Burkhardt

RECOMMENDED PROCEDURES

for the Examination of Sea Water and Shellfish

FOURTH EDITION 1970

The American Public Health Association, Inc.
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RECOMMENDED PROCEDURES FOR THE EXAMINATION OF SEA WATER AND SHELLFISH

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INTRODUCTION

Recognizing the public health and economic importance of a clean supply of safe shellfish and the necessity for uniform methods for the bacteriologic examination of shellfish, the Laboratory Section of the American Public Health Association appointed a Committee on Standard Methods for the Examination of Shellfish, which made its first report in 1910. The methods developed by this committee, published in 1922, and again with a few changes in 1933, were in use until 1943. The second complete revision, prepared in 1942 and modified in 1947, involved major changes in the methods for shellfish examination. A third complete revision was made in 1962, which added new technics and tests reflecting research in the field from 1947 through 1962.

The Fourth Edition contains methods for bacteriologic, chemical and physical tests for sea water, as well as microbiologic tests for shellfish. The committee has prescribed, whenever possible, methods and technics identical to those in the twelfth editions of *Standard Methods for the Examination of Dairy Products* (1967) and *Standard Methods for the Examination of Water and Wastewater* (1965), both published by the American Public Health Association. Reference to these two books, for certain toxicologic testing of glassware and media, may be necessary for users of *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.

Bacteriologic examinations have served a definite purpose in sanitary surveys of shellfish-growing areas and have aided in the assessment of the sanitary quality of shellfish as harvested or marketed. The Fourth Edition places special emphasis on the application of the various tests for the determination of coliform and fecal coliform organisms. These tests, combined, have indicated pollution probabilities in the shellfish growing areas and potentially dangerous contamination of the product during harvesting, washing, packing and marketing. The plate count has been used as an indicator of general sanitation and of adverse conditions during processing, storage and transportation. Chemical and physical tests for such parameters as salinity and temperature accompany the bacteriologic tests for shellfish and growing areas. Since naturally occurring biotoxins may be present at certain seasons in various geographic locations, the toxin assay methods have been inserted.

The Fourth Edition has been revised to include several methods that may be found in other publications. Quite often these methods are not

readily available to laboratory personnel in the field. Part II contains the shorter tests for oxygen, salinity and temperature necessary as an adjunct to the sanitary survey of shellfish-growing waters. In Part III, the bacteriologic procedures for the determination of coliform, fecal coliform and total plate count in sea water and shellfish may be found. The more common membrane filter procedures for enumerating coliform, fecal coliform and fecal streptococci in sea water are offered in Part IV. One method each for the detection of paralytic shellfish poisoning (PSP) and Ciguetera toxins is presented in Part V. While the method for PSP has been published elsewhere, it is included here as a readily available guide for conducting the test.

The procedures set forth for the examination of sea water and shellfish are intended to describe methods applicable to water and sanitary surveys of shellfish-growing areas and to bacteriologic surveys of commercial shellfish operations. The tests included are for index organisms indicative of fecal contamination and, as such, indicate a possible danger of transmission of enteric diseases. Specific methods for the detection and enumeration of certain pathogens such as salmonella, shigella and enteric viruses have not been established to the satisfaction of the committee. Interpretation of the results of the tests in this manual, in terms of the possible significance of indicator organisms, requires evaluation through a sanitary survey. Until such time as methodology for detecting and enumerating enteric pathogens can be standardized, the possibility of transmitting enteric diseases requires epidemiologic analysis.

The various tests in this manual have remained recommended procedures. Several of the tests have not been performed in all geographic areas where shellfish are grown or processed. It is the hope of the committee that these published methods will be evaluated by all interested groups within a relatively short time. As more experience with each test is accumulated, the acceptable and confirmed procedures can then be published as standard methods.

RECOMMENDED PROCEDURES FOR THE EXAMINATION OF SEA WATER AND SHELLFISH

PART 1

APPARATUS AND MEDIA

A. LABORATORY APPARATUS

1.0 Incubators

Incubators shall maintain a uniform and constant temperature at all times in all areas in use and must not vary more than ±0.5 C in the areas used. This can be accomplished by the use of water-jacketed or anhydric types of incubators with thermostatically controlled low-temperature electric heating units properly located and insulated in or adjacent to walls or floors of the chamber, and preferably equipped with mechanical means of circulating air.

Incubators equipped with high-temperature heating units are unsatisfactory since such sources of heat, when improperly placed, frequently cause overheating and excessive drying of the agar, with consequent failure of colony formation. Incubators so heated may be made to operate satisfactorily by replacing the high-temperature units with suitable wiring arranged to operate at a lower temperature and by installation of mechanical means of air circulation. It is desirable, where ordinary room temperatures vary excessively, that laboratory incubators be kept in special rooms which may be maintained at a few degrees below the recommended incubator temperature.

Special incubating rooms, well insulated, and equipped with properly distributed heating units and with forced air circulation, may be used provided that they conform to required temperature limits. When such

rooms are used, daily range of temperature in areas where plates or tubes are incubated should be recorded.

Incubators shall be provided with shelves so spaced as to assure uniformity of temperature throughout the chamber. One inch of space should be provided between adjacent stacks of culture dishes and between walls and stacks. Culture dishes should not be stacked over four high.

The thermometers employed should be accurate ones which are regularly checked against a thermometer certified by the National Bureau of Standards. The bulbs of the accurate thermometers shall be continuously immersed in liquid (glycerine, water, or mineral oil). The thermometers shall be maintained at representative locations within the incubator and daily readings of the temperature shall be recorded. In addition, it is desirable to maintain a recording thermometer within the incubator on the middle shelf to record temperature variations over a 24-hr period. Temperature variations within the incubator when filled to maximum capacity should be frequently recorded from top and bottom shelves.

2.0 Hot-Air Sterilizing Ovens

Hot-air sterilizing ovens shall be of sufficient size to prevent crowding of the interior, constructed to give uniform and adequate sterilizing temperatures, and equipped with suitable thermometers capable of registering accurately in the range 160 to 180 C. The use of a temperature-recording instrument is optional but desirable.

3.0 Autoclaves

Autoclaves shall be of sufficient size to prevent crowding of the interior; constructed to provide uniform temperatures within the chambers up to and including the sterilizing temperature of 121 C; and equipped with accurate thermometers with the bulb properly located on the exhaust line so as to register the minimum temperature within the sterilizing chambers. (A temperature-recording instrument is optional.) Pressure gauges and properly adjusted safety valves should be connected directly to either the saturated steam power lines or to a suitable steam generator. The autoclave should be capable of reaching the desired temperature within 30 min.

4.0 Colony Counters

Standard Quebec colony counter, dark-field model, is preferred. One equivalent may be used provided that similar magnification and visibility are attained.

5.0 pH Equipment

Electrometric pH meters, with an accuracy of 0.1 pH unit, shall be used for the determination of pH values in media.

6.0 Balances

Balances providing a sensitivity of at least 0.1 g at a load of 150 g shall be used with appropriate weights. An analytical balance having a sensitivity of 1 mg under a load of 10 g shall be used for weighing less than 2 g of materials.

7.0 Media Preparation Utensils

Utensils shall be of borosilicate glassware or other suitable noncorrosive material such as stainless steel, and shall be clean and free of foreign residues. Metalware should not contain toxic materials—copper, zinc, antimony, chromium or detergents—which might contaminate the media.

8.0 Pipets

Pipets may be of any convenient size provided it is found by actual test that they deliver accurately and readily the required amount in the manner in which they are used. The error of calibration shall not exceed 2.5%. If tip delivery, use pipets with unbroken tips and with graduations distinctly marked. Discard pipets with damaged tips. Calibrated and marked bacteriologic transfer pipets may be required in order to satisfy water quality enforcement regulations. Pipets conforming to the APHA standards as given in *Standard Methods for the Examination of Dairy Products* (1967) may be used. Do not use pipets larger than 10 ml to deliver 1 ml. Do not use pipets larger than 1 ml to deliver 0.1 ml.

9.0 Pipet Containers

Metal boxes, preferably of aluminum or stainless steel, are preferred, end measurement 2 to 3 inches round, square, or rectangular, length about 16 inches. Paper wrappings may be used, provided that no dele-

terious or toxic materials adhere to the pipets. Copper cans or boxes should not be used as containers.

10.0 Dilution Bottles or Tubes

Bottles or tubes of resistant glass, preferably borosilicate, closed with glass stoppers, rubber stoppers, or screw caps equipped with liners that do not produce toxic or bacteriostatic compounds on sterilization, shall be used. Cotton plugs shall not be used as closures. Graduation levels shall be indelibly marked on side of dilution bottles. Plastic bottles constructed of nontoxic materials and consisting of acceptable sizes may be substituted for glass.

11.0 Petri Dishes

Petri dishes 100 mm in diameter with the side wall of the bottom at least 15 mm high shall be used with glass or porous tops as preferred. The bottom of the dish shall be free from bubbles and scratches and shall be flat so that the medium will be of uniform thickness throughout the plate. Plastic petri dishes, when found to be satisfactory and when sterilized by the manufacturer, may be substituted for glass dishes for single use only. Glass petri dishes may be sterilized and stored in metal cans (aluminum or stainless steel, but not copper), or may be wrapped in paper, preferably best-quality sulfate pulp (kraft), prior to sterilization.

12.0 Fermentation Tubes

A fermentation tube of any type may be used, provided it is of suitable size to permit conformance to the requirement for concentration of nutritive ingredients as described subsequently. Metal or plastic tube closures are preferred provided that neither volatile nor toxic or bacteriostatic compounds are produced on sterilization. Cotton plugs should not be used when the fermentation tube is to be employed in the fecal coliform test in conjunction with an elevated temperature water bath.

13.0 Sample Bottles

Bottles of glass or other materials resistant to the solvent action of water, of any suitable size and shape, may be used in the collection of the sample, provided that the bottles (a) are capable of being properly washed and sterilized; (b) will contain a sufficient volume of sample for all the required tests plus adequate space to allow for effective shaking; and (c) will maintain samples uncontaminated until the examinations

are complete. Ground glass-stoppered bottles, preferably widemouth, of resistant glass are recommended for use as water-sample bottles.

Plastic sample bottles may be used provided they can be repeatedly sterilized at 121 C for 15 min without distortion and also provided they do not produce toxic or bacteriostatic compounds on sterilization.

Metal or plastic screw-cap closures equipped with liners may be used on sample bottles provided such caps and liners do not produce toxic or bacteriostatic compounds on sterilization.

The tops and necks of sample bottles shall be covered with metal foil, rubberized cloth, or heavy impermeable paper before sterilization.

B. WASHING AND STERILIZATION

1.0 Washing

All glassware must be thoroughly cleaned, using a suitable detergent and hot water (160 F), rinsed in hot water (180 F) to remove all traces of residual washing compound, and then rinsed for a succession of four rinses with complete change of water each time. Final rinse should be distilled water. The effectiveness of the rinse should be established by testing as described in the latest edition of *Standard Methods for the Examination of Water and Wastewater* (1965).

2.0 Sterilization

Glassware, except when in metal containers, shall be sterilized for not less than 60 min at a temperature of 170 C, unless it is known by means of recording thermometers that the oven temperatures are uniform, under which exceptional conditions 160 C may be used. Glassware in metal containers shall be heated to a temperature of 170 C for not less than 2 hr.

Sample bottles (except plastic) may be sterilized either as above or in an autoclave at 121 C for 30 min.

Where available, plastic bottles may be sterilized with low-temperature ethylene oxide gas. Precaution should be taken to assure that all gas has been removed from the container before using.

C. MATERIALS

1.0 Water

Distilled water which has been tested and found free from traces of dissolved metals and bactericidal or inhibitory compounds shall be used

for preparation of culture media and reagents. Bactericidal and inhibitory compounds should periodically be measured by a biologic test procedure outlined in the latest edition of *Standard Methods for the Examination of Water and Wastewater* (1965).

D. PREPARATION OF CULTURE MEDIA

1.0 Storage of Culture Media

Culture media may be stored in any clean, dry space where excessive evaporation and danger of contamination have been eliminated.

Liquid media in fermentation tubes, if stored at refrigeration or even moderately low temperatures, may dissolve sufficient air to produce a bubble in the tube when incubated at 35 C. It is imperative, therefore, that fermentation tubes which have been at a low temperature be incubated overnight before use and those tubes containing air discarded.

Liquid media fermentation tubes may be stored at approximately 25 C; but since evaporation may proceed rapidly under these conditions, resulting in marked changes in concentration of the ingredients, storage at this temperature should not exceed a period of one week.

2.0 Adjustment of Reaction

The reaction of culture media shall be expressed as pH values. The increase in the hydrogen ion concentration (decrease in pH) during sterilization will vary slightly with the individual sterilizer in use, and the initial reaction required to obtain the correct final reaction shall be determined. The decrease in the pH reading will usually be 0.1 to 0.2, but may occasionally be as great as 0.4. When buffering salts such as phosphates are present in the media, decrease in pH value as determined will be negligible.

Tests to control the pH before sterilization shall be made with a pH meter. Calculate how much of approximately one normal NaOH solution must be added to the bulk of the medium in order to reach the prescribed reaction. After adding the correct amount and thoroughly mixing, check the reaction and adjust if necessary. The required final reaction is given in the directions for preparing each medium. If specific reaction is not prescribed, adjustment is not necessary.

3.0 Sterilization

All media, except carbohydrate broths or those with other specifications, shall be sterilized in the autoclave at 121 C for 15 min after the temperature has reached 121 C. When the pressure reaches zero, the medium shall be removed from the autoclave and cooled quickly to avoid decomposition. To permit uniform heating and rapid cooling, materials should be packed loosely and in small containers.

Carbohydrate liquid media may usually be sterilized in the autoclave at 121 C for 10 min; the autoclave temperature shall never exceed 121 C.

It is sometimes preferable to prepare a 10-20% solution of carbohydrate in distilled water and to sterilize by filtering through a membrane filter or other device.

E. MEDIA

To provide uniformity the use of dehydrated media is mandatory.

This section contains formulas for all culture media, solutions, and reagents mentioned in both the official and the tentative methods for the examination of sea water and shellfish. For more ease of reference they are organized into the following four groups and are listed alphabetically in each of the groups:

1) Media for the Presumptive and Completed Tests for Coliform Organisms and for the Standard Plate Count
2) Media for Differentiation of Coliform Organisms
3) Media and Solutions for the Enumeration of Coliform, Fecal Coliform Organisms and Fecal Streptococci by the Membrane Filter Procedure
4) Other Solutions and Reagents.

1.0 MEDIA FOR THE PRESUMPTIVE AND COMPLETED TESTS FOR COLIFORM ORGANISMS AND FOR THE STANDARD PLATE COUNT

1.1 Brilliant Green Lactose Bile Broth 2%

Ingredient	Amount	Unit
Peptone or gelysate	10.0	g
Lactose	10.0	g
Oxgall	20.0	g
Brilliant green	0.0133	g
Distilled water to make	1,000	ml

Final reaction should be pH 7.2.

Distribute in fermentation tubes and sterilize as directed (Part ID, Sec. 3.0).

The reaction after sterilization (determined by electrometric method) should be not less than pH 7.1 and not more than pH 7.4.

1.2 Endo Agar

Formula I

Stock Agar

Beef extract	5.0 g
Peptone or gelysate	10.0 g
Lactose	10.0 g
Agar, bacteriologic grade	30.0 g
Distilled water	1,000 ml

Final reaction should be pH 7.4.

Suspend 41.5 g in a liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for one min. Dispense and sterilize as directed (Part ID, Sec. 3.0). Cool to 45 C and resuspend precipitate by gentle rotation of flask before pouring.

Formula II

Base Medium

Peptone or gelysate	10.0 g
Lactose	10.0 g
Potassium phosphate, dibasic, K_2HPO_4	3.5 g
Agar, bacteriologic grade	20.0 g
Distilled water to make	1,000 ml

Final pH should be 7.5 after sterilization.

1.3 Eosin Methylene Blue Agar

Peptone or gelysate	10.0 g
Lactose	10.0 g
Potassium phosphate, dibasic, K_2HPO_4	2.0 g
Agar, bacteriologic grade	15.0 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled water	1,000 ml

Adjustment of pH not necessary.

1.4 Lactose Broth

Beef extract	3.0 g
Peptone or gelysate	5.0 g
Lactose	5.0 g
Distilled water	1,000 ml

pH should be between 6.8 and 7, preferably 6.9.

When fermentation tubes for the examination of 10-ml or 100-ml portions of sample are prepared, the lactose broth medium must be of such strength that the addition of that volume of sample to the medium in the fermentation tube will not reduce the concentration of ingredients in the mixture below that in the standard lactose broth medium. Where dehydrated medium is used, the proper concentration of ingredients may be obtained by using the amounts of dehydrated products shown in the following tabulation:

Inoculum ml	Medium in Tube ml	Medium and Inoculum ml	Amount of Dehydrated Lactose Broth Used per 1,000 ml
1	10 or more	11 or more	13.0 g
10	10	20	26.0 g
10	20	30	19.5 g
100	50	150	39.0 g
100	35	135	50.1 g
100	20	120	78.0 g

1.5 Lauryl Tryptose Broth

Tryptose or biosate	20.0 g
Lactose	5.0 g
Potassium phosphate, dibasic, K_2HPO_4	2.75 g
Potassium phosphate, monobasic, KH_2PO_4	2.75 g
Sodium Chloride, NaCl	5.0 g
Sodium lauryl sulfate	0.1 g
Distilled water	1,000 ml

Final pH should be approximately 6.8.

Single strength may be used for inocula of 1 ml or less. In the examination of 10-ml or 100-ml portions of sample, the strength of the lauryl sulfate medium must be maintained. The addition of any volume of sample to the medium in the fermentation tube must not reduce the concentration of ingredients in the mixture below that in the single-strength medium when 1 ml or less of inoculum is used. When dehydrated me-

dium is employed, the proper concentration of ingredients may be obtained by using the amounts shown in the following tabulation:

Inoculum ml	Amount of Medium per Tube ml	Volume of Medium and Inoculum ml	Dehydrated Medium per 1,000 ml
1 or less	10	11	35.6 g
10	20	30	53.4 g
10	30	40	47.3 g
100	35	135	137.1 g
100	50	150	106.8 g

1.6 Nutrient Agar

Beef extract	3.0 g
Peptone	5.0 g
Agar, bacteriologic grade	15.0 g
Distilled water	1,000 ml

Final pH should be 6.8 ±0.1.

1.7 Nutrient Broth

Formula I

Beef extract	3.5 g
Peptone	5.0 g
Distilled water	1,000 ml

Formula II

Beef extract	3.0 g
Trypticase, or equivalent	2.5 g
Thiotone, or equivalent	2.5 g
Distilled water	1,000 ml

Final pH should be between 6.8 and 7.0.

1.8 Plate Count Agar

Tryptone or trypticase	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar, bacteriologic grade	15.0 g
Distilled water	1,000 ml

Final pH should be pH 7.0±0.1.

To desired amount of cold distilled water in suitable container, add appropriate amount of dehydrated base medium. Allow to soak about 3-5 min and then bring mixture into complete solution with minimal delay either by boiling above an asbestos-centered wire gauze over a free flame (double boiler preferred), stirring frequently to prevent burning on bottom of container, or by exposure in steam chest to actively flowing steam. Determine pH of medium and adjust reaction if necessary. Dispense into bottles, tubes, or flasks. Sterilize as directed (Part ID, Sec. 3.0). Final reaction should be pH 7.0 ± 0.1.

2.0 MEDIA FOR DIFFERENTIATION OF COLIFORM ORGANISMS

2.1 E C Medium

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts, Bacto No. 3 or equivalent	1.5 g
Potassium phosphate, dibasic, K_2HPO_4	4.0 g
Potassium phosphate, monobasic, KH_2PO_4	1.5 g
Sodium chloride	5.0 g
Distilled water	1,000 ml

Final pH should be 6.9 after sterilization.

2.2 Indole Test Medium (Tryptophane Broth)

Tryptone or trypticase, or equivalent	10.0 g
Distilled water	1,000 ml

Add the peptone to the cold distilled water. Heat while stirring to obtain complete solution. Distribute in 5-ml portions in culture tubes and sterilize by autoclaving as directed (Part ID, Sec. 3.0).

2.3 Buffered Glucose Broth (MR-VP)

Polypeptone, or equivalent	5.0 g
Dextrose	5.0 g
Potassium phosphate, dibasic, K_2HPO_4	5.0 g

Distribute 10-ml portions in culture tubes and sterilize in the autoclave at 121 C for 12-15 min, provided that the total time of exposure to heat is not longer than 30 min.

2.4 Sodium Citrate Test Medium

Ingredient	Amount	Unit
Sodium ammonium phosphate, $NaNH_4PO_4 \cdot 4H_2O$	1.5	g
Potassium phosphate, monobasic, KH_2PO_4	1.0	g
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.2	g
Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$	3.0	g
Distilled water	1,000	ml

Dissolve the dehydrated medium in the distilled water. Distribute into culture tubes in 5-ml amounts and sterilize as directed (Part ID, Sec. 3.0).

