen fixation	100 ml 100 ml	Manganese sulfate Alkali-iodide azide
Imhoff cone	2	1 litre
Waterproof ink and pen		For outdoor recording
Clipboard for standard forms, including penholder	5	Locally produced

Fig. 1

STANDARD CLIPBOARD FOR USE IN FIELD AND LABORATORY



ANNEX III

CULTIVATION SUBSTRATES AND TEST REAGENTS FOR MICROBIOLOGICAL EXAMINATIONS

This annex contains an alphabetically ordered list of substrates and reagents, their make-up and their specific reactions. While discouraging the use of self-prepared substrates, as the accuracy, precision and comparability of results can easily be jeopardized, the list is presented in an effort to assist in safe identification of a product and to improve general knowledge of examination processes. The use of identical substrates and reagents by a number of collaborating or corresponding institutes may provide opportunities for centralized bulk purchases at reduced cost per unit. Where feasible, we have striven to recommend products that are commercially available both for the sake of comparability, and also as the constituents are more likely to be highly standardized. Mention of proprietary names in this context does not imply that the products are endorsed or recommended by WHO or UNEP in preference to others of a similar nature that are not mentioned. Substrates and reagents which are not commercially available are indicated by an asterisk*.

Once again, it must be emphasized that directions for use issued by manufacturers must always be strictly adhered to.

Arabinose broth*

Peptone	10.0 g
Meat extract	5.0 g
Na_2HPO_4	2.0 g
Distilled water	1 litre

Adjust pH to 7.4, autoclave 15 minutes at 121°C, then add 5.0 g arabinose and 12 ml 0.2% autoclaved aqueous solution of bromothymol blue.

To obtain a 2% NaCl arabinose add 20 g of NaCl and similarly for other % of NaCl.

Reactions: Positive: yellow colour
Negative: substrate colour

Applications: Vibrios, Yersinia

Arginine broth*

Base medium is that used for Lysine. Add 10.0 g L-arginine sterilized in boiling water. Adjust pH to colourless medium. Dispense 3 - 4 ml in small test tubes and cover with liquefied paraffin.

Reactions: Positive: amethyst-like colour before 4 days
Negative: colourless or yellow after 4 days

Applications: Vibrios, Shigella, Yersinia

Bismuth sulfite agar

Peptone	10 g
Meat extract	5 g
D(+) glucose	5 g
Ferro-sulfate	0.3 g
Na_2HPO_4	4 g
Brilliant green	0.025 g
Bismuth sulfite	8 g

Agar	15 g
Distilled water	1 litre

The pH is adjusted to 7.6 ± 0.1. Thick plates must be prepared, e.g. 20 ml substrate per plate, should be used. The plates must be dried free from condensate before use.

Application Salmonellas

Blood agar

Tryptose	10.0 g
Beef extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1 litre

Above is the base medium. Before use, 5% of sterile sheep or cattle blood is added to the liquefied substrate before pouring on the plates. Store blood at 4°C no longer than two weeks. Prevent coagulation by adding oxylate or citrate.

Applications Salmonellas, Vibrios, Shigella, Yersinia.

Brilliant green lactose saccharose agar

Meat extract	5	g
Peptone	10.0	g
Lactose	10.0	g
Sucrose	10.0	g
NaCl	3.0	g
Na_2HPO_4	2.0	g
Phenol red	0.08	g
Brilliant green	0.0125	g
Agar	12.0	g
Distilled water	1	litre

Adjust pH to 6.9. Autoclave at 121° C for 15 minutes. To prevent swarming, add 8 mg sodium sulfadiazine per 100 ml substrate or 0.3% alkylbenzene sulfonate (Teepol). The plates must be dried until no condensate is left.

Application Salmonellas

Bromothymol blue

Pure bromothymol blue	1.0	g
0.1 N NaOH	25	ml
Distilled water	475	ml

Autoclave 15 minutes at 121°C.

Applications Sugar fermenting processes

Cellobiose broth*

Same as arabinose, with cellobiose replacing arabinose.

Application Yersinia

Cytochrome oxydase reagent*

Ascorbic acid	10 mg
Sterile distilled water	10 ml
Tetramethylparaphenyldianim hydrochloride	100 mg

Storage in refrigerator. Ascorbic acid is added to prevent oxidation of the reagent.

Procedure Observe reactions in a cytochrome oxydase-moistened filter paper. Streak suspect colonies onto the surface.

Reactions Positive: blue colour within 10 seconds

Negative: no blue colour within 10 seconds

Applications Total coliforms, Vibrios, Shigella, Yersinia.

Fluid Yersinia peptone broth (selective)*

Peptone	20.0 g
NaCl	5.0 g
Na_2HPO_4, 12 H_2O	29.5 ml, 0.5 M
NaH_2PO_4	4.0 ml, 0.5 M
Distilled water	1 litre

Adjust pH to 7.6. Autoclave 15 min at 121°C. Immediately before use, add from a 40% sterilized aqueous urea solution to obtain a final urea concentration of 1.0%. Sterilize urea by filtering through a 0.22 µ filter. Also just before use add Teepol (ABS) to a final broth concentration = 0.2%, which will ensure that gram-positive bacteria will not grow.

