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Techniques of Water-Resources Investigations
of the United States Geological Survey

Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

L.J. Britton and P.E. Greeson, Editors

This report supersedes TWRI 5A4, published in 1977, entitled "Methods for collection and analysis of aquatic biological and microbiological samples," edited by P.E. Greeson and others.

Revised 1987
Book 5

LABORATORY ANALYSIS

DEPARTMENT OF THE INTERIOR

MANUEL LUJAN, JR., *Secretary*

U.S. GEOLOGICAL SURVEY

Dallas L. Peck, *Director*

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PREFACE

The series of chapters on techniques describes methods used by the U.S. Geological Survey for planning and conducting water-resources investigations. The material is arranged under major subject headings called books and is further subdivided into sections and chapters. Book 5 is on laboratory analysis. Section A is on water. The unit of publication, the chapter, is limited to a narrow field of subject matter. "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" is the fourth chapter to be published under Section A of Book 5. The chapter number includes the letter of the section.

This chapter was prepared by several aquatic biologists and microbiologists of the U.S. Geological Survey to provide accurate and precise methods for the collection and analysis of aquatic biological and microbiological samples.

Use of brand, firm, and trade names in this chapter is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey.

This chapter supersedes "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" edited by P.E. Greeson, T.A. Ehlke, G.A. Irwin, B.W. Lium, and K.V. Slack (U.S. Geological Survey Techniques of Water-Resources Investigations, Book 5, Chapter A4, 1977) and also supersedes "A Supplement to—Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" by P.E. Greeson (U.S. Geological Survey Techniques of Water-Resources Investigations, Book 5, Chapter A4), Open-File Report 79-1279, 1979.

TECHNIQUES OF WATER-RESOURCES INVESTIGATIONS OF THE U.S. GEOLOGICAL SURVEY

The U.S. Geological Survey publishes a series of manuals describing procedures for planning and conducting specialized work in water-resources investigations. The manuals published to date are listed below and may be ordered by mail from the **U.S. Geological Survey, Books and Open-File Reports, Federal Center, Box 25425, Denver, Colorado 80225** (an authorized agent of the Superintendent of Documents, Government Printing Office).

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CONVERSION FACTORS

Metric units (International System) in this report may be converted to inch-pound units by using the following conversion factors:

Multiply metric unit	*By*	*To obtain inch-pound unit*
centimeter (cm)	0.3937	inch
cubic meter (m^3)	35.31	cubic foot
gram (g)	0.03527	ounce, avoirdupois
gram per cubic meter (g/m^3)	62.45×10^{-6}	pound per cubic foot
gram per cubic meter per hour [(g/m^3)/h]	62.45×10^{-6}	pound per cubic foot per hour
kilogram (kg)	2.205	pound, avoirdupois
kilogram per square centimeter (kg/cm^2)	14.22	pound per square inch
liter (L)	0.2642	gallon
meter (m)	3.281	foot
meter per second (m/s)	3.281	foot per second
microgram (μg)	35.27×10^{-8}	ounce, avoirdupois
microliter (μL)	26.42×10^{-8}	gallon
micrometer (μm)	39.37×10^{-6}	inch
milligram (mg)	35.27×10^{-5}	ounce, avoirdupois
milliliter (mL)	26.42×10^{-5}	gallon
millimeter (mm)	0.03937	inch
square centimeter (cm^2)	0.155	square inch
square kilometer (km^2)	0.3861	square mile
square meter (m^2)	10.76	square foot
square millimeter (mm^2)	1.550×10^{-3}	square inch

Inch-pound units in this report may be converted to metric units (International System) by using the following conversion factors:

Multiply inch-pound unit	*By*	*To obtain metric unit*
acre-foot (acre-ft)	1,233	cubic meter
cubic foot per second (ft^3/s)	0.028317	cubic meter per second
foot (ft)	0.3048	meter
inch (in.)	25.4	millimeter
mile (mi)	1.609	kilometer
ounce, fluid	0.02957	liter
pound, avoirdupois (lb)	453.6	gram
pound per square inch (psi)	703.1	kilogram per square meter
square inch (in^2)	6.452	square centimeter
square mile (mi^2)	2.59	square kilometer

