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Field application of the membrane filter method for bacteriological quality determination of water

Charles Liston Gambill

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FIELD APPLICATION OF THE MEMBRANE FILTER METHOD FOR BACTERIOLOGICAL QUALITY DETERMINATION OF WATER

BY

CHARLES LISTON GAMBILL

A

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Approved by

J. Kenneth Roberts (advisor)

W. T. Schrenk

E. R. Carlton

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CHAPTER I

I. INTRODUCTION

An important phase of engineering is quality control of a process. In the bacteriological examination of water quality control is one of the most important of many processes. Many instruments, techniques, devices and procedures have been developed in the last sixty years to analyze the quality of a water from a bacteriological standpoint.

Whipple, Smith, Sedwick, Fuller, McGrady, Jordan and their associates, along with many others, have been pioneers in this field.

In current procedures the bacteriological analysis of a water is performed by analyzing a sample for presence of organisms of the coliform group of bacteria. The basis for this selection will be discussed later in this study. The currently accepted procedure is outlined in the tenth edition of Standard Methods for the Examination of Water, Sewage and Industrial Wastes (1). The standard tests for the coliform group includes the Presumptive Test, Confirmed Test and Completed Test. The number of positive findings of the coliform group of organisms is computed and recorded in terms of the "Most Probable Number" (MPN).

Since 1950 extensive research has been conducted by the United States Public Health Service and many other research agencies using a membrane filter for determination of the presence of the coliform organism group in a water.

(1) All references are in bibliography.

The purpose of this thesis is to demonstrate the definite advantages to be realized when using the membrane filter method as an accepted procedure for the bacteriological analysis of a water; and specifically when used under emergency or field conditions such as military units in the field. These same advantages would apply to Civil Defense and other such agencies when operating under emergency or field conditions.

Data from actual tests showing techniques, equipment and procedures used and augmented by other data available from research papers will be utilized.

CHAPTER II

LITERATURE REVIEW

This review is presented in three parts: a brief history of the bacteriological methods of water analysis, the theory of the "Most Probable Number", and the procedures currently recognized as standard by the American Public Health Association, American Water Works Association and Federation of Sewage and Industrial Wastes Associations.

I. HISTORY OF BACTERIOLOGICAL METHODS

Prior to about 1890 the chemical analysis of a water and a consideration of conditions at the source were the chief items used to judge the sanitary quality of a water (2). In 1894 members of the American Public Health Association recognized the need for using standard methods in the bacteriological examination (3) (4).

At a meeting of the American Public Health Association in 1899, a committee was appointed and charged with the extension of standard procedures to all methods involved in the analysis of water (1). This committee published its report in 1905 and as such became the first edition of "Standard Methods of Water Analysis". These standards covered not only bacteriological methods for the examination of water but also covered physical, chemical, and microscopical methods.

Revisions and additions were made and published as successive editions of Standard Method of Water Analysis. The second edition was dated 1912; the third 1917; the fourth 1920; and the fifth 1923.

The sixth edition was published in 1925 under the joint auspices of the American Public Health Association and the American Water Works Association. This edition was the first to use the inclusive title, Standard Methods for the Examination of Water and Sewage. The current edition is the tenth edition, dated 1955.

II. THEORY OF MOST PROBABLE NUMBER

Specific disease germs are difficult to isolate from water unless they are fairly numerous. It is possible for water to be heavily polluted with sewage without any specific disease germs being present. All sewage water, however, is potentially dangerous because, where fecal matter exists, disease germs are likely to appear at any time.

Escherich was the first to isolate the 'Bacterium coli' (Escherichia coli) (5). In 1885 he isolated it from the feces of a cholera patient. Subsequently it was found to be a normal inhabitant of the intestinal track of man and many other animals, and also appeared regularly in their excreta. Because of its abundance in the intestinal track, it was logically assumed that water could not become polluted with fecal material without this organism being present. Hence, its presence in a water is considered prima facie evidence of pollution with fecal material.

Escherichi coli, originally regarded as a single species has since proved to be a group made up of a number of species (6). All these possess certain characteristics in common and are defined in Standard Methods for the Examination of Water, Sewage and Industrial

Wastes as including all of the aerobic and facultative anaerobic gram - negative non spore forming bacilli which ferment lactose with gas formation within 48 hours at 35°C (1). All organisms with such characteristics are now designated as members of the coliform group. The organisms of the coliform group are practically never dangerous in themselves, but as indicators of the probable presence of disease germs they are particularly significant.

Because of extreme variations in quality of waters, i.e., dissolved minerals and gases, turbidity, degrees of pollution, no one method has been developed that is fully satisfactory for the examination of all waters. However, a "standard" method has been developed and is generally accepted as standards for public water suppliers. These standards are published as U. S. Public Health Service Drinking Water Standards, 1946.

The custom has developed to report the results of the coliform test as a Most Probable Number (MPN) per 100 milliliters of sample.

The value or number obtained using the "Most Probable Number" method is defined by Hoskins and Butterfield as "that bacterial density, which if it had been actually present in the sample under examination, would, more frequently than any other, have given the observed analytical results". (7)

In 1915 M. H. McGrady pointed out that the frequency of appearance of gas producing organisms in the portion drawn from a sample is an exponential function of the number of such organisms in the sample. He further demonstrated that for any combination of results obtained by testing one or more portions of one or several

dilutions of a sample, a single Most Probable Number (MPN) per 100 ml of sample is the density of organisms most likely for that combination of results. This density may not correspond to the density actually present in a given sample, but in the long run the Most Probable Numbers will represent more closely than will any other series of numbers the densities of gas producing organisms in the sample examined.

In 1917, A. Wohlman and H. L. Weaver showed how, by a few approximations, McCrady's basic equation could be more readily solved. Also in 1917 M. Greenwood and G. U. Yule, apparently unaware of McCrady's work, produced a basic equation similar to the others. The solution of the various equations proposed for determining the MPN is q uite laborious, consequently tables of computed MPN's corresponding to various fermentation tube results have been prepared. A table prepared by J. H. Hoskins in 1933-34 indicating the MPN for evaluation of Coli-Aerogenes tests by Fermentation Tube Method provides for the widest variety of combinations of both dilutions and tubes.

This table is utilized in the current edition of Standard Methods for the Examination of Water, Sewage and Industrial Wastes.

The accuracy of the most probable number has been the subject of many studies, both experimental and mathematical.

Halvorson and Ziegler showed the accuracy of the MPN, when derived from single dilution results, is dependent on both the number of tubes used and on the bacterial density (8). In further stud

study they calculated the frequencies which different possible combinations of tube results would be obtained if they used 10 tubes of 3 dilutions each. They used a geometric series in the repeated examination of suspensions of various bacterial densities. From these frequencies and the Most Probable Numbers corresponding to various expected results, frequency distribution curves were drawn to show the frequency of occurance of percentage deviations of the Most Probable Number from the mode corresponding to the bacterial density. The results showed when three dilutions are used the accuracy of the observed Most Probable Number for the range considered, is almost independent of the bacterial density.

C. Eisenhart and P. W. Wilson showed that a closer accuracy of Most Probable Number may be obtained by assuming the logarithms of the Most Probable Number to be normally distributed with a median limiting standard deviation of 0.166, for ten tubes, about the logarithms of the assumed density than by considering the standard deviation of the MPN itself (9).

The aforementioned studies are only a portion of many made to determine the accuracy of the Most Probable Number which, at its best, represents the best estimate of bacterial density available when using this method.

III. CURRENT STANDARDS FOR MOST PROBABLE NUMBER METHOD

For current standards using the Most Probable Number Method for Department of the Army units, Department of the Army Technical Bulletin MED 229, 17 December 1957, (Sanitary Control of Water Supplies for Fixed Installations) provides a guide and standards for sanitary control of water supplies for fixed (military) installations.

The portion of this bulletin pertaining to bacteriological examination of water is as follows:

a. Procedure. For the procedures covering the bacteriological examination of water samples, seem TM 8-227/AFM 160-14, and Standard Methods for the Examination of Water and Sewage, Tenth Edition, 1955 (see app.).

b. Standard Portion. The standard portion of water for the application of the bacteriological test will be 10 milliliters.

c. Standard Sample.

(1) The standard sample for the application of the bacteriological test will consist of five standard 10-milliliter portions.

(2) Examination of 1.0 ml. and 0.1 ml. portions as outlined in paragraph 410, TM 8-227/AFM 160-14, may also be included. These portions, however, should not be used in the evaluation of finished-water quality. This procedure may be used to estimate the most probable number of bacteria in the raw or finished water. Tables for determining MPN are contained in the text, "Standard Methods for the Examination of Water and Sewage", Tenth Edition, 1955.

d. Reporting of Bacteriological Results.

(1) Laboratory results will be reported on DD Form 686, and will be forwarded to the medical officer submitting the sample with a duplicate copy furnished for the post engineer or installations engineer. This report shows

whether or not coliform bacteria were found in each portion of the sample tested, with pertinent remarks as outlined in paragraph 411, TM 8-227/AFM 160-14.

e. Interpretation of Results.

(1) The laboratory will not interpret the results of the bacteriological examination of water samples. This will be the responsibility of the medical officer submitting the sample and will be on the basis of the following minimum requirements:

(a) Requirement No. 1. Of all the standard 10-milliliter portions examined per month not more than 10 percent will show the presence of organisms of the coliform group. (For an immediate evaluation at any time during any current month, the preceding 30-day period will be used).

(b) Requirement No. 2. Three or more of the five portions of a standard sample may show the presence of organisms of the coliform group only in case the observation is not repeated in--

1. Consecutive samples taken at the same outlet.

2. More than five percent of the standard samples when 20 or more have been examined per month.

3. More than one standard sample when less than 20 samples have been examined per month.

(c) Special Samples. In event that coliform organisms are found in any of the samples examined, special samples will be collected from the same outlet in accordance with suggested remedial action plan outlined in paragraph 15.

(2) To illustrate how bacteriological results of water samples may be interpreted in accordance with the two minimum requirements, assume that at a certain installation 30 standard samples were collected during the month. Of this total, three samples were reported by the laboratory as having three positive portions

each. Since 30 standard samples consist of 150 standard portions, this would mean that nine portions or six percent of the total standard portions are positive. According to Requirement No. 1, this percentage falls within the limit of 10 percent. However, three of the standard samples out of 30 contained three positive portions each. This amounts to 10 percent which exceeds the limit of five percent as established in Requirement No. 2. The water supply at this installation has failed to meet the minimum requirements of bacteriological quality.

(3) When the membrane filter technique is used, procedures and reporting will be as described in the text, "Standard Methods for the Examination of Water and Sewage".