Application Yersinia

Gram reagents*

Methyl violet solution (0.2 g methyl violet must be dissolved in 100 ml distilled water and then filtered).

Lugol's solution (1.0 g I and 3.0 g KI in 4.0 ml distilled water; then add distilled water to 100 ml and store in a dark place).

Alcohol, 96%.

Neutral red (1.0 g neutral red is dissolved in 100 ml distilled water. Boil and add 0.1 ml acetic acid and filter).

Procedure

(1) fix bacteria colonies on a micro-slide by moving the slide over a flame;

(2) dye with methyl violet for one minute;

(3) remove methyl violet and rinse in Lugol's solution for one minute.

(4) remove Lugol's solution and apply alcohol for a few seconds;

(5) rinse with distilled water;

(6) dye with neutral red for one minute

(7) rinse with distilled water;

(8) dry on filter paper.

Reactions — Gram positive = blue - dark
Gram negative = red

Application — General, for most bacteria.

Hajna's GN - broth

Yeast extract	1.0 g
Di-ammonium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	2.0 g
Sodium chloride	5.0 g
Magnesium sulfate	0.5 g
Sodium citrate	5.0 g
Sodium deoxycholate	0.5 g
Distilled water	1 litre

Adjust pH to 7.0 ± 0.2.

Hugh Leifson agar*

Peptone	2.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Agar	3.0 g
Bromothymol Blue	0.08 g
Distilled water	1 litre

Adjust pH to 7.1. Autoclave at 121°C for 15 minutes.

Preparation of bromothymol blue: Pure bromide
Thymol blue = 1.0 g; 0.1 N NaOH = 25 ml;
Distilled water = 475 ml; autoclave 15 minutes at 121°C

To the above Hugh Leifson base medium is added 20 ml 50% sterile glucose solution. The substrate is dispensed in 5 ml portions in small test tubes. Place suspect strains in two tubes, and cover one by sterile paraffin to obtain anaerobic growth.

Reactions

(1) both tubes yellow = fermentative bacteria;

(2) only aerobic tube yellow = oxidative bacteria;

(3) both tubes blue green or blue = no degradation of glucose.

Applications — Vibrios, Yersinia.

Inositole broth*

Same as arabinose, with inositole replacing arabinose.

Reactions — Positive: yellow colour
Negative: substrate colour

Application — Vibrios

KF streptococcus agar

Proteose peptone No. 3 or polypeptone	10.0 g
Yeast extract	10.0 g
Sodium chloride	5.0 g
Sodium glycerophosphate	10.0 g
Maltose	20.0 g
Lactose	1.0 g
Sodium azide	0.4 g
Agar	20.0 g
Distilled water	1 litre

Mix 7.64 g of dehydrated medium with 100 ml of distilled water in a flask. Heat in a boiling water bath to dissolve the agar. After solution is complete, heat for an additional five minutes. Cool to 50 to 60°C and add 1 ml sterile aqueous 1% solution of 2, 3, 5 triphenyl tetazolium chloride/100 ml. Adjust pH to 7.2 with 10% Na_2CO_3 if necessary. The medium may be maintained at between 45 and 50°C for up to 4 hrs. before plates are poured. Poured plates may be stored in the dark up to 30 days when maintained at between 2 and 10°C.

Application — Faecal streptococcus

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 together with incubation in peptone water to demonstrate indole production.

Reactions — Positive: red coloured surface after addition of the reagent
Negative: no red colour develops

Applications — Faecal coliforms, Salmonellas, Vibrios, Shigella.

Lysine decarboxylation (base) medium

Peptone (Evans)	5.0 g
Beef extract	5.0 g
Dextrose	0.5 g

Bromo cresol purple (0.6 ml 1.6%)	0.01 g
Cresol red (2.5 ml 0.2%)	0.005 g
Pyridoxal hydrochloride	5.0 mg
Distilled water	1 litre

Adjust pH to 6.0. Add 10 g L-lysine to 1000 ml of the above autoclaved base medium. Adjust pH to colourless medium. Place 3 - 4 ml in each test tube and cover by 1 cm sterile paraffin. Autoclave 15 minutes at 121°C.

Reactions — Positive: amethyst-like colour before four days
Negative: no change or colourless after four days

Applications — Salmonellas, Vibrios, Shigella, Yersinia.

M-endo-agar-MF

Polypeptone	10 g
Thiopeptone	5 g
Casitone	5 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 sulfate	.05 g
Sodium desoxycholate	.10 g
Sodium sulfite	2.10 g
Basic fuchsine	1.05 g
Distilled water	1 litre
Agar	15 g

Rehydrate in 1 litre 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.

The finished medium should be stored in the dark at 4°C and any unused medium discarded after 96 hours.

Application — Total coliforms

M-FC-agar

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	.1 g
Distilled water	1 litre
Agar	15.0 g

Rehydrate in the distilled water containing 10 ml of 1% rosolic acid in 0.2 N NaOH. Heat the medium to boiling point and promptly remove from heat. Cool to below 45°C (Do not sterilize by autoclaving!). Final pH should be 7.4.