2.5 Simmons' Citrate Agar

Ingredient	Amount	Unit
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.2	g
Ammonium phosphate, monobasic, $NH_4H_2PO_4$	1.0	g
Potassium phosphate, dibasic, K_2HPO_4	1.0	g
Sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$	2.0	g
Sodium chloride, NaCl	5.0	g
Agar, bacteriologic grade	15.0	g
Bromthymol blue	0.08	g
Distilled water	1,000	ml

Suspend ingredients in cold distilled water and heat to boiling to dissolve the medium completely. Distribute in tubes or flasks and autoclave at 121 C for 15 min. Cool medium in slanting position.

Reaction after sterilization, pH 6.8.

3.0 MEDIA AND SOLUTIONS FOR THE ENUMERATION OF COLIFORM AND FECAL STREPTOCOCCI BY THE MEMBRANE FILTER PROCEDURE

3.1 MF-Endo Medium

Ingredient	Amount	Unit
Tryptose or polypeptone or equivalent	10.0	g
Thiopeptone or thiotone or equivalent	5.0	g
Casitone or trypticase or equivalent	5.0	g
Yeast extract	1.5	g
Lactose	12.5	g
Sodium chloride, NaCl	5.0	g
Potassium phosphate, dibasic, K_2HPO_4	4.375	g
Potassium phosphate, monobasic, KH_2PO_4	1.375	g
Sodium lauryl sulfate	0.05	g
Sodium desoxycholate	0.10	g
Sodium sulfite	2.10	g
Basic fuchsin	1.05	g
Ethyl alcohol (95% C_2H_5OH)	20.0	ml
Distilled water	1,000	ml

Add the ethanol to the distilled water. Dissolve the other ingredients in the water-alcohol mixture by heating to the boiling point. Do not allow to boil more than 5 sec. Do not submit to steam under pressure. The pH after solution should be between 7.1 and 7.3.

The formulated medium shall be stored at 2-10 C and any unused medium discarded after 96 hr.

3.12 LES MF Holding Medium—Coliform

Dissolve the following in 1 liter distilled water:

Ingredient	Amount	Unit
Tryptone	3.0	g
M-Endo broth MF	3.0	g
Potassium phosphate, dibasic, K_2HPO_4	3.0	g
Sodium benzoate	1.0	g
Sulfanilamide	1.0	g
Para-aminobenzoic acid	1.2	g
Cycloheximide	0.5	g

Heat to boiling until all ingredients are dissolved and make up lost volume with distilled water. Distribute into fermentation tubes and autoclave as directed previously. No pH adjustment is required.

3.13 LES Endo Agar Medium

Dissolve the following in 1 liter cold distilled water to which 20 ml 95% ethyl alcohol have been added:

Ingredient	Amount	Unit
Yeast extract	1.2	g
Casitone or trypticase	3.7	g
Thiopeptone or thiotone	3.7	g
Tryptose	7.5	g
Lactose	9.4	g
Dipotassium hydrogen phosphate, K_2HPO_4	3.3	g
Potassium dihydrogen phosphate, KH_2PO_4	1.0	g
Sodium chloride, NaCl	3.7	g
Sodium desoxycholate	0.1	g
Sodium lauryl sulfate	0.05	g
Sodium sulfite	1.6	g
Basic fuchsin	0.8	g
Agar	15.0	g

Heat to boiling to complete solution. Cool to 45-50 C and dispense in 4-ml quantities into the lower section of 60-mm glass or plastic dishes. If dishes of any other size are used, adjust the quantity to give an equiva-

lent depth. Plates may be stored up to two weeks in the refrigerator. Do not expose to direct sunlight.

3.2 M-FC Broth Base

Tryptose or Biosate	1.0 g
Proteose peptone #3 or polypeptone	0.5 g
Yeast extract	0.3 g
Sodium chloride, NaCl	0.5 g
Lactose	1.25 g
Bile salts	0.15 g
Aniline blue (water blue)	0.01 g
Distilled water	100 ml

Rosolic Acid Solution

Rosolic acid	1.0 g
Sodium hydroxide (0.2*N*)	100 ml

(Rosolic acid salt solution may be stored for two weeks in the dark in the refrigerator. Discard if it changes from a red to muddy brown color.)

Suspend the M-FC broth base in 100 ml distilled water. Add 1 ml of the Rosolic acid salt solution. Heat to boiling (DO NOT AUTOCLAVE). Cool and use. Final pH 7.4±0.1.

3.3 M-enterococcus Agar

M-enterococcus agar contains the following ingredients per liter of distilled water:

Tryptose, trypticase or thiotone	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dipotassium hydrogen phosphate, K_2PHO_4	4.0 g
Sodium azide	0.4 g
Agar	10.0 g
2,3,5-triphenyltetrazolium chloride	0.1 g

pH after heating should be 7.2. Sterilize by bringing to a boil.

4.0 SOLUTIONS AND REAGENTS

Stock prep.

4.1 Buffered Dilution Water

4.11 Stock Phosphate Buffer Solution

Potassium phosphate, monobasic, KH_2PO_4	34.0 g
Distilled water	500 ml
Sodium hydroxide, NaOH	1*N*
Distilled water to make	1,000 ml

Dissolve the potassium phosphate in 500 ml distilled water. Adjust to pH 7.2 with 1*N* sodium hydroxide and make up to 1 liter with distilled water. Approximately 175 ml of the sodium hydroxide solution are required to adjust the stock buffer to pH 7.2.

4.12 Final Phosphate Buffered Dilution Water

Stock phosphate buffer solution	1.25 ml
Distilled water	1,000 ml

Dilution bottles or tubes shall be filled with the proper amount of dilution water so that after sterilization they will contain the quantity desired with a tolerance of 2%. The dilution bottles or tubes shall be sterilized in the autoclave as directed (Part ID, Sec. 3.0).

4.2 Peptone Water

Peptone or gelysate	5.0 g
Distilled water	1,000 ml

Dissolve peptone in distilled water. Dispense in amounts to provide 99±2.0 ml or 9±0.2 ml after autoclaving at 121 C for 15 min.

4.3 Indole Test Reagent

Paradimethyl-amino-benzaldehyde, m.p. 74-75 C	5.0 g
Iso Amyl alcohol, ACS, $CH_3(CH_2)_4OH$	75 ml
Concentrated hydrochloric acid, HCl	25 ml

Dissolve the paradimethyl-amino-benzaldehyde in the iso amyl alcohol and then add the concentrated hydrochloric acid. The final solution should have a light yellow color.

Laboratory reagent iso amyl alcohol is recommended. Normal amyl alcohol may be substituted for the iso. Some brands of paradimethyl-amino-benzaldehyde are not satisfactory and some brands become unsatisfactory on aging.

The amyl alcohol solution should have a pH value less than 6.0. Both amyl alcohol and benzaldehyde compound should be purchased in as small amounts as consistent with the volume of work to be done.

4.4 Methyl Red Indicator Solution

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml

Dissolve methyl red in alcohol. Make up to 500 ml with distilled water.

4.5 Voges-Proskauer Reagents

a) α-Naphthol Solution

α-Naphthol, purified m.p. 92.5 C or higher	5 g
Absolute ethyl alcohol, C_2H_5OH	100 ml

This solution should be freshly prepared.

b) Potassium Hydroxide Solution

Potassium hydroxide, KOH	40 g
Distilled water	100 ml

PART II

PROCEDURES FOR THE PHYSICAL AND CHEMICAL EXAMINATION OF ESTUARINE WATERS

A. INTRODUCTION

The procedures described herein are intended for the physical and chemical examination of estuarine waters. Those tests outlined are normally used in conjunction with bacteriologic analyses of shellfish-growing areas. Physical and chemical data are needed for interpretation of the significance of the bacteriologic data. The tests presented here may be performed in the field or as rapid methods in the laboratory.

B. COLLECTION OF SAMPLES

Samples of sea water for physical and chemical examination should be collected in clean bottles. Bottles of glass or other material resistant to the solvent action of water may be used. A 2-liter sample should be taken for complete physical and chemical examinations. Devices from several manufacturers are available to take samples at varying depths. The following considerations should be made in selecting a sampler:

1. Bottle must flush thoroughly so as not to contaminate a depth sample by dragging down surface water.
2. Bottle must seal positively.
3. Bottle must be light enough to facilitate handling from boat.
4. Bottle must be large enough to secure an adequate volume of sample.

The shorter the time lapse between collection of the sample and its analysis, the more reliable will be the analytical results.

C. PHYSICAL AND CHEMICAL EXAMINATION

1.0 Dissolved Oxygen

1.1 Introduction—Dissolved oxygen may be measured either by the titration method or by using an oxygen analyzer. The oxygen analyzer incorporates a membrane-surfaced probe connected to a galvanic cell. Since most oxygen analyzers are still in the developmental stage, standardization of the individual oxygen analyzer should be made by comparison with the titration method before using for the routine analysis of sea water.

1.2 Determination by titration—

1.21 *General discussion:* The method described here is a modification of the classic Winkler procedure. This method can be used for dissolved oxygen concentrations ranging from 0.005 to 8 mg/L. Oxygen concentrations below 0.1 mg/L will be slightly but not significantly low.

1.22 *Reagents:* Unless otherwise noted, all reagents should be of analytical reagent grade.

1.23 *Manganese sulfate solution:* Dissolve 480 g $MnSO_4 \cdot 4H_2O$ or 365 g $MnSO_4 \cdot H_2O$ in distilled water and dilute to 1 liter.

1.24 *Alkaline iodide solution:* Dissolve 500 g of NaOH in 500 ml of distilled water. Dissolve 500 g of KI in 500 ml of distilled water and mix the two solutions. The NaOH is preferably of analytical reagent grade, but may be of high commercial quality.

1.25 *Starch indicator solution:* Suspend 2 g of soluble starch in 400 ml of water. Add an approximate 20% solution of NaOH, vigorously stirring until the solution becomes clear. Allow the solution to stand for 1 to 2 hr. Add concentrated HCl until the solution is *just* acid to litmus paper. Then add 2 ml glacial acetic acid. Dilute to 1 liter with distilled water. Discard the solution when the end point is no longer a pure blue but takes on a green or brownish tint.

1.26 *Standard sodium thiosulfate solution:*

A. Dissolve 2.2 g reagent grade $Na_2S_2O_3 \cdot 5H_2O$ and 0.1 g of Na_2CO_3 in 1 liter of water. Add 1 drop of CS_2 per liter as a preservative. The thiosulfate solution should be prepared in quantity and stored in a dark well-stoppered bottle below 25 C.

B. IODATE SOLUTION (0.01*N*): Dry analytical grade $KH(IO_3)_2$ at 105 C for 1 hr. Cool in dessicator. Weigh out 0.3250 g. Dissolve the salt in 300 ml distilled water by warming the solution. Cool, transfer to a 1-liter volumetric flask and bring up to the mark with distilled water. This solution is stable indefinitely.

C. CALIBRATION OF THIOSULFATE SOLUTION: Fill a 300-ml B.O.D. bottle with sea water. Add 1.0 ml concentrated H_2SO_4 and 1.0 ml alkaline iodide solution. (Add below surface of water and allow sample to overflow in order to avoid trapping air bubbles in bottle.) Mix thoroughly. Add 1.0 ml $MnSO_4 \cdot H_2O$ solution and mix. Withdraw 50-ml aliquots to titration flasks. Use one or two flasks for blank determination. To the other flasks add 5.00 ml of 0.0100*N* $KH(IO_3)_2$ from a clean calibrated 5-ml pipet. Allow the iodine liberation to proceed for at least 2 min but not more than 5 min. All solutions should be held below 25 C and out of direct sunlight. Titrate the iodine with the thiosulfate solution. When V is the titer in milliliters, then:

$$F = 5.00/V$$

The factor "F" should not vary over a period of time. The determination, using the mean of at least 5 titrations, should be made at the discretion of the analyst.

1.3 Procedure—

1.31 *Sampling procedure and storage:*

1.311 Rinse B.O.D. bottle twice with the sample being analyzed. If the sample is obtained from a reversing bottle, a length of rubber tubing should be taken from the top to the bottom of the B.O.D. bottle and sea water introduced in such a way as to minimize turbulence and agitation of the sample. The end of the rubber tube must always be kept beneath the surface of the water as the bottle is filled.

1.312 Allow the water to overflow fom the top of the B.O.D. bottle, which must be stoppered at once. Allow a volume equal to one-third of the volume of the B.O.D. bottle to overflow before stoppering. No air should remain in the bottle.

1.313 B.O.D. bottles must be filled immediately or in not more than 15 min after the sample is drawn.

1.314 The analysis should be made in less than 1 hr. Samples should be stored in the dark or in subdued light to minimize photosynthesis by any phytoplankton that may be present.

1.315 If the analysis must be delayed, add 1.0 ml of manganese sulfate reagent and 1.0 ml alkaline iodide solution to the sample in the B.O.D. bottle. Restopper immediately and mix (10 min) the contents thoroughly until the precipitated manganese manganic hydroxide is evenly dispersed. No air bubbles should be trapped in the bottle. The remainder of the analysis may be completed at a later time.

1.32 *Titration:*

1.321 Add 1.0 ml manganese sulfate reagent and 1.0 ml alkaline iodide solution to the sample as described in Sec. 1.315. (DO NOT REPEAT this step if sample was held in storage and delayed titration was used.)

1.322 Shake bottles to distribute the precipitate. Allow sample to warm to room temperature while precipitate has settled at least one-third of the way to the bottom of the bottle.

1.323 Add 1.0 ml concentrated (sp gr 1.84) sulfuric acid, restopper the bottle and mix so that all the precipitate dissolves. No air should be trapped in the bottle.

1.324 Within an hour of acidification transfer 50 ml of solution to a 250-ml Erlenmeyer flask by means of a pipet. Titrate *at once* with standard thiosulfate solution until a very pale straw color remains. Add 5 ml of starch indicator and continue the titration to the first disappearance of the blue color.

1.33 *Calculation:* Subtract any blank correction (see C. 1.26c above) from the titration to obtain the corrected titration (V ml) and calculate the oxygen content of a sample from the following formula where 50.0 ml aliquot is taken from a 300-ml B.O.D. bottle.

$$\text{mg at } O_2/l = 0.1006 \cdot F \cdot V$$

The "F" value is obtained from Sec. C. 1.26c.

2.0 Salinity

2.1 Introduction—The salinity of sea water has been defined as the total amount of solid material, in grams, contained in one kilogram of sea water when all the carbonate has been converted to oxide, the bromine and iodine replaced by chlorine, and all organic matter completely oxidized. This numerical value of salinity is slightly lower than the amount of dissolved solids in grams per kilogram of sea water.

The chloride ion concentration in sea water can be measured by chemical titration against a standard solution of silver nitrate. Thus, chlorinity is defined as parts per thousand of the mass of chlorine, bromine and iodine in one thousand grams of sea water solution, assuming that iodine and bromine have been replaced by chlorine. Chlorosity is obtained by multiplying the chlorinity of a water sample by its density at 20 C.

The relationship between salinity and chlorinity has been stated by Knudsen's empirical formula: $\text{Salinity} = 0.03 + 1.805 \times \text{chlorinity}$.

Note that this equation is only definitive and may not be applicable to all waters in a true chemical connotation. For further reference on the relationship of salinity, chlorinity and chlorosity, please refer to the books by Sverdrup, Johnson and Fleming (1942) or Von Arx (1962) cited at the end of Part II.

Salinity measurements are based on any one of four technics: (1) electrical conductivity, (2) density, (3) silver nitrate titration, and (4) measurement of refractivity. The refractivity method is seldom used and will not be considered at this time.

2.2 Salinity Measurement by Electrical Conductivity—Measurements of the salinity by electrical conductivity have been employed for several years. "Conductivity" cells are now available with or without electrodes. Those without electrodes are battery operated and may be used in an area remote from electrical supplies. Both types of cells have been used for field determinations of salinity. Due to the relatively high concentration of the ions and the effect that temperature has upon the conductivity, the apparatus and technic employed are rather complicated. Each instrument must be standardized using sea water samples *of known salinity* (determined by silver nitrate titrations).

Many instruments are available on the market. The types of instruments procured may be guided by local requirements and budgetary allotment.

2.3 Salinity Measurement by Density (Hydrometric Method)—The hydrometric method is one of the simpler methods of salinity determination. It is well suited for shoreline and small-boat observations. The precision of this method is ± 0.1 parts per thousand salinity.

2.31 *Apparatus:*

2.311 HYDROMETER JAR. Approximately 400-mm height with inside diameter 45 mm; or 500-ml graduated cylinder; or rubber-stoppered

transparent plastic tube, 400 mm high with inside diameter 45 mm open on one end.

2.312 THERMOMETER. Graduated in 0.2 C divisions.

2.313 SEA WATER HYDROMETER.* For complete salinity range in estuaries 3 are needed with respective ranges of: 0.996-1.011, 1.010-1.021, and 1.020-1.031. Hydrometer division should be .002. One set of hydrometers should be calibrated by the National Bureau of Standards for specific gravity of NaCl solutions at 15/4 C.

2.314 SEA WATER TEMPERATURE AND DENSITY REDUCTION TABLES. Special Publication No. 298, Coast and Geodetic Survey, U.S. Department of Commerce, 1953. Supt. of Documents. U.S. Government Printing Office, Washington, D.C. 20 cents.

2.32 *Procedure:*

2.321 Fill a suitable cylinder ⅘ full of sea-water sample.

2.322 Place the appropriate hydrometer and the thermometer in the cylinder, making certain that the cylinder is in a vertical position.

2.323 Read and record the temperature.

2.324 Read and record specific gravity after the hydrometer stabilizes. The fourth decimal place must be estimated.

2.325 Make temperature corrections for specific-gravity reading from factors listed in Table 1 (Appendix).

2.33 *Calculation:*

2.331 Determine salinity from Table 2 (Appendix). Locate corrected density and read salinity from opposite column. Report salinity in parts per thousand (‰).

2.4 **Salinity Measurements by Titration**—The following technic for the determination of salinity by the titration method has been adopted for work in estuarine waters and surface waters of coastal inlets. Chlorinity and salinity measurements are a function of the chlorosity of a water sample by the titration method. The chlorosity is standardized against a sea-water standard of known chlorosity. This technic is not as precise as the longer classic Knudsen titration. However, the method is less time-consuming and is correct to about 0.05 to 0.1 ‰ salinity. The range of salinities that may be tested by this method is 4 to 40 ‰.

* May be purchased from Emil Greiner Co., 20 North Moore St., N.Y., N.Y. 10004.

2.41 *Sampling Procedure and Storage:*

2.411 The sample bottle should be a standard 8-oz bottle with a No. 6 size cork stopper. The cork stopper should be soaked from 30 to 40 sec in melted paraffin wax, drained and dried. Excess wax should be removed.

2.412 Rinse bottle 3 times and then fill to shoulder.

2.413 Seal the bottle by forcing the waxed cork down to a few millimeters below the level of the neck. If samples are not to be examined within two days, dip the neck of the bottle in melted wax. No change in salinity will occur over an extended period of time.

2.414 Unsealed samples should be titrated within a few minutes. No titration of the open samples should be delayed for more than 1 hr.

2.42 *Apparatus and Reagents:*

2.421 Automatic 10-ml pipet.

2.422 Automatic zero-adjusting 35-ml buret. (Top of buret is lubricated with a trace of paraffin stopcock grease. DO NOT USE SILICONE.)

2.423 Tall-form 200-ml beakers. (Keep clean by soaking at periodic intervals in cold 5% sodium hydroxide in methyl alcohol followed by rinse in nitric acid and then in distilled water.)

2.424 STANDARD SEA WATER. Standard sea water of a known chlorinity ("Eau de Mer Normale") may be obtained from the Depot d'Eau Normale, Laboratoire Hydrographique, Charlottenlund Slot, Copenhagen, Denmark. (Local distributors may be found in *Sources of Limnological and Oceanographic Apparatus and Supplies*, Special Publication No. 1 of the Committee on Apparatus and Supplies, American Society of Limnology and Oceanography, Vol. IX, April 1964.)

A secondary standard may be prepared by obtaining a large volume (10 liters or more) of filtered sea water with a chlorinity near 18 ‰. (It is preferable to collect sea-water samples below 50 M in the open ocean.) Stabilize this sample by adding a few crystals of thymol. Fill sample bottles rapidly and stopper as described in 1.31. The mean of 10 or more sample titrations is used as the chlorosity (20 C) of this secondary-standard sea water.*

* Secondary standards are generally available at local university oceanography departments.

2.425 SILVER NITRATE SOLUTION (approx. 0.28N). Dissolve 48.5 g reagent grade silver nitrate, $AgNO_3$, in 500 ml distilled water. Make up to 1 liter in a volumetric flask. Store in a glass-stoppered brown glass bottle at room temperature.

2.426 POTASSIUM CHROMATE INDICATOR. Dissolve 63 g of CP potassium chromate in 100 ml distilled water. Add a few drops of 0.28N silver nitrate solution until, after stirring, the red silver chromate precipitate persists. Allow the precipitate to settle and filter through fine filter paper (Whatman No. 42). Store in glass dropper bottle at room temperature.

2.427 STANDARD SODIUM CHLORIDE. Dry approximately 35 g CP sodium chloride to constant weight at 100 C. Cool and weigh out 29.674 g. Dissolve in distilled water and dilute to 1 liter. Two solutions should be prepared in order to check one against the other.