Degree Celsius (°C) may be converted to degree Fahrenheit (°F) by using the following equation:

$$°F=9/5(°C)+32.$$

Degree Fahrenheit (°F) may be converted to degree Celsius (°C) by using the following equation:

$$°C=5/9(°F-32).$$

The following terms and abbreviations also are used in this report:

disintegrations per minute (dpm)
gram per liter (g/L)
gram per milliliter (g/mL)
liter per milligram multiplied by centimeter (L/mg)×cm
lumens per square meter (lumens/m^2)
microcurie (μCi)
microcurie per microgram (μCi/μg)
microcurie per milliliter (μCi/mL)
microgram-atoms per liter (μg-atoms/L)
microgram per liter (μg/L)
microgram per milliliter (μg/mL)
millicurie (mCi)
milligram carbon per cubic meter per day [mg(C/m^3)/d]
milligram carbon per cubic meter per hour [mg(C/m^3)/h]
milligram carbon per square meter per day [mg(C/m^2)/d]
milligram oxygen per cubic meter per day [mg(O_2/m^3)/d]
milligram oxygen per cubic meter per hour [mg(O_2/m^3)/h]
milligram oxygen per square meter per day [mg(O_2/m^2)/d]
milligram per cubic meter (mg/m^3)
milligram per liter (mg/L)
milligram per liter per acre-foot [(mg/L)/acre-ft]
milligram per square meter (mg/m^2)
milliliter per minute (mL/min)
millivolt (mV)
nanometer (nm)
revolutions per minute (r/min)
volt (V)
Watt (W)

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

L.J. Britton and P.E. Greeson, Editors

Abstract

Chapter A4 contains methods used by the U.S. Geological Survey to collect, preserve, and analyze water to determine its biological and microbiological properties. Part 1 consists of detailed descriptions of more than 45 individual methods, including those for bacteria, phytoplankton, zooplankton, seston, periphyton, macrophytes, benthic invertebrates, fish and other vertebrates, cellular contents, productivity, and bioassays. Each method is summarized, and the applications, interferences, apparatus, reagents, analyses, calculations, reporting of results, precisions, and references are given. Part 2 consists of a glossary. Part 3 is a list of taxonomic references.

Introduction

The U.S. Department of the Interior has the basic responsibility for the appraisal, conservation, and efficient use of the Nation's natural resources, including water as a resource, as well as water involved in the use and development of other resources. As one of the several agencies of the U.S. Department of the Interior, the U.S. Geological Survey's primary responsibility in relation to water is to assess its availability and use as a natural resource. The U.S. Geological Survey's responsibility for water appraisal includes not only assessments of the location, quantity, and availability of water but also determinations of water quality. Inherent in this responsibility is the need for extensive water-quality studies related to the physical, chemical, and biological adequacy of natural and developed surface- and ground-water resources. Included, also, is the need for supporting research to increase the effectiveness of these studies.

As part of its mission, the U.S. Geological Survey is responsible for providing a large part of the water-quality data (for rivers, lakes, and ground water) used by planners, developers, water-quality managers, and pollution-control agencies. A high degree of reliability and standardization of these data is of paramount importance.

This chapter provides accurate and precise methods for the collection and analysis of aquatic biological and microbiological samples, primarily from freshwater. Although excellent and authoritative manuals on aquatic biological analyses are available, their methods and procedures often are diverse. The purpose of this chapter is to provide, in a single publication, the methods used by the U.S. Geological Survey in conducting biological investigations.

The work of the U.S. Geological Survey in aquatic biology and microbiology ranges from research to the collection of biological information from onsite investigations and from a nationwide network of water-quality stations. The objectives vary so widely that it is impractical to tailor methods to fit all possible requirements. In general, the methods described here apply to the collection of biological information.

In order to keep users informed of revised and new techniques in the field of aquatic biology, this chapter is updated periodically. It is clear from the accelerating rate of publication of reports on the subject of aquatic biology that new and improved methods are being developed in response to man's increasing awareness of his environment. A technique that represents the state-of-the-art today may be outdated tomorrow. The author of a manual of techniques may have the impression of taking a "grab sample" from a changing stream of new developments, although it is possible to a degree to integrate the experience of the past and to select the most appropriate methods from an ever-growing number of methods.