The finished medium should be stored at 2 to 10°C and any unused medium discarded after 96 hours

Application Faecal coliforms

MacConkey broth

	Single Strength	Double Strength
Sodium taurocholate	5 g	10 g
Lactose	10 g	20 g
NaCl	5 g	10 g
Peptone	20 g	40 g
Distilled water	1 litre	1 litre

Dissolve by shaking. Adjust pH to 7.1, and add 2 ml of an alcohol solution (e.g. bremocresol purple (1%)) to the single strength medium and 4 ml to the double strength medium.

Autoclave before any use at 110°C for 15 minutes. The gas inside the Durham tubes (inverted vials) contained in the test tubes will disappear during autoclaving.

Application Total coliforms, Faecal coliforms.

Nessler's reagent*

Mix 5.0 g KI and 5 ml distilled and boiled water. Add cooled saturated solution of $HgCl_2$ until precipitate no longer redissolves on shaking. Add 40 ml 9N NacH. Dilute by distilled water until 100 ml. Let the mixture stand for 24 hours before use.

Application Vibrios (nitrate bouillon)

Nitrate bouillon*

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	1.0 g
Distilled water	1 litre

After 96 hours add 1 ml of reagents A & B:

Reagent A: 0.8% sulfanilic acid in 5 N acetic acid

Reagent B: 0.6% dimethyl -x-naphtylamine in 5 N acetic acid

Reactions Positive: red colour (nitrite present)

Negative: no red colour

Application Vibrios

Note No nitrite means either no bacterial nitrate reduction or further reduction of nitrite to ammonia. The absence of nitrate reduction can be demonstrated by adding powdered Zn (5mg/ml of culture) and then

testing for red colouring. The presence of ammonia can be demonstrated by Nessler's reagent (dark brown or yellow precipitate upon adding a few drops of the reagent).

Ornithine decarboxylation medium*

The base medium is that used for lysine base medium. Add 10.0 g of ornithine sterilized in distilled water by boiling to 1000 ml autoclaved base medium. Adjust pH to colourless medium. Dispense 3 - 4 ml in small test tubes and cover with 1 - 2 cm sterile paraffin.

Reactions: Positive: amethyst colour before four days
Negative: colourless or yellow after four days

Applications: Vibrios, Yersinia.

Peptone water

Tryptone	10.0 g
NaCl	5.0 g
Distilled water	1 litre

Dispense in test tubes in 5 ml amounts and autoclave at 121°C for 15 minutes. Adjust pH to 7.0 - 7.4.

Reactions: Cf. Kovac's reagents, used to test for indole production.

Applications: Faecal coliforms, Salmonellas, Vibrios.

Phenol red mannitol agar

Proteose peptone	10.0 g
Beef extract	1.0 g
D - mannitol	10.0 g
Sodium chloride	5.0 g
Phenol red	0.025 g
Agar	15.0 g
Distilled water	1 litre

Dissolve by heating. Autoclave 15 minutes at 121°C. Adjust pH to 7.4. Avoid swarming of Proteus by adding .3% alkylbenzene sulfonate (Teepol) to the agar.

Application: Salmonellas

Phenylalamine agar NCA

DL - phenylalamine	2.0 g
Yeast extract	3.0 g
Na_2HPO_4	1.0 g
NaCl	5.0 g
Agar	20.0 g
Distilled water	1 litre

Dissolve ingredients by heating in water. Filter, place in test tubes and sterilize at 115°C for 15 minutes. Solidify in tilted tubes to obtain a long agar surface.

Reactions — Positive: green surface colour upon addition of 10% aqueous ferric chloride

Negative: no green colouring

Applications — Yersinia

Phosphate buffer (pH = 7.2)*

K_2HPO_4	3 g
KH_2PO_4	1 g
Distilled water	1 litre

Autoclaving at 121°C for 15 minutes.

Application — All dilution series

Plate count agar

Peptone	5 g
Yeast extract	3 g
Agar	15 g
Distilled water	1 litre

Dissolve by heating and agitate gently. Adjust pH to 7.0.

Application — Total heterotrophic bacteria.

Preuss potassium tetrathionate broth

Peptone	8.6 g
NaCl	6.4 g
Potassium tetrathionate	20.0 g
Crystal violet	0.005 g
Distilled water	1 litre

The pH is adjusted to 6.5 ± 0.1. Sterile substrate powders are dissolved in sterilized water without heating. Do not autoclave the prepared medium. The selective capacity may improve by a a adding .3% alkylbenzene sulfonate (Teepol).

Application — Salmonellas

Pre-enrichment medium*

(Non-selective buffer solution)

Peptone	10.0 g
NaCl	5.0 g
Na_2HPO_4, 12 H_2O	9.0 g

KH_2PO_4	1.5 g
Distilled water	1 litre

Dissolve by heating. Autoclave 15 minutes at 121°C. Adjust pH to 7.2.

Application Salmonellas

Pteridine discs

Ether	5 ml
99% alcohol	5 ml
2 - 4 - diamino - 6 - 7 - diisopropylpteridine (Vibriostaticum O/12A)	150 mg

Cork the tube while dissolving, then add discs and let soak. When thoroughly soaked the discs are dried in sterile Petri dishes. After drying, the discs are ready for use on solid media (e.g. blood agar).

Application Vibrios

Rhamnose broth

Same as Arabinose with Rhamnose replacing Arabinose.