(a) The standard sample for this test will be not less than 50 milliliters, when the membrane filter procedure is used, which will be filtered through one or more membranes so that the total colony count (coliforms plus non-coliforms) on each filter will not exceed 400. The use of standard samples larger than 50 milliliters will provide more information than smaller samples, but the total colony count per filter will not in any case exceed 400.

(b) The sample must be freed of any disinfecting agent at the time of its collection.

(c) The arithmetic mean density of all standard samples examined per month by the membrane filter procedure will not exceed one per one hundred milliliters.

(d) Utilizing the membrane filter procedure, greater than the average number of coliform colonies will occasionally be found in a single standard sample. This will be permissible, provided the numbers of coliform colonies per standard sample are not greater than three per fifty milliliters, four per one hundred milliliters, seven per 200 milliliters, thirteen per five hundred milliliters, or 22 per one thousand milliliters in:

1. Any two consecutive standard samples.

2. More than five percent of the standard samples when 20 or more samples have been examined per month.

3. One standard sample when less than 20 samples have been examined per month. Provided further, that when one standard sample shows a larger number of colonies than are permissible, as indicated above, daily samples from the same sampling point will be collected and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.

CHAPTER III

THE MEMBRANE FILTER

The value of the membrane filter and its application in the field of water analysis and sewage disposal has been rather slow in being recognized. However, at present several agencies, including both private and governmental, are engaged in extensive research efforts to determine the many fields in which the membrane filter may be effectively utilized.

I. HISTORICAL BACKGROUND

More than sixty years ago Sanarelli performed a series of experiments using a membrane filter, essentially the same as the membrane filter in use today (10). However, H. Bechhald conducted the first systematic study of the physical-chemical properties of a number of such membranes (10).

Since 1911 several authors from different European countries have conducted investigations with respect to the properties of porous collodion membranes.

Zsigmondy and Bachman, 1916-18, contributed studies on the production methods of porous collodion membranes. These studies led to production of membranes on a commercial scale. For many years the Membran-Filtergeselbschaft, Sartorious Werke, Goettingen, Germany produced membrane filters. In 1922 Zsigmondy was granted a U. S. patent on his production methods.

Up until 1930 one of the primary difficulties encountered in producing the membrane filter was that of controlling the pore sizes

on individual filters. In the 1930's J. Elford of England and P. Grabur of France developed methods for the production of membrane filters with controlled pore sizes. From research records it appears that from the early 1930's until during World War II little effort was made toward industrial production of membranes except in Russia and Germany.

Shortly after World War II Dr. Alfred Goetz of the U. S. visited Germany under the auspices of the Joint Intelligence Objective Agency of the Armed Services. There he conducted an investigation of the German development of the membrane filter. The results of Dr. Goetz's investigation are contained in Fiat Final Report 1312 (11).

In 1955, the 10th edition of "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" included methods for testing for coliform bacteria by the membrane filter method. Use of the membrane filter is designated a tentative method in that publication.

On October 23, 1956, the Federal Government published a declaration of intent to amend the Drinking Water Standards under the Interstate Quarantine Regulations. "The Federal Register" published an amendment permitting use of the membrane filter procedure in the bacteriological examination of water subject to Federal Regulation. This proposed amendment became official March 1, 1957.

II. THEORY OF MEMBRANE FILTER

The membrane filter as used in water bacteriology is a thin cellulosic membrane disk about 0.15 millimeters in thickness and approximately 47 millimeters in diameter.

Procedures for production vary with the manufacturer, however, in general a cellulose ester such as cellulose nitrate is dissolved in a solvent. Water or some other liquid insoluble in the cellulose solution is added. This forms an emulsion having great uniformity in the distribution and size of droplets of the insoluble liquid. The emulsion is cast on plates and dried under rigidly controlled conditions with respect to temperature and humidity. The droplets of insoluble fluid retain their size and identity in the dried film and become the pores in the finished membrane. The dried porous film is then cut into disks of desired size.

The pores of the filter are esentially direct channels through the filter occupying from 80 to 85% of the total volume of the filter. The number of pores may be further demonstrated when it is noted there are approximately 50,000,000 pores of uniform size opening of 0.45 microns ($\pm$ 0.02 microns) per square centimeter of filter surface. Since it is difficult to visualize such small sizes as a comparison of bacteria size to pore size the majority of coliform fall with limits of 0.5 to 1.5 microns in size. Because of the high ratio of pores to solid matrix, the flow of liquid through the filter is rapid. Membrane filters are wettable and also free of soluble chemical substances inhibitory to bacterial growth. The pore openings

are approximately perpendicular to the filter surface area and are a bit smaller on one side of the surface than on the other. Consequently, when a liquid is strained through a filter, entering the small side of the pore opening, a 'screening action' is obtained.

Results of investigations by A. Goetz and Tsuneishi show that when the number of particles retained on a filter is plotted against total thickness of the filter, all visible particles are retained within the first 15 microns of the filter (10).

Basic procedure in the use of the membrane filter in coliform tests is:

a. A suitable volume of sample is strained through a membrane filter.

b. The filter is then placed on a pad saturated with a culture media.

c. The innoculated filter is incubated under prescribed conditions.

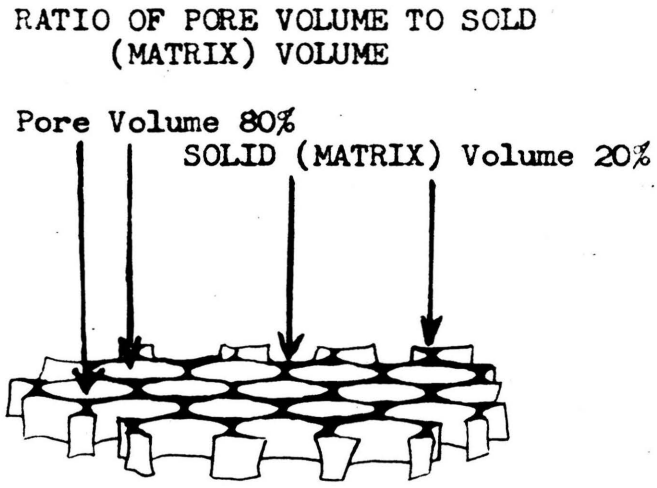
d. After incubation the number of coliform and non-coliform bacteria present in the sample are determined by a direct count of the number of colonies that developed on the inoculated membrane filter.

Two kinds of filters for bacteriological testing of water are presently available in this country. These are:

a. Millipore Filters, Type HA, white grid marked, 47 mm in diameter.

b. Bac-T-Flex Green Grid Flexible Membrane Filters.

Figure 1 presents a sectional view of a membrane filter.



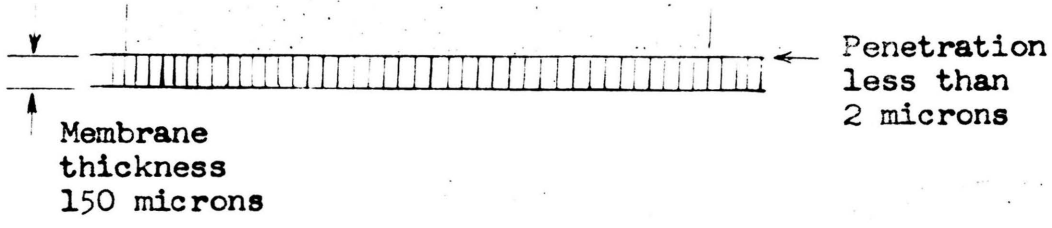


FIGURE 1

MICROSCOPIC AND SUBMICROSCOPIC PARTICLES
ARE "SCREENED" FROM FLUIDS PASSING
THROUGH A MEMBRANE FILTER

III. MEMBRANE FILTER EQUIPMENT

A portion of the items used in the examination of water by the membrane filter method are standard items used in normal bacteriological laboratory work. Other items used are designed for specific use in the membrane filter method.

Equipment utilized is as follows:

A. Filter Holding Unit

The filter holding unit is a device for supporting the membrane filter and for holding the sample until it passes through the filter. The holding unit is in two parts and during filtration the sample passes from the upper element to the lower element.

a. The lower element, or filter base, as it is often called, supports the membrane filter on a plate. This plate, about 50 mm in diameter, may be either fine screen or a porous disk that allows free passage of liquids. The outer edge around this plate is a smooth non-porous surface. When filtering a sample the membrane filter disk is placed on this porous screen with the outer edges of the disk covering the smooth non-porous surface around the edge of the plate. The lower element includes a fitting to permit the unit to be mounted in a suction flask.

b. The upper element, usually called the funnel, contains the sample until it is drawn through the membrane filter. The outer edge of the lower portion of this element is a flat ring that rests on the outer edge of the membrane filter disk and also directly over the smooth non-porous edge of the lower element.

c. The upper and lower elements are fastened together by a locking ring or clamps.

Several types of filter holding units have been commercially developed for use in the examination of liquids.

a. The "Coli 5" Apparatus (Figure 2a).

This is a metal unit with a sintered glass membrane support plate. Two interchangeable top sections are supplied and each one fits the lower element by means of a bayonet joint and locking ring. One top section is a short cylindrical element with a 30 ml capacity; the other is funnel-shaped, with about 800 ml capacity.

b. The Millipore Pyrex Filter Holder (Figure 2b).

This unit is made of glass, the upper element having a 250 ml capacity. The assembled filter holder is joined with a spring loaded clamp which engages on flat surfaces encircling the upper and lower elements.

c. Millipore Standard Hydrosol Filter Holder (Figure 2c).

Most components of this unit are made of stainless steel. The porous membrane support plate is either carbon or a fine-mesh stainless steel screen. The upper element is a cylinder 4 1/2 inches in diameter, constricted to a narrow cylinder at the bottom to fit the lower element. Capacity of the upper element is about 1 liter. The assembled filter holding unit is joined by a bayonet and locking ring.

d. The Sabro Membrane Filter Holder (Figure 2d).