A methods manual is only one of several tools available to the investigator. At best, it can indicate "how to." It can never indicate "what to" nor can it indicate what a specific numerical value means. Entire volumes have been written on subjects (for example, primary productivity) to which this chapter can devote only a few pages. It is emphasized that the successful investigator must keep abreast of the new developments, both in methodology and in the understanding of aquatic ecosystems.

Safety procedures, especially with use of hazardous chemicals or equipment, micro-organisms that may produce human disease, water that may contain bacteria, and radioactive substances, should be recognized, and manufacturers' instructions should be followed when using the methods in this chapter. Special attention is called to a number of hazardous materials within the individual methods; this serves to emphasize safety concerns.

Part 1: Description of Methods

BACTERIA

Introduction

Bacteria can be collected, observed, and counted directly using the highest resolution of the light microscope. A method for counting total bacteria by epifluorescence is included in this chapter; however, the method is somewhat difficult and may not be appropriate for general use. Of far greater applicability are methods whereby the bacteria in a measured volume of water are placed in contact with material on which they can grow. After a suitable time, each bacterium in the sample will multiply into an easily visible colony. The number of colonies is extrapolated from the number of bacteria in the original sample.

The first method in the following section provides an approximation of the total bacterial population. Because all culture methods are selective, a total count of the bacteria in a habitat is impossible using this technique. However, uniform methods permit comparison of results by different investigators. The remaining methods given are designed to be selective for specific groups of bacteria. These methods will provide an estimate of the number of bacteria in an environment, but no information is obtained about the activity of the organisms in the ecosystem being studied.

Most-probable-number (MPN) methods, using multiple-dilution tubes, can be used to estimate the size of a bacterial population without counting either single cells or colonies (Meynell and Meynell, 1970). Several dilutions of a sample are made and aliquots are inoculated into suitable media. The method requires either that the media be selective for a specific group of bacteria and allow only those organisms to grow or that some readily identifiable product be produced. The dilutions, including the most dilute samples used, need to contain no bacterial cells of the type under study (dilution to extinction). Based on the distribution of positive and negative cultures, the MPN of bacteria in the original sample is calculated.

MPN tables are included with each applicable method. These tables are based on those published in "Standard Methods" by the American Public Health Association and others (1985); however, the tables have been modified to include the procedures specified in "Techniques of Water-Resources Investigations" methods. All MPN tables use 1-, 0.1-, and 0.01-mL sample volumes and express MPN per 1 or 100 mL depending on how the count is to be reported. Examples included with each method illustrate the calculation of MPN if sample volumes other than 1, 0.1, and 0.01 mL are used.

The membrane-filter (MF) method has attained widespread application in microbiology principally because it is simple and quick to perform (Bordner and others, 1977). Also, it is statistically more reliable than the MPN method. A brief discussion of the merits and limitations of the MF method is appropriate here; precision and accuracy depend to a great extent on careful attention to procedural details.

Membrane filters used in microbiology are inert plastic films about 125 μm thick. The membranes are available in a variety of chemical types, each designed for a particular application. It is imperative that the analyst select a type intended for bacterial application. Whatever the type, the membrane is about 80 percent void with pores of uniform size. Pore sizes of 0.45 or 0.7 μm (Green and others, 1975; Sladek and others, 1975; American Public Health Association and others, 1985) are the most common sizes used in microbiology because the type of bacteria most often counted is larger than 0.5 μm. Membranes with pore size less than 0.45 μm are available but are used less commonly in microbiology because of their susceptibility to clogging. Filters are manufactured in many sizes from about 13 to 293 mm in diameter, but only the 47-mm diameter size is used commonly in microbiology. The useful shelf life of membrane filters is 1 year (American Public Health Association and others, 1985).