Application Yersinia

Selenite cystine enrichment broth

Peptone	5.0 g
L-cystine	0.01 g
Lactose	4.0 g
Anhydrous di-sodium hydrogen phosphate	2.0 g
Sodium selenite	4.0 g
Distilled water	1 litre

Adjust pH to 7.0 ± 0.2. Do not autoclave, and dissolve at less than 70°C.

Application Salmonellas

Sulfide-indole-motility (SIM) substrate

Peptone from casein	20.0 g
Peptone from meat	6.6 g
Ammonium iron (III) citrate	.2 g
Sodium thiosulfate	.2 g
Agar	3.0 g

Autoclave 15 minutes at 121°C. Adjust pH to 7.3.

Reactions (1) Motility is indicated by diffuse turbidity of the medium around the puncture line. Growth limited to the puncture line means non-motility.

(2) H_2S production is shown by blackening along the puncture line or throughout the medium.

(3) The indole reaction is tested after (1) and (2) applying Kovac's reagent (q.v.)

Application: Shigella, Yersinia.

Simmons' citrate agar

Ammonium dihydrogen phosphate	1.0 g
Di-potassium hydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulfate	0.2 g
Bromothymol blue	0.08 g
Agar	12.0 g

Autoclave 15 minutes at 121°C. Adjust pH to 6.9.

Reactions: Positive: blue colour before four days
Negative: substrate colour after four days

Applications: Shigella, Yersinia.

Solid Yersinia peptone agar (selective)*

Peptone	20.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1 litre

Before use add Teepol (ABS) to a final concentration of 0.3% which will ensure no growth of gram positive bacteria.

Application: Yersinia

Starch-salt agar*

Trypticase-peptone	10.0 g
Yeast extract	3.0 g
NaCl	50.0 g
Agar	15.0 g
Distilled water	1 litre

Before use, 5 g soluble starch is added to the base medium after heating in distilled water. In addition, 15 ml of an autoclaved aqueous 0.2% Bromothymol blue solution is added.

Reactions: Positive: yellow colour
Negative: substrate colour

Application: Vibrios

Sucrose broth

Same as arabinose, with sucrose replacing arabinose.

Applications Vibrios, Yersinia.

Sugar free agar (for sample shipment)*

Meat extract	5.0 g
Peptone	10.0 g
Na_2HPO_4, 12 H_2O	2.0 g
Agar	15.0 g
Distilled water	1 litre

Autoclave 15 minutes at 121°C. Adjust pH to 7.4. Place 5 ml portions in small test tubes. Inoculate stab cultures. Seal for storage by use of melted paraffin.

Application Salmonellas

Sugar solution*

Saccharose	2000 g
Distilled water	1530 ml

Dissolve by boiling. Add 1.53 ml 0.1% Tween 80. Adjust pH to 11 by adding 4N NaOH.

Application Parasite separation.

Sulfite agar*

Tryptone	15.0 g
Yeast extract	10.0 g
Agar	15.0 g
Distilled water	1 litre

Just before use add to 200 ml of liquefied agar:

2 ml sterile 5% ferric citrate
2 ml sterile 5% sodium sulfite

Place approximately 4 ml substrate in small test tubes. Inoculate by stab culture technique.

Reactions Positive: blackening along stab or all over

Negative: no blackening

Application Salmonellas

Taurocholate-tellurite-peptone substrate

Trypticase	10.0 g
NaCl	10.0 g
Sodium taurocholate	5.0 g

Na_2CO_3	1.0	g
Distilled water	1	litre

Adjust pH to 9.2 ± 0.1. Autoclave 15 minutes at 121°C. 1 ml of filter-sterilized potassium tellurite in water is added before use.

Applications Vibrio Chol. and NAG.

Teepol

Purchase preferably Teepol 515 (alkyl benzene sulfonate) or 610 (alkyl sulfonate) or Lutensit ABA. It may be difficult to obtain because it is not yet very widely known or applied.

Applications Yersinia, salmonellas, vibrios, viruses

Thiosulfate-citrate-bile-salt-sucrose-sugar (TCBS) agar

Peptone	10	g
Yeast extract	5	g
Sodium citrate	10	g
Sodium thiosulfate	10	g
Ox bile (dried)	5	g
Sodium cholate	3	g
Sucrose	20	g
NaCl	10	
Ferric citrate	1	
Thymol blue	0.04	g
Bromothymol blue	0.04	g
Agar	14	
Distilled water	1	litre

Adjust pH to 8.6 ± 0.1. Sterilize by boiling, but avoid autoclaving.

Application Vibrios

Triple sugar iron (TSI) agar

Meat extract	3.0	g
Yeast extract	3.0	g
Peptone from casein	15.0	g
Peptone from meat	5.0	g
Lactose	10.0	g
Sucrose	10.0	g
D(+) glucose	1.0	g
Ammonium trivalent iron citrate	0.5	g
Sodium chloride	5.0	g
Sodium thiosulfate	0.5	g

Phenol red	0.024 g
Agar	12.0 g
Distilled water	1 litre

Adjust pH to 7.4. Autoclave 15 minutes at 121°C. Dispense 6 - 8 ml portions into small tubes, and allow to solidify to obtain a 3 cm deep butt and a 3 cm long surface slant.