This unit is primarily stainless steel in construction. The lower element is a stainless steel cup with a metal cover which acts as a combination suction chamber, filtrate receiver and filter supporting

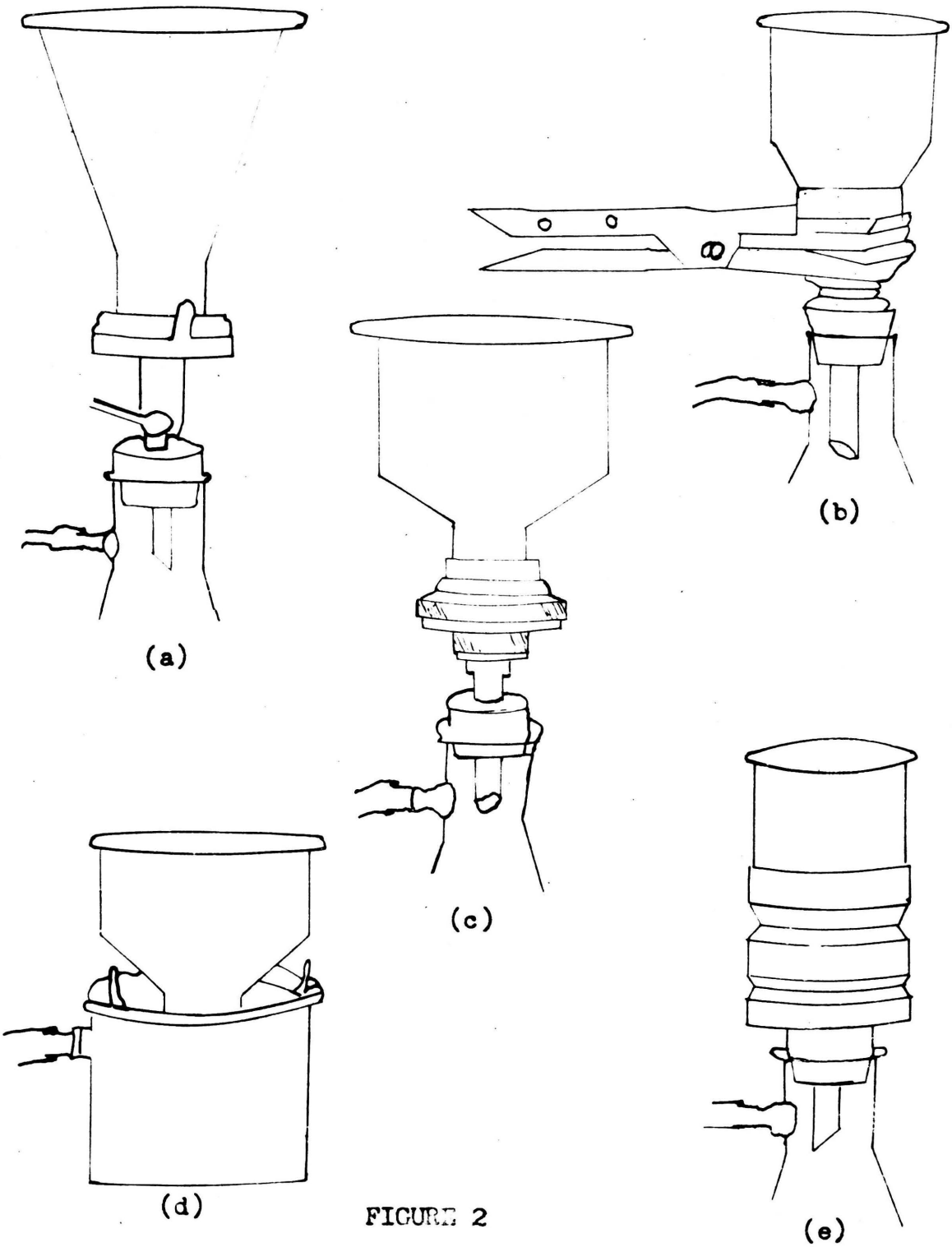


FIGURE 2

TYPES OF FILTER HOLDER UNITS
(a) COLI "5" UNIT; (b) MILLIPORE PYREX HOLDER; (c) MILLIPORE STANDARD HYDROSOL UNIT; (d) SABRO HOLDER; (e) ISOPOR UNIT

element. The cover is fitted with a rubber gasket to insure an air tight fit of the cover on the top of the cup. A porous sintered stainless steel membrane support disk is mounted in the center of the cover. A valve is located on the side of the cup to which a pumping device can be attached. Capacity of the upper element is about 500 ml. The assembled filter holding unit is joined by a locking ring at the base of the upper element, which engages on three spring clamps in the covering plate of the lower elements.

e. Isopor Membrane Filter Holder (Figure 2e).

This is a metal unit, with the upper element being made of a stainless steel cylinder with inner graduations of 50, 100, 250 and 500 ml levels. The upper element is also equipped with a locking ring for assembly of the unit for sample filtration. The membrane support plate in the lower element is a porous carbon disk mounted in stainless steel. An aluminum plate on the lower element serves as a cap when the unit is assembled for sterilization by the formaldehyde method.

For care and maintenance of the filter holding units it is recommended they be polished with a silicone preparation about every two months. This hydrophobic coating prevents metal or glass from being wetted and minimizes sample retention on surfaces of the filter holding assembly. SURGeoSIL or a comparable commercial product is a satisfactory silicone preparation. The metal portion of all components should be protected from scratches or other damage. Particular care should be taken with that portion of holding units that come into contact with the filter disk.

The locking rings used in some filter holding units have two or more small wheels or rollers which engage on parts of the filter holding assembly. Occasional cleaning or adjustment of these rings is necessary to insure that the rollers turn freely.

IV. FILTER EQUIPMENT ACCESSORIES

A. Culture Containers.

Most membrane filters are incubated in individual containers. Almost any type or form of culture container is acceptable if it is made of impervious, bacteriologically inert material. The prime requirement is the culture container should be large enough to permit the membrane filters to lie flat. The following are widely accepted:

1. Glass Petri Dishes — Conventional borosilicate glass culture dishes. For routine work the 60 mm x 15 mm petri dishes are ideal in size.

2. Metal Cans — One or two ounce metal ointment cans make very satisfactory culture containers. For field use culture containers of this type are particularly suitable. The cost of these containers is very reasonable and with reasonable care to prevent rusting, such boxes may be used up to a dozen times before they must be discarded.

3. Plastic Petri Dishes — Plastic containers of the proper size have been developed for use with the membrane filter. Their cost is reasonable, however, they cannot be heat sterilized and probable use on a single service basis is the most feasible.

B. Vacuum Facilities.

Water can be filtered through a membrane filter by gravity alone, but the filtration rate is too slow for practical purposes. For routine work in the laboratory an electric vacuum pump may be used.

A water pump, or the so called "aspirator" gives a satisfactory vacuum, provided a reasonably high water pressure is available. For use in the field a small hand pump, such as a bicycle pump, in which the leather valves have been reversed, works very well. This size pump is small and very convenient for carrying.

C. Suction Flask.

Several types of filter holding apparatus are fitted in a suction flask for sample filtration. For equipment that does not provide a suction flask, any size flask may be used, however, a one liter size is most satisfactory. Between the suction flask and the vacuum facility a piece of latex rubber tubing is used. A rubber tubing with a wall thickness of 3/32 inch will not collapse under vacuum.

If the vacuum device is operating continuously when connected to the suction flask it will be necessary to use a pinch clamp to cut off the vacuum during intervals when samples are not actually being filtered.

D. Ring Stand with Split Ring.

When the filter holding unit is disassembled after sample filtration, the worker's hands should be free to manipulate the membrane filter. It is desirable to have a convenient location to place the upper element during this operation. A split ring stand is very convenient for this purpose. The funnel element may be placed on the laboratory bench in an inverted manner, however, precautions must be taken to prevent bacterial contamination.

E. Graduated Cylinders.

Conventional 50 ml or 100 ml graduated borosilicate glass cylinders are satisfactory for measurement of samples greater than 20 ml.

F. Pipettes and Eye Droppers.

Graduated Mohr pipettes are used for measurement of small samples and for dispensing culture media. For laboratory work pipettes should be available in 1 ml and 10 ml sizes. For field use a small eye dropper is very satisfactory.

G. Forceps and Alcohol Jar.

All manipulation of membrane filters is with sterile forceps. For sterilization, both in the field and laboratory, forceps should be kept with their tips immersed in ethanol or methanol. When the forceps are used, they are removed from the container and the alcohol is burned off.

H, Dilution Water.

For rinsing the filter during filtration of samples, sterile buffered distilled water as described in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" for bacteriological examination of water is used in membrane filter methods. When operating in the field and buffered distilled water is not readily available, water that has already passed through a membrane filter may be used for rinse purposes between filtration operations.

I. Incubation Facilities.

Both temperature and humidity requirements must be recognized in the incubation facilities provided. Incubation temperatures

for cultivation of coliform bacteria on membrane filters must be at or near 35°C. Recently successful incubation has been demonstrated at 32°C. From a humidity standpoint, membrane filter cultures must be incubated in an atmosphere maintained at or very near 100% relative humidity. These temperature and humidity requirements can be satisfied in any of several types of equipment. A conventional type incubator may be used with a high degree of success. A covered vegetable crisper may be placed in the incubator and water saturated cotton kept in the crisper. The cultures are placed inside the vegetable crisper during incubation. A constant temperature water bath can be modified to make an excellent incubator. A belt type apparatus containing the cultures to be incubated may be worn around the waist to provide adequate temperature for incubation. Culture containers should be sealed or closed when worn around the waist. By virtue of being tightly closed when worn around the waist control of humidity does not present as great a problem.

V. STERILIZATION OF MEMBRANE FILTER EQUIPMENT AND ACCESSORIES

Recommended procedures for sterilization of the membrane f'lter and accessories are as follows:

A. Filter Holding Unit

The filter holding unit should be sterile at the beginning of each filtration series. Provided there is no unintentional contamination of the unit, the filter does not need to be sterilized again during a filtration series unless there is an interruption of thirty minutes or longer. Should such an interruption occur in a filtration series it is necessary then to sterilize the unit before reusing. After each filtration the funnel walls are flushed with sterile water to rid them of contamination. Repeated tests by competent authorities have shown that when the funnel walls are properly flushed with sterile water there is no cross contamination of later samples.

Several methods may be utilized to sterilize the filter holding unit. They are:

a. The preferred method is by sterilization in the autoclave. The funnel and receptable are wrapped separately in Kraft paper and sterilized in the autoclave for 15 minutes at 121°C. At the end of 15 minutes the steam pressure is released rapidly to encourage drying of the filter holding unit.

b. The unit may be sterilized by immersing it in boiling water 2 to 10 minutes.

c. The unit may be sterilized by holding it 30 minutes in a flowing steam sterilizer.

d. Some units (Millipore stainless unit and Isoper unit) are available with accessories permitting anhydrous sterilization with formaldehyde. Methanol is introduced into a porous plate and the filter holding unit placed over the ignited methanol. By closing the top of the unit the methanol is incompletely oxidized inside the holding unit, resulting in the generation of formaldehyde which is bactericidal. The filter holding unit is kept closed for at least 15 minutes to insure complete sterilization.