This standard should also be standardized against Copenhagen water. In addition, the sodium chloride should be standardized with the secondary sea-water standard at periodic intervals.

2.43 *Standardization of Silver Nitrate Solution:*

2.431 Pipet 25 ml of standard salt solution at room temperature into a 150-ml Erlenmeyer flask.

2.432 Add 6 drops of the saturated potassium chromate indicator.

2.433 Titrate with silver nitrate solution in a yellow light until red silver chromate just precipitates.

2.434 Stopper the flask with a rubber stopper and shake vigorously until the curds of silver chloride are broken.

2.435 Wash down the rubber stopper with distilled water and continue the titration to the brown end point.

2.436 The normality of the silver nitrate is calculated from the following formula:

$$N = \frac{12.69}{A}$$

where N = Normality of silver nitrate, A = Amount (ml) of silver nitrate used for the titration of the standard salt solution.

Standardization titrating should be performed on at least 5 standard sodium chloride aliquots. The mean of the amount (ml) from these titrations should be used as A in the above formula.

2.437 The chlorosity equivalent of 1 ml silver nitrate is calculated by the following formula:

$$ClEq = N \times 0.355$$

where ClEq = Chlorosity equivalent of 1 ml silver nitrate and N = Normality of silver nitrate.

2.44 *Procedure for Titration of Samples of Sea Water:*

2.441 Allow sea water to equilibrate to same temperature as that of the silver nitrate solution.

2.442 Pipet 25 ml of sea water into a 150-ml Erlenmeyer flask.

2.443 Add 6 drops of potassium chromate solution.

2.444 Titrate with silver nitrate using the same procedure as noted in 2.431-2.437 for standardization of silver nitrate solution.

2.445 The chlorosity of the water is determined as follows:

$$Cl_o = d\ ClEq \times 40$$

where Cl_o = Chlorosity of the sea water, d = ml of silver nitrate required for the titration and ClEq = chlorosity equivalent per *liter* of silver nitrate.

2.446 To convert chlorosity to chlorinity refer to Table 3 (Appendix) and make proper correction. Record chlorinity as Cl ‰.

2.447 Calculate salinity by reading salinity (S ‰) directly from the corresponding chlorinity in Table 4 (Appendix).

3.0 Hydrogen Ion Concentration

3.1 Water samples shall have all pH determinations made by a pH meter.

4.0 Temperature

Three types of temperature-measuring devices are used in estuarine waters. The conventional laboratory thermometer may be used for surface samples. Subsurface temperatures may be determined by: (1) conventional thermometer using insulated sample bottle, (2) reversing thermometer, or (3) continuous recorder such as a thermograph. In each type, the accuracy of the thermometer must be within ±0.05 C of a Bureau of Standards thermometer.

4.1 Conventional Thermometer—

4.11 The thermometer must have a scale marked for every tenth of a degree. The scale should be etched upon the glass of the capillary. The thermometer should be of small thermal capacity in order to attain rapid equilibrium.

4.12 The thermometer should be checked for calibration errors against a National Bureau of Standards thermometer with the scale immersed to the height of the mercury column.

4.13 Samples taken from a vessel must be obtained as far away as possible from discharge outlets of the hull and, if the vessel is under way, they should be taken near the bow so as to avoid the churned-up water of the wake.

4.14 Subsurface samples may be collected in an insulated bottle. The temperature should be taken with a conventional thermometer *immediately* after the surfacing of the sample bottle.

4.2 Reversing Thermometer—The thermometers normally used for measuring temperatures at subsurface levels are of the reversing type. They are generally mounted on water-sample bottle apparatus so that temperatures and the water sample are obtained at the same level. Sverdrup *et al* (1942) have given a description of both protected and unprotected reversing thermometers. Readings of the protected reversing thermometers must be corrected for changes due to differences between the temperature at reversal and the temperature at which the thermometer is read. The following formula is used to calculate the correction:

$$\Delta T = \left[\frac{(T^1 - t)\ \ (T^1 + Vo)}{K}\right] \quad \left[\frac{1 + (T - t)\ \ (T^1 + Vo)}{K}\right] + L$$

where:

ΔT = Correction to be added algebraically to uncorrected reading,
T^1 = Uncorrected reading of thermometer at reversal,
t = Temperature at which thermometer is read,
Vo = Volume of the small bulb end of the capillary up to the 0 C graduation,
K = A constant depending upon the relative thermal expansion of mercury and the type of glass used in the thermometer (K usually has a value of 6100),
L = Calibration correction of the thermometer which depends on T^1.

When large numbers of observations are to be made it is convenient to prepare graphs for the thermometer from which the value of ΔT can be obtained for any values of T^1 and t in which the calibration correction is included.

4.3 Temperature Using Thermograph—Temperature measurement by methods described in 4.1 and 4.2 yield observations at discrete points in space and time. The methods are also time-consuming. In recent years many devices have been developed for obtaining continuous temperature observations at a given depth. These thermographs may be used at shore stations or on board a vessel. Generally the initial cost of the thermograph or bathythermograph will determine whether or not this type of instrument should be used in an estuarine study. In any case, each instrument should be calibrated with a National Bureau of Standards thermometer before use in the field.

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PART III

PROCEDURES FOR THE BACTERIOLOGIC EXAMINATION OF SEA WATER AND SHELLFISH

A. EXAMINATION OF SEA WATER

1.0 Collection and Transportation of Sample

Samples of sea water for bacteriologic examination shall be collected in clean sterile bottles. A description of suitable bottles and their preparation may be found in Part I, Sections A and B. The bottles should be fully protected against contamination both during sampling and after collection.

Surface samples may be collected without the aid of special sampling devices. Keep the sampling bottle unopened until immediately before filling. During sampling, the stopper and neck of the bottle should be protected from contamination. In collecting the sample, hold the bottle near the base and plunge it neck downward below the surface. It should then be tilted with the neck pointed slightly upward and, during filling, the bottle should be pushed horizontally forward in a direction away from the hand to avoid contamination. In order to facilitate shaking, do not fill bottle completely full.

A number of mechanical devices are available for the collection of samples below the surface. The more popular of these utilize the sample bottle and are so designed that, upon reaching the desired depth, the stopper may be raised to fill the bottle. Such devices are useful up to depths of 60 ft. Beyond that the hydrostatic pressure makes it impossible to remove the stopper. When such samplers are not applicable, the capillary tube-water samplers in general use in oceanographic work may be employed.

A sampler for depths up to 60 ft should have the following features:

1) It should be of simple mechanical design.

2) The sampler should preferably hold a sterile sampling bottle with mechanical devices to raise and lower the stopper.
3) The design should be such that the sample container will not be contaminated during collection or manipulation in preparation for sampling.
4) The surfaces in contact with the sample should not be of metal or other materials which are bactericidal.
5) Sampler should be capable of being sterilized between uses to prevent contamination of the sample.

Whenever possible, the bacteriologic examination should be initiated immediately after collection and preferably within 1 hr after collection. When conditions necessitate delay in the initiation of the bacteriologic examination, the samples shall be kept at a temperature at or below 10 C until examined. In no case shall samples be tested if they have been held more than 30 hr.

2.0 Tests for Members of the Coliform Group

The coliform group of organisms as determined by the multi-tube test shall include all of the aerobic and facultative anaerobic gram-negative nonspore-forming rods which ferment lactose with gas formation within 48 hr at 35 C.

2.1 Presumptive Test—Lactose broth or lauryl sulfate lactose broth, in fermentation tubes, shall be used as the Presumptive Test medium. Dilutions shall be made in sterile phosphate buffered dilution water or 0.5% sterile peptone water.

Prior to transfer of the sample to the culture tube or dilution bottle or tube, the sample bottle shall be shaken vigorously 25 complete up-and-down movements of about 1 ft in 7 sec. Each dilution bottle or tube, after the addition of the measured portion of the sample, shall be shaken vigorously 25 times as above before the subsample is removed for transfer to a culture tube or dilution bottle (Figure 1, Appendix).

2.11 *Procedure:*

Inoculate:

a) Each of 5 tubes of double-strength Presumptive Test broth with 10 ml of sample *

* Formulations of lactose broth other than double strength may be used for 10 and 100-ml inoculations. Refer to Part I for recommended formulations of lauryl sulfate or lactose broth when 10- or 100-ml portions are inoculated.

b) Each of 5 tubes of single-strength Presumptive Test broth with 1 ml of sample

c) Each of 5 tubes of single-strength Presumptive Test broth with 0.1 ml of sample.

In conducting bacteriologic surveys of shellfish-growing areas where stations are to be sampled on repeated occasions, the use of 3 tubes per dilution may be justified in order to save time and material. Decision concerning the choice of 3 or 5 tubes would depend to a great extent on the degree of accuracy desired. In no case should less than 3 tubes per dilution be used.

In order to avoid indeterminate results, extension of the dilutions suggested above may be necessary. The amounts of sample selected for inoculation shall be such that the largest portions will give positive results in all or the majority of tubes, and the smallest portions will give negative results in all or the majority of tubes. To attain this with any degree of assurance with samples of doubtful quality, it is usually necessary to inoculate at least 4 decimal dilutions.

Incubate the fermentation tubes at 35 C $\pm$ 0.5 C. Examine each tube at the end of 24 $\pm$ 2 hr and if no gas is formed return tubes to the incubator and examine again at the end of 48 $\pm$ 3 hr. Record the presence or absence of gas formation at each examination of the tubes regardless of the amount of gas produced. Formation of gas may be recognized by the presence of a bubble of gas in the upper end of the inverted fermentation tube. The appearance of an air bubble must not be confused with actual gas production. If the bubble in the inner tube is a result of fermentation, the broth medium will be cloudy and active fermentation may be shown by continued appearance of small bubbles of gas throughout the medium when the tube is tapped or gently shaken. Absence of gas formation at the end of 48 hr incubation constitutes a negative test for coliform-group organisms.

2.2 Confirmed Test—Except as indicated below, all Presumptive Test fermentation tubes showing any amount of gas at the end of 24 or 48 hr of incubation shall be subjected to the Confirmed Test. When active fermentation appears in the fermentation tube before expiration of the 24-hr period of incubation, it is advisable to transfer these cultures without waiting for completion of the full period.

When samples from sources are frequently examined on a routine basis, and when the frequency of false-positive Presumptive Tests is

known to be low, it may not be necessary to confirm all positive presumptive tubes, especially the 24-hr tubes. When 3 or more replicate portions of a series of 3 or more decimal dilutions of a sample are planted, an acceptable alternate procedure would be as follows:

1. Select the tubes of the highest dilution (smallest volume) in which all the tubes show gas production in 24 hr.
2. Submit all these tubes to the Confirmed Test as well as every one of the gas-positive tubes in all the higher dilutions.
3. If there are no dilutions in which less than all tubes show gas production, all gas-positive tubes of the highest dilution and of the next-to-the-highest shall be submitted to the Confirmed Test.
4. Submit to the Confirmed Test all tubes of all dilutions in which gas is produced only at the end of 48-hr incubation.
5. If fewer than 3 portions of any dilution or volume or if fewer than 3 decimal dilutions of the original sample are planted, all tubes producing gas during 24 or 48 hr incubation shall be submitted to the Confirmed Test.
6. All tubes producing gas that have not been submitted to the Confirmed Test shall be recorded as containing organisms of the coliform group, even though all the Confirmed Tests may yield negative results.

2.21 *Procedure:*

1) Mix the Presumptive Test fermentation tube by gentle shaking or rotating.

2) Transfer one loopful of the medium to a fermentation tube containing brilliant green lactose bile broth.*

Care must be exercised to assure the transfer of a full loopful of viable culture. The loop must be cool before final loading. Should it be desired to quench the loop in the culture medium, this may be done in the medium to which the transfer is to be made. If done in the presumptive broth, the loop should be emptied of its initial charge by application to the side of the tube above the level of the medium and then reloaded.

3) Incubate the inoculated tubes for 48 hr $\pm$ 3 hr at 35 C $\pm$ 0.5 C.

4) The formation and presence of gas in any amount in the inverted

* In addition to platinum-iridium wire loops, transfer may be made by nontoxic sterile applicator sticks or sterile 3-mm aluminum or stainless steel loops.

vial of the brilliant green lactose bile broth tube within 48±3 hr constitutes a Confirmed Test for coliform group organisms (Figure 2, Appendix).

2.3 Completed Test for Coliform Organisms—

2.31 *Procedures:*

A. Streak one or more plates of endo or Levine's methylene blue agar from each primary fermentation tube showing gas formation or from each brilliant green lactose bile broth fermentation tube showing gas formation. The plates should be so streaked as to insure the presence of some discrete colonies, separated by at least 0.5 cm.

1—Use an inoculating needle slightly curved at the tip (curved section approximately 10 mm in length).

2—Tap and incline the fermentation tube to avoid picking up membrane or scum on the needle.

3—Insert the sterilized needle into the liquid in the tube to a depth of 5.0 mm.

4—Lift the needle from under the surface of the liquid and shake it to remove droplets of medium.

5—Streak the plate, bringing only the curved section of the needle in contact with the agar surface.

B. Invert the plate with plastic or glass cover and incubate at 35 C±0.5 C for 24 hr±2 hr.

C. Three types of colonies will develop on endo or Eosin methylene blue agar on incubation within 24 hr:

1—Typical coliform: nucleated with or without metallic sheen

2—Atypical: opaque, unnucleated, or pink

3—Noncoliform: all others.

Pick one or more typical coliform colonies from each of the plates. If no typical coliform colonies are present, pick two or more atypical colonies considered most likely to consist of coliform organisms from each of the plates.

Inoculate separate lactose broth tubes and nutrient agar slants from each picking. The use of a colony counter or other means of equivalent magnification is recommended to assist in picking. Choose, if possible, well-isolated colonies, separated by at least 0.5 cm from other colonies.

Touch only the end of the inoculating needle to the top of the colony in order to minimize the possibility of transferring a mixed culture.

D. Incubate the agar slants and the lactose broth tubes at 35 C±0.5 C for 24±2 hr. Record the gas formation in the lactose broth tube and, if no gas is found, incubate an additional 24±2 hr.

E. Examine, microscopically, gram-stained preparations from the agar slants corresponding to the gas-positive lactose broth tubes and record gas production.

2.4 Results—The formation of gas in the lactose broth tube and the demonstration of gram-negative nonspore-forming rods in the agar slant constitute a Positive Completed Test for coliform organisms (Figure 3, Appendix).

The absence of gas formation in the lactose broth or the failure to demonstrate gram-negative nonspore-forming rods in the agar culture constitutes a Negative Completed Test.

2.5 Computing and Recording the Results—Results of tests for coliform organisms or for various members of the coliform group of organisms by the multi-tube dilution method are to be reported in terms of the "Most Probable Number" (MPN) index. It should be realized that the MPN is not a precise enumeration of the numbers of bacteria in any given volume of sample. The precision and confidence limits of the test using any given number of tubes and of the MPN method of estimating densities have been determined and are available from reports listed in the bibliography at the end of Part III. The accuracy of the result is dependent upon the number of portions of each dilution planted in the multi-tube fermentation procedure. For example, the approximate lower and upper 95% confidence limits, when 3 tubes each of 3 dilutions are planted, may be estimated as 21% of the MPN for the lower and 395% for the upper. By comparison, the approximate 95% confidence limit for 5 tubes in each of 3 decimal dilutions has been stated to be 29% for the lower and 290% for the upper.

The MPN results for a variety of planting series and positive results are shown in the tabulation below. The quantities indicated at the heads of the columns merely indicate decimal dilutions and need not be considered as indicative of the volumes planted.

The figures may be used in computing the MPN in larger or smaller portion plantings in the following manner: If, instead of portions of 10, 1.0, and 0.1 ml a combination of portions of 100, 10, and 1 ml is used,

the MPN may be recorded as 0.1 times the figure in the tabulation (Tables 9, 10 or 11, Appendix).

On the other hand, should a combination of corresponding portions of 1.0, 0.1 and 0.01 ml be planted, record 10 times the figure in the tabulation; should a combination of portions of 0.1, 0.01, and 0.001 ml be planted, record 100 times the figure in the tabulation.

When more than 3 dilutions are employed in a decimal series of dilutions, the results from only 3 of them are significant. To select the 3 dilutions to be used in determining the MPN index, using the system of 5 tubes of each dilution as an example, the highest dilution which gives positive results in all of the 5 portions tested (no lower dilution giving any negative results) and the 2 next succeeding high dilutions should be chosen. The results of these 3 volumes should then be used in the computation of the MPN index. In examples given below, significant dilution results are shown in boldface. The number in the numerator represents positive tubes; that in the denominator, the total tubes planted.

	1 ml or g	0.1 ml or g	0.01 ml or g	0.001 ml or g
(a)	5/5	**5/5**	**2/5**	**0/5**
(b)	**5/5**	**4/5**	**2/5**	0/5
(c)	**0/5**	**1/5**	**0/5**	0/5

In example (c) above, the first 3 dilutions should be taken in order to throw the positive result into the middle dilution.

When a case arises such as is shown below in (d), where a positive occurs in a dilution higher than the 3 chosen according to the rule, it should be included in the result of the highest chosen dilution, making the result read as in (e).

	1 ml or g	0.1 ml or g	0.01 ml or g	0.001 ml or g
(d)	**5/5**	**3/5**	**1/5**	1/5
(e)	**5/5**	**3/5**	**2/5**	0/5

2.6 Where individual species are to be identified, see Appendix.

3.0 Multi-Tube Test for Members of the Fecal Coliform Group in Sea Water

3.1 Introduction—Although methodology to differentiate rapidly and absolutely coliforms of fecal origin from those originating in nonfecal sources is currently unavailable, practical methods have been developed which favor the selection and growth of fecal coliform organisms while eliminating many, but not all, of the types generally considered to have little or no public health significance. The elevated temperature test using the Eijkman reaction as modified by Perry and Hajna, and by Geldreich *et al* has been shown to have the necessary sensitivity and specificity needed for a reliable laboratory confirmatory test. From a practical standpoint, gas production under the conditions of the test is an indication of the presence of recent fecal contamination. No gas production is interpreted as indicative of a source other than the intestinal tract of warm-blooded animals and is considered nonfecal in origin.

The multiple-tube enrichment method with confirmation of gas-positive tubes in EC Media for 24±2 hr in a circulating covered water bath at 44.5 C±0.2 C is the method to be used for shellfish and sea water and is described in this section.

3.2 Presumptive Test—See Part III A, Sec. 2.1.*

3.3 Confirmed Test—All Presumptive Test fermentation tubes showing any amount of gas at the end of the 24- or 48-hr incubation period shall be subjected to the Confirmed Test. When active fermentation appears in the fermentation tube before the expiration of the 24-hr period of incubation, transfer these cultures without waiting for completion of the full period.

3.31 *Procedure:*

1) Mix the Presumptive Test fermentation tube by gentle shaking or swirling.
2) Transfer one loopful (using a 3-mm inside diameter loop) of the medium to the fermentation tube containing EC Broth (Part III A, Sec. 2.11).

* At times, should it be desirable to avoid indeterminate results, it will be necessary to plant 100-ml portions. It is more important to obtain real values at the low MPN levels of fecal coliforms, since these organisms represent only a portion of the total coliform population, and their presence has greater sanitary significance.

Extreme care must be exercised to assure the transfer of a full loopful of viable culture. The loop must be cool before final loading.

3) Incubate the inoculated EC fermentation tubes at 44.5 C±0.2 C in a circulating covered water bath for 24±2 hr. All EC tubes must be placed in the water bath within 30 min after inoculating. Submerge the broth tubes in the bath so that the water level is above the highest level of the medium. The thermometer used in the water bath shall be subdivided to at least 0.2 C, preferably 0.1 C, and shall be checked for accuracy at the test temperature with a Bureau of Standards thermometer or one of equivalent accuracy. It is desirable to equip the water bath with a recording thermometer to record automatically the temperature variations throughout the entire period of incubation. In the absence of a recording thermometer, positive and negative controls using a known 44.5 C gas-positive *Escherichia coli* culture and a known 44.5 C gas-negative *Aerobacter aerogenes* or other coliform biotype culture should be included in each water bath at time of use.

4) Record the presence or absence of gas formation in the EC tubes after 24±2 hr incubation at 44.5 C±0.2 C. Gas production in the fermentation tube within 24±2 hr is considered a Positive Confirmed Test for fecal coliform organisms. Tubes which fail to produce gas within 24±2 hr are considered negative for fecal coliform organisms.

3.4 Expression of Results—The fecal coliform MPN is computed in the same manner as the coliform MPN (see Part III A, Sec. 2.5).

4.0 Standard Plate Count

4.1 Plating—In preparing plates, enough sample amounts shall be planted to give from 30 to 300 colonies per plate, and the aim shall be to have at least 2 plates giving colonies between these limits. Use not less than 4 plates per sample, 2 from each of 2 dilutions. With samples of unknown plate count density 3 or 4 dilutions might be indicated.

It is not desirable to plant more than 1 ml per plate. Therefore, when the total number of colonies developing from 1 ml is less than 30, it will be necessary to record the results as observed.