Bacterial analysis begins with sample collection, which is described in a general way in this introduction. Media and equipment preparation are described with each specific method. At some point in each method, a sample aliquot is passed through a filter. Membrane filters have a rapid flow rate initially due to the large void volume, but the filter will clog quickly if the sample is turbid. For this and other reasons, the MF method generally is not suitable for turbid water. Even with relatively clear water, sample filtration generally is limited to about 100 to 250 mL per filter. If it is necessary to filter a larger volume of sample, as with the isolation of *Salmonella*, it is permissible to divide a sample volume between several filters.

After filtration, the bacteria may be arrayed singly, paired, or in chains on the surface of the membrane. They cannot be seen without magnification; therefore, the filters must be incubated for a time sufficient for the individual cells to grow into visible colonies. After filtration, the filter is aseptically placed in a petri dish containing solid (agar) medium. Use of broth media is not recommended in the Water Resources Division because optimum cell growth depends on an adequate nutrient supply, and solid (agar) media have been found

to yield larger colony counts than broth-grown media cultures. This is due to the larger volume (6.5 mL compared to 1.8 mL) of medium used in the agar technique.

Incubation is allowed to proceed at 35 °C for 24 to 48 hours for total coliform and fecal streptococcal bacteria or at 44.5 °C for 24 hours for fecal coliform bacteria. It is very important that the temperature be held within the limits established for each method. Recent work (Green and others, 1975) indicated that many more cells are retained on the surface of the membrane than actually grow. During incubation, the petri dishes generally will lose moisture and dry. This is particularly true of dry (air) incubators at 44.5 ± 0.2 °C. The result of drying serves to inhibit bacterial growth, thus underestimating the true population. To prevent dryness, the petri dishes should be checked for proper sealing before incubation. Cracked dishes should be discarded.

When the individual cells have multiplied to visible colonial size (usually 24- to 48-hour incubation), the colonies must be counted. The counting procedure is based on enumerating all colonies of a specific color, regardless of size or shape. Each bacterial method has different colony identification criteria. After a count has been made, the result is calculated and reported in terms of number of colonies per milliliter or 100 mL of sample.

Media used in many of the methods described in this manual are commercially available in a pre-mixed, dehydrated form. Unopened containers of nutrient media should not be stored for more than 1 year. The shelf life of opened containers of media is highly variable; to extend the shelf life of opened containers, the media should be stored in a dessicator.

Collection

If valid information about the number and type of bacteria present in an environment is to be obtained, care must be taken before, during, and after sampling. A valid sample will be representative of the organisms present at the site under study and will be uncontaminated by extraneous organisms. After such a soil or water sample has been obtained, it must be processed as quickly as possible and carefully maintained so the bacterial populations do not change extensively.

The study objective is of overriding importance, and the final determination of the best sampling method, frequency of sample collection, and number and distribution of sampling sites is left to the judgment of the investigator. The sites and methods used for sampling of bacteria need to correspond as closely as possible to those selected for chemical and other biological sampling.

Some of the general guidelines for collecting soil and water samples given by Guy and Norman (1970), Wood (1976, p. 1-7), and Hem (1985) can be applied to microbiological work. However, collecting valid samples for bacterial analysis is more difficult because extra care is required to avoid contamination and because micro-organisms rarely are distributed randomly. Bacteria within any habitat or microhabitat probably will have a clumped or patchy distribution. Localized differences in chemical and physical characteristics, such as Eh, pH, temperature, nutrient availability, and dissolved-oxygen concentration, will affect the size and distribution of the bacterial population.

Although guidelines for sample collection are provided in this section, it is impossible to provide detailed instructions on sample collection for all possible circumstances. More extensive discussions of microbiological sampling are given in the following:

1. Surface water—Rodina (1972), Collins and others (1973), and Skinner and Shewan (1977).
2. Ground water—Dunlap and McNabb (1973), Dunlap and others (1977), and Scalf and others (1981).
3. Soil—Black (1965), Parkinson and others (1971), and Williams and Gray (1973).

Surface water

The location of sampling sites and the frequency of sampling are critical factors in obtaining meaningful data about bacterial density in any water body. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary laterally, with depth, and with time of day. Generally, multiple samples collected at different depths and sites within a study area yield more reliable data than do single samples. Water in small, fast-flowing streams is likely to be well mixed. A point sample, collected at a single transverse position located at the centroid of flow, may be adequate (Goerlitz and Brown, 1972).