B. Sterilization of Membranes and Absorbent Pads.

Membranes may be procured in units of 100 in a package or in units of 10 in Kraft envelopes. If units of 100 membranes are used they should be repackaged into smaller lots prior to sterilization. Each membrane unit consists of one membrane filter and one absorbent pad. Several methods may be utilized to sterilize the membrane and pads:

a. Sterilization in the autoclave is preferred. Ten minutes at 121°C is recommended. After 10 minutes holding period the steam pressure is released rapidly to encourage drying of the membranes and absorbent pads.

b. In an emergency, membrane filters may be sterilized by immersion in boiling water for 10 minutes. When this method is used the membrane filters and absorbent pads should be separated so they will not stick to one another. The boiling water method is used only as an emergency means and should not be the general practice.

When packages of 100 units are repackaged in smaller units, it is general practice to put 10 to 20 membranes in a petri dish or ointment can and the like number of pads in a separate container for sterilization.

C. Glassware and Metal Ointment Cans.

Sterilization at 170°C for at least one hour is desirable for the glassware and metal ointment cans used. The opening of graduated cylinders should be covered with paper or metal foil prior to sterilization.

Glassware and "ointment cans" may be sterilized in the autoclave. They should be sterilized for 15 minutes at 121°C.

When the metal ointment cans are sterilized, it is best to loosen the top of each can before sterilization. After sterilization allow the cans to cool and replace the tops. The reason for this is quite often the ointment cans have lacquer on them and during sterilization it may soften and upon cooling tend to seal the lid of the can to the bottom.

D. Plastic Culture Containers

Plastic containers cannot be heat sterilized because of the thermolabile characteristics of the plastic. Plastic containers may be sterilized by immersion in a 70% solution of ethanol in water for at least 30 minutes. Exposure to ultraviolet light is another method of sterilization of plastic culture containers.

VI. CULTURE MEDIA USED WITH MEMBRANE FILTERS

Filtration of a water sample through a membrane filter results in particles of suspended matter and bacteria being retained on the filter surface. When a suitable culture medium is made available for growth the bacteria suspended on the filter can be grown in place. The bacteria are cultivated by placing the membrane filter on an absorbent pad that has been saturated with a suitable medium. The culture medium diffuses through the pores of the filter and when combined with proper temperature, time and humidity requirements results in the development of the bacterial colony. In principle each bacterial cell originates a single bacterial colony.

Due to the selective absorptive property of the membrane filter some culture media that has proven satisfactory for the cultures of agar plate cultures does not perform well when used with membrane filters. In the process of diffusion through the filter pores some components of the culture medium may be reduced in concentration or completely removed. This results in a different composition of culture medium at point in which it is available for bacterial growth than it was when first introduced to the absorbent pad under the filter.

Culture media for use with membrane filters have been developed for the following purposes:

a. "Total" bacterial counts
b. Media for bacterial indicators of pollution
c. Presence of coliform organisms
d. Presence of enterococcus group

Membrane filter media for the development, differentiation and enumeration of special groups of bacteria are based on a differential

principle, i.e., one in which the bacteria favored for a particular media are easily identified. To accomplish this principle, components of a differential medium for membrane filter cultures includes the following:

a. Substances favoring growth of the bacteria for which medium is designed. This includes the addition of yeast or meat extracts, fermentable carbohydrates and peptones.

b. Selective inhibitors that will prevent growth of the maximum number of the bacteria in which there is no interest as to quantity or type and at the same time have a minimum adverse effect on growth of the kind of bacteria for which the medium is designed.

c. A differential indicator system. The purpose of the indicator system is to cause the desired bacterial groups to produce characteristic colonies that may be easily recognized when present on a filter containing several types of bacterial colonies. This differentiation is accomplished by including in the medium a substance which is chemically changed by the organisms to be differentiated and which gives visible evidence of this differentiation so that each bacterial colony may be easily identified. The normal procedure is to include a pH indicator in the medium. The accumulation of organic acids in the bacteria changes the pH of the bacterial colony resulting in a change in color of the colony and surrounding culture medium.

There are several methods of using media with the membrane filter. They are:

a. A single stage medium. After filtration of a water sample the membrane filter is placed on a saturated absorbent pad and

left there throughout the incubation period. During incubation the medium diffuses through the pores of the membrane to cultivate growth of the bacteria. Upon completion of incubation the cultural results are examined and interpreted directly.

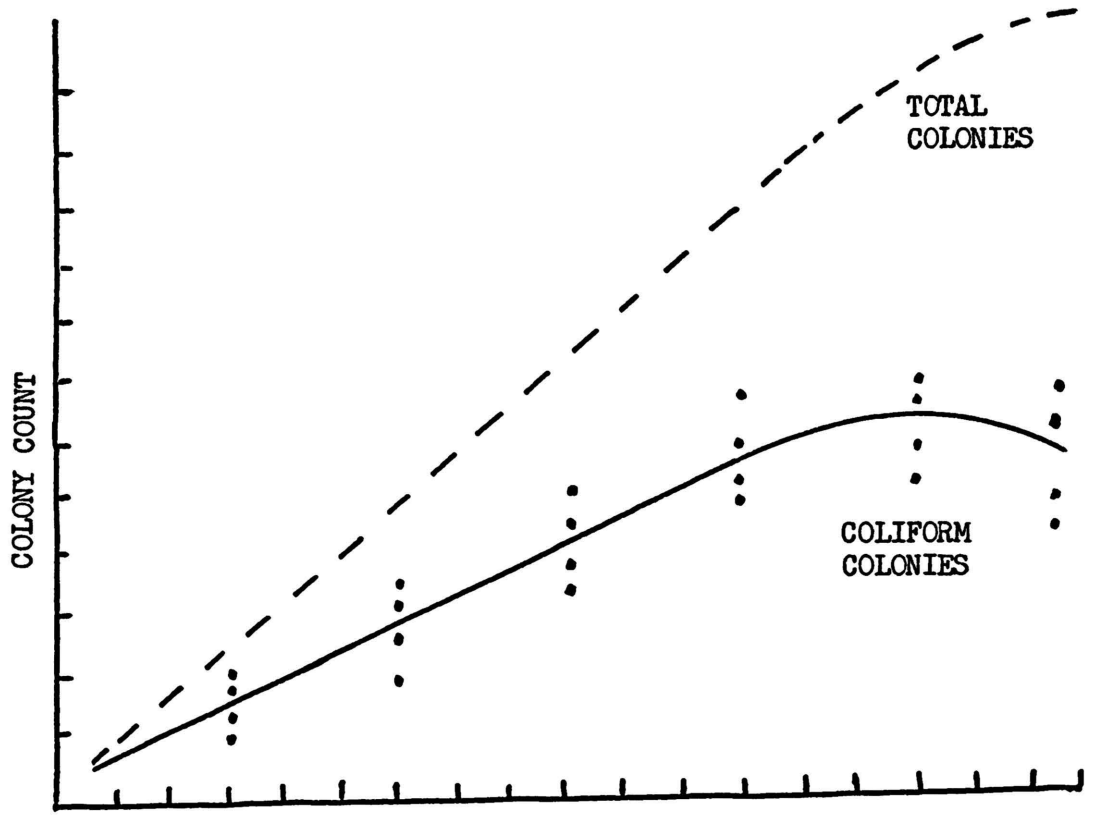
b. A two stage medium. After filtration of a water sample the membrane filter is placed on an absorbent pad that has been saturated with an enrichment type medium. Its purpose is to furnish all the bacteria an enriched nourishment during the early stages of growth. After the membrane filter has been incubated on the enriched medium for approximately two hours it is transferred to another pad that has been saturated with a differential medium and the incubation is continued. A fact unique with membrane filter methods is the membrane filter can be transferred from one culture medium to another without disturbing the bacteria colonies that are on the filter. Any number of transfers may be made as long as the surface of the filter is not disturbed.

c. A multi stage media involves the use of multi stage techniques. After filtration of a water sample the membrane filter is temporarily placed on an absorbent pad that has been saturated with a bacteriostatic agent. In presence of such a substance bacterial growth is inhibited, or slowed greatly, but the organisms are not killed. This type medium is particularly useful when it is desired to mail or transport the membrane filter a distance before normal incubation is begun. After the desired non-growth period is concluded the membrane filter is then transferred to a pad saturated with a development type medium, either one or two stage, and incubated.

VII. SAMPLE FILTRATION VOLUMES

A wide range of sample volumes can be tested using the membrane filter technique. Generally, the only limitations on sample volumes are the amount of suspended matter in the sample and the bacterial density.

While this method of testing does lend itself to a range of volume samples the graph in Figure 3 illustrates the characteristic pattern of colony counts when replicate filtrations are made of a series of sample volumes in coliform tests with EHC Endo Medium (12).



Courtesy Public Health Service

The line designated "Total Colonies" in Figure 3 includes both coliform and non-coliform colonies. The line designated "Coliform Colonies" refers only to differentiated colonies having the typical color and sheen characteristics of coliform colonies on the medium. For a portion of the range of sample volumes there is a proportional relationship between sample volume and colony count. This relationship fails at higher colony counts. With total colony count this failure can be attributed to simple crowding of bacteria on the membrane filter. With the coliform colony count the failure of the relationship is attributed to crowding of the colonies and also the supression of sheen production of coliform colonies due to the presence of excessive numbers of non-coliform colonies.

In waters containing a relatively large amount of suspended matter and a low bacteria count, difficulties can be encountered. The suspended material is deposited on the filter during filtration and when incubated, results in development of colonies tending to run together. This makes counting difficult or even impossible. The most satisfactory solution in this situation is filtration of a desired sample volume in several increments.

Occasionally samples of water will appear to be relatively free of suspended matter but upon filtration the pores block or clog very quickly. This is due to the presence of suspended matter of colloidal size and again the most satisfactory solution 's to filter samples in small increments.

For compliance with Federal Interstate Quarantine Drinking Water Standards, the standard sample volume is defined: "The standard sample for the bacteriological tests shall consist of not less than fifty milliliters, when the membrane filter procedure is used, which shall be filtered through one or more membranes so that the total colony count (coliform plus non-coliforms) on each filter shall not exceed 400. The use of standard samples larger than fifty milliliters will provide more information than smaller samples but the total colony count in any case shall not exceed 400". (13).

For compliance with Interstate Drinking Water Standards, the arithmetic mean density of all standard samples examined per month must not exceed one coliform per 100 milliliter of sample examined.

When testing waters known to contain coliform bacteria consideration must be given to filtration of a sample volume that will produce less than 400 total colonies and also permit easy identification of the differential coliform colonies.

Reliable quantitative results are obtained when sample volume sizes are selected that result in the production of 20 to 60 coliform colonies and not more than 250 to 350 total colonies. With a minimum of 20 coliform colonies on a single membrane filter the statistical error due to random sampling variations will be reduced. With an upper limit of 60 coliform colonies per membrane filter a distinct colony differentiation is present.