4.2 Procedure—

1) Prior to the transfer of the sample to the dilution bottle or plates, the sample bottle shall be shaken vigorously 25 complete up-and-down movements of about 1 ft in 7 sec. Each dilution bottle or tube, after the addition of the measured portion of the sample, shall be shaken as above before the subsample is removed for transfer to the plates or dilution bottle.

Add the appropriate amount of sample to the culture dish, using the dilutions prepared for the inoculation of the presumptive tubes. In the examination of highly turbid waters, 0.1 ml inocula of the original sample should not be measured directly. One ml of a 1:10 dilution should be used.

Cool melted medium promptly to about 45 C (113 F) and hold at 44-46 C (111-115 F) in water bath or incubator until used. Extend thermometer into water in a separate container similar to that used for medium and expose to same conditions as the medium to serve as a temperature control. Do not depend upon sense of touch to indicate proper temperature of medium when pouring agar.

Prepare control plates to check on the sterility of the dilution water, the sterilized plates and the agar medium.

2) Add not less than 10 ml of liquefied plate count agar medium (Part I E, Sec. 1.8) at a temperature of 44-46 C to the inoculated dish. Flame the lips of test tubes or flasks containing the liquefied medium before pouring into the dish. Lift the cover of the culture dish just enough for the introduction of either the pipet or the culture medium. Melt only such quantities of agar medium as will be used within 3 hr.

3) Mix the medium and sample by tilting and rotating the dish. A mechanical rotator designed for this purpose may be used. Not more than 20 min shall elapse between plating of the sample and pouring of the culture medium.

4) The agar shall be solidified as rapidly as possible after pouring and then placed immediately in the incubator. Glass-covered or plastic culture dishes shall be inverted before placing in the incubator.

5) Incubate at a temperature of 35 C±0.5 C for 48±3 hr.

4.3 Counting Colonies—Count colonies with the aid of magnification under uniform and properly controlled artificial illumination. The Quebec colony counter, dark-field model, is preferred, equipped with a guide plate ruled in square centimeters. Other counting aids which pro-

vide equivalent conditions may be used. Avoid mistaking particles of undissolved medium or precipitated matter in plates for pinpoint colonies. To distinguish colonies from dirt specks and other foreign matter, examine doubtful objects carefully, using higher magnification where required.

4.31 *Selecting and Counting Plates:*

a) Select spreader-free plates with 30-300 colonies. Count all colonies including those of pinpoint size on selected plates, record dilution or dilutions used and report total colonies as basis for Standard Plate Count (see Table 5, Sample 1011).

b) If only 1 plate of a certain dilution yields 30-300 colonies, count other duplicate plates, unless excluded by (d) and (g) below and include such counts in the average (arithmetic) (see Table 5, Sample 1012). Where 1 or more duplicate plates from consecutive decimal dilutions are to be counted, determine average count per dilution before proceeding as in (c) (see Table 5, Sample Nos. 1013, 1014 and 1015).

c) If plates from two consecutive decimal dilutions yield 30-300 colonies each, compute estimated count per ml for each dilution by multiplying colonies per plate by dilution used. Report the arithmetic average as Standard Plate Count per ml unless the higher computed count is more than twice the lower one, in which case report lower computed count as Standard Plate Count per ml (see Table 5, Sample Nos. 1014 and 1015).

d) If spreaders occur on plate(s) selected, count colonies on representative portions thereof only when colonies are well distributed in spreader-free areas and the area covered by spreader(s) (including total repressed growth area, if any) does not exceed ½ of plate. Where repressed growth area alone exceeds ¼ of total area, report test as directed in (g) below.

e) Where there is no plate with 30-300 colonies, and one or more plates with more than 300 colonies, use such plate(s) having nearest 300 colonies, counting and estimating numbers per plate as in Sec. 4.32 below (see Table 5, Sample No. 1016).

f) If plates from all dilutions yield less than 30 colonies each, record actual number of colonies on the lowest dilution unless excluded by (d) and (g) but report computed count as "less than" 30 times corresponding dilution. For example, if the 1:100 dilution has been used, report Standard Plate Count per ml as "less than 3000" (<3000). (See Table 5, Sample No. 1017.)

g) If all plates from any sample show no colonies, or have excessive spreader growth, or are known to be contaminated, or are otherwise unsatisfactory, report result as "No Colonies" (NC), or "Spreader" (Spr), or "Laboratory Accident" (LA), or "Growth Inhibitors" (GI), or in other appropriate terms. In plates which show no growth, or proportionately less growth in lower dilutions, there may be a tendency on the part of the analyst to suspect the presence of inhibitory material in the sample under examination, but such cannot be interpreted as evidence of inhibitors.

4.32 *Estimating Colonies on Crowded Plates:* Where colonies per plate appreciably exceed 300, count colonies in portions of plate representative of colony distribution and estimate therefrom total number per plate. Where there are less than 10 colonies per sq cm, count colonies in from 12 to 14 (13 preferred) such areas, selecting, if representative, 6 (or 7) consecutive squares diagonally across the plate and 6 (or 7) consecutive squares at right angles thereto. Provided no squares are recounted, initial selection of squares to be counted may be vertical and remain at right angles thereto. Sum of colonies in 13 representative sq cm multiplied by 5 yields estimated colonies per plate when area of plate is 65 sq cm.

Where there are more than 10 colonies per sq cm, count colonies in 4 such representative squares, multiply average number found per sq cm by appropriate factor to determine estimated colonies per plate. Because the average inside diameter of the bottom of recommended petri dish is 91 mm (area 65 sq cm), multiply average number of colonies per sq cm by 65. To avoid errors among estimates caused by unequal distribution of colonies depending upon varying depths of media in plates, use dishes with flat bottoms. CAUTION: Where pressed glass dishes or single-use plastic dishes with a smaller diameter are used, laboratories should determine the actual diameter and use a corrected factor for computing.

4.33 *Counting Spreading Colonies:* When counting of spreading colonies cannot be avoided, count each of the three distinct types as one source. The first type is a chain of colonies not too distinctly separated, a condition which appears to be caused by disintegration of a bacterial clump when petri dish is rotated to mix agar with diluted sample. Where

only one such chain exists, count it as a single colony. Where one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chain(s) as a separate colony.

The second type is the spreader that develops in a film of water between agar and the bottom of dish. The third type forms in a film of water at the edge or over the surface of the agar. The two latter types develop largely because of accumulation of moisture at point from which the spreader originates. When diluted sample is uniformly distributed throughout the medium, the above-mentioned bacterial foci rarely develop into spreading colonies. Any laboratory with 5% or more of plates more than ¼ covered with spreaders should take immediate steps to eliminate the trouble. Note condition for reporting results as laboratory accidents, where spreader(s) covers more than ½ of plate.

4.4 Computing Standard Plate Count—

(a) To compute Standard Plate Count, multiply total colonies or average of estimated number/plate by the reciprocal of the dilution used. Examples of typical computations appear in Table 5. Because of limitations of method, do not report estimates by this method as showing number of bacteria per ml (or per g). Computed figure is an estimate of total colonies that would have developed per ml of water on the medium under specified growth conditions if an entire ml of sample had been plated. To abridge this awkward statement, the numerical estimate of colonies per plate is multiplied by proper dilution factor and result recorded as a Standard Plate Count per ml — (SPC per ml ——) at 35 C, or Standard Plate Count per g —— (SPC per g ——) at 35 C.

(b) To avoid creating fictitious ideas of accuracy, when reporting Standard Plate Counts per ml record only first two left-hand digits, raising second digit to next higher number only when third digit from left is 5, 6, 7, 8 or 9 and using 0's for each successive digit toward right from second digit (see examples in Table 5).

B. EXAMINATION OF SHELLFISH

1.0 Collection and Transportation of Samples

Samples of shell stock and shucked, unfrozen shellfish should be examined within 6 hr after collection and in no case shall they be examined if they have been held more than 24 hr after collection. The report of the examination shall include a record of the time elapsed between collection and examination.

Individual containers of shellfish samples shall be marked for identification and the same mark shall be put in its proper place on the descriptive form which accompanies the sample.

A history and description of the shellfish shall accompany the sample to the laboratory. This shall include:

1—Date, time, and place of collection
2—The area from which the shellfish were harvested
3—The date and time of harvesting
4—The conditions of storage between harvesting and collection.

All of this information may not be obtainable for shellfish samples collected in market areas. In such case, the identification of the shipper, the date of shipment, and the harvesting area should be determined as well as the date, time and place of collection.

1.1 Shell Stock (Shellfish in the Shell)—Samples of shellfish shall be collected in clean containers. The container shall be waterproof, and shall be durable enough to withstand the cutting action of the shellfish and abrasion during transportation. Waterproof paper bags, paraffined cardboard cups or plastic bags are suitable types of containers. A tin can with a tight lid is also suitable.

Shell-stock samples shall be kept in dry storage at a temperature above freezing but lower than 10 C (32-50 F) until examined. Shell stock shall not be allowed to come in contact with ice.

In general, a minimum of 12 shellfish shall be taken in order to obtain a representative sample and to allow for the selection of sound animals suitable for shucking. With most species, allowing for the necessary culls, approximately 200 g of shell liquor and meats will be obtained.

Because of their larger size, 10 to 12 of certain species, such as the Pacific oyster, *Crassostrea gigas*, the surf clam, *Mactra solidissima*, and certain larger sizes of the hard clam, *Mercenaria mercenaria*, may produce more than 200 g of shell liquor and meats. It is desirable to use at least 10 animals for a sample. Therefore, blender jars of a larger size than those ordinarily used may be indicated. Certain blenders will accept ordinary mason jars and thus a two-quart container should be used for the blending of the species.*

* Where two-quart containers are not available, the 10-12 shellfish should be ground for 30 sec. Then 200 g of this meat homogenate should be blended with 200 g sterile buffered phosphate water or 0.5% sterile peptone water for 60 sec.

On the other hand, 10 or 12 of certain other species, such as the Olympia oyster, *Ostrea lurida,* and small sizes of the Pacific little neck clams, *Protothaca staminea* and *Tapes japonica,* may produce much less than 100 g of shell liquor and meats. Blender containers of smaller size are indicated, but even when 1 or ½-pint jars are used, as many as 20 to 30 of these species would be required to produce an adequate volume for proper blending.

1.2 Shucked Shellfish—A sterile widemouth jar of a suitable capacity with a watertight closure is an acceptable container for samples of shucked shellfish taken in shucking houses, repacking establishments or bulk shipments in the market. The shellfish may be transferred to the sample jar with sterile forceps or spoon. Samples of the final product of shucking houses or repacking establishments may be taken in the final packing cans or containers. The comments pertaining to species of various sizes in the section on shell stock applies to shucked shellfish. Consumer-size packages are acceptable for examination, provided that they contain an adequate number of animals.

Samples of shucked shellfish shall be refrigerated immediately after collection by packing in crushed ice and they shall be so kept until examined.

1.3 Frozen Shucked Shellfish—If the package contains an adequate number of animals (10 to 12), one or two packages may be taken as a sample. Samples from larger blocks may be taken by coring with a suitable instrument or by quartering, using sterile technic. Cores or quartered samples shall be transferred to sterile widemouth jars for transportation to the laboratory.

It is desirable to keep samples of frozen shucked shellfish in the frozen state at temperatures close to those at which the commercial stock was maintained. When this is not possible, samples of frozen shucked shellfish shall be packed in crushed ice and kept so until examined.

2.0 Preparation of Sample for Examination

2.1 Shellfish in the Shell—

2.11 *Cleaning the Shells:*

The hands of the examiner must be thoroughly scrubbed with soap and water.

Scrape off all growth, loose material from the shell and scrub the shell stock with a sterile stiff brush under running water of drinking-water

quality, paying particular attention to the crevices at the junctions of the shells. Place the cleaned shell stock in clean containers or on clean towels and allow to drain in the air.

2.12 *Removal of Shell Contents:* Before starting the removal of shell contents, the hands of the examiner must be thoroughly scrubbed with soap and water and rinsed with 70% alcohol.

Open the shellfish as directed below, collecting the appropriate quantities of shell liquor and meats in a sterile blender or other suitable sterile container.

2.121 OYSTERS. Hold the oyster in the hand or on a fresh clean paper towel on the bench with the deep shell on the bottom. Using a sterile oyster knife, insert the point between the shells on the ventral side (at the right when the hinge is pointed away from the examiner), about ¼ the distance from the hinge to the bill. Entry may also be made at the bill after making a small opening with a sterile instrument similar to bone-cutting forceps.

Cut the adductor muscle from the upper flat shell and pry the shell wide enough to drain the shell liquor into a sterile tared beaker, widemouth jar, or blender jar. The upper shell may then be pried loose at the hinge, discarded, and the meats transferred to the beaker or jar after severing the muscle attachment to the lower shell.

2.122 HARD CLAMS. Entry into the hard clam, *Mercenaria mercenaria* or the Pacific little-neck clam, is best done with a sterile, thin-bladed knife similar to a paring knife. To open the clam, hold it in the hand, place the edge of the knife at the junction of the bills, and force it between the shells with a squeezing motion. An alternative method is to nibble a small hole in the bill with sterile bone-cutting forceps, and with the knife sever the 2 adductor muscles.

Drain the shell liquor into the sample container. Cut the adductor muscles from the shells and transfer the body of the animal to the sample container.

2.123 OTHER CLAMS. The soft clam, *Mya arenaria,* the Pacific butter clam, *Saxidomus giganteus,* the surf clam, *Mactra solidissima,* and similar species may be shucked with a sterile paring knife, entering at the siphon end and cutting the adductor muscles first from the top valve and then from the bottom valve.

2.124 MUSSELS. Mussels, *Volsella* and *Mytilus* species, may be en-

tered at the byssal opening. The byssal threads should be removed during the cleansing of the shell. The knife may be inserted and the shells spread apart with a twisting motion, allowing the draining of the shell liquor. Cut away the many attachments from the shell.

2.2 Shucked Shellfish—Transfer a suitable quantity from a sample jar to a sterile tared blender jar or other container, using a sterile spoon (Part III B, Sec. 1.2).

2.3 Dilution and Grinding—Weigh the sample to the nearest gram. Transfer the weighed samples to a sterile blender jar and add an equal amount, by weight, of sterile phosphate buffered dilution water or 0.5% sterile peptone water. (A dilution of equal amounts by weight of shucked pack of certain species of shellfish results, after grinding in a laboratory blender, in a mixture which is of too heavy a consistency for pipetting. The meats of the hard clam, the surf clam, and the butter clam often are of this characteristic. Transfer such species, undiluted, to culture tubes. In these cases, the use of greater proportions of dilution water would be permissible. Addition of 3 parts by weight of dilution water to 1 part of the weighed sample is suggested. With such dilutions, 4 ml of the ground sample would be equal to 1 g of shellfish and thus should be used in the inoculation procedure, Part III B, Sec. 3.1. (Adjustment of Presumptive Broth Concentration should be made.)

Grind for 60–120 sec in a laboratory blender operating at approximately 14,000 RPM. The optimum grinding time between these limits will vary with make of machine, condition of machine, species of shellfish, and probably the physical state of the meats. In general, a grinding time of 60–90 sec will be found to be optimum for all species. Excessive grinding in smaller containers should be avoided to prevent overheating.

3.0 Test for Members of the Coliform Group

3.1 Presumptive Test—The ground sample shall be cultured *within 2 min after the completion of the grinding period.* On standing, separation in the sample takes place. Therefore, to avoid gross errors in volumetric measurement of the suspension, the sample shall be thoroughly mixed immediately before transfer to the presumptive medium if there has been any delay between grinding and culturing of the sample.

Lactose broth or lauryl tryptose broth, single strength in fermentation tubes, shall be used as the Presumptive Test medium. Dilutions shall be made in sterile phosphate buffered dilution water or 0.5% sterile

peptone water. All dilutions just prior to pipetting shall be shaken 25 times through an arc of 1 ft in a time interval not exceeding 7 sec.

3.11 *Procedure:*

INOCULATE:

a. Each of 5 tubes with 2 ml of the ground sample (equal to 1 g of shellfish).

b. Each of 5 tubes with 1 ml of a 1:10 dilution of the shellfish sample (equal to 0.1 g of shellfish).
NOTE: The 1:10 dilution may be made by adding 20 ml of the ground sample to 80 ml of sterile phosphate buffered dilution water or 0.5% sterile peptone water.

c. Each of 5 tubes with 1 ml of a 1:100 dilution of the shellfish sample (equal to 0.01 g of shellfish).
NOTE: The 1:100 dilution may be made by adding 11 ml of the 1:10 dilution prepared in (b) above to 99 ml of dilution water.

d. Each of 5 tubes with 1 ml of a 1:1000 dilution of the shellfish sample (equal to 0.001 g of shellfish).
NOTE: The 1:1000 dilution may be made by adding 1 ml of the 1:10 dilution prepared in (b) above to 99 ml of dilution water.

In order to avoid indeterminate results, extension of the dilutions suggested above may be necessary. The amounts of sample selected for inoculation shall be such that the largest portions will give positive results in all or the majority of tubes and the smallest portions will give negative results in all or the majority of tubes. To attain this with any degree of assurance with samples of doubtful quality, it will be necessary to inoculate additional decimal dilutions.

3.12 Incubate the fermentation tubes at 35 C ± 0.5 C. Proceed with the Presumptive Test as directed under Examination of Sea Water (Part III A, Sec. 2.1).

3.2 Confirmed Test—Proceed with the Confirmed Test as directed under Examination of Sea Water (Part III A, Sec. 2.2).

3.3 Completed Test—Proceed with the Completed Test as directed under Examination of Sea Water (Part III A, Sec. 2.3).

3.4 Expression of Results—The coliform density shall be expressed as "Most Probable Number" (MPN) per 100 g of sample. Compute the

MPN as directed in the Examination of Sea Water (Part III A, Sec. 2.5).

3.5 Where individual coliform species are to be identified see Appendix.

4.0 Tests for Fecal Coliforms in Shellfish

4.1 Presumptive Test—The Presumptive Test for fecal coliforms is conducted in the same manner as the Presumptive Test for coliform organisms (Part III B, Sec. 3.1).

4.2 Confirmed Test—Proceed with the Confirmed Test as directed under Examination of Sea Water (Part III A, Sec. 3.3).

4.3 Where individual fecal coliform species are to be identified see Appendix.

4.4 Expression of Results—The fecal coliform density in shellfish shall be expressed as "Most Probable Number" (MPN) per 100 g of sample. Compute the MPN as directed in Examination of Sea Water (Part III A, Sec. 2.5).

5.0 Standard Plate Count

The Standard Plate Count of samples of shellfish shall be conducted as directed under Examination of Sea Water (Part III A, Sec. 4.0) except that the plates shall be incubated for 48 ± 3 hr rather than 24 hr ± 2 hr. In the examination of shellfish, 0.1 ml inocula of the original sample shall not be used. One ml of an appropriate dilution shall be used. Express the results as Standard Plate Count per g of sample 35 C, 48 hr. Ordinarily it is not desirable to plant more than 0.1 g of shellfish in a plate because of excess turbidity produced by larger quantities of sample. Therefore, when the total number of colonies developing from 0.1 g is less than 30 it will be necessary to record the results as observed.

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PART IV

MEMBRANE FILTER METHODS FOR THE BACTERIOLOGIC EXAMINATION OF SEA WATER

EXAMINATION OF SEA WATER

1.0 Membrane Filter Procedure for Enumeration of Coliform Group Organisms

This procedure was presented as a standard technic for the enumeration of coliforms in fresh water in the Twelfth Edition of *Standard Methods for the Examination of Water and Wastewater* (1965). Since that time it has proved its value as a direct, reproducible, and rapid procedure for establishing coliform densities in fresh water. It has also been found useful and applicable for the enumeration of coliforms in some sea waters. Its limitations are chiefly in waters which have a high turbidity, large numbers of noncoliforms as related to low coliform counts, or which contain toxic substances.

1.1 Laboratory Apparatus and Materials—All general laboratory apparatus should meet specifications outlined in Part I, Section A.

1.11 *Culture dishes:* Culture dishes of the petri dish type should be used. The bottom of the dish should be flat and 5–6 cm in diameter so that the absorbent pad for nutrient will lie flat. The glass should be borosilicate or equivalent grade. Clean culture dishes may be wrapped singly or in convenient numbers, in metal foil or a suitable paper substitute, prior to sterilization. Suitable sterile nontoxic plastic dishes may be used.

1.12 *Filtration units:* The filter-holding assembly should consist of a seamless funnel which fastens to a receptacle bearing a porous plate for support of the filter membrane. The parts should be so designed that the funnel unit can be attached to the receptacle by means of a convenient locking device. The construction should be such as to insure that the membrane filter will be securely held on the porous plate of the receptacle without mechanical damage and that all the fluid will pass through the membrane in the filtration of the sample. The filter-holding assembly may be constructed of glass, porcelain, or any noncorrosive nontoxic bacteriologically inert metal. It is recommended that the two parts of the assembly be wrapped separately in heavy wrapping paper for sterilization and storage until use.

For filtration, the receptacle of the filter-holding assembly is mounted in a one-liter filtering flask with a side tube or in some other suitable device such that pressure differential can be drawn on the filter membrane. The filter flask should be connected by the side arm to an electric vacuum pump, a filter pump operating on water pressure, a hand aspirator, or to other means of securing a pressure differential.