To collect a sample of water at the surface, open a sterile milk dilution bottle or equivalent sample container, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly rotating the bottle until the neck points slightly upward. The mouth of the bottle must be directed into the current. If there is no current, as in a lake, a current should be created artificially by pushing the bottle horizontally forward away from the hand (American Public Health Association and others, 1985).

Several types of microbiological sampling apparatus are available that collect a water sample at depth. Samplers of the Kemmerer or Van Dorn type have been used, but their use is discouraged; most of these devices are not autoclavable, and the metallic parts, if present, can have bacteriocidal effects if they remain in contact with the sample for a prolonged period of time. Niskin and ZoBell samplers (Rodina, 1972) collect a sample in either a sterile plastic bag or a sterile bottle. All of these sampling devices are triggered by a messenger and collect samples at one point in the water column.

Samplers, such as the D-77 and DH-80, available from the Federal Interagency Sedimentation Project, St. Anthony Falls, Hydraulic Laboratory, Minneapolis, Minn., can be used for collecting depth-integrated samples from flowing water. The sampler's nozzle and chamber are autoclavable.

Ground water

Obtaining a valid sample of ground water for microbiological examination requires care in well construction and sampling technique. During well construction, the potential for contamination by the extraneous introduction of nutrients and bacteria needs to be minimized.

Generally, the water in the casing and in proximity to the well is not representative of the ground water at a distance from the well. Oxidation-reduction and nutrient conditions generally are different near the well where bacteria may be present in greater numbers than in the aquifer some distance from the well. There is no general rule for the number of times that water in the well casing must be cleared before collecting water samples for bacterial analysis. The volume of pumping necessary will depend on site-specific conditions and the purpose of the investigation. Public-supply, industrial, or irrigation wells, which are pumped continuously, may give the most representative sample of aquifer water.

The possibility of external contamination during sampling can be lessened by sterilizing all materials that will come in contact with the water sample; however, this may be difficult during some onsite conditions. Water within 25 ft of land surface can be collected by peristaltic and other low-volume suction pumps fitted with sterile hoses. For studies that require water samples collected deeper than 25 ft, other types of pumps must be used. Gas-powered, all-glass pumps that can be heat sterilized have been developed, but these are fragile and require special care (Tomson and others, 1980). Gas-powered squeeze pumps that fit into small-diameter wells and that may be autoclaved also have been developed (Koopman, 1979). Portable submersible pumps commonly are the most convenient sampling devices. Although they may be difficult or impossible to sterilize, these pumps can be disinfected by recirculating a chlorine solution.

Soil and sediment

Collect soil samples using sterile procedures and place in sterile glass, polypropylene or teflon bottles, or Whirl-Pak bags. Avoid exposing soil samples to heat or drying. If the sample is not processed on the day of collection, it may be stored at 4 °C for 1 to 2 weeks in the closed container, provided that the container is pinholed for aeration. Just prior to processing, pass the entire sample through a 10-mesh sieve (2,000 μm) and mix thoroughly before taking an aliquot for analysis. If desired, a separate subsample may be taken for determination of dry weight (Clark, 1965).

Bottom-material sampling devices suitable for use in anaerobic environments are available. The simplest device, useful in soft muds and mucks, consists of a length of thin-wall plastic or metal tubing. The tube is pushed into the soil to the desired depth, and the open end is sealed with a rubber stopper. The entire assembly then is withdrawn. The core should remain in place because of the suction effect exerted by the closed air chamber above the core. In deep water, a remote-operating core sampler, such as the K-B type (Wildlife Supply Co., or equivalent), may be required. Fine-grained material may be sampled by inserting a large bore hypodermic syringe or cannula through holes drilled through the side of the coring tube. If a core is to be subdivided, remove contaminants from the coring device by trimming the perimeter of the core with sterile instruments.