When testing treated water it will probably not be possible to have high coliform colony counts; in those cases it is recommended that 200 ml, or 300 ml or 100 ml be filtered. For unpolluted surface water a volume in the range of 0.01 ml to 10 ml should be filtered.

If previous bacteriological data are available, then sample filtration volumes can be computed.

1. First, determine the arithmetic means of total bacterial counts known from previous data. Then determine the volume of sample, which on the average can be expected to produce 20 coliform colonies. This is the Basic Test Quantity (BTQ).

Example: Previous data shows that a given source has an average coliform density of 160 per 100 ml.

$$\frac{20}{\text{Av. Coliforms per 100 ml}} \times 100 = \text{BTQ}$$

$\frac{20}{160} \times 100 = 12.5$ ml. This is the Basic Test Quantity

2. If three samples are to be filtered (preferred), then filter 1/3 the BTQ, the BTQ and three times the BTQ.

Thus, from the example in 1, above:

The computed amounts for filtration are 4.2 ml, 12.5 ml and 37.5 ml. For convenience in measurement, filter 4 ml, 12 ml and 37 ml.

3. If two sample volumes are to be filtered, then filter 1/2 the BTQ and 1.5 times the BTQ.

Thus, from the example in 1, above:

The computed amounts for filtration are 6.3 ml and 18.7 ml. For convenience, filter 6 ml and 19 ml.

4. If one filtration is to be made (not generally recommended), filter only the amount determined to be the Basic Test Quantity.

Thus, from the above example:

Filter 13 ml. (rounded off from 12.5 ml)

VIII. COLIFORM COUNTING PROCEDURES

The technique of counting coliform colonies on a membrane filter can be accomplished by any person with a minimum of training. Membrane filters manufactured by Millipore Filter Corp., subdivide the effective filtering area into squares equal to 1/100 the effective filtering area. (9.6 cm^2 for 47 mm diameter filters). Grid imprinted Bacti-Flex membrane filters, manufactured by Schleicher and Schuell, subdivide the effective filtering area into squares equal to 1/20 the effective filtering area.

The grid lines imprinted on these membranes can be used as guide lines in counting. On membrane filters in which the colony count is high and well distributed over the entire surface of the filter, the colonies on a selected number of grid squares may be counted and extended to a figure representing the number covering the entire filter area.

Plain membranes can be purchased, however, caution must be utilized in counting colonies on a plain membrane because of duplicate counting of individual colonies. Also, there is no means for estimating the colony count on a plain membrane.

For best results in counting the colonies the surface of the membrane filter should be perpendicular to an overhead light source. Then by observing each colony from an angle the colonies with a metallic luster (sheen) will be easily distinguished. All colonies which present a sheen appearance, even a "pin point" amount, should be counted as coliform colonies. A small hand magnifying glass will aid materially in counting the colonies.

Counting difficulties most often experienced by untrained personnel are:

a. Water condensate confused as bacterial growth.

b. Turbidity particles confused as colonies.

c. Difficulty in counting confluent colonies.

Colony counts from individual filtrations on membrane filters will represent the number of coliform per volume of sample filtrated. However, all recording of results should be extended to indicate the proportionate number of coliform per 100 ml for that particular volume sample.

CHAPTER IV

DISCUSSION OF EXPERIMENTS

The purpose of this investigation was to determine the feasibility and reliability of using the membrane filter method for field determination of the quality of a water. A realistic approach as possible was undertaken during the experimental phase. A United States Army Engineer Unit stationed at Fort Leonard Wood, Missouri, established a series of water points thereby providing treated water as well as raw water from which sample volumes could be obtained for the experiments. The water points were established by the 62nd Engineer Battalion (Constr.) and were located at different sites on the Big Piney River; all points established being within the physical boundary limits of Fort Leonard Wood.

The equipment used for treatment of the raw river water was a portable water purification unit consisting of a diatomite filter, capacity of fifty gallons per minute, and accessories. This item of equipment is a standard item of equipment authorized for engineer units that are responsible for furnishing potable water to troops in the field. The personnel that operated this equipment during the testing period were those normally assigned as operators of the purification equipment. There was no special training provided for the personnel for operation of the equipment during the tests.

To provide an additional source of raw water, experiments were also performed on sample volumes taken from the Little Piney River at Newburg, Missouri.

I. EQUIPMENT USED FOR THE EXPERIMENTS

The equipment used for conduct of the membrane filter test was:

a. One Millipore Hydrosol Standard Filter Holder.

b. A Suction Flask, one liter in size.

c. A Vacuum Source. For work in the field a hand operated vacuum pump was used.

d. Water Sample Bottles. Those used were of a wide mouth variety and had either screw caps or gound glass stoppers.

e. Dilution and Rinse Water Bottles. The dilution and rinse water bottles were of a resistant glass with glass stoppers or screw caps.

f. Pipettes, Graduated Cylinders and Eye Droppers. Graduated pipettes 2 ml and 10 ml in size were used. The graduated cylinders used were 25 ml and 50 ml in size. In the field a glass eye dropper was used in lieu of a 2 ml pipette.

g. Containers for Culture Medium. The culture medium was mixed and transported in 30 ml capacity glass bottles with screw caps.

h. Culture Containers. Glass petri dishes 60 by 15 mm were used. Also round two ounce metal ointment cans were used.

i. Rubber Tubing. Latex rubber tubing with a 3/16" inside diameter and 3/32 inch wall thickness was used.

j. Small alcohol jar with forceps. All manipulation of membrane filters was done with forceps. During a filtration series the tips of the forceps were immersed in methanol and when ready to be used the alcohol was burned off the tip.

k. Ring Stand with Split Ring. During a filtration series the split ring was used to hold the funnel in an inverted position while removing or placing a new membrane filter on the lower element.

l. Gas burner or alcohol burner. Used to burn the methanol off tips of the forceps.

m. Incubation Facilities. Two methods of incubation were used during the experiment. One was a standard laboratory incubator and the other was a belt worn around the waist. A vegetable crisper containing cotton saturated with water was placed in the incubator to provide 100% humidity.

n. Small Hand Magnifying Glass. An aid in counting coliform colonies on incubated membrane filters.

o. Light Source. For examination and counting of incubated cultures in the field a flashlight was adequate. In the laboratory a fluorescent light was used.

II. MATERIALS

Materials used during conduct of the test were:

(a) Membrane Filter.

The membrane filters used for the tests were Millipore Filters, type HA, pore size 0.45 micron $\pm$ 0.02 micron, grid marked, white, 47 mm in size.

(b) Absorbent (Nutrient Pad).

White absorbent pads the same size as the membrane filters and provided by the Millipore Filter Corporation were used.

(c) Buffered Distilled Water.

Buffered distilled water as described in Standard Methods for the Examination of Water, Sewage and Industrial Wastes for bacteriological examination of water was used as rinse water between sample filtrations.

(d) Methanol.

Used in the field for sterilization.

(e) Culture Medium.

All experiments were performed using a commercial, single stage, dehydrated medium prepared by Difco Laboratories. The ingredients per liter of the medium, M-Endo-MF, are as follows:

Bacto - Yeast Extract	1.5 g
Bacto - Casitone	5.0 g
Bacto - Thiopeptone	5.0 g
Bacto - Tryptose	10.0 g
Bacto - Lactose	12.5 g
Sodium Desoxycholate	0.1 g
Dipotassium Phosphate	4.375 g
Monopotassium Phosphate	1.375 g
Sodium Chloride	5.0 g
Sodium Lauryl Sulfate	0.05 g
Sodium Sulphite	2.1 g
Bacto Basic Fuchsin	1.05 g

(f) Ethanol.

Ethanol was combined with distilled water and the dehydrated medium to furnish the liquid medium.

III. PROCEDURE FOR EXPERIMENTS

The 62nd Engineer Patallion (Constr.) established a water point at various times between 1 October 1958 and 30 March 1959. Each time a complete portable treatment plant was established. Sample filtration volumes were taken from both the raw water and water that had been treated. A complete treatment unit was established each time a treated water sample volume was to be filtrated. This was done in an attempt to insure the treated water would be the same product as that produced for actual troop use in the field or in a civil disaster emergency. The plant operators continuously checked the residual chlorine content of the finished water to insure it met required standards.

Two methods were alternately utilized in sterilizing the filter holding unit. When sterilized in the laboratory the filter unit was wrapped in Kraft paper and sterilized in the autoclave 15 minutes at 121°C. The upper and lower elements were wrapped separately. When the filter unit was sterilized on the site in the field a small amount of methanol was poured on the wick in the sterilizer base. The methanol was lighted and the upper element placed over the base. The lower element was screwed into the cover and the cover placed on the upper element. The lower element that was screwed into the cover was then inside the upper element. With the unit closed the methanol was not completely oxidized inside, resulting in the formation of formaldehyde which is bactericidal. The filter holding unit was kept closed for 15 minutes to insure sterilization. In Figure 4 the filter holding unit is assembled for sterilization in the field.

FIGURE 4

FILTER UNIT IS PREPARED FOR STERILIZATION BY USING METHANOL. THE FUNNEL ELEMENT IS PLACED IN THE STERILIZER BASE AND THE COVER PLACED ON THE FUNNEL.

All glassware was sterilized for not less than one hour at 170° C. Petri dishes were wrapped in groups of three for convenience. Graduated cylinders and pipettes were wrapped individually prior to sterilization.

Ointment cans were sterilized for at least one hour at 170°C. with the tops of the cans loosened and placed in the sterilizer separately. The reason for this being the cans are lacquered and if they are left closed during heat sterilization the lacquer softens and tends to stick the covers to the bottoms of the cans. Immediately after the cans were cool the tops were replaced and the cans wrapped in groups of three for convenience.

The medium was prepared according to the manufacturer's directions. 48 grams of dehydrated medium added to one liter of distilled water containing 10.5 grams of ethanol constitute one liter of liquid medium. For ethanol, commercial grain alcohol (190 proof) was used. To sterilize the mixture it was heated to boiling.

Membrane filters were procured from the manufacturer in packages of one hundred each. The packages were broken down into quantities of ten membrane filters per three ounce ointment can and ten absorbent pads per three ounce ointment can. The entire group of one hundred filters and one hundred absorbent pads (twenty ointment cans) were sterilized at one time. By keeping the containers closed until the items were used in the field it was not necessary to sterilize them again. The membrane filters and absorbent pads were sterilized by placing them in the autoclave for ten minutes at 121°C. After sterilization the steam pressure was released rapidly to assist in drying the filters.

Upon reaching the test site in the field the following procedure was followed:

(a) A flat working surface was selected. This varied from a small folding table to a large log or bed of a truck.

(b) Placed in a row the sterile culture containers to be used for that series of tests. They were ointment cans, petri dishes, or a combination of the two. Figure 5 depicts the layout of a typical setup for a filtration series.

(c) Using a wax pencil the culture containers were numbered to correspond with the membrane filter numbers shown on the data sheet.

(d) A small bottle of methanol was set out and the tips of the forceps remained immersed in this when not being used. A small amount of methanol was poured on the wick of the sterilizer base and lighted.

(e) The forceps were removed from alcohol and ignited to burn off the alcohol. Then using the sterile forceps for manipulation, one sterile absorbent pad was placed in each culture container.

(f) Using a sterile eye dropper (or 2 ml pipettes) about 2 ml of culture medium was placed on each absorbent pad. Sufficient medium was applied so that when the culture container was tipped, a good sized drop of culture medium freely drained out of the absorbent pad. Each culture container remained covered except when it was necessary to have it removed for work with that particular container.

(g) The filter holder suction flask was placed in a suitable working space and the rubber hose from the vacuum pump attached.

(h) The lower element of the filter unit was unwrapped and placed in the vacuum flask.

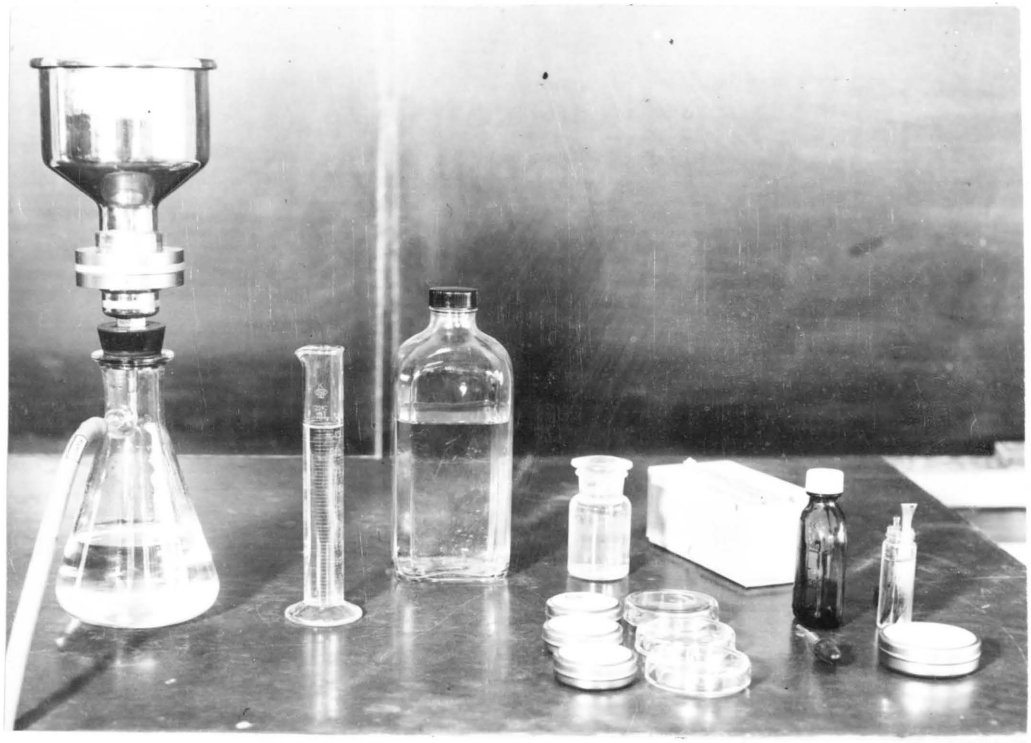


FIGURE 5

LAYOUT OF EQUIPMENT FOR A FILTRATION SERIES

(i) Using sterile forceps, a sterile membrane filter, grid side up was placed on the lower element of the filter holder. The upper element was joined to the lower element by turning the locking ring.

(j) The measured test volume was delivered into the funnel. If the sample volume was less than 10 ml, it was preceded by about 10 ml of sterile water. When the sample was 10 ml or larger it was not necessary to use sterile water first.

(k) The suction pump was operated to aid filtration of the sample through the membrane filter.

(l) The funnel walls were rinsed with approximately 20 ml of sterile water.

(m) The upper element was unlocked and placed in an inverted position.

(n) Using sterile forceps the membrane was placed on the appropriate saturated absorbent pad. The membrane filter was rolled on the saturated pad to avoid entrapment of air bubbles between the membrane and the underlying absorbent pad.

(o) The remaining filtrations in the series were completed using the same procedure as described. When there was a delay between successive filtrations of thirty minutes or longer the filter element was resterilized.

(p) The culture containers were inverted and placed in the incubator. If the containers were to be worn around the waist, they were placed in the belt.

(q) After incubation for 20 hours, ± 2 hours, the culture containers were removed from the incubator or waist, examined and the coliform colonies counted. When counting the coliform colonies in the laboratory a fluorescent lamp was used as shown in Figure 6.

In counting the coliform colonies each filter was counted twice. Between counts the filter was rotated one half turn. The value of using a small hand magnifying glass is demonstrated in Figure 7.

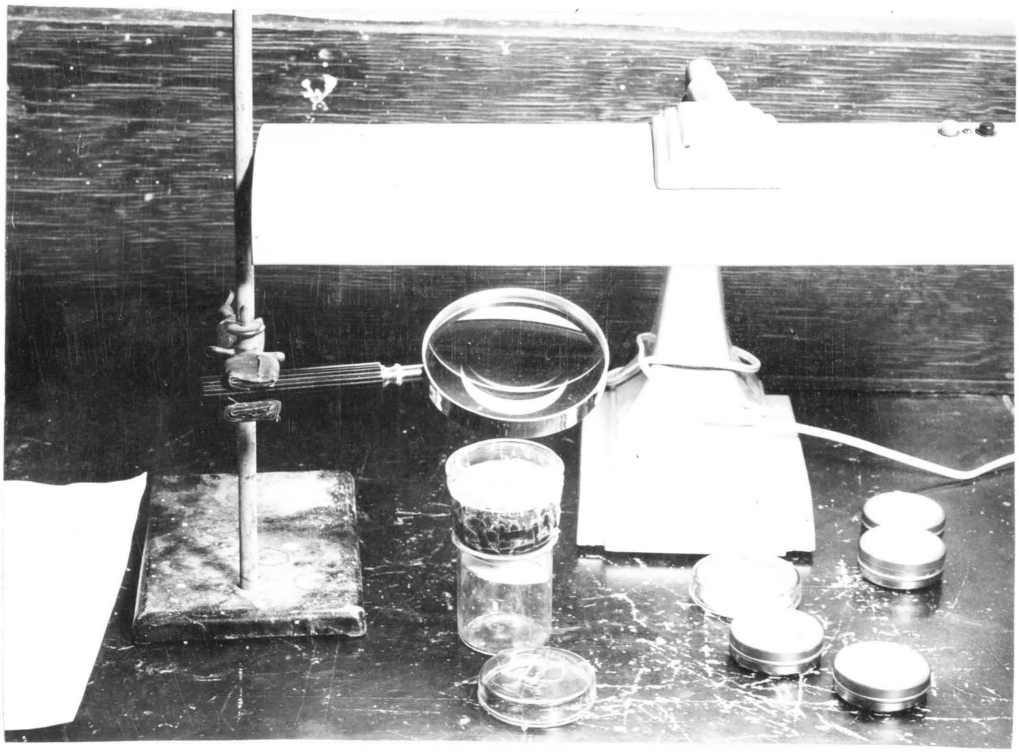


FIGURE 6

EQUIPMENT LAYOUT FOR COUNTING COLIFORM COLONIES

FIGURE 7

USING A MAGNIFYING GLASS TO AID
IN COUNTING COLIFORM COLONIES

CHAPTER V

DISCUSSION OF RESULTS

The tests were conducted over a period of eight months. This period of time permitted the membrane filter method to be used in the field during all types of weather, thereby permitting judgment of its performance under varying conditions. Performance can be judged not only from the effect of changing conditions of raw water in a stream but also the effect of severe cold on the filtration process when it is performed in the field.

Forty three filtrations of treated water produced by the 62nd Engineer Batallion (Constr.) were analyzed. The sample volume of each filtration varied from 100 ml to 600 ml. The membrane filter showed no evidence of clogging of the pores when filtering sample volumes of 600 ml. The plated membranes were incubated in both petri dishes and ointment cans using the conventional incubator and body as sources of heat. No coliform colonies were detected on any of the incubated cultures.

The results of the tests performed on raw water can best be analyzed from the following aspects.

(a) Culture containers

(b) Culture medium

(c) Incubation methods

(d) Turbidity, freezing and photographing.

I. CULTURE CONTAINERS

Two types of culture containers were utilized. They were 15 by 60 mm glass petri dishes and two ounce ointment cans. During the incubation period the membrane filter cultures must be incubated at or very near 100% relative humidity. The glass culture dishes fit very loosely when they are closed, so unless the individual dishes are sealed, control of the humidity becomes an important item. It is feasible to seal individual petri dishes and incubate them in a thermos bottle (14). However, to accomplish this in the field on a permanent basis would involve additional work as well as additional materials and time.

Two ounce ointment cans were utilized in conjunction with glass petri dishes. The results of the separate tests are tabulated in Table I. Duplicate sample volumes were run in a series of tests. In general the average coliform count per 100 ml of sample volume on cultures incubated in a petri dish was slightly higher than the colony count of those cultures incubated in an ointment can.

The metal ointment cans containing the cultures were incubated by two methods. One plated membrane filter was placed on a saturated nutrient pad in an ointment can and placed in a conventional incubator. Since the can was tightly closed it was not placed in the vegetable crisper inside the incubator to insure a surroundirg atmosphere near 100% relative humidity. The duplicate plated membrane filter of this series was placed on a saturated nutrient pad in an ointment can. The can was then closed and placed in a belt and worn around the waist for

TABLE I

COMPARISON OF COLIFORM COLONY COUNTS WHEN CULTURES ARE INCUBATED IN DIFFERENT TYPE CONTAINERS

SERIES NUMBER		PETRI DISH	OINTMENT CAN
		(No. of coliform colonies per 100 ml sample volume)	
1	Big Piney River	487	358
2	Big Piney River	128	112
3	Big Piney River	121	120
4	Big Piney River	174	166
5	Little Piney River	23	24
6	Little Piney River	24	22
7	Little Piney River	28	23
8	Little Piney River	26	25
9	Little Piney River	21	22
10	Little Piney River	6	4
11	Big Piney River	4	5
12	Big Piney River	54	59
13	Big Piney River	112	106
14	Little Piney River	81	80

20 hours. The belt is an insulated device made of cotton cloth and fastens around the waist with three snaps. On the inside front of the belt are two pockets, i.e., one on each side, each of which will hold three two ounce ointment cans. A flap with a snap on it is provided to keep the ointment cans in the pockets. The cans are placed next to the body when the belt is worn. The only insulation between the cans and body is one thickness of cloth that forms the pocket, however, that portion of the belt covering the outside of the cans is insulated with a layer of cotton. This is to maintain the temperature as constant as possible and also as near body temperature as possible. Figure 8 demonstrates a belt containing ointment cans being worn around the waist. When wearing a belt containing two or four ointment cans there is no discomfort. However, when wearing a belt containing six ointment cans there is a slight discomfort during sleeping hours. Since the belt must be worn for approximately twenty hours a portion of the wearing time will be during hours of normal sleep.

Table II presents a comparison of a series of duplicate filtration volumes in which the membrane filters were incubated in ointment cans, one in a conventional incubator and the other by body temperature.

The number of coliform colonies developed on the cultures that were incubated by wearing them around the waist is slightly higher than the number produced on the duplicate filters that were incubated in the conventional incubator. This increase in number of colonies per 100 ml of sample volume may possibly be due to a lower incubation temperature when the culture is incubated by wearing it around the

FIGURE 8

AN INCUBATION BELT BEING WORN AROUND THE WAIST

TABLE II

COMPARISON OF THE NUMBER OF COLIFORM COLONIES PER 100 ml OF SAMPLE VOLUME FOR A SERIES OF DUPLICATE FILTRATIONS

SERIES NUMBER	METHOD OF INCUBATION OF OINTMENT CAN CONTAINING CULTURE	
	Conventional Incubator	Belt around Waist
1	358	359
2	125	117
3	133	200
4	27	23
5	20	24
6	21	26
7	28	24
8	25	20
9	91	97
10	55	62
11	118	132
12	82	81

waist. Through normal body movement and during periods of changing clothes there may be short increments of time when a portion of the maximum amount of body heat is lost through ventilation and not transferred to the culture container. The fact that higher coliform counts have been indicated when an incubation temperature of 32°C is compared with 35°C has been recognized by other personnel doing research work with the membrane filter (14).

II. CULTURE MEDIUM

An ideal liquid culture medium is one in which the medium will retain the ability to perform its function regardless of the physical hazards it may undergo. A culture medium that is to be included in a field kit should continue to give good performance after freezing and thawing prior to use, withstand aging, and exposure to heat and sunlight.

The culture medium used for this series of experiments was a commercial dehydrated medium, Difco's M Endo MF. To determine its performance after freezing and thawing a series of two freeze-thaw experiments were performed.

50 ml of fresh liquid medium was prepared and then 25 ml of this portion was frozen for a three hour period. The frozen medium was then thawed and a duplicate set of three filtrations each were performed using the frozen-thawed medium for one-half the filtrations and the non frozen medium for the other half. To provide for uniformity during incubation, all the cultures were incubated in metal ointment cans in a conventional incubator. Detailed examination of all incubated cultures revealed very little, if any, difference in the development of coliform colonies on all the cultures. Also the difference in the number of coliform colonies developed on the plated membranes using the frozen-thawed medium was approximately the same as the number that developed on the duplicate membranes using the non-frozen medium. Results of these tests are tabulated in Table III.

TABLE III

RESULTS OF DUPLICATE FILTRATIONS USING A FROZEN-THAWED MEDIUM AND A NON-FROZEN MEDIUM

Series Number	Average coliform colonies per 100 ml sample volume	
	Non-frozen Medium	Frozen-thawed Medium
1	197	174
2	221	207

TABLE IV

AVERAGE COLIFORM DENSITIES FOR A SERIES OF FILTRATIONS USING FRESH AND OLD MEDIUM

Series Number	Age of Medium				
	Fresh	2 days old	3 days old	4 days old	5 days old
1	24				
2	23	22			
3	25		11		
4	26			7	
5	22				1

To determine the ability of the liquid culture medium to perform its function as it ages a series of duplicate filtrations were performed. In one set fresh culture medium was used and in the duplicate set a fresh medium was used the first day and portions from this same culture medium were used on succeeding days. Thus, for each calendar day the liquid culture medium was one day older. The number of coliform colonies developed on both sets of filtrations on the first and second day did not vary much. However, on the third day, the number of coliform colonies developed on the plated membrane filters using the old medium was substantially lower than the number developed on plated membrane filters using the fresh medium. When using a five day old culture medium there was an average of only one coliform colony per 100 ml of sample volume developed on a membrane filter compared with an average of twenty-two colonies per 100 ml sample volume when using a fresh culture medium. Results of this tests are indicated in Table IV.

III. INCUBATION

The two methods of incubation were observed very closely for the value of the membrane filter method in the field would be enhanced considerably if body incubation of cultures was feasible. The method of wearing the ointment cans around the waist has already been described. The number of coliform colony counts per 100 ml of sample volume on a series of duplicate filtrations is illustrated in Table V. The temperature of the conventional incubator was maintained at 35°C for a portion of the series and at 33°C for the remainder of the tests.

TABLE V

COLIFORM COLONY COUNTS PER 100 ML SAMPLE VOLUME FOR DUPLICATE VOLUME FILTRATION'S

Temperature of Laboratory Incubation in °C	Ointment cans containing cultures incubated by	
	Conventional Incubator	Body Incubation
35°	376	333
35°	330	383
35°	105	114
35°	116	121
35°	132	140
35°	180	223
35°	205	218
35°	13	14
35°	15	16
35°	21	26
35°	25	20
33°	20	24
33°	27	25
33°	28	24
33°	27	20
33°	45	45
33°	168	163
33°	90	102
33°	53	87
33°	90	83
33°	74	72

In general, the total number of coliform colonies that developed on a plated membrane incubated at 35°C was slightly less than the number that developed on a duplicate plated membrane that was incubated by body temperature. This was also the case in the duplicate filtrations that were incubated at 33°C and by the body. However, as shown in Table V there were isolated cases in both instances in which the opposite was noted.

IV. TURBIDITY, FREEZING AND PHOTOGRAPHING

During the experiments conducted turbidity or algae growth never presented any problem. In some research work this factor has been reported as a disadvantage in the use of the membrane filter (15). The highest turbidity encountered during the tests was 65 parts per million. In general the turbidity range of the Big Piney and Little Piney Rivers ranges from 10 to 15 parts per million.

Experiments were conducted in open weather during December and the air temperature ranged as low as 15°F. When the temperature was this low a plated membrane filter would freeze while transferring it from the filter unit to a saturated absorbent pad. When an attempt was made to roll the filter onto the pad it broke in half. This same effect was encountered in an air temperature of 29°F with a slight wind blowing. Any tests performed in the field should be conducted in a sheltered area in which the air temperature is above 32°F.

Incubated membrane filters can be photographed and retained as permanent records. Figures 9 and 10 are photographs of a sample volume taken from the Little Piney River on 29 April 1959. The photograph in Figure 9, is an ordinary photograph. The coliform colonies with their characteristic metallic sheen appear to stand out more than the non-coliform colonies, however, because of light reflection from small particles of turbidity and some non-coliform colonies it is difficult to accurately count each coliform colony in the photograph. In an attempt to eliminate all false sheen appearing colonies in a photograph a red filter placed in front of the camera lens was used.

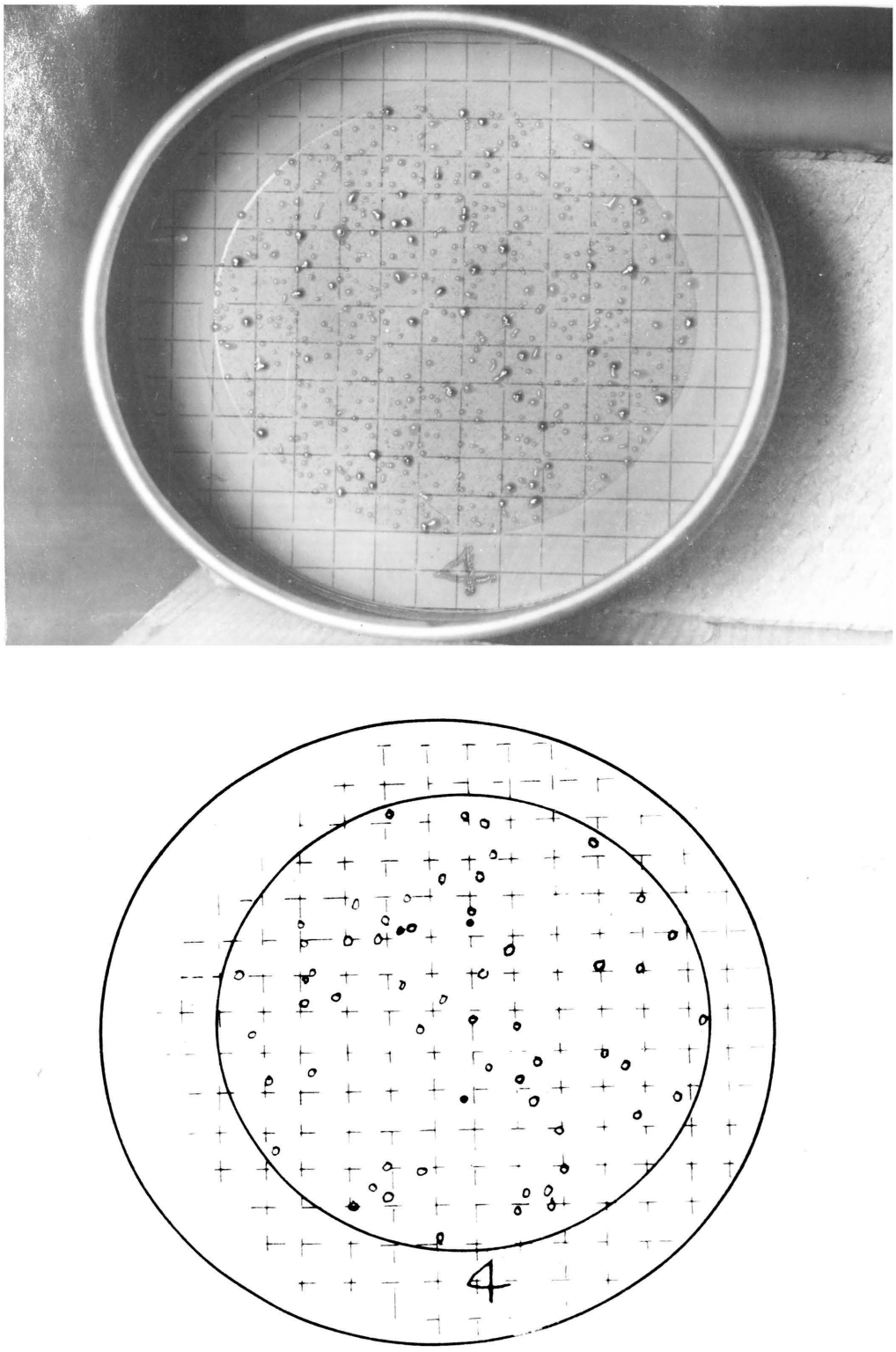
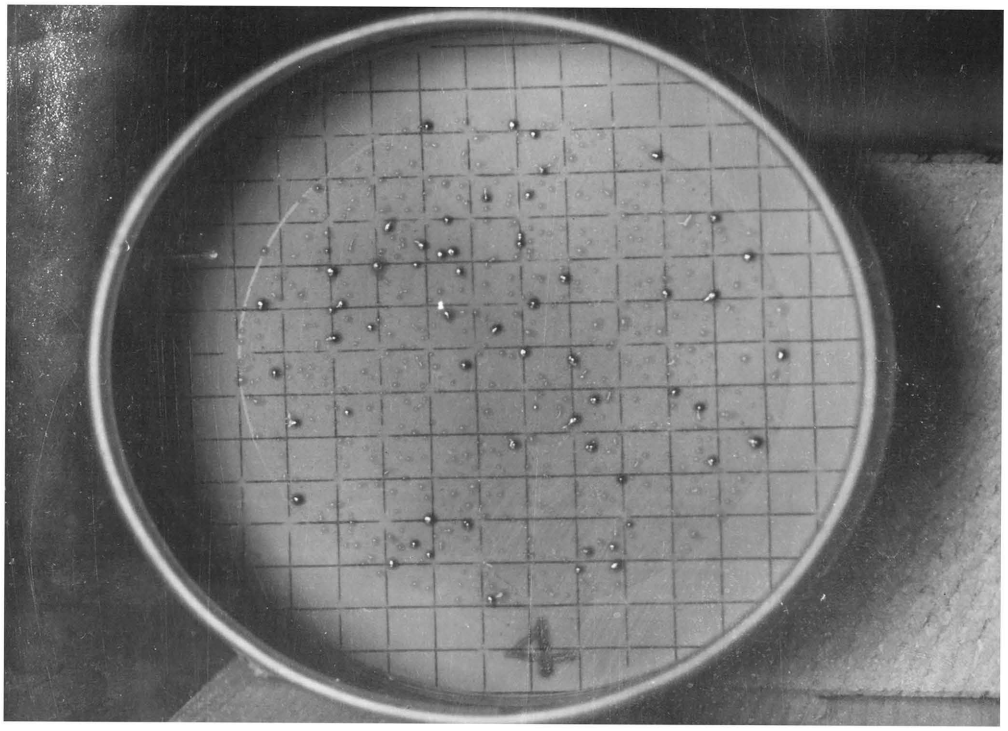


FIGURE 9

ORDINARY PHOTOGRAPH OF INCUBATED CULTURE SAMPLE TAKEN FROM LITTLE PINEY RIVER, APRIL 29, 1959. COLIFORM COLONIES APPEARING ON FILTER ARE INDICATED IN DRAWING



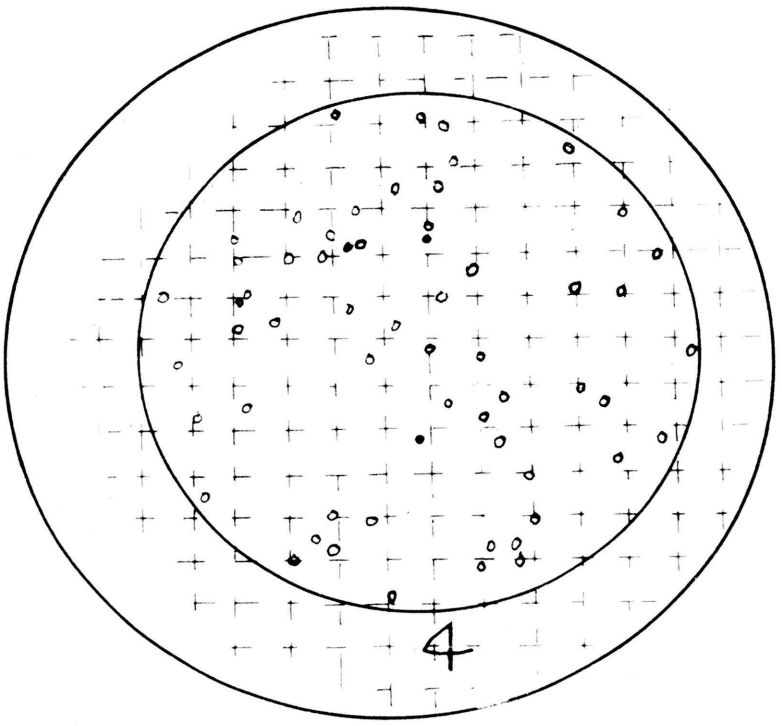


FIGURE 10

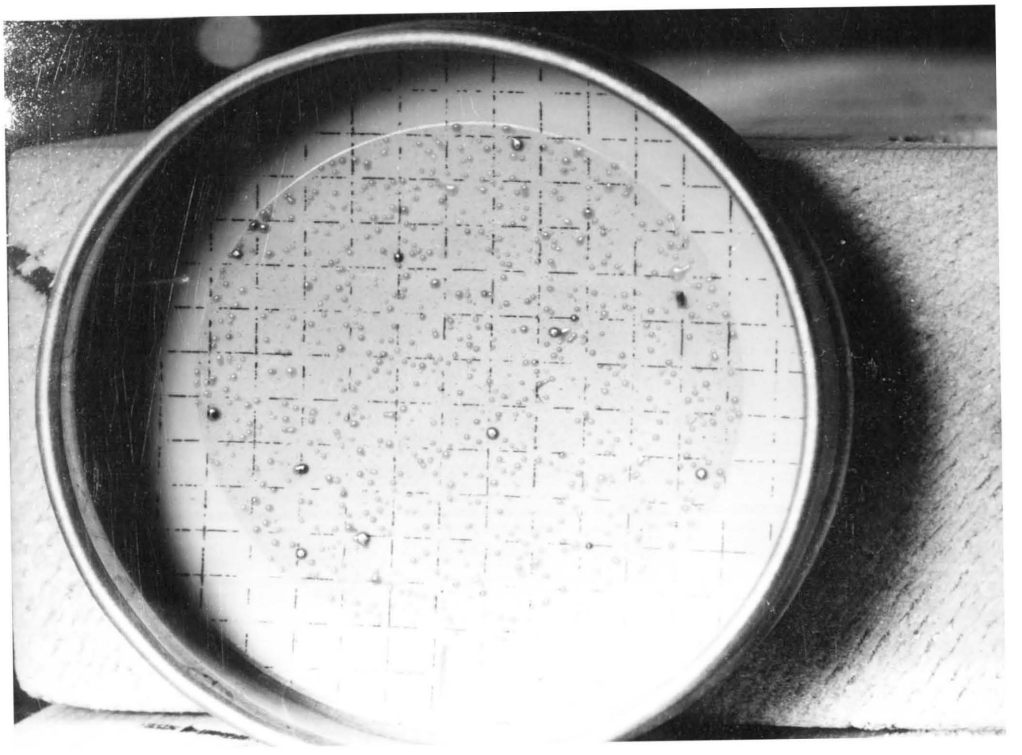
PHOTOGRAPH OF SAME INCUBATED CULTURE SHOWN IN FIGURE 9. RED FILTER USED WITH CAMERA. COLIFORM COLONIES APPEARING ON FILTER ARE INDICATED IN DRAWING

Figure 10 is a photograph of the same incubated membrane filter shown in Figure 9, however, only the coliform colonies have the distinct appearance. The majority of the non-coliform colonies are dark red in appearance. By using a filter approximately the same color as these colonies their appearance in a photograph is toned down but is not completely eliminated. This will still permit a total colony count from a photographed incubated membrane filter to be made when it is desired. To accurately identify the coliform colonies they are indicated in the circle below the photograph.

The photographs were taken with a speed graphic 4" x 5" camera using Kodak Royal Pan sheet film. Filter used was WRATTEN, A-25 Red. Exposure time, 1/10 second at f-16 without filter and 1 second at f-16 with the filter. Effective stop opening due to bellows extension was f-39. Light source was a number 2 photoflood light in an aluminum reflector place at an angle of 20° to axis of the camera. Distance from light to subject was 27 inches, lens to subject 8.5 inches and from lens to film was 13 inches.

Figures 11, 12 and 13 are photographs of incubated membrane filters plated with samples taken from the Big Piney River on 4 March 1959.

These photographs represent permanent records of the condition of a given water at a definite time. Permanent records as these should prove very valuable to municipal agencies for future reference. From a military standpoint permanent records in the form of photographs would permit quick formation of adequate plans for water treatment facilities in any area desired.



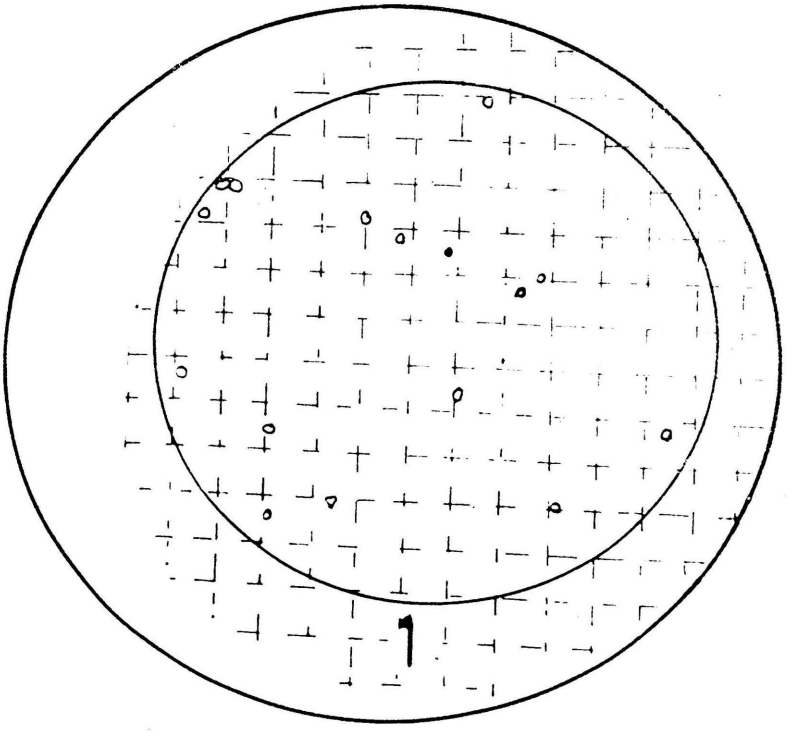