The filtration units should be sterile at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted if there is an interval of 30 min or longer between sample filtrations. After such interruption, any further sample filtration is treated as a new filtration series and requires a resterilization of all membrane filter holders in use. Rapid decontamination of this equipment between successive samples may be done by use of an ultraviolet (U.V.) sterilizer. In this sterilization procedure, a 2-min exposure of the filtration unit to U.V. radiation is sufficient. Do not subject membrane filter culture preparations to any random U.V. radiation leaks that might occur from such sterilization cabinets. Some measure of eye protection is recommended. Either safety glasses or prescription ground glasses are adequate for eye protection from stray radiation if the U.V. sterilization cabinet is not light tight during exposure interval.

1.13 *Filter membranes:* Only those filter membranes may be employed which have been found by complete laboratory tests to provide full bacterial retention, stability in use, freedom from chemicals inimical to the growth and development of bacteria, and satisfactory speed of filtration. They should preferably be grid-marked. Different brands of membrane filters meeting these specifications can be obtained from manufacturers and suppliers of laboratory equipment. The manufacturer's directions for handling and sterilization of the filters should be followed. Suitable packaged filters designed for autoclave sterilization, or if desired, presterilized, can be purchased.

1.14 *Absorbent pads:* Absorbent pads for nutrients should be discs of filter paper or other material known to be of high quality and free of sulfites or other substances that could inhibit bacterial growth. They should be approximately 48 mm in diameter and of such thickness that they will absorb 1.8 to 2.2 ml of nutrient. Presterilized absorbent pads or those subsequently sterilized in the laboratory should release less than one milligram of total acidity when titrated to the phenolphthalein end point, pH 8.3, using 0.02*N* NaOH. The pads may be simultaneously sterilized with membrane filters available in resealable kraft envelopes or separately in other suitable containers. They must be free of visible moisture prior to use.

1.15 *Forceps:* Forceps should be round-tipped without corrugations on the inner sides of the tips. They may be sterilized before use in a flame or by dipping in 95% ethyl or absolute methyl alcohol and then igniting the fluid.

1.16 *Incubators:* Facilities for incubation of membrane filter cultures must provide a temperature of 35 C±0.5 C and maintain a high level of humidity.

1.17 *Microscope and light source:* Membrane filter colonies are best counted with a magnification of 10–15 diameters and with the light source adjusted to give maximum sheen appearance. A binocular wide-field dissecting microscope is recommended as the best optical system. However, a small fluorescent lamp with magnifier is acceptable. Colony differentiation is best observed with diffused daylight developed from cool, white fluorescent lamps. The use of a microscope illuminator with optical system for light concentration from an incandescent light source is specifically unsatisfactory for coliform colony discernment on Endo-type media.

1.18 *Culture Media:* MF endo medium or LES endo medium (Part I E, Sec. 3.1 and 3.13).

1.2 Procedure—

1.21 *Selection of sample size:* The size of sample will be governed by the expected bacterial density and should result in the growth of about 50 coliform colonies and not more than 200 colonies of all types. All waters should be examined by the filtration of three aliquot volumes depending on the expected bacterial density. When less than 20 ml of sample (diluted or undiluted) is to be filtered, a small amount of sterile dilution water should first be added to the funnel prior to filtration. This

increase in water volume aids the uniform dispersion of the bacterial suspension over the entire effective filtering surface.

1.22 *Culture procedure:* Place a sterile absorbent pad in bottom half of sterile culture dish and pipet enough MF endo medium or LES holding medium (1.8–2.0 ml) to saturate the pad. Any surplus medium should be removed.

1.23 *Filtration of sample:* Using sterile forceps, place a sterile filter over the porous plate of the apparatus, grid side up. Carefully place the matched funnel unit over the receptacle and lock it into place. The filtration is then accomplished by passing the sample through the filter under partial vacuum. The filter should be rinsed by the filtration of three volumes of 20–30 ml of sterile buffered distilled water or 0.5% peptone between samples. Unlock and remove the funnel, immediately remove the filter by sterile forceps and place it on the sterile pad or agar with a rolling motion to avoid the entrapment of air. Where M-endo broth is used, incubate the inverted dish with filter for 22–24 hr at 35 C±0.5 C in an incubator with high humidity. High humidity may be obtained by placing inverted dishes on a rack in a sealed plastic container with ½ inch water in the bottom of the container. Incubate the LES holding medium for 1½–2 hr at 35 C±0.5 C. Then transfer to LES endo agar medium and incubate for 18–20 hr at 35 C±0.5 C.

1.24 *Counting:* The typical coliform colony has a pink to dark red color with a metallic sheen. The sheen area may vary from a small pinhead size to complete coverage of the colony surface. The count is best made with the aid of a low-power (10–15 magnification) binocular wide-field dissecting or stereoscopic microscope, using a fluorescent light source adjusted to give maximum sheen.

Coliform organisms may occasionally produce colonies which are not typical in color or sheen. If only atypical forms are found, their identity as coliform bacteria should be confirmed by transfer of doubtful colonies to brilliant green bile broth. Gas formation within 48 hr incubation at 35 C is deemed evidence of coliform colonies.

1.25 *Calculation of coliform density:* The calculated coliform density is reported in terms of (total) coliform per 100 ml. The computation is derived from the membrane filter count within the 20–80 coliform colony range and is made by use of the following equation:

$$\text{(Total) Coliform Colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

If the membrane filter counts are individually less than 20, all such counts should be totaled and the value based on the total volume of sample examined. For example, if duplicate 50-ml portions contained 5 and 3 coliform colonies, the count would be reported as 8 coliforms per 100 ml. However, if 10, 1.0, and 0.1 portions were examined with, respectively, 19, 3, and 1 coliforms, the result would be reported as 210 coliforms per 100 ml. This is calculated by $\frac{(19+3+1)(100)}{11.1}=207$. The number of coliforms should not be recorded with more than two significant figures per 100 ml.

When there are excessive colonies on the membrane filter the report should be "TNTC" (Too Numerous To Count) or if there is growth without well-defined colonies the report should be "Confluent." In either case a new sample should be requested and more appropriate volumes selected for filtration.

2.0 Membrane Filter Procedure for Enumeration of Fecal Coliforms

Determination of fecal coliform densities in estuarine water or sea water may also be effected by the following membrane filter technic which has been reported to have a 93% accuracy for differentiating coliforms of warm-blooded animals and coliforms from other sources. This procedure uses an enriched lactose medium that depends on rosolic acid and on a water-bath incubation temperature of 44.5 C$\pm$0.2 C for its selectivity (Part IV, Sec. 2.3).

2.1 Culture Medium—M-FC broth base plus rosolic acid solution (Part I E, Sec. 3.2). The rosolic acid should be prepared and added to the sterile base medium as outlined and must be discarded when the solution changes from dark red to muddy brown.

2.2 Culture Dishes—Tight-fitting plastic dishes are essential if these membrane filter cultures are to be submerged in a water bath during incubation. Enclosing groups of fecal coliform cultures in plastic bags is recommended to reduce further the occurrence of leakage during submersion.

2.3 Incubators—The specificity of the fecal coliform test is directly related to the incubation temperature. A temperature tolerance of $\pm$0.2 C can be obtained with most types of water baths that also are equipped with a circulation system and a cover to prevent heat loss.

It has also been found possible to maintain this temperature tolerance in a good quality water-jacketed air incubator (44.5 C$\pm$0.2 C). Daily controls with EC gas-positive *E. coli* and EC-negative *A. aerogenes* should be included with each set of tests.

2.4 Procedure—

2.41 *Selection of sample size:* The volume of water to be examined by the membrane filter technic must receive careful consideration before filtration is started. When the bacterial level of the sample is totally unknown, it is necessary to filter several decimal quantities of sample to establish the true fecal coliform density. The best method is to estimate the ideal quantity expected to yield a countable membrane, and to use two additional quantities representing one-tenth and ten times this quantity, respectively. Sample quantities that will yield counts between 20–60 fecal coliform colonies result in greater accuracy of density determination.

2.42 *Filtration of sample:* Observe the same procedure and precautions as prescribed under Part IV, Sec. 1.0.

2.43 *Culture procedure:* Place a sterile absorbent pad in each culture dish and pipet approximately 2 ml of M-FC medium prepared as directed under Media Specifications (Part I E) to saturate the pad. Carefully remove any surplus liquid from the culture dish. The prepared filter is then placed on the medium-impregnated pad as described under Section 1.23 above.

2.44 *Incubation:* The prepared cultures are inverted in waterproof plastic bags for protection during submersion in the water bath for the 24-hr incubation period at 44.5 C$\pm$0.2 C. The dishes must be anchored below the water surface during incubation to maintain critical temperature requirements. All prepared cultures should be placed in incubators within 20 min after filtration.

2.45 *Counting:* Colonies produced by fecal coliforms are blue in color. The nonfecal coliform colonies are gray to cream colored. Background color on the membrane filter will vary from a yellowish cream to faint blue, depending on the age of the rosolic acid salt reagent. Normally, few nonfecal coliform colonies will be observed on M-FC medium because of the selective action of the elevated temperature and the addition of the rosolic acid salt reagent. The colony count is best made with the aid of a low-power (10–15 magnification) binocular wide-field dissecting microscope or other optical device.

2.46 *Calculation of fecal coliform density:* The density is computed from the sample quantities that produced membrane filter counts within the desired 20–60 fecal coliform colony range. This colony density range is more restrictive than the 20–80 total coliform range because of larger colony growth on M-FC medium. Proceed with the calculation as stated (Part IV A, Sec. 1.25). Record densities as fecal coliforms per 100 ml.

3.0 Membrane Filter Procedure for Enumeration of Fecal Streptococcal Group Organisms

The terms "fecal Streptococcus" and "Enterococcus" have been used somewhat synonymously in recent years and there have been varying opinions as to what species, varieties or biotypes of streptococci are included when these terms are employed.

On the basis of new concepts of speciation of the fecal streptococci it is suggested that the terms "fecal Streptococcus" and "Lancefield's Group D Streptococcus" be considered as synonymous and that the use of these terms be restricted to denote the following species or their varieties used as indicators of fecal contamination: *S. faecalis; S. faecalis,* var. *liquefaciens; S. faecalis* var. *zymogens; S. durans, S. faecium, S. bovis,* and *S. equinus.* All but the latter two species meet the criteria of enterococci. Other varieties or biotypes of *S. faecalis* and *S. faecium* have been reported, but their nomenclature and taxonomic position in the fecal streptococcal group await further investigation.

The membrane filter technic affords direct counts of fecal streptococci in water.

3.1 Laboratory apparatus—See Part IV A, Sec. 1.1.

3.2 Culture medium M-enterococcus agar—(Refer to Part I E, Sec. 3.3.) Pour or pipet 7–10 ml liquefied medium into culture dishes. Flame the surface if necessary to eliminate bubbles.

3.3 Procedure—

3.31 *Selection of sample size and filtration:* Filter samples of water through the sterile membrane to attain 20 to 100 colonies on the membrane surface. It may be necessary to filter amounts varying from 100 to 10, 1, 0.1 ml, depending on the amount of pollution in the water sample. Transfer the filter directly to the agar medium in the petri dishes, avoiding air bubbles.

3.32 *Incubation:* Invert culture plates and incubate at 35 C±0.5 C for 48 hr.

3.4 Counting—Colonies produced by fecal streptococci are dark red to pink in color. The count is best made with the aid of a low-power (10–15 magnification) binocular wide-field dissecting or stereoscopic microscope.

3.5 Calculation of fecal streptococcal density—The density is computed from the sample quantities that produce membrane filter counts within the desired 20–100 fecal streptococcus colony range. This colony density range is greater than the 20–80 total coliform range because of the increased selectivity of fecal streptococci media. Proceed with the calculation as stated under 1.25 above. Record densities as fecal streptococci per 100 ml.

3.6 Confirmed Test—If further confirmation of subgroup identification is indicated, methods as described in the twelfth edition of *Standard Methods for the Examination of Water and Wastewater* (1965) should be used.

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PART V

BIOASSAY FOR SHELLFISH TOXINS

A. BIOASSAY FOR PARALYTIC SHELLFISH POISON *

1.0 Introduction

The association of marine dinoflagellates of the genus *Gonyaulax* with a paralytic toxin found in molluscan shellfish, particularly along the Atlantic and Pacific coasts of North America, is well documented. In 1937, Sommer and Meyer identified *Gonyaulax catenella* as the source of paralytic shellfish poison found in California mussels. They developed the first practical bioassay, in which a mouse unit was defined as the minimum amount of poison required to kill a 20-gram mouse in 15 min when 1.0 ml of shellfish extract is injected intraperitoneally. A similar assay method was described in 1947 by Canadian workers. Other studies have shown chemical and physical similarities between poisons isolated from toxic shellfish and *G. catenella* cultures, and have provided further proof that the toxin in California mussels and Alaska butter clams is derived from *G. catenella*. An allied species, *G. acatenella*, has been shown to be a source of shellfish toxicity in British Columbia, while *G. tamarensis* has been demonstrated to be the dinoflagellate species associated with PSP on the Atlantic coast of Canada.

The bioassay procedure was modified by Schantz, McFarren and others, who used purified shellfish poison as a reference standard, thus permitting the expression of the response of mice in terms of a definite weight of poison in the reference standard. Collaborative studies by various laboratories have shown reasonably close agreement, and the bioassay procedure was first published in 1960 as an official method of the Association of Official Agricultural Chemists.

* Adapted from A.O.A.C. See bibliography at end of Part V.

1.1 Materials—

1.11 *Paralytic shellfish poison standard solution* (100 μg per ml): Request for PSP standard solution (100 μg/ml) should be submitted to Division of Criteria and Standards, Bureau of Water Hygiene, ECA, 12720 Twinbrook Parkway, Rockville, Maryland 20852.

1.12 *Paralytic shellfish poison reference solution* (1 μg/ml): Dilute 1 ml standard solution to 100 ml with distilled water. Solution is stable several weeks at 3-4 C. Final pH should be between 2.0 to 4.0.

1.13 *Mice:* Healthy mice, 19–21 g, from a stock colony are used for routine assays. If <19 or >21 g, apply correction factor to obtain true death time (see Table 6). Do not use mice weighing > 23 grams. Do not reuse mice.

1.2 Standardization of Bioassay—

1.21 Dilute 10 ml aliquots of 1 μg/ml reference solution with 10, 15, 20, 25 and 30 ml distilled water, respectively, until intraperitoneal injection of 1 ml doses into a few test mice causes a median death time of 5–7 min; pH of dilutions should be 2–4 and must not be > 4.5. Test additional dilutions in 1 ml increments of distilled water, *e.g.* if 10 ml diluted with 25 ml distilled water kills mice in 5–7 min, test dilutions diluted 10+24 and 10+26. Care should be taken to maintain these dilutions between a pH of 2.0 to 4.0.

1.22 Inject group of 10 mice with each of 2, or preferably 3, dilutions that fall within median death time of 5–7 min. Give one (1) ml dose to each mouse by intraperitoneal injection and determine death time as time elapsed from completion of injection to the last gasping breath of mouse.

1.23 Repeat assay 1 or 2 days later, using dilutions prepared above which differed by 1 ml increments of distilled water. Then repeat entire test, starting with testing of dilutions prepared from newly prepared reference solution.

1.24 Calculate median death time for each group of 10 mice used on each dilution. If all groups of 10 mice injected with any one dilution gave median death times of <5 or >7 min, disregard results from this dilution in subsequent calculations. On the other hand, if any of the groups of 10 mice injected with one dilution gave a median death time falling between 5 and 7 min, include all groups of 10 mice used on that dilution, even though some of the median death times may be <5 or >7 min. From the median death time for each group of 10 mice in each of the selected dilutions, determine the number of mouse units per ml from Table 6. Divide the calculated μg poison per 1 ml by the mouse units per ml to obtain conversion factor (CF value) expressing μg poison equivalent to 1 mouse unit. Calculate the average of the individual CF values, and use this average value as a reference point to check routine assays. Individual CF values may vary significantly within a single laboratory if technics and mice are not rigidly controlled. This situation will require continued use of reference standard or secondary standard, depending on the volume of assay work performed.

1.3 Use of Standard with Routine Assay of Shellfish—

1.31 Check CF value periodically. If shellfish products are assayed less than once a week, determine CF value on each day assays are performed by injecting 5 mice with an appropriate dilution of the reference standard. If assays are made on several days during each week, only one check need be made each week on a dilution of the standard such that the median death time falls within 5–7 min. The CF value thus determined should check with the average CF value within ±20%. If it does not check within this range, complete group of 10 mice by adding 5 mice to the 5 mice already injected, and inject a second group of 10 mice with the same dilution of standard. Average the CF value determined for the second group with that of the first group. Take the resulting value as the new CF value. A variation of >20% represents a significant change in the response of mice to poison, or in the technic of assay. Changes of this type require a change in the CF value.

1.32 Repeated checks of CF value ordinarily produce consistent results within ±20%. If wider variations are found frequently, the possibility of uncontrolled or unrecognized variables in the method should be investigated before proceeding with routine assays.

1.4 Preparation of Sample—

1.41 *Clams, oysters and mussels:* Thoroughly clean outside of shellfish with fresh water. Open by cutting adductor muscles. Rinse inside with fresh water to remove sand or other foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anesthetics before opening shell, and do not cut or damage body of mollusk at this stage. Collect ca. 100–150 g of meats in a glazed dish. As soon as possible, transfer meats to a

No. 10 sieve without layering, and let drain for 5 min. Pick out pieces of shell, and discard drainings. Grind in household-type grinder with ⅛–¼" holes, or in a blender until homogeneous.

1.42 *Scallops:* Separate edible portion (adductor muscle) and apply test to this portion alone. Drain and grind as in 1.41.

1.43 *Canned shellfish:* Place entire contents of can (meat and liquid) in blender, and blend until homogeneous. For large cans, drain meat in large Büchner or sieve and collect all liquid. Determine weight of meat and volume of liquid. Recombine portion of each in proportionate quantities. Blend recombined portions in blender until homogeneous.

1.5 Extraction—

1.51 Weigh 100 g of well-mixed material into a tared beaker. Add 100 ml 0.1*N** HCl, stir thoroughly, and check pH (should be <4.0, preferably ca. 3.0). If necessary, adjust pH as indicated below. Heat the mixture, boil gently 5 min, and let cool to room temperature. Adjust cooled mixture to pH 2.0–4.0 (never >4.5) as determined by BDH Universal Indicator, phenol blue, Congo red paper, or pH meter. To lower pH, add 5*N* HCl dropwise with stirring; to raise pH, add 0.1*N* NaOH dropwise with constant stirring to prevent local alkalinization and consequent destruction of poison. Transfer mixture to graduated cylinder and dilute to 200 ml.

1.52 Return mixture to beaker, stir to homogeneity, and let settle until portion of supernatant is translucent and can be decanted free of solid particles large enough to block a 26-gauge hypodermic needle. If necessary, centrifuge mixture or supernatant for 5 min at 3,000 RPM or filter through paper. Only enough liquid to perform bioassay is necessary.

1.6 Mouse Test—

1.61 Inoculate each test mouse intraperitoneally with 1 ml of the acid extract. Note the time of inoculation and observe mice carefully for time of death as indicated by the last gasping breath. Record death time from stopwatch or clock with a sweep second hand. One mouse may be used for the initial determination, but 2 or 3 are preferred. If the death time, or the median death time of several mice is <5 min,

* Some laboratories advocate the use of varying normalities of HCl, depending on the species of shellfish to be extracted, *e.g.*, extracts of *Mya arenaria* prepared with 0.18*N* HCl seldom require pH adjustment.

make a dilution to obtain death times of 5–7 min. If the death time of 1 or 2 mice injected with undiluted sample is >7 min, a total of 3 or more mice for each dilution must be inoculated to establish the toxicity of the sample. If large dilutions are necessary, adjust the pH of the dilution by the dropwise addition of dilute HCl (0.1 or 0.101*N*) to pH 2.0–4.0, never >4.5. Inoculate 3 mice with a dilution that gives death times of 5–7 min.

1.7 Calculation of Toxicity—

1.71 Determine median death times of mice, including survivors, and (from Table 6) determine corresponding number of mouse units. If test animals weigh <19 g or >21 g, make correction for each mouse by multiplying mouse units corresponding to death time for that mouse by the weight correction factor for that mouse (from Table 7) and then determine the median mouse unit for the group. (Consider the death time of survivors as >60 min or equivalent to <0.875 mouse units in calculating the median.) Convert mouse units to μg poison per ml by multiplying by the CF value.

1.72 μg poison per 100 g meat = (μg per ml × dilution factor) × 200.

1.73 Consider any value >80 μg per 100 g as hazardous and unsafe for human consumption.

B. METHOD FOR THE BIOASSAY OF *Gymnodinium breve* TOXIN(S) IN SHELLFISH *

2.0 Introduction

Toxicologic evidence strongly incriminated the toxigenic marine dinoflagellate *Gymnodinium breve* as being the source of a toxic substance composed of a fast-acting and a slow-acting toxic component. This toxin(s) has been detected in edible oysters and clams exposed to *G. breve* "red tides" along the Florida west coast. The bioassay of *G. breve* toxin(s) in shellfish was originally developed by McFarren *et al.* (1965) using toxic shellfish collected during a *G. breve* "red tide" in the spring of 1963. At that time, several persons became mildly ill after eating shellfish that had been exposed to the *G. breve* "red tide" conditions.