Sample containers

Samples for microbiological examination must be collected and held in containers that have been carefully cleaned and sterilized by autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for at least 15 minutes. Narrow-mouth bottles (milk dilution) are the preferred sample containers. Caps or stoppers must be loosened during autoclaving to allow the steam to contact all surfaces. Alternatively, dry glassware may be sterilized in a hot air oven at 170 °C for a minimum of 2 hours. Presterilized plastic bags (Whirl-Pak, or equivalent) are commercially available and may be suitable for soil or bottom-material samples but are not recommended for collection of water samples for bacterial analysis.

Sample containers must be constructed of a material that can be sterilized and that is resistant to the solvent action of water. Borosilicate glass or plastic that can be autoclaved without distortion or the production of toxic compounds are acceptable materials. Containers made of polypropylene and teflon are autoclavable.

Containers may be of any suitable size and shape and must allow a sufficient volume of sample to be collected and must maintain the sample uncontaminated until analyses are complete. When the sample is collected, ample air space must be left in the container to facilitate mixing of the sample by shaking.

Bottle closures must be water tight. Ground-glass-stoppered bottles are acceptable, as are bottles with plastic screwcap closures, provided that, during sterilization, no bacteriostatic or nutritive compounds are produced.

Dechlorination

A dechlorinating agent should be added to sample bottles used to collect water containing residual chlorine. Sodium thiosulfate is a satisfactory dechlorinating agent that will neutralize any residual chlorine and prevent continuing bacteriocidal action prior to sample processing. Add 0.1 mL of a 10-percent solution of sodium thiosulfate to each 120-mL sample container prior to sterilization (American Public Health Association and others, 1985). This concentration of sodium thiosulfate will neutralize a sample containing about 15 mg/L of residual chlorine.

Chelating agent

A chelating agent should be added to water samples suspected of containing greater than 0.01 mg/L of heavy metals,

such as copper, nickel, or zinc. Add 0.3 mL of a 15-percent solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt to each 120-mL sample bottle prior to sterilization (Bordner and others, 1978).

Preservation and storage

A general rule in working with micro-organisms is that the more rapidly the samples are processed, the more accurate the results will be. The chemical and biological characteristics of the sample will change during storage and no longer will be representative of conditions at the sampling site. Therefore, microbiological analysis should begin as soon as possible after collection, preferably within 1 hour and not more than 6 hours. Samples should be iced or refrigerated, but never frozen, and kept in the dark during the holding period. Sample containers should not be totally immersed in water during storage. Under no circumstances should samples be exposed to direct sunlight. If it is impossible to transport the sample to the laboratory within the required period of time, onsite analytical procedures should be considered.

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Standard plate count (membrane-filter method)

(B-0001-85)

Parameter and Code:
Total plate count, TPC medium, 35 °C, 24 hours (colonies/mL): 31751

The standard plate count is an empirical method for estimating the aerobic, heterotrophic bacterial population in a water sample. Because the nutrient and environmental requirements of certain bacteria are unique, the colony counts derived by this method generally underestimate the natural population. Anaerobic bacteria and many species of autotrophic bacteria will not grow on the specified medium, and for these, other methods must be used.

1. Applications

The method is applicable for all water with a dissolved-solids concentration of less than 20,000 mg/L. The test is performed using the agar-plate method (Bordner and others, 1978; American Public Health Association and others, 1985).

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on tryptone glucose extract (TPC) agar. After incubation at 35±0.5 °C for 24±2 hours, the colonies are counted. Staining is used to enhance the contrast between the bacterial colonies and the filter.

3. Interferences

3.1 Suspended materials may not permit the filtration of sample volumes sufficient to produce significant results. Water samples with a large suspended-solids concentration may be divided between two or more membrane filters.

3.2 Some species of bacteria and fungi exhibit a spreading type of growth, and a single colony may cover the entire surface of the filter, obscuring other colonies.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 *Alcohol burner*, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 *Aluminum seals*, one piece, 20 mm.

4.3 *Bottles*, milk dilution, screwcap.

4.4 *Bottles*, serum.

4.5 *Crimper*, for attaching aluminum seals.

4.6 *Decapper*, for removing aluminum seals from spent tubes.

4.7 *Filter-holder assembly** and *syringe* that has a *two-way valve** or *vacuum hand pump*.

4.8 *Forceps**, stainless steel, smooth tips.