Reactions
Red/red: no carbohydrate fermentation
Red/yellow: glucose fermentation only
Red/yellow: glucose, lactose and/or sucrose fermentation

Blackening means H_2S production. Gas may be produced along the stab.

Applications Salmonellas, Shigella.

Tryptone 1% broth

Tryptone	10.0 g
Distilled water	1 litre

Autoclave 15 minutes at 121°C. To obtain 2%, 4%, 8%, or 10% NaCl, add 20 g, 40 g, 80 g, or 100 g NaCl respectively to the above base medium. Autoclaving is again necessary after preparation.

Reaction Growth indicated by increased turbidity.
No growth leaves a clear medium.

Application Vibrios.

Tryptone sulfite neomycin, agar

Peptone	15.0 g
Yeast extract	10.0 g
Sodium sulfite	1.0 g
Trivalent iron citrate	0.5 g
Polymyxin B sulfate	0.02 g
Neomycin sulfate	0.05 g
Agar	13.5 g
Distilled water	1 litre

Autoclave at 121°C for only 10 minutes. Adjust pH to 7.2.

Application Cl perfringens.

Urea broth

Yeast extract	.1 g
K PO	9.1 g
Na_2HPO_4	9.5 g
Urea	20.0 g
Phenol red	0.01 g
Distilled water	1 litre

Sterilize by passing through a 0.22 γ membrane filter. Adjust pH to 6.8. Dispense in small tubes, 3 - 4 ml each.

Reactions — Positive: red colour after 1 - 4 days

Negative: still yellow after 4 days

Applications — Salmonellas, Shigella, Yersinia.

VP-broth*

Protease - Peptone	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1 litre

Autoclave 15 minutes at 121°C. Add 5 g glucose as an autoclaved aqueous solution.

Procedure — After 3 - 4 days' incubation 2.0 ml 6% alcoholic alpha-naphtol and 0.8 ml 40% KOH are added to 4 ml VP-broth.

Reactions — Positive: red colour after a few minutes

Negative: substrate colour

Applications — Vibrios, Yersinia.

Vibrio parahaemolyticus - selective salt meat broth*

Veal infusion broth	1 litre
NaCl	55.0 g
Starch, soluble	5.0 g
Distilled water	1 litre

Dissolve 5.0 g of soluble starch by boiling in water and add to the substrate, which may be applied with or without addition of 0.2% alkyl benzene sulfonate (Teepol). Adjust pH to 7.4±0.1. Autoclave 15 minutes at 121°C.

Application — V. parahaemolyticus.

Xylose broth*

Same as arabinose with two exceptions:

(1) Xylose replaces arabinose

(2) Sterilization must be done by 0.22 γ filtration, not by autoclaving the broth.

Application — Yersinia.

Xylose-lysine-desoxycholate, agar

D (+) xylose	3.5 g
L (+) lysine	5.0 g
Lactose	7.5 g
Saccharose	7.5 g
NaCl	5.0 g

Yeast extract	3.0	g
Sodium desoxycholate	2.5	g
Sodium thiosulfate	6.8	g
Ferric ammonium citrate	0.8	g
Phenol red	0.08	g
Agar	13.5	g
Distilled water	1	litre

Adjust pH to 7.4 ± 0.1.

Application Shigella.

ANNEX IV

STANDARD FORMS FOR DATA RECORDING IN FIELD AND LABORATORY

A number of standard forms have been prepared to help ease work in the field and laboratory. The use of these forms will ensure good record-keeping and safe handling of results, and the forms may also serve as checklists during sampling and analyses. The forms are:

FORM 1	Sample tag or label
FORM 2	Reference point, currents
FORM 3	Reference point, hydrography
FORM 4	Multiple point sampling
FORM 5	Pollution source sampling
FORM 6	Microbiological laboratory examination, various
FORM 7	Total coliform, most probable number
FORM 8	Total and faecal coliforms, most probable number
FORM 9	Quantitative pathogen examination, most probable number
FORM 10	Qualitative pathogen examination, most probable number
FORM 11	Periodical summary sheet

The forms may be used directly or with minor modifications to record basic results. Before reproduction, the name and address of the responsible institution should be printed on the forms.

Form 1

IDENTIFICATION LABEL FOR
SAMPLE CONTAINERS

COASTAL MONITORING IDENTIFICATION LABEL FORM 1
Institution: Address: Telephone:
Area name:
Point identification:
Date: Time:
Medium: Parameter:
Sampling depth (m)
Temperature: °C Salinity: °/oo
Container No.
Signature:
Remarks:

Notes:

(1) Preferably use self-adhesive labels or tags with printed text, including full address and telephone of the institution responsible for the sampling.

(2) A container identification (indelible, preferably printed code) is indispensable for safe handling of numerous samples and subsequent laboratory analysis. Any locally used identification is acceptable.

(3) Area name according to the monitoring map identification (see Figs 5a and 5b). Similarly point identification (use letter and number only).

(4) Medium: For example, water, sediment, mollusc, sewage, etc.

(5) Parameter: For example, microbiological, oxygen, salinity, biological oxygen demand, etc.

(6) Salinity is normally determined by salinometer. If determined by hydrometer, enter specific gravity under remarks and calculate salinity later.