The bioassay is based on the relationship of dose to the death time

* Taken in toto from Cummins and Hill. See bibliography at end of Part V.

of mice injected intraperitoneally with crude toxic residues extracted from shellfish with diethyl ether. The two toxic components of the residue have not been purified; therefore, relative toxicity is expressed in terms of mouse units. One mouse unit is that amount of crude toxic residue that, on the average, will kill 50% of the test animals (20 g mice) in 930 min.

2.1 Apparatus—

a) Electric blender
b) Analytical balance
c) Centrifuge (explosion proof) and 250-ml centrifuge cups
d) Steam bath
e) Chemical hood
f) 1000-ml separatory funnel
g) 400-ml and 100-ml beakers
h) 1-ml glass syringes; 23 gauge needles
i) Watch or timer

2.2 Reagents—

a) Hydrochloric acid, conc. (12*N*)
b) Sodium chloride (reagent grade)
c) Cottonseed oil, *e.g.*, Wesson® oil
d) Diethyl ether (purified for fat extraction)

2.3 Test Animals—

2.31 *Mice:* Healthy mice, weighing 20 g ± 1 g from stock colony used for routine assay. Lighter or heavier mice can be used; however, do not use mice weighing <10 g or >25 g. Do not reuse mice.

2.4 Preparation of Sample—

2.41 Clean and shuck shellfish; drain off shell liquor; homogenize shellfish meats in blender.

2.5 Extraction—

2.51 Weigh 100 g of homogenate into a tared 400-ml beaker. Add 1 ml conc HCl and 5 g of NaCl to the shellfish homogenate. Heat to boiling (stirring frequently) and cook for 5 min. Cool.

2.52 Transfer the cooked homogenate to a 250-ml centrifuge cup. Add 100 ml of diethyl ether, stopper and shake vigorously for 5 min

(venting frequently). Centrifuge at 2000 RPM for 10 to 15 min. Transfer the ether extract to a 1000-ml separatory funnel.

2.53 Extract the shellfish homogenate 3 additional times with 100 ml of diethyl ether, transferring each ether extract to the 1000-ml separatory funnel. Drain off any emulsion from the combined ether extracts. Transfer the ether extract to a 400-ml beaker.

2.54 Evaporate most of the ether on a steam bath under a chemical hood. Quantitatively transfer the remaining ether extract to a tared 100-ml beaker. Evaporate ether. (NOTE: Ensure that no ether remains after the final evaporation step.)

2.6 Mouse Assay—

2.61 Accurately weigh viscous oil residue remaining. On the assumption that the density of the residue is the same as cottonseed oil, *e.g.*, Wesson® oil (0.917 g $\simeq$ 1 ml), gravimetrically bring the residue up to 9.17 g (10 ml). Since the resulting solution of the toxin in cottonseed oil can now be readily handled, any subsequent dilution can be made on a volume basis. CAUTION: Mix well before injection.

2.62 Inject 1 ml of the residue-cottonseed oil mixture intraperitoneally into each of two mice weighing approximately 20 g. Record the time of injection. Observe the mice for 24 hr. Determine the death time as the time elapsed from the time of injection to the last gasping breath of a mouse. Record the death time of each mouse surviving a given period of *continuous* observation as greater than (>) the duration of that *continuous* observation period (in minutes). For example, assign a death time of >930 min to those mice that survive a 930-min (15.5 hr) *continuous* observation period, but die (unobserved) or survive the required overall 24-hr observation period.

2.63 If the first 2 mice do not die within 360 min, inject 3 more mice and observe the 5 mice for 24 hr.

2.64 If the first 2 mice die in less than 110 min, a dilution is made with cottonseed oil and each of 2 mice injected. [NOTE: Recommended dilution 1:1.25, *e.g.*, add 2 ml of cottonseed oil to the remaining 8 ml of the residue-cottonseed oil mixture; if required, repeat the identical dilution-injection sequence.] When a dilution is found that causes the mice to die between 110 and 360 min after injection, 3 more mice are each injected as previously described and observed for 24 hr. [NOTE: The duration of the *continuous* observation period will dictate the lower limit of the assay's sensitivity. For example, 10 mouse units/100 g

shellfish meats is the least amount of toxin that usually can be quantified using 20-gram mice. This level of sensitivity can only be achieved by continuously observing the mice for 930 min (15.5 hr). Under some circumstances, 20 mouse units/100 g shellfish meats will be the lower limit of sensitivity using 20-gram mice, since for the sake of accuracy and expediency, the mice will not be continuously observed longer than 360 min (6 hr).]

2.7 Calculation of Toxicity—

2.71 Determine the number of mouse units per ml corresponding to the death time of each of the 5 mice injected (Table 7). Assign an indeterminate *less than* (<) to those mouse units corrresponding to indeterminate death times.

2.72 Adjust the mouse units for differences in the weight of each mouse in the following manner: Multiply the number of mouse units per ml by the weight correction factor for that mouse (Table 8), *e.g.*, the number of mouse units per ml corresponding to a >930-min death time of a 22-gram mouse would be (<1.0 mouse unit/ml) × (1.12 weight correction factor for 22-gram mouse) = <1.12 mouse unit per ml.

2.73 Determine the *mean* number of mouse units per ml if 100% mortality is observed and death times are determinate. Determine the *median* number of mouse units per ml if 100% mortality is observed and death times are indeterminate. Determine the *median* number of mouse units per ml if *less than* 100% mortality is observed (include survivors).

2.74 Calculate the total number of mouse units per 100 g shellfish meats in the following manner: Multiply the *mean* or *median* number of mouse units per ml injected by the dilution factor (if applicable) and by 10 (residue originally made up to 10 ml with cottonseed oil), *i.e.*, mouse units per 100 g shellfish meats = (mouse units per ml) × (dilution factor) × (10).

2.8 Reporting Procedure—

2.81 When relative toxicity is determinate, *REPORT:* number of mouse units/100 g shellfish meats. When relative toxicity is indeterminate (*e.g.*, <10 mouse units/100 g shellfish meats), *REPORT:* (i) (*number of mice*) mice died in 24 hr; toxin detectable, <10 mouse units/100 g shellfish meats; or (ii) no mice died in 24 hr; toxin not detectable, <10 mouse units/100 g shellfish meats.

2.82 Consider any detectable level of toxin per 100 g shellfish meats as potentially unsafe for human consumption.

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APPENDIX

Appendix

Differentiation of Coliform and Fecal Coliform Organisms

The differentiation of coliform and fecal coliform organisms is included primarily to allow identification of individual species isolated by the Completed Tests described in Part III of the manual. Normally the routine tests associated with sanitary surveys of shellfish-growing waters or commercial processing of shellfish would not necessitate the use of these differential tests. There may be an occasion when the knowledge of species present may assist in the determination of whether a public health hazard exists.

The reaction classifications have not been listed for each species. References for these combined reactions may be found in Edwards and Ewing (1962) or Kaufman (1966).

1.0 Procedure

The results of the differential tests are made inutile by failure to obtain pure cultures before inoculation of the differential media. Any doubt that the culture consists of only one species should be eliminated by replating on Levine's eosin methylene blue agar or endo agar.

Inoculate each of the media listed below with cultures taken from the agar slant or lactose broth tube at the conclusion of the Completed Test discussed in Part III. The inoculation should not be too heavy and an inoculating needle should be used. Special precautions should be made to transfer a minimum amount of inoculum into the citrate medium.

a) One tube of tryptone broth
b) One tube of Koser's citrate medium or Simmons' citrate agar
c) Two tubes of MR-VP medium.

2.0 Differential Tests

2.1 Indole test—Incubate the inoculated tryptone broth at 35 C $\pm$ 0.5 C for 24$\pm$2 hr. Add 0.3 ml Indole reagent to 5 ml of the tryp-

tone broth culture. Allow to stand 10 min. The appearance of a dark red layer on the surface indicates a *Positive Indole test.*

2.2 Methyl Red Test—Incubate the MR-VP medium at 35 C ± 0.5 C for 5 days. Add 5 drops of methyl red indicator solution to 5 ml of the MR-VP broth culture.

A distinct red color is a *Methyl Red Positive.* A *Methyl Red Negative* results in a distinct yellow color, and intermediate colors are questionable.

2.3 Voges-Proskauer Test—Incubate the MR-VP medium at 35 C ± 0.5 C for 48 hr. Add 0.6 ml of a-Naphthol solution to 1 ml of the MR-VP broth culture. Shake, then add 0.2 ml 40% KOH. Allow to stand 2–4 hr. Development of a red color constitutes a *Positive Test.*

2.4 Citrate Utilization Test—Incubate the Koser citrate medium at 35 C ± 0.5 C for 72 hr. Turbidity in the Koser's citrate tube, indicating growth, represents a *Positive Test.*

Or, incubate the Simmons' medium at 35 C ± 0.5 C for 48 hr. Growth on the medium with change in color from green to deep blue constitutes a *Positive Test.*

Figure 1—Presumptive Test

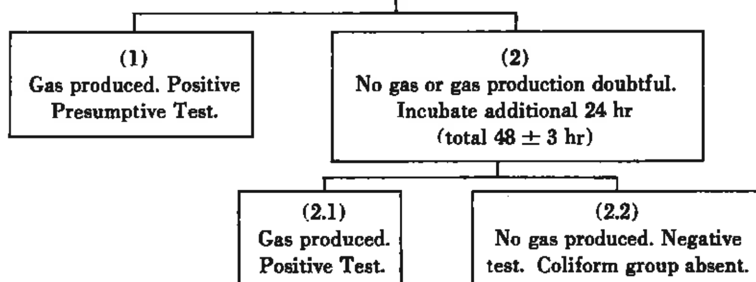


Figure 2—Confirmed Test

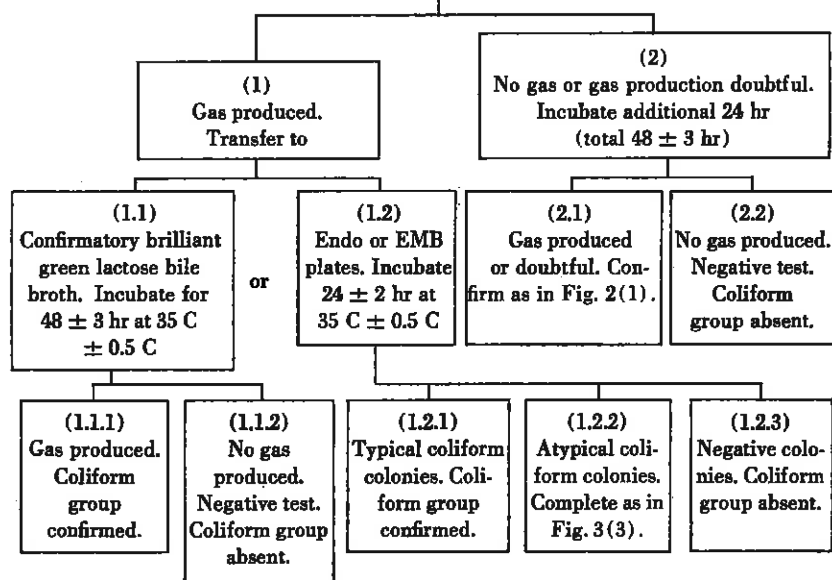
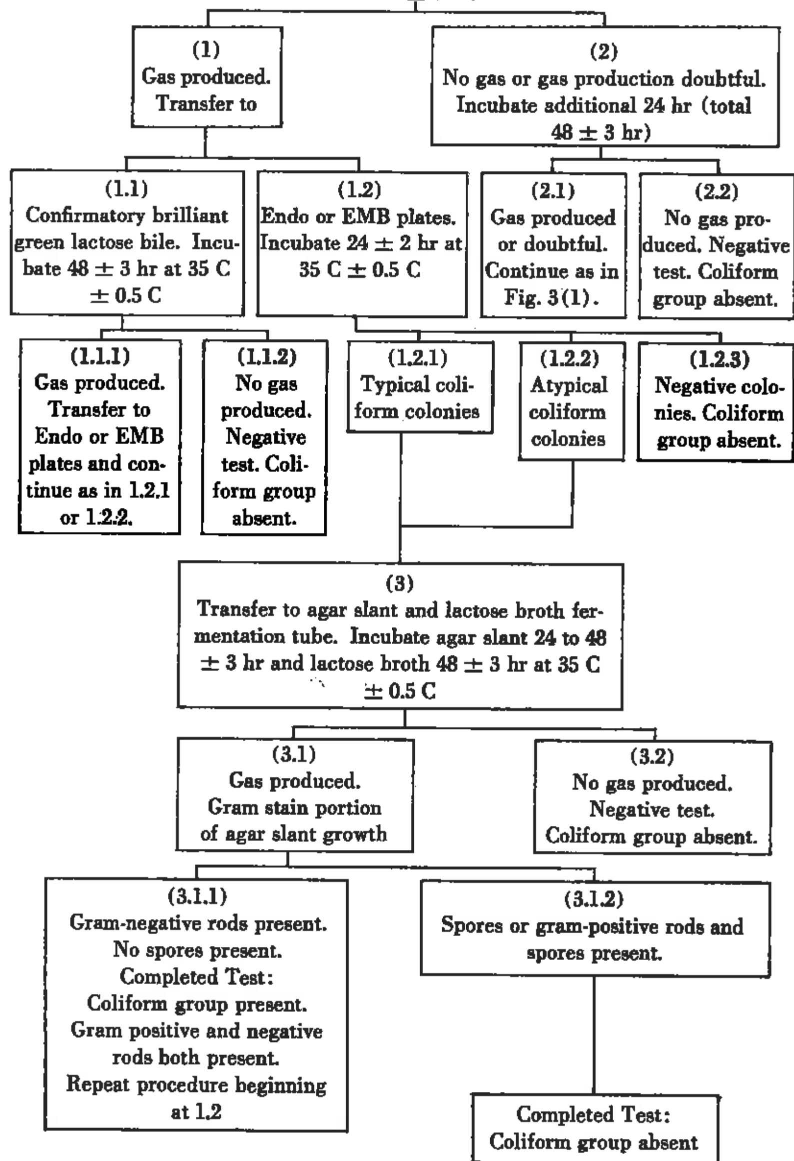


Figure 3—Completed Test



TABLES

Table 1—Differences to convert hydrometer readings at any temperature centrigrade to density at 15 C (59F)

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

SOURCE: Zerbe, W. B. and C. B. Taylor (1953).

(continued)

Table 1—(*continued*)

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					

(*continued*)

Table 1—(*continued*)

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													

(*continued*)

Table 1—(*concluded*)

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 2—Corresponding densities and salinities *

Density	Salinity	Density	Salinity	Density	Salinity	Density	Salinity
0.9991	0.0	1.0026	4.5	1.0061	9.0	1.0096	13.6
0.9992	0.0	1.0027	4.6	1.0062	9.2	1.0097	13.7
0.9993	0.2	1.0028	4.7	1.0063	9.3	1.0098	13.9
0.9994	0.3	1.0029	4.8	1.0064	9.4	1.0099	14.0
0.9995	0.4	1.0030	5.0	1.0065	9.6	1.0100	14.1
0.9996	0.6	1.0031	5.1	1.0066	9.7	1.0101	14.2
0.9997	0.7	1.0032	5.2	1.0067	9.8	1.0102	14.4
0.9998	0.8	1.0033	5.4	1.0068	9.9	1.0103	14.5
0.9999	0.9	1.0034	5.5	1.0069	10.1	1.0104	14.6
1.0000	1.1	1.0035	5.6	1.0070	10.2	1.0105	14.8
1.0001	1.2	1.0036	5.8	1.0071	10.3	1.0106	14.9
1.0002	1.3	1.0037	5.9	1.0072	10.5	1.0107	15.0
1.0003	1.5	1.0038	6.0	1.0073	10.6	1.0108	15.2
1.0004	1.6	1.0039	6.2	1.0074	10.7	1.0109	15.3
1.0005	1.7	1.0040	6.3	1.0075	10.8	1.0110	15.4
1.0006	1.9	1.0041	6.4	1.0076	11.0	1.0111	15.6
1.0007	2.0	1.0042	6.6	1.0077	11.1	1.0112	15.7
1.0008	2.1	1.0043	6.7	1.0078	11.2	1.0113	15.8
1.0009	2.2	1.0044	6.8	1.0079	11.4	1.0114	16.0
1.0010	2.4	1.0045	6.9	1.0080	11.5	1.0115	16.1
1.0011	2.5	1.0046	7.1	1.0081	11.6	1.0116	16.2
1.0012	2.6	1.0047	7.2	1.0082	11.8	1.0117	16.3
1.0013	2.8	1.0048	7.3	1.0083	11.9	1.0118	16.5
1.0014	2.9	1.0049	7.5	1.0084	12.0	1.0119	16.6
1.0015	3.0	1.0050	7.6	1.0085	12.2	1.0120	16.7
1.0016	3.2	1.0051	7.7	1.0086	12.3	1.0121	16.9
1.0017	3.3	1.0052	7.9	1.0087	12.4	1.0122	17.0
1.0018	3.4	1.0053	8.0	1.0088	12.6	1.0123	17.1
1.0019	3.5	1.0054	8.1	1.0089	12.7	1.0124	17.3
1.0020	3.7	1.0055	8.2	1.0090	12.8	1.0125	17.4
1.0021	3.8	1.0056	8.4	1.0091	12.9	1.0126	17.5
1.0022	3.9	1.0057	8.5	1.0092	13.1	1.0127	17.7
1.0023	4.1	1.0058	8.6	1.0093	13.2	1.0128	17.8
1.0024	4.2	1.0059	8.8	1.0094	13.3	1.0129	17.9
1.0025	4.3	1.0060	8.9	1.0095	13.5	1.0130	18.0

* Density at 15 C. Salinity in parts per 1,000.

(continued)

Table 2—(*continued*)

Density	Salinity	Density	Salinity	Density	Salinity	Density	Salinity
1.0131	18.2	1.0166	22.7	1.0201	27.3	1.0236	31.9
1.0132	18.3	1.0167	22.9	1.0202	27.5	1.0237	32.0
1.0133	18.4	1.0168	23.0	1.0203	27.6	1.0238	32.1
1.0134	18.6	1.0169	23.1	1.0204	27.7	1.0239	32.3
1.0135	18.7	1.0170	23.3	1.0205	27.8	1.0240	32.4
1.0136	18.8	1.0171	23.4	1.0206	28.0	1.0241	32.5
1.0137	19.0	1.0172	23.5	1.0207	28.1	1.0242	32.7
1.0138	19.1	1.0173	23.7	1.0208	28.2	1.0243	32.8
1.0139	19.2	1.0174	23.8	1.0209	28.4	1.0244	32.9
1.0140	19.3	1.0175	23.9	1.0210	28.5	1.0245	33.1
1.0141	19.5	1.0176	24.1	1.0211	28.6	1.0246	33.2
1.0142	19.6	1.0177	24.2	1.0212	28.8	1.0247	33.3
1.0143	19.7	1.0178	24.3	1.0213	28.9	1.0248	33.5
1.0144	19.9	1.0179	24.4	1.0214	29.0	1.0249	33.6
1.0145	20.0	1.0180	24.6	1.0215	29.1	1.0250	33.7
1.0146	20.1	1.0181	24.7	1.0216	29.3	1.0251	33.8
1.0147	20.3	1.0182	24.8	1.0217	29.4	1.0252	34.0
1.0148	20.4	1.0183	25.0	1.0218	29.5	1.0253	34.1
1.0149	20.5	1.0184	25.1	1.0219	29.7	1.0254	34.2
1.0150	20.6	1.0185	25.2	1.0220	29.8	1.0255	34.4
1.0151	20.8	1.0186	25.4	1.0221	29.9	1.0256	34.5
1.0152	20.9	1.0187	25.5	1.0222	30.1	1.0257	34.6
1.0153	21.0	1.0188	25.6	1.0223	30.2	1.0258	34.8
1.0154	21.2	1.0189	25.8	1.0224	30.3	1.0259	34.9
1.0155	21.3	1.0190	25.9	1.0225	30.4	1.0260	35.0
1.0156	21.4	1.0191	26.0	1.0226	30.6	1.0261	35.1
1.0157	21.6	1.0192	26.1	1.0227	30.7	1.0262	35.3
1.0158	21.7	1.0193	26.3	1.0228	30.8	1.0263	35.4
1.0159	21.8	1.0194	26.4	1.0229	31.0	1.0264	35.5
1.0160	22.0	1.0195	26.5	1.0230	31.1	1.0265	35.7
1.0161	22.1	1.0196	26.7	1.0231	31.2	1.0266	35.8
1.0162	22.2	1.0197	26.8	1.0232	31.4	1.0267	35.9
1.0163	22.4	1.0198	26.9	1.0233	31.5	1.0268	36.0
1.0164	22.5	1.0199	27.1	1.0234	31.6	1.0269	36.2
1.0165	22.6	1.0200	27.2	1.0235	31.8	1.0270	36.3

(continued)

Table 2—*continued*

Density	Salinity	Density	Salinity	Density	Salinity	Density	Salinity
1.0271	36.4	1.0286	38.4	1.0301	40.3	1.0316	42.3
1.0272	36.6	1.0287	38.5	1.0302	40.4	1.0317	42.4
1.0273	36.7	1.0288	38.6	1.0303	40.6	1.0318	42.5
1.0274	36.8	1.0289	38.8	1.0304	40.7	1.0319	42.7
1.0275	37.0	1.0290	38.9	1.0305	40.8	1.0320	42.8
1.0276	37.1	1.0291	39.0	1.0306	41.0		
1.0277	37.2	1.0292	39.2	1.0307	41.1		
1.0278	37.3	1.0293	39.3	1.0308	41.2		
1.0279	37.5	1.0294	39.4	1.0309	41.4		
1.0280	37.6	1.0295	39.6	1.0310	41.5		
1.0281	37.7	1.0296	39.7	1.0311	41.6		
1.0282	37.9	1.0297	39.8	1.0312	41.7		
1.0283	38.0	1.0298	39.9	1.0313	41.9		
1.0284	38.1	1.0299	40.1	1.0314	42.0		
1.0285	38.2	1.0300	40.2	1.0315	42.1		

Table 3—Conversion of chlorosity C_0 at 20_0C to cholorinity Cl ‰

Calculated Chlorosity	Subtract for Chlorinity	Calculated Chlorosity	Subtract for Chlorinity
9.95—10.35	—0.12	15.88—16.17	—0.31
10.36—10.75	—0.13	16.18—16.32	—0.32
10.76—11.15	—0.14	16.33—16.62	—0.33
11.16—11.46	—0.15	16.63—16.82	—0.34
11.47—11.76	—0.16	16.83—17.11	—0.35
11.77—12.06	—0.17	17.12—17.32	—0.36
12.07—12.46	—0.18	17.33—17.57	—0.37
12.47—12.86	—0.19	17.58—17.82	—0.38
12.87—13.07	—0.20	17.83—18.02	—0.39
13.08—13.37	—0.21	18.03—18.27	—0.40
13.38—13.67	—0.22	18.28—18.47	—0.41
13.68—14.02	—0.23	18.48—18.67	—0.42
14.03—14.27	—0.24	18.68—18.97	—0.43
14.28—14.52	—0.25	18.98—19.17	—0.44
14.53—14.82	—0.26	19.18—19.32	—0.45
14.83—15.09	—0.27	19.33—19.52	—0.46
15.10—15.37	—0.28	19.53—19.77	—0.47
15.38—15.68	—0.29	19.78—19.97	—0.48
15.69—15.87	—0.30		

Table 4—Conversion of chlorosity to salinity

Conversion of 20 C chlorosity, $Cl/\text{liter}_{(20)}$, to salinity, $S‰$, from the expression

$$S‰ = 0.03 + [1.8050 \times Cl/\text{liter}_{(20)} \times 1/\rho_{(20)}]$$

where $\rho_{(20)}$ is the density of sea water at chlorosity $Cl/\text{liter}_{(20)}$.