4.9 *Graduated cylinders*, 100-mL capacity.

4.10 *Hypodermic syringes*, sterile, 1-mL capacity, equipped with 26-gauge, ⅜-in. needles.

4.11 *Hypodermic syringes*, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 *Incubator**, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or *heaterblock* (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator that has more precise temperature regulation is satisfactory for laboratory use.

4.13 *Membrane filters*, white, grid, sterile, 0.45-μm pore size, 47-mm diameter, and *absorbent pads*.

4.14 *Microscope*, binocular wide-field dissecting-type, and *fluorescent lamp*.

4.15 *Pipets*, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 *Pipets*, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 *Pipettor*, or *pi-pump*, for use with 1- and 10-mL pipets.

4.18 *Plastic petri dishes with covers*, disposable, sterile, 50×12 mm.

4.19 *Rubber stoppers*, 13×20 mm.

4.20 *Sample-collection apparatus*. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 *Sterilizer*, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing

for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks that prevent removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 *Thermometer*, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Buffered dilution water.* Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 *N* sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 *Distilled or deionized water.*

5.3 *Ethyl alcohol*, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 *Methyl alcohol*, absolute, for sterilizing filter-holder assembly.

5.5 *Methylene blue staining solution.* Add 3 g methylene blue dye to 300 mL of 95-percent ethyl alcohol. Dissolve