Form 2

CURRENTS

COASTAL MONITORING / REFERENCE POINT VESSEL / FORM 2					
Institution and country					
Area:			Date:		
Point:			Sheet:		
Time hrs	Depth m	Direction from		Velocity cm/s	
Remarks:					

Form 3

HYDROGRAPHIC DATA

COASTAL MONITORING / REFERENCE POINT VESSEL / FORM 3

Institution and country:

Area: | Date:

Point: | Sheet no:

1. Air temp °C
2. Wind, m/s
3. Wind, direction from
4. Wave height, m
5. Secchi disc, m

Time hrs	Depth m	Salinity* °/co	Temp °C	Oxygen bottle no.**, %

Notes:

* If salinity is determined hydrometrically, the specific gravity is entered here. No. of parts per thousand is determined later.

** If oxygen is determined by sampling, the bottle number should be entered here, and percentage entered after laboratory analysis.

Form 4 MULTIPLE POINT SAMPLING

COASTAL MONITORING / MULTIPLE POINT SAMPLING/FORM 4

Institution and country:

Area: Map:

Date: Sheet No:

Point no	Time hrs	Medium	Parameter	Depth m	Container No.	Total depth m	Garber* classif visually	Cool** box °C

Notes: Medium abbreviation: Wat-er, Sed-iment, Mol-lusc,
Parameter abbreviation: Ox-ygen, Mic-robiology,
** Only occasional reading, e.g. every 2 hours.
* See Table 2, Section 5.

Form 5 POLLUTION SOURCE SAMPLING

COASTAL MONITORING / POLLUTION SOURCE SAMPLING / FORM 5

Institution and country:

Area: Date:
Map: Sheet no:

Point no	Time hrs	Pollution Source* type	BOD Cont. No. mg/c	Settleable Matter Container No. ml/l	Parameter** Sample volume Container No.		

Notes: *Source: river, prim-ary, sec-ondary, ter-tiary or raw sewage, etc.

**Parameter, e.g. bact-eria, virus, par-asites.

Form 6

MICROBIOLOGICAL LABORATORY EXAMINATION
(MEMBRANE FILTRATION, POUR PLATE, SPREAD PLATE OR STANDING TUBE METHODS)

COASTAL MONITORING / LABORATORY EXAMINATION / FORM 6											
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 of Colonies in the 20 - 80 range only: 50											
10											
1											
10^{-1}											
10^{-2}											
10^{-3}											
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:											

Form 7

TOTAL COLIFORM LABORATORY EXAMINATION
(MOST PROBABLE NO. METHOD)

COASTAL MONITORING / LABORATORY EXAMINATION / FORM 7

Institution and country										Organism: Total coliform Area: Year: Sheet no:									
Point no																			
Date d																			
Time h																			
Medium																			
Depth m																			
Temperature °C																			
Salinity °/oo																			
Container no																			
Start incubation d																			
h																			
Sample test amount unit: g or ml	1a	1b	1a	1b	1a	1b	1a	1b		1a	1b	1a	1b	1a	1b	1a	1b		
10																			
Number of positive tubes for the sample test amount indicated 1																			
10^{-1}																			
10^{-2}																			
10^{-3}																			
10^{-4}																			
10^{-5}																			
10^{-6}																			
10^{-7}																			
Date and time of ceasing incubation																			
Most probable no. / .....*																			
Examiner's signature																			

Notes: * Use 100 ml for water, 100 g for shellfish, and 1 g for sediment.
1a: Reading after 24 hours at 36°C in MacConkey
1b: Reading after 48 hours at 36°C in MacConkey

Remarks:

Form 8 TOTAL AND FAECAL COLIFORM LABORATORY EXAMINATION (MOST PROBABLE NO. METHOD)

COASTAL MONITORING / LABORATORY EXAMINATION / FORM 8																
Institution and country:								Organism: Coliform, total and faecal Area: Year: Sheet no:								
Point	no															
Date	d															
Time	h															
Medium																
Depth	m															
Temperature	°C															
Salinity	‰															
Container	no															
Start incubation	d															
	h															
Sample test amount unit:	g or ml	1a	1b	2a	2b	1a	1b	2a	2b	1a	1b	2a	2b	1a	1b	2a	2b
Number of positive tubes for the sample test amount indicated	10																
	1																
	10^{-1}																
	10^{-2}																
	10^{-3}																
	10^{-4}																
	10^{-5}																
	10^{-6}																
	10^{-7}																
Stop incubation d & h																
Date and time of stopping incubation																
Total coli, most probable no. / .....*																
Faecal coli, most probable no. / .....*																
Examiner's signature																

Notes: * Use 100 ml for water, 100 g for shellfish, and 1 g for sediment.
1a Reading after 24 hrs at 36°C in MacConkey
1b Reading after 48 hours at 36°C in MacConkey
2a Reading after 24 hrs at 44°C in MacConkey
2b Reading after 24 hrs at 44°C in peptone water

Remarks:

Form 9 QUANTITATIVE PATHOGEN LABORATORY EXAMINATION

COASTAL MONITORING / LABORATORY EXAMINATION / FORM 9										
Institution and country			Organism:							
			Area :							
			Year :							
			Sheet no:							
Point	no									
Date	d									
Time	h									
Medium										
Depth	m									
Temperature	°C									
Salinity	‰									
Container	no									
Sample test amount unit	g or ml									
Number of positive tubes for the sample test amount indicated	..... x 10^{3}									
	..... x 10^{2}									
	..... x 10^{1}									
	..... x 1									
	..... x 10^{-1}									
	..... x 10^{-2}									
	..... x 10^{-3}									
	..... x 10^{-4}									
	..... x 10^{-5}									
	..... x 10^{-6}									
Most probable no. / .....*										
Examiner's signature										
Notes: * Use 100 ml water, 100 g shellfish, and 1 g sediment										
Remarks:										

Form 10 QUALITATIVE PATHOGEN LABORATORY EXAMINATION

COASTAL MONITORING / LABORATORY EXAMINATION / FORM 10

Institution and country:

Organism :
Area :
Year :
Sheet no :

Point no. / Depth (m)	Date / Time	Temp °C / Salin. °/oo	Sign container no.	Medium	Positive / Negative	Remarks

Form 11 PERIODICAL REPORTS FOR INDIVIDUAL YEARS, DATES, POINTS, MEDIA AND ORGANISMS

COASTAL MONITORING / PERIODICAL SUMMARY / FORM 11

Institution and country

Area: | Organism:

Point, longitude: ....°....'....".... | Medium:

Point, latitude: ....°....'....".... | Year:

Sheet No:

Date	Reference point				Sampling point				Reference point				Sampling point				Date
	Wind from		Current from		Temp	Salin	Depth	Count	Wind from		Current from		Temp	Salin	Depth	Count	
	°	m/s	°	cm/s	°C	°/oo	m	*	°	m/s	°	cm/s	°C	°/oo	m	*	

Notes: (1) Give wind and current directions in compass degrees, ± 10° approximately.

* Counts are per 100 ml for water, per 100 g for shellfish, and per g for sediments.

ANNEX V

GENERAL REQUIREMENTS AND QUALITY CONTROL FOR MICROBIOLOGICAL LABORATORIES

The following recommendations apply to the minimum programme (see Chapters 3 and 6).

1. Laboratory personnel training

The analyst (laboratory technician) should have one year's experience in water microbiology before being assigned to work independently on major examination programmes or parts thereof.

The supervisor of the laboratory should hold at least a Bachelor's Degree in microbiology and should have had considerable technical field and laboratory training.

Extension courses are recommended on a regular basis, for example by sending personnel to specialized laboratories for in-service training, when new procedures or organisms to be examined are introduced.

Control of good laboratory performance is commented on below (section 4, quality control programme), but it should be noted that good records in the laboratory quality control programme may significantly characterize the skills of the personnel.

2. Laboratory equipment, supplies and materials

pH meter	Must measure accurately per 0.1 pH unit. Must be clean and calibrated for each period of use with pH = 7.0 standard buffer. Date commercial buffers on initial use (sticker on the container).
Balances	Must be clean, not corroded, tear out and detect accurately according to specifications (± 0.3 mg for analytical balance, ± 50 mg for others). Balances should carry a sticker to verify maintenance records.
Thermometers	Must be checked regularly against a certified thermometer (glass thermometers yearly, metal thermometers quarterly). Liquid columns must be without separation.
Incubators	Must be of sufficient size to allow good air or water circulation (even temperature distribution). Must be checked daily for temperature stability. Built-in thermometers must be checked as indicated for thermometers. Must be checked daily when in use for correct humidity control (air incubators).
Autoclave	Must be of sufficient size to prevent crowding of items to be sterilized. Must be checked daily for correct temperatures on the exhaust side and timing. Must reach 121°C (when filled) within 15 minutes and should not require more than 12 minutes for depressurization.
Dry air sterilizer (hot air oven)	Must be checked daily for correct temperature and stability. Thermometer should be checked separately as indicated above.
Refrigerator	Must be checked for preset temperature in the 1-8°C range. Must be cleaned and disinfected regularly, e.g., on a weekly or monthly basis depending on use.
Filtration equipment	Membrane filter equipment should be non-leaking and uncorroded. All reusable units must be suitable for use in an autoclave. Preferably use sterile single packed filters. If not, make sure that rinsable membranes are sterilized at 121° for at least 10 minutes. Rinsable filters are normally not to be recommended.
Glassware Plastic ware	Must be checked for stains and spots. If cleaning does not help, the item must be discarded.
Metal utensils	Metal utensils must be stainless steel.
Measuring equipment	Pipettes must deliver the required volume quickly and accurately (tolerance ± 2.5%). Pipettes should not be badly etched, and marks should be clearly legible.

3. Laboratory practice

Sterilization — The following sterilization procedures are required:

Item	Method and temperature/time
All glassware and plastic items	Dry sterilization 140°C for 3 hours, 160°C for 2 hours
All substrates and media in general, but always follow manufacturers' directions	Autoclaving 121°C for 15 minutes
Carbohydrate-containing media	Autoclaving, 121° C, maximum 12 minutes

Distilled or deionized water — Although distilled or deionized water may be acceptable for routine chemistry, there is a good chance that the water contains enough of some constituent to be toxic or stimulatory to microorganisms. The microbiologist should test the quality of the laboratory-pure water or have it tested by a State-authorized laboratory.