$Cl/\text{liter}_{(20)}$	$S‰$	$Cl/\text{liter}_{(20)}$	$S‰$	$Cl/\text{liter}_{(20)}$	$S‰$	$Cl/\text{liter}_{(20)}$	$S‰$
2.00	3.64	.35	.27	2.70	4.89	.05	.52
.01	.66	.36	.29	.71	.91	.06	.54
.02	.68	.37	.30	.72	.93	.07	.56
.03	.69	.38	.32	.73	.95	.08	.57
.04	.71	.39	.34	.74	.97	.09	.59
.05	.73	2.40	4.36	.75	4.98	3.10	5.61
.06	.75	.41	.37	.76	5.00	.11	.63
.07	.77	.42	.39	.77	.02	.12	.65
.08	.78	.43	.41	.78	.04	.13	.66
.09	.80	.44	.43	.79	.06	.14	.68
2.10	3.82	.45	.45	2.80	5.07	.15	.70
.11	.84	.46	.46	.81	.09	.16	.72
.12	.86	.47	.48	.82	.11	.17	.74
.13	.87	.48	.50	.83	.13	.18	.75
.14	.89	.49	.52	.84	.14	.19	.77
.15	.91	2.50	4.54	.85	.16	3.20	5.79
.16	.93	.51	.55	.86	.18	.21	.81
.17	.95	.52	.57	.87	.20	.22	.82
.18	.96	.53	.59	.88	.22	.23	.84
.19	3.98	.54	.61	.89	.24	.24	.86
2.20	4.00	.55	.63	2.90	5.25	.25	.88
.21	.02	.56	.64	.91	.27	.26	.90
.22	.03	.57	.66	.92	.29	.27	.91
.23	.05	.58	.68	.93	.31	.28	.93
.24	.07	.59	.70	.94	.32	.29	.95
.25	.09	2.60	4.71	.95	.34	3.30	5.97
.26	.11	.61	.73	.96	.36	.31	5.99
.27	.12	.62	.75	.97	.38	.32	6.00
.28	.14	.63	.77	.98	.40	.33	.02
.29	.16	.64	.79	.99	.41	.34	.04
2.30	4.18	.65	.80	3.00	5.43	.35	.06
.31	.20	.66	.82	.01	.45	.36	.08
.32	.21	.67	.84	.02	.47	.37	.09
.33	.23	.68	.86	.03	.48	.38	.11
.34	.25	.69	.88	.04	.50	.39	.13

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
3.40	6.15	3.80	6.86	4.20	7.58	4.60	8.29
.41	.16	.81	.88	.21	.60	.61	.31
.42	.18	.82	.90	.22	.61	.62	.33
.43	.20	.83	.92	.23	.63	.63	.35
.44	.22	.84	.93	.24	.65	.64	.36
.45	.24	.85	.95	.25	.67	.65	.38
.46	.25	.86	.97	.26	.68	.66	.40
.47	.27	.87	6.98	.27	.70	.67	.42
.48	.29	.88	7.01	.28	.72	.68	.44
.49	.31	.89	.02	.29	.74	.69	.45
3.50	6.33	3.90	7.04	4.30	7.76	4.70	8.47
.51	.34	.91	.06	.31	.77	.71	.49
.52	.36	.92	.08	.32	.79	.72	.51
.53	.38	.93	.10	.33	.81	.73	.52
.54	.40	.94	.11	.34	.83	.74	.54
.55	.42	.95	.13	.35	.85	.75	.56
.56	.43	.96	.15	.36	.86	.76	.58
.57	.45	.97	.17	.37	.88	.77	.60
.58	.47	.98	.18	.38	.90	.78	.61
.59	.49	.99	.20	.39	.92	.79	.63
3.60	6.50	4.00	7.22	4.40	7.93	4.80	8.65
.61	.52	.01	.24	.41	.95	.81	.67
.62	.54	.02	.26	.42	.97	.82	.69
.63	.56	.03	.27	.43	7.99	.83	.70
.64	.58	.04	.29	.44	8.01	.84	.72
.65	.59	.05	.31	.45	.02	.85	.74
.66	.61	.06	.33	.46	.04	.86	.76
.67	.63	.07	.35	.47	.06	.87	.77
.68	.65	.08	.36	.48	.08	.88	.79
.69	.67	.09	.38	.49	.10	.89	.81
3.70	6.68	4.10	7.40	4.50	8.11	4.90	8.83
.71	.70	.11	.42	.51	.13	.91	.85
.72	.72	.12	.43	.52	.15	.92	.86
.73	.74	.13	.45	.53	.17	.93	.88
.74	.76	.14	.47	.54	.18	.94	.90
.75	.77	.15	.49	.55	.20	.95	.92
.76	.79	.16	.51	.56	.22	.96	.94
.77	.81	.17	.52	.57	.24	.97	.95
.78	.83	.18	.54	.58	.26	.98	.97
.79	.84	.19	.56	.59	.27	.99	.99

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
5.00	9.01	5.40	9.72	5.80	10.43	6.20	11.14
.01	.02	.41	.74	.81	.45	.21	.16
.02	.04	.42	.75	.82	.47	.22	.18
.03	.06	.43	.77	.83	.48	.23	.20
.04	.08	.44	.79	.84	.50	.24	.21
.05	.10	.45	.81	.85	.52	.25	.23
.06	.11	.46	.83	.86	.54	.26	.25
.07	.13	.47	.84	.87	.56	.27	.27
.08	.15	.48	.86	.88	.57	.28	.28
.09	.17	.49	.88	.89	.59	.29	.30
5.10	9.18	5.50	9.90	5.90	10.61	6.30	11.32
.11	.20	.51	.91	.91	.63	.31	.34
.12	.22	.52	.93	.92	.64	.32	.36
.13	.24	.53	.95	.93	.66	.33	.37
.14	.26	.54	.97	.94	.68	.34	.39
.15	.27	.55	.99	.95	.70	.35	.41
.16	.29	.56	10.00	.96	.72	.36	.43
.17	.31	.57	.02	.97	.73	.37	.44
.18	.33	.58	.04	.98	.75	.38	.46
.19	.34	.59	.06	.99	.77	.39	.48
5.20	9.36	5.60	10.07	6.00	10.79	6.40	11.50
.21	.38	.61	.09	.01	.81	.41	.52
.22	.40	.62	.11	.02	.82	.42	.53
.23	.42	.63	.13	.03	.84	.43	.55
.24	.43	.64	.15	.04	.86	.44	.57
.25	.45	.65	.16	.05	.88	.45	.59
.26	.47	.66	.18	.06	.89	.46	.60
.27	.49	.67	.20	.07	.91	.47	.62
.28	.50	.68	.22	.08	.93	.48	.64
.29	.52	.69	.24	.09	.95	.49	.66
5.30	9.54	5.70	10.25	6.10	10.97	6.50	11.68
.31	.56	.71	.27	.11	10.98	.51	.69
.32	.58	.72	.29	.12	11.00	.52	.71
.33	.59	.73	.31	.13	.02	.53	.73
.34	.61	.74	.32	.14	.04	.54	.75
.35	.63	.75	.34	.15	.05	.55	.76
.36	.65	.76	.36	.16	.07	.56	.78
.37	.67	.77	.38	.17	.09	.57	.80
.38	.68	.78	.40	.18	.11	.58	.82
.39	.70	.79	.41	.19	.12	.59	.84

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
6.60	11.85	7.00	12.56	7.40	13.27	7.80	13.98
.61	.87	.01	.58	.41	.29	.81	14.00
.62	.89	.02	.60	.42	.31	.82	.02
.63	.91	.03	.62	.43	.33	.83	.03
.64	.92	.04	.63	.44	.34	.84	.05
.65	.94	.05	.65	.45	.36	.85	.07
.66	.96	.06	.67	.46	.38	.86	.09
.67	11.98	.07	.69	.47	.40	.87	.11
.68	12.00	.08	.71	.48	.41	.88	.12
.69	.01	.09	.72	.49	.43	.89	.14
6.70	12.03	7.10	12.74	7.50	13.45	7.90	14.16
.71	.05	.11	.76	.51	.47	.91	.18
.72	.07	.12	.78	.52	.49	.92	.19
.73	.08	.13	.79	.53	.50	.93	.21
.74	.10	.14	.81	.54	.52	.94	.23
.75	.12	.15	.83	.55	.54	.95	.25
.76	.14	.16	.85	.56	.56	.96	.27
.77	.16	.17	.86	.57	.57	.97	.28
.78	.17	.18	.88	.58	.59	.98	.30
.79	.19	.19	.90	.59	.61	.99	.32
6.80	12.21	7.20	12.92	7.60	13.63	8.00	14.34
.81	.23	.21	.94	.61	.65	.01	.35
.82	.24	.22	.95	.62	.66	.02	.37
.83	.26	.23	.97	.63	.68	.03	.39
.84	.28	.24	12.99	.64	.70	.04	.41
.85	.30	.25	13.01	.65	.72	.05	.42
.86	.31	.26	.02	.66	.73	.06	.44
.87	.33	.27	.04	.67	.75	.07	.46
.88	.35	.28	.06	.68	.77	.08	.48
.89	.37	.29	.08	.69	.79	.09	.50
6.90	12.39	7.30	13.10	7.70	13.80	8.10	14.51
.91	.40	.31	.11	.71	.82	.11	.53
.92	.42	.32	.13	.72	.84	.12	.55
.93	.44	.33	.15	.73	.86	.13	.57
.94	.46	.34	.17	.74	.88	.14	.58
.95	.47	.35	.18	.75	.89	.15	.60
.96	.49	.36	.20	.76	.91	.16	.62
.97	.51	.37	.22	.77	.93	.17	.64
.98	.53	.38	.24	.78	.95	.18	.65
.99	.55	.39	.25	.79	.96	.19	.67

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
8.20	14.69	8.60	15.40	9.00	16.10	9.40	16.81
.21	.71	.61	.41	.01	.12	.41	.82
.22	.72	.62	.43	.02	.14	.42	.84
.23	.74	.63	.45	.03	.16	.43	.86
.24	.76	.64	.47	.04	.17	.44	.88
.25	.78	.65	.48	.05	.19	.45	.89
.26	.80	.66	.50	.06	.21	.46	.91
.27	.81	.67	.52	.07	.23	.47	.93
.28	.83	.68	.54	.08	.24	.48	.95
.29	.85	.69	.56	.09	.26	.49	.96
8.30	14.87	8.70	15.57	9.10	16.28	9.50	16.98
.31	.88	.71	.59	.11	.30	.51	17.00
.32	.90	.72	.61	.12	.31	.52	.02
.33	.92	.73	.63	.13	.33	.53	.03
.34	.94	.74	.64	.14	.35	.54	.05
.35	.95	.75	.66	.15	.37	.55	.07
.36	.97	.76	.68	.16	.38	.56	.09
.37	14.99	.77	.70	.17	.40	.57	.11
.38	15.01	.78	.71	.18	.42	.58	.12
.39	.03	.79	.73	.19	.44	.59	.14
8..40	15.04	8.80	15.75	9.20	16.45	9.60	17.16
.41	.06	.81	.77	.21	.47	.61	.18
.42	.08	.82	.79	.22	.49	.62	.19
.43	.10	.83	.80	.23	.51	.63	.21
.44	.11	.84	.82	.24	.53	.64	.23
.45	.13	.85	.84	.25	.54	.65	.25
.46	.15	.86	.86	.26	.56	.66	.26
.47	.17	.87	.87	.27	.58	.67	.28
.48	.18	.88	.89	.28	.60	.68	.30
.49	.20	.89	.91	.29	.61	.69	.32
8.50	15.22	8.90	15.93	9.30	16.63	9.70	17.33
.51	.24	.91	.94	.31	.65	.71	.35
.52	.25	.92	.96	.32	.67	.72	.37
.53	.27	.93	15.98	.33	.68	.73	.39
.54	.29	.94	16.00	.34	.70	.74	.40
.55	.31	.95	.01	.35	.72	.75	.42
.56	.33	.96	.03	.36	.74	.76	.44
.57	.34	.97	.05	.37	.75	.77	.46
.58	.36	.98	.07	.38	.77	.78	.47
.59	.38	.99	.09	.39	.79	.79	.49

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
9.80	17.51	10.20	18.22	10.60	18.92	11.00	19.62
.81	.53	.21	.23	.61	.94	.01	.64
.82	.54	.22	.25	.62	.96	.02	.66
.83	.56	.23	.27	.63	.97	.03	.68
.84	.58	.24	.29	.64	18.99	.04	.69
.85	.60	.25	.30	.65	19.01	.05	.71
.86	.62	.26	.32	.66	.03	.06	.73
.87	.63	.27	.34	.67	.04	.07	.75
.88	.65	.28	.36	.68	.06	.08	.76
.89	.67	.29	.38	.69	.08	.09	.78
9.90	17.69	10.30	18.39	10.70	19.10	11.10	19.80
.91	.70	.31	.41	.71	.11	.11	.82
.92	.72	.32	.43	.72	.13	.12	.83
.93	.74	.33	.45	.73	.15	.13	.85
.94	.76	.34	.46	.74	.17	.14	.87
.95	.77	.35	.48	.75	.18	.15	.89
.96	.79	.36	.50	.76	.20	.16	.90
.97	.81	.37	.52	.77	.22	.17	.92
.98	.83	.38	.53	.78	.24	.18	.94
.99	.85	.39	.55	.79	.25	.19	.96
10.00	17.87	10.40	18.57	10.80	19.27	11.20	19.97
.01	.88	.41	.59	.81	.29	.21	19.99
.02	.90	.42	.60	.82	.31	.22	20.01
.03	.92	.43	.62	.83	.32	.23	.03
.04	.94	.44	.64	.84	.34	.24	.04
.05	.95	.45	.66	.85	.36	.25	.06
.06	.97	.46	.67	.86	.38	.26	.08
.07	17.99	.47	.69	.87	.39	.27	.10
.08	18.01	.48	.71	.88	.41	.28	.11
.09	.02	.49	.73	.89	.43	.29	.13
10.10	18.04	10.50	18.74	10.90	19.45	11.30	20.15
.11	.06	.51	.76	.91	.47	.31	.17
.12	.08	.52	.78	.92	.48	.32	.18
.13	.09	.53	.80	.93	.50	.33	.20
.14	.11	.54	.81	.94	.52	.34	.22
.15	.13	.55	.83	.95	.54	.35	.24
.16	.15	.56	.85	.96	.55	.36	.26
.17	.16	.77	.87	.97	.57	.37	.27
.18	.18	.58	.88	.98	.59	.38	.29
.19	.20	.59	.90	.99	.61	.39	.31

Table 4—(*continued*)

Cl/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰	*Cl*/liter$_{(20)}$	*S*‰
11.40	20.33	11.80	21.03	12.20	21.73	12.60	22.43
.41	.34	.81	.04	.21	.75	.61	.44
.42	.36	.82	.06	.22	.76	.62	.46
.43	.38	.83	.08	.23	.78	.63	.48
.44	.40	.84	.10	.24	.80	.64	.50
.45	.41	.85	.11	.25	.82	.65	.51
.46	.43	.86	.13	.26	.83	.66	.53
.47	.45	.87	.15	.27	.85	.67	.55
.48	.47	.88	.17	.28	.87	.68	.57
.49	.48	.89	.18	.29	.89	.69	.58
11.50	20.50	11.90	21.20	12.30	21.90	12.70	22.60
.51	.52	.91	.22	.31	.92	.71	.62
.52	.54	.92	.24	.32	.94	.72	.64
.53	.55	.93	.26	.33	.96	.73	.65
.54	.57	.94	.27	.34	.97	.74	.67
.55	.59	.95	.29	.35	21.99	.75	.69
.56	.61	.96	.31	.36	22.01	.76	.71
.57	.62	.97	.33	.37	.03	.77	.72
.58	.64	.98	.34	.38	.04	.78	.74
.59	66	.99	.36	.39	.06	.79	.76
11.60	20.68	12.00	21.38	12.40	22.08	12.80	22.78
.61	.69	.01	.40	.41	.09	.81	.79
.62	.71	.02	.41	.42	.11	.82	.81
.63	.73	.03	.43	.43	.13	.83	.83
.64	.75	.04	.45	.44	.15	.84	.85
.65	.76	.05	.47	.45	.16	.85	.86
.66	.78	.06	.48	.46	.18	.86	.88
.67	.80	.07	.50	.47	.20	.87	.90
.68	.82	.08	.52	.48	.22	.88	.92
.69	.83	.09	.54	.49	.23	.89	.93
11.70	20.85	12.10	21.55	12.50	22.25	12.90	22.95
.71	.87	.11	.57	.51	.27	.91	.97
.72	.89	.12	.59	.52	.29	.92	22.99
.73	.90	.13	.61	.53	.30	.93	23.00
.74	.92	.14	.62	.54	.32	.94	.02
.75	.94	.15	.64	.55	.34	.95	.04
.76	.96	.16	.66	.56	.36	.96	.06
.77	.97	.17	.68	.57	.37	.97	.07
.78	20.99	.18	.69	.58	.39	.98	.09
.79	21.01	.19	.71	.59	.41	.99	.11

Table 4—(*continued*)