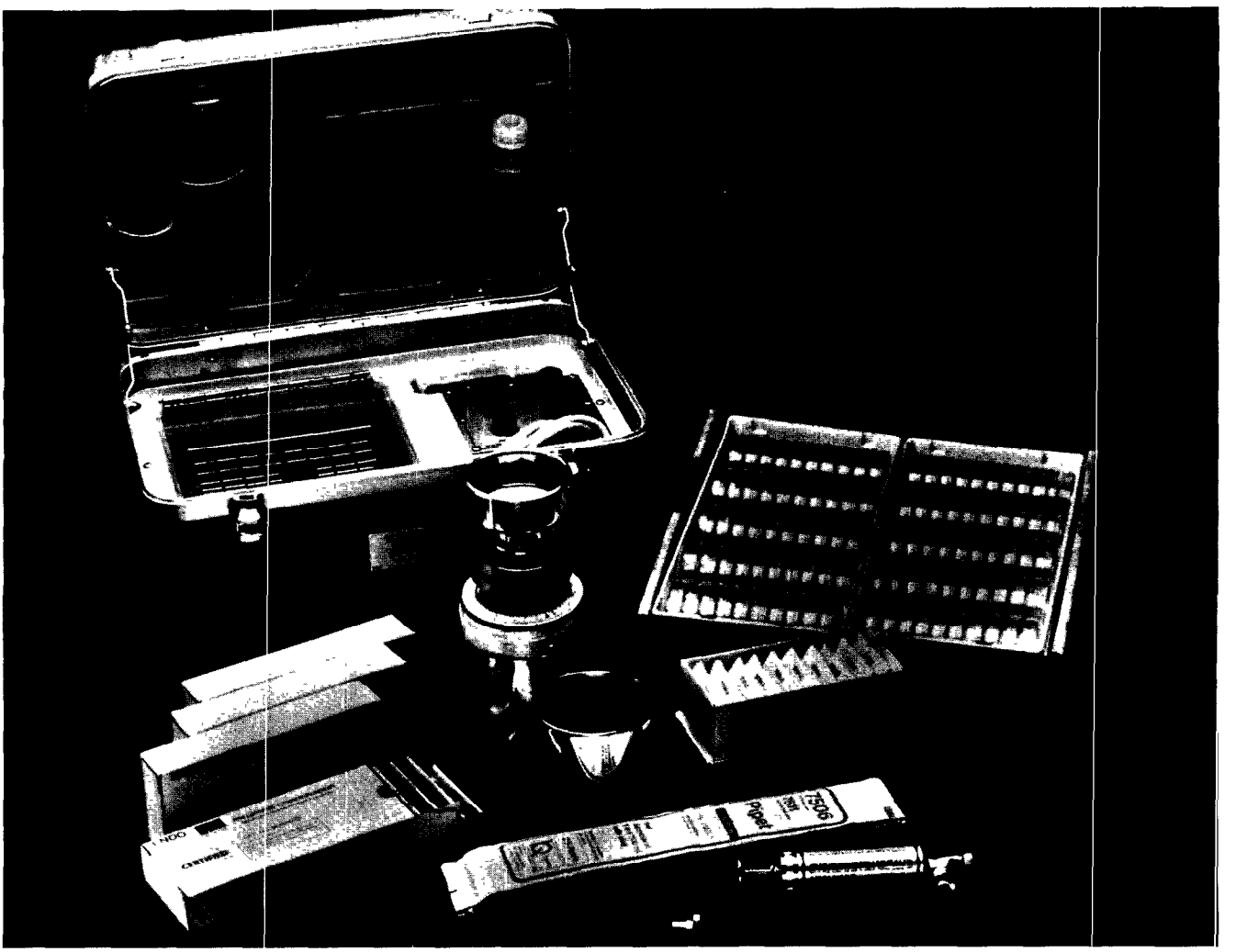

Figure 1.—Portable water laboratory. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

0.1 g of potassium hydroxide (KOH) in 1 L of distilled water. Add to the alcoholic methylene blue solution and mix well.

5.6 *Tryptone glucose extract agar.* Prepare medium according to manufacturer's instructions, using agar. Heat while stirring vigorously until the solution becomes clear. Remove from heat immediately when clear. (Prevent scorching or boiling over of the medium.) The agar must be dispensed into suitably capped containers and sterilized in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes before the medium is added to presterilized petri dishes (see 6.1).

6. Analysis

The volume of the sample to be filtered depends on the expected bacterial density of the water being tested, but the volume should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 150 colonies. When there are no existing data on the bacterial density of a given sample, the quantities must be determined by trial. The following guidelines may be helpful for unknown water: unpolluted ground water, 10- and 50-mL samples; unpolluted surface water, 0.001-, 0.01-, 0.1-, and 1-mL samples.

6.1 Pour the agar medium at 45 to 50 °C into a petri dish bottom to a depth of about 4 mm (6–7 mL). Replace petri dish tops (not tightly, to prevent excessive condensation) and allow agar to solidify.

6.2 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize

Figure 2.—Portable heaterblock incubator. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.3 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps—Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.4 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.4.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.4.2 If the volume of the sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.4.3 If the volume of original water sample is less than 1 mL, proceed as in 6.4.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.5 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.6 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.7 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.8 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.9 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.10 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles (indicated by bulges) are present under the filter, remove the filter using sterile forceps and roll onto the agar again.

6.11 Close the plastic petri dish by firmly pressing down on the top.

6.12 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35±0.5 °C for 24±2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 After incubation, saturate an absorbent pad with 1.8 mL of methylene blue staining solution.

6.14 Transfer incubated filter with developed colonies to the newly saturated pad and wait 15 minutes.

6.15 Count the colonies, which will be dark blue against a lighter color background. The counts are best made using 10× to 15× magnification. Illumination is not critical.

6.16 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 150, use the equation:

$$\text{Colonies/mL} = \frac{\text{Number of colonies counted}}{\text{Volume of original sample filtered (milliliters)}}.$$

7.2 If all filters have colony counts less than the ideal of 20 colonies or greater than 150 colonies, calculate using the equations in 7.5 for only those filters having at least one colony but not having colonies too numerous to count. Report

results as number per milliliter, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters contain colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 150 for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\text{Volume filter 1} + \text{Volume filter 2}}{\text{Volume sum}} \qquad \frac{\text{Colony count filter 1} + \text{Colony count filter 2}}{\text{Colony count sum}}$$

$$\text{Colonies/mL} = \frac{\text{Colony count sum}}{\text{Volume sum (milliliters)}}.$$

Note 5: Do not calculate the total colonies per milliliter for each volume filtered and then average the results.

8. Reporting of results

Report number of colonies per milliliter to two significant figures and designate as "standard plate count at 35 °C." Never report a count as less than one.

9. Precision

No numerical precision data are available.

10. Sources of information

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