Washing — Washing must provide clean glassware without stains or spotting. Detergents must be non-toxic.

Incubation — Dishes should have tight-fitting lids to prevent drying out, particularly if humidity control of the air incubator is suspect or non-existent.
Dishes should always be incubated upside down to prevent condensation water on the substrate surface.
In water-bath incubators the dishes should be immersed only inside plastic bags. The water level should always be above that of the substrate inside immersed test tubes.

Check sterility of media and handling — Always use non-inoculated specimen parallel with inoculated test tubes or dishes in order to check sterility of media, containers and handling. If non-sterility is detected, the examination must be repeated in its entirety after complete checking and sterilization procedures and new sampling.

Culture containers — Tubes or other culture containers should always be of sufficient size to ensure less than 3/4 filling.

Pipetting — Always use a Peleus suction ball when pipetting, and avoid risk of infection by not using the mouth for suction.

Substrates Reagents — Specifications as to storage, handling, (and preparation) of culture media and reagents are given in Annex III. Always follow manufacturers' directions to the letter.
Dehydrated media containers are kept tightly closed and stored in a cool dry place. Dehydrated media are not used if discoloured or caked.
Media for maximum probable number tests are prepared in clean, smooth-surfaced glass or stainless steel utensils. Laboratory-pure water is used and solution of the media is completed by heating, to dissolve before dispensing to culture tubes or bottles.
The membrane filter media are heated in a boiling water bath for five minutes, to allow complete solution. A pH check is made on each batch of medium after preparation and sterilization. The pH must be within 0.2 units of the specified value.
Membrane filter broths and agar media are stored under refrigeration and normally used within one week.

Substrates Reagents — Media for maximum probable number tests are prepared in tubes and plugged with cotton, and must be used within one week. If they are refrigerated after sterilization, they are incubated overnight at 35°C to confirm usability. Tubes showing growth or gas bubbles are discarded.

	Any laboratory media in screw-cap tubes may be stored for up to three months, provided the media are stored in the dark and media evaporation is not excessive (0.5 ml per 10 ml total volume). Storage of media in ampoules is limited to the shelf-life expiry dates given by manufacturers.
Refrigeration	Samples are refrigerated whenever possible during transit and laboratory storage (as well as outside after sampling and before receipt in the laboratory).
Aging	Samples taken more than 30 hours earlier should not be accepted for analysis, even if they have been properly cooled.
Unidentified samples	Samples received without satisfactory identification (see Chapter 9 and Annex IV) should be discarded.

4. Quality control programme

4.1 General

A formal, written programme for laboratory quality control is to be maintained and made available for review. Management, supervisors, and analysts should participate in establishing and organizing the quality control programme. Each participant should have a copy of the quality control programme as a whole and a detailed guide of his own portion.

4.2 Analytical control methods

A record of analytical control tests such as replicate analyses and parallel testing should be kept. Data should be kept for at least two years and should be available for inspection. The analytical control tests should consist, for example, of:

(a) Duplicate analyses by different persons, for example by splitting one sample of 200 ml into two 100 ml samples, etc.; several organisms and analysts could be involved. This should be done on a quarterly basis.

(b) Colony counting by more than one analyst of the same specimen. This should be done on a quarterly basis.

(c) Reference laboratory analyses of a few samples that are also analysed locally, to compare results between the local and the specialized laboratory.

4.3 Media and equipment control methods

Quality control records of materials and equipment should be completed by entering information on the unit checked, the results of the check (e.g. the temperature observed), and the initials of the person making the check.

A written record on media, materials and equipment should be maintained, and should be available for inspection.

Operating temperatures of incubators, waterbaths, hot air ovens, and refrigerators must be checked daily and the controls adjusted as needed. Autoclave temperature and pressure readings are recorded for each sterilization cycle.

The following quality checks must be made: balances must be calibrated annually; temperatures must be calibrated annually; pH meters are calibrated at each use; and quality analyses of the laboratory pure water are made monthly.

Records must be made available for inspection of batches of sterilized media lot numbers, date, sterilization time-temperature, final pH, and technicians' identity.

Membrane filters are certified by the manufacturer for use in water analysis. Certification must include data on ink toxicity, recovery, retention and absence of growth-promoting substances.

Media are ordered on a basis of twelve-month needs. Bottles are dated on receipt and when first opened. Except for large volume uses, media are purchased in 100-200 g bottles to extend shelf life. Bottles of media are used within six months after opening. (Shelf-life of unopened bottles must not exceed two years.)

Lot numbers of membrane filters and dates of receipt are recorded.

Testing is carried out on media and membranes to determine recovery and performance, as compared with a previous acceptable lot.

Heat-sensitive tapes should be used during sterilization.

Service contracts (or approved internal protocols) should be maintained on balance, autoclave, water still, etc., and the service records entered in a log book.

ANNEX VI

AGENDA

1. Selection of monitoring areas
2. Characteristics of the monitoring areas
3. Parameters to be measured (physical, chemical, biological, microbiological)
4. Monitoring procedures
5. Data processing
6. Criteria for recreational beaches

ANNEX VII

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