$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰
13.00	23.13	13.40	23.83	13.80	24.52	14.20	25.22
.01	.14	.41	.84	.81	.54	.21	.24
.02	.16	.42	.86	.82	.56	.22	.26
.03	.18	.43	.88	.83	.58	.23	.27
.04	.20	.44	.89	.84	.59	.24	.29
.05	.21	.45	.91	.85	.61	.25	.31
.06	.23	.46	.93	.86	.63	.26	.32
.07	.25	.47	.95	.87	.65	.27	.34
.08	.27	.48	.96	.88	.66	.28	.36
.09	.28	.49	.98	.89	.68	.29	.38
13.10	23.30	13.50	24.00	13.90	24.70	14.30	25.39
.11	.32	.51	.02	.91	.72	.31	.41
.12	.34	.52	.03	.92	.73	.32	.43
.13	.35	.53	.05	.93	.75	.33	.45
.14	.37	.54	.07	.94	.77	.34	.46
.15	.39	.55	.09	.95	.79	.35	.48
.16	.41	.56	.10	.96	.80	.36	.50
.17	.42	.57	.12	.97	.82	.37	.52
.18	.44	.58	.14	.98	.84	.38	.53
.19	.46	.59	.16	.99	.85	.39	.55
13.20	23.48	13.60	24.17	14.00	24.87	14.40	25.57
.21	.49	.61	.19	.01	.89	.41	.59
.22	.51	.62	.21	.02	.91	.42	.60
.23	.53	.63	.23	.03	.92	.43	.62
.24	.55	.64	.24	.04	.94	.44	.64
.25	.56	.65	.26	.05	.96	.45	.66
.26	.58	.66	.28	.06	.98	.46	.67
.27	.60	.67	.30	.07	24.99	.47	.69
.28	.62	.68	.31	.08	25.01	.48	.71
.29	.63	.69	.33	.09	.03	.49	.72
13.30	23.65	13.70	24.35	14.10	25.05	14.50	25.74
.31	.67	.71	.37	.11	.06	.51	.76
.32	.69	.72	.38	.12	.08	.52	.78
.33	.70	.73	.40	.13	.10	.53	.79
.34	.72	.74	.42	.14	.12	.54	.81
.35	.74	.75	.44	.15	.13	.55	.83
.36	.76	.76	.45	.16	.15	.56	.85
.37	.77	.77	.47	.17	.17	.57	.86
.38	.79	.78	.49	.18	.19	.58	.88
.39	.81	.79	.51	.19	.20	.59	.90

Table 4—(*continued*)

$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰	$Cl/\text{liter}_{(20)}$	S‰
14.60	25.92	15.00	26.61	15.40	27.31	15.80	28.00
.61	.93	.01	.63	.41	.32	.81	.02
.62	.95	.02	.65	.42	.34	.82	.03
.63	.97	.03	.66	.43	.36	.83	.05
.64	25.99	.04	.68	.44	.38	.84	.07
.65	26.00	.05	.70	.45	.39	.85	.09
.66	.02	.06	.72	.46	.41	.86	.10
.67	.04	.07	.73	.47	.43	.87	.12
.68	.06	.08	.75	.48	.44	.88	.14
.69	.07	.09	.77	.49	.46	.89	.16
14.70	26.09	15.10	26.79	15.50	27.48	15.90	28.17
.71	.11	.11	.80	.51	.50	.91	.19
.72	.13	.12	.82	.52	.51	.92	.21
.73	.14	.13	.84	.53	.53	.93	.23
.74	.16	.14	.86	.54	.55	.94	.24
.75	.18	.15	.87	.55	.57	.95	.26
.76	.19	.16	.89	.56	.58	.96	.28
.77	.21	.17	.91	.57	.60	.97	.29
.78	.23	.18	.92	.58	.62	.98	.31
.79	.25	.19	.94	.59	.64	.99	.33
14.80	26.26	15.20	26.96	15.60	27.65	16.00	28.35
.81	.28	.21	.98	.61	.67	.01	.36
.82	.30	.22	26.99	.62	.69	.02	.38
.83	.32	.23	27.01	.63	.71	.03	.40
.84	.33	.24	.03	.64	.72	.04	.42
.85	.35	.25	.05	.65	.74	.05	.43
.86	.37	.26	.06	.66	.76	.06	.45
.87	.39	.27	.08	.67	.77	.07	.47
.88	.40	.28	.10	.68	.79	.08	.49
.89	.42	.29	.12	.69	.81	.09	.50
14.90	26.44	15.30	27.13	15.70	27.83	16.10	28.52
.91	.46	.31	.15	.71	.84	.11	.54
.92	.47	.32	.17	.72	.86	.12	.55
.93	.49	.33	.18	.73	.88	.13	.57
.94	.51	.34	.20	.74	.90	.14	.59
.95	.53	.35	.22	.75	.91	.15	.61
.96	.54	.36	.24	.76	.93	.16	.62
.97	.56	.37	.25	.77	.95	.17	.64
.98	.58	.38	.27	.78	.97	.18	.66
.99	.59	.39	.29	.79	.98	.19	.68

Table 4—(*continued*)

Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$
16.20	28.69	16.60	29.39	17.00	30.08	17.40	30.77
.21	.71	.61	.40	.01	.09	.41	.79
.22	.73	.62	.42	.02	.11	.42	.80
.23	.75	.63	.44	.03	.13	.43	.82
.24	.76	.64	.45	.04	.15	.44	.84
.25	.78	.65	.47	.05	.16	.45	.85
.26	.80	.66	.49	.06	.18	.46	.87
.27	.82	.67	.51	.07	.20	.47	.89
.28	.83	.68	.52	.08	.22	.48	.91
.29	.85	.69	.54	.09	.23	.49	.92
16.30	28.87	16.70	29.56	17.10	30.25	17.50	30.94
.31	.88	.71	.58	.11	.27	.51	.96
.32	.90	.72	.59	.12	.28	.52	.98
.33	.92	.73	.61	.13	.30	.53	30.99
.34	.94	.74	.63	.14	.32	.54	31.01
.35	.95	.75	.65	.15	.34	.55	.03
.36	.97	.76	.66	.16	.35	.56	.04
.37	28.99	.77	.68	.17	.37	.57	.06
.38	29.00	.78	.70	.18	.39	.58	.08
.39	.02	.79	.71	.19	.41	.59	.10
16.40	29.04	16.80	29.73	17.20	30.42	17.60	31.11
.41	.06	.81	.75	.21	.44	.61	.13
.42	.07	.82	.77	.22	.46	.62	.15
.43	.09	.83	.78	.23	.47	.63	.17
.44	.11	.84	.80	.24	.49	.64	.18
.45	.13	.85	.82	.25	.51	.65	.20
.46	.14	.86	.84	.26	.53	.66	.22
.47	.16	.87	.85	.27	.54	.67	.23
.48	.18	.88	.87	.28	.56	.68	.25
.49	.20	.89	.89	.29	.58	.69	.27
16.50	29.21	16.90	29.90	17.30	30.60	17.70	31.29
.51	.23	.91	.92	.31	.61	.71	.30
.52	.25	.92	.94	.32	.63	.72	.32
.53	.26	.93	.96	.33	.65	.73	.34
.54	.28	.94	.97	.34	.66	.74	.36
.55	.30	.95	29.99	.35	.68	.75	.37
.56	.32	.96	30.01	.36	.70	.76	.39
.57	.33	.97	.03	.37	.72	.77	.41
.58	.35	.98	.04	.38	.73	.78	.42
.59	.37	.99	.06	.39	.75	.79	.44

Table 4—(*continued*)

Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$	Cl/liter$_{(20)}$	$S‰$
17.80	31.46	18.20	32.15	18.60	32.84	19.00	33.53
.81	.48	.21	.17	.61	.86	.01	.54
.82	.49	.22	.18	.62	.87	.02	.56
.83	.51	.23	.20	.63	.89	.03	.58
.84	.53	.24	.22	.64	.91	.04	.60
.85	.55	.25	.23	.65	.92	.05	.61
.86	.56	.26	.25	.66	.94	.06	.63
.87	.58	.27	.27	.67	.96	.07	.65
.88	.60	.28	.29	.68	.98	.08	.67
.89	.61	.29	.30	.69	32.99	.09	.68
17.90	31.63	18.30	32.32	18.70	33.01	19.10	33.70
.91	.65	.31	.34	.71	.03	.11	.72
.92	.67	.32	.36	.72	.05	.12	.73
.93	.68	.33	.37	.73	.06	.13	.75
.94	.70	.34	.39	.74	.08	.14	.77
.95	.72	.35	.41	.75	.10	.15	.79
.96	.74	.36	.42	.76	.11	.16	.80
.97	.75	.37	.44	.77	.13	.17	.82
.98	.77	.38	.46	.78	.15	.18	.84
.99	.79	.39	.48	.79	.17	.19	.85
18.00	31.80	18.40	32.49	18.80	33.18	19.20	33.87
.01	.82	.41	.51	.81	.20	.21	.89
.02	.84	.42	.53	.82	.22	.22	.91
.03	.86	.43	.55	.83	.23	.23	.92
.04	.87	.44	.56	.84	.25	.24	.94
.05	.89	.45	.58	.85	.27	.25	.96
.06	.91	.46	.60	.86	.29	.26	.97
.07	.92	.47	.61	.87	.30	.27	33.99
.08	.94	.48	.63	.88	.32	.28	34.01
.09	.96	.49	.65	.89	.34	.29	.03
18.10	31.98	18.50	32.67	18.90	33.36	19.30	34.04
.11	31.99	.51	.68	.91	.37	.31	.06
.12	32.01	.52	.70	.92	.39	.32	.08
.13	.03	.53	.72	.93	.41	.33	.09
.14	.05	.54	.73	.94	.42	.34	.11
.15	.06	.55	.75	.95	.44	.35	.13
.16	.08	.56	.77	.96	.46	.36	.15
.17	.10	.57	.79	.97	.48	.37	.16
.18	.11	.58	.80	.98	.49	.38	.18
.19	.13	.59	.82	.99	.51	.39	.20

Table 4—(*continued*)

Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰
19.40	34.22	19.80	34.90	20.20	35.59	20.60	36.28
.41	.23	.81	.92	.21	.61	.61	.30
.42	.25	.82	.94	.22	.63	.62	.31
.43	.27	.83	.95	.23	.64	.63	.33
.44	.28	.84	.97	.24	.66	.64	.35
.45	.30	.85	34.99	.25	.68	.65	.36
.46	.32	.86	35.01	.26	.70	.66	.38
.47	.34	.87	.02	.27	.71	.67	.40
.48	.35	.88	.04	.28	.73	.68	.41
.49	.37	.89	.06	.29	.74	.69	.43
19.50	34.39	19.90	35.07	20.30	35.76	20.70	36.45
.51	.40	.91	.09	.31	.78	.71	.47
.52	.42	.92	.11	.32	.80	.72	.48
.53	.44	.93	.13	.33	.82	.73	.50
.54	.46	.94	.14	.34	.83	.74	.52
.55	.47	.95	.16	.35	.85	.75	.53
.56	.49	.96	.18	.36	.87	.76	.55
.57	.51	.97	.19	.37	.88	.77	.57
.58	.52	.98	.21	.38	.90	.78	.59
.59	.54	.99	.23	.39	.92	.79	.60
19.60	34.56	20.00	35.25	20.40	35.93	20.80	36.62
.61	.58	.01	.27	.41	.95	.81	.64
.62	.59	.02	.28	.42	.97	.82	.65
.63	.61	.03	.30	.43	35.99	.83	.67
.64	.63	.04	.32	.44	36.00	.84	.69
.65	.64	.05	.34	.45	.02	.85	.71
.66	.66	.06	.35	.46	.04	.86	.72
.67	.68	.07	.37	.47	.06	.87	.74
.68	.70	.08	.39	.48	.07	.88	.76
.69	.71	.09	.40	.49	.09	.89	.77
19.70	34.73	20.10	35.42	20.50	36.11	20.90	36.79
.71	.75	.11	.44	.51	.12	.91	.81
.72	.77	.12	.46	.52	.14	.92	.83
.73	.78	.13	.47	.53	.16	.93	.84
.74	.80	.14	.50	.54	.18	.94	.86
.75	.82	.15	.51	.55	.19	.95	.88
.76	.83	.16	.52	.56	.21	.96	.89
.77	.85	.17	.54	.57	.23	.97	.91
.78	.87	.18	.56	.58	.24	.98	.93
.79	.89	.19	.58	.59	.26	.99	.94

Table 4—(*concluded*)

Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰	Cl/liter$_{(20)}$	S‰
21.00	36.96	.25	.39	21.50	37.82	.75	.24
.01	36.98	.26	.40	.51	.83	.76	.26
.02	37.00	.27	.42	.52	.85	.77	.28
.03	.01	.28	.44	.53	.87	.78	.29
.04	.03	.29	.46	.54	.89	.79	.31
.05	.05	21.30	37.47	.55	.90	21.80	38.33
.06	.06	.31	.49	.56	.92	.81	.34
.07	.08	.32	.51	.57	.94	.82	.36
.08	.10	.33	.53	.58	.95	.83	.38
.09	.12	.34	.54	.59	.97	.84	.40
21.10	37.13	.35	.56	21.60	37.99	.85	.41
.11	.15	.36	.58	.61	38.00	.86	.43
.12	.17	.37	.59	.62	.02	.87	.45
.13	.18	.38	.61	.63	.04	.88	.46
.14	.20	.39	.63	.64	.06	.89	.48
.15	.22	21.40	37.65	.65	.07	21.90	38.50
.16	.24	.41	.66	.66	.09	.91	.51
.17	.25	.42	.68	.67	.11	.92	.53
.18	.27	.43	.70	.68	.12	.93	.55
.19	.29	.44	.71	.69	.14	.94	.57
21.20	37.30	.45	.73	21.70	38.16	.95	.58
.21	.32	.46	.75	.71	.17	.96	.60
.22	.34	.47	.77	.72	.19	.97	.62
.23	.36	.48	.78	.73	.21	.98	.63
.24	.37	.49	.80	.74	.23	.99	.65
						22.00	38.67

SOURCE: Reproduced with permission from *A Manual of Sea Water Analysis* by the Director General of Printing and Publishing, Department of Public Printing and Stationery, Ottawa, Canada.

Table 5—Examples for computing standard plate count

Sample No.	Colonies per Dilution 1:100	Colonies per Dilution 1:1,000	Count Ratio *	Standard Plate Count at 35 C	Rule(s) Applicable †
1011	175	16			
	208	17		19,000	(a)
1012	322	23			
	278	29		30,000	(a), (b)
1013	296	40			
	378	24		33,000	(a), (b)
1014	138	42			
	162	30	2.4	15,000	(a), (b), (c)
1015	274	35			
	230	Spreader ‡	1.4	30,000	(a), (b), (c), (g)
1016	325	22			
	329	25		33,000	(a), (e)
1017	18	0			
	21	0		<3,000	(f)

* Ratio of the greater to the lesser plate count, as applied to plates from consecutive dilutions having between 30 and 300 colonies.

† See Part IIIA, Sec. 4.31 in text.

‡ Spreader and adjoining area of repressed growth covering more than ½ of the plate.

Table 6—Death time: mouse unit relations for paralytic shellfish poison (acid)

Death Time *	Mouse Units	Death Time *	Mouse Units	Death Time *	Mouse Units	Death Time *	Mouse Units
1:00	100.0	3:00	3.70	55	1.96	30	1.13
10	66.2	05	3.57	5:00	1.92	10:00	1.11
15	38.3	10	3.43	05	1.89	30	1.09
20	26.4	15	3.31	10	1.86	11:09	1.075
25	20.7	20	3.19	15	1.83	30	1.06
30	16.5	25	3.08	20	1.80	12:00	1.05
35	13.9	30	2.98	30	1.74	13	1.03
40	11.9	35	2.88	40	1.69	14	1.015
45	10.4	40	2.79	45	1.67	15	1.000
50	9.33	45	2.71	50	1.64	16	0.99
55	8.42	50	2.63	6:00	1.60	17	0.98
2:00	7.67	55	2.56	15	1.54	18	0.972
05	7.04	4:00	2.50	30	1.48	19	0.965
10	6.52	05	2.44	45	1.43	20	0.96
15	6.06	10	2.38	7:00	1.39	21	0.954
20	5.66	15	2.32	15	1.35	22	0.948
25	5.32	20	2.26	30	1.31	23	0.942
30	5.00	25	2.21	45	1.28	24	0.937
35	4.73	30	2.16	8:00	1.25	25	0.934
40	4.48	35	2.12	15	1.22	30	0.917
45	4.26	40	2.08	30	1.20	40	0.898
50	4.06	45	2.04	45	1.18	60	0.875
55	3.88	50	2.00	9:00	1.16		

* In minutes.

Table 7—Correction table for weight of mice

Weight of Mice (Grams)	Mouse Units	Weight of Mice (Grams)	Mouse Units
10	0.50	17	0.88
10.5	0.53	17.5	0.905
11	0.56	18	0.93
11.5	0.59	18.5	0.95
12	0.62	19	0.97
12.5	0.65	19.5	0.985
13	0.675	20	1.000
13.5	0.70	20.5	1.015
14	0.73	21	1.03
14.5	0.76	21.5	1.04
15	0.785	22	1.05
15.5	0.81	22.5	1.06
16	0.84	23	1.07
16.5	0.86		

Table 8—Relationship of dose to death time and weight of mice injected with *Gymnodinium breve* toxin(s) extracted from shellfish

Death time in minutes (20 gram mice)	Mouse units per 1 ml	Mouse weight correction	
		Weight of mice in grams	Correction factor
8	10.0	10	0.39
10	9.0	11	0.45
12	8.0	12	0.51
14	7.0	13	0.57
16	6.0	14	0.63
18	5.0	15	0.69
20	4.5	16	0.75
30	4.0	17	0.81
38	3.8	18	0.87
45	3.6	19	0.94
60	3.4	20	1.00
83	3.2	21	1.06
105	3.0	22	1.12
140	2.8	23	1.18
180	2.6	24	1.24
234	2.4	25	1.30
300	2.2	26	1.36
360	2.0	27	1.39
435	1.8	28	1.41
540	1.6	29	1.42
645	1.4	30	1.43
780	1.2	—	—
930	1.0	—	—

SOURCE: Data from McFarren *et al.* 1965.

Table 9—Most probable numbers per 100 ml of sample, planting 3 portions in each of 3 dilutions in geometric series

Number of Positive Tubes			MPN	Number of Positive Tubes			MPN	Number of Positive Tubes			MPN	Number of Positive Tubes			MPN
10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml	
0	0	0		1	0	0	3.6	2	0	0	9.1	3	0	0	23
0	0	1	3.0	1	0	1	7.2	2	0	1	14	3	0	1	39
0	0	2	6.0	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9.0	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3.0	1	1	0	7.3	2	1	0	15	3	1	0	43
0	1	1	6.1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9.2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6.2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9.3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9.4	1	3	0	16	2	3	0	29	3	3	0	240
0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1,100
0	3	3	19	1	3	3	29	2	3	3	53				

Table 10—Most probable numbers per 100 ml of sample, planting 4 portions in each of 3 dilutions in geometric series

Number of Positive Tubes			MPN	Number of Positive Tubes			MPN	Number of Positive Tubes			MPN	Number of Positive Tubes			MPN	Number of Positive Tubes			MPN
10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml	
0	0	0		1	0	0	2.6	2	0	0	6.0	3	0	0	11	4	0	0	23
0	0	1	2.3	1	0	1	5.1	2	0	1	9.1	3	0	1	16	4	0	1	34
0	0	2	4.5	1	0	2	7.8	2	0	2	12	3	0	2	20	4	0	2	50
0	0	3	6.8	1	0	3	10	2	0	3	16	3	0	3	26	4	0	3	71
0	0	4	9.0	1	0	4	13	2	0	4	19	3	0	4	31	4	0	4	95
0	1	0	2.3	1	1	0	5.2	2	1	0	9.3	3	1	0	16	4	1	0	36
0	1	1	4.6	1	1	1	7.9	2	1	1	13	3	1	1	21	4	1	1	55
0	1	2	6.8	1	1	2	11	2	1	2	16	3	1	2	26	4	1	2	81
0	1	3	9.1	1	1	3	13	2	1	3	20	3	1	3	32	4	1	3	110
0	1	4	11	1	1	4	16	2	1	4	23	3	1	4	38	4	1	4	140
0	2	0	4.6	1	2	0	8.0	2	2	0	13	3	2	0	21	4	2	0	62
0	2	1	6.9	1	2	1	11	2	2	1	16	3	2	1	27	4	2	1	94
0	2	2	9.2	1	2	2	13	2	2	2	20	3	2	2	33	4	2	2	130
0	2	3	12	1	2	3	16	2	2	3	24	3	2	3	40	4	2	3	170
0	2	4	14	1	2	4	19	2	2	4	28	3	2	4	47	4	4	4	210
0	3	0	7.0	1	3	0	11	2	3	0	17	3	3	0	28	4	3	0	110
0	3	1	9.3	1	3	1	14	2	3	1	20	3	3	1	34	4	3	1	160
0	3	2	12	1	3	2	16	2	3	2	24	3	3	2	41	4	3	2	220
0	3	3	14	1	3	3	19	2	3	3	28	3	3	3	48	4	3	3	280
0	3	4	16	1	3	4	22	2	3	4	32	3	3	4	56	4	3	4	360
0	4	0	9.4	1	4	0	14	2	4	0	21	3	4	0	35	4	4	0	240
0	4	1	12	1	4	1	17	2	4	1	25	3	4	1	43	4	4	1	390
0	4	2	14	1	4	2	20	2	4	2	29	3	4	2	50	4	4	2	700
0	4	3	17	1	4	3	23	2	4	3	33	3	4	3	59	4	4	3	1,400
0	4	4	19	1	4	4	26	2	4	4	37	3	4	4	67				

Table 11—Most probable numbers per 100 ml of sample, planting 5 portions in each of 3 dilutions in geometric series

No. Positive Tubes			MPN	No. Positive Tubes			MPN	No. Positive Tubes			MPN	No. Positive Tubes			MPN	No. Positive Tubes			MPN	No. Positive Tubes			MPN
10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml		10 ml	1 ml	0.1 ml	
0	0	0		1	0	0	2.0	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4.0	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6.0	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8.0	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9.0	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4.0	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1,600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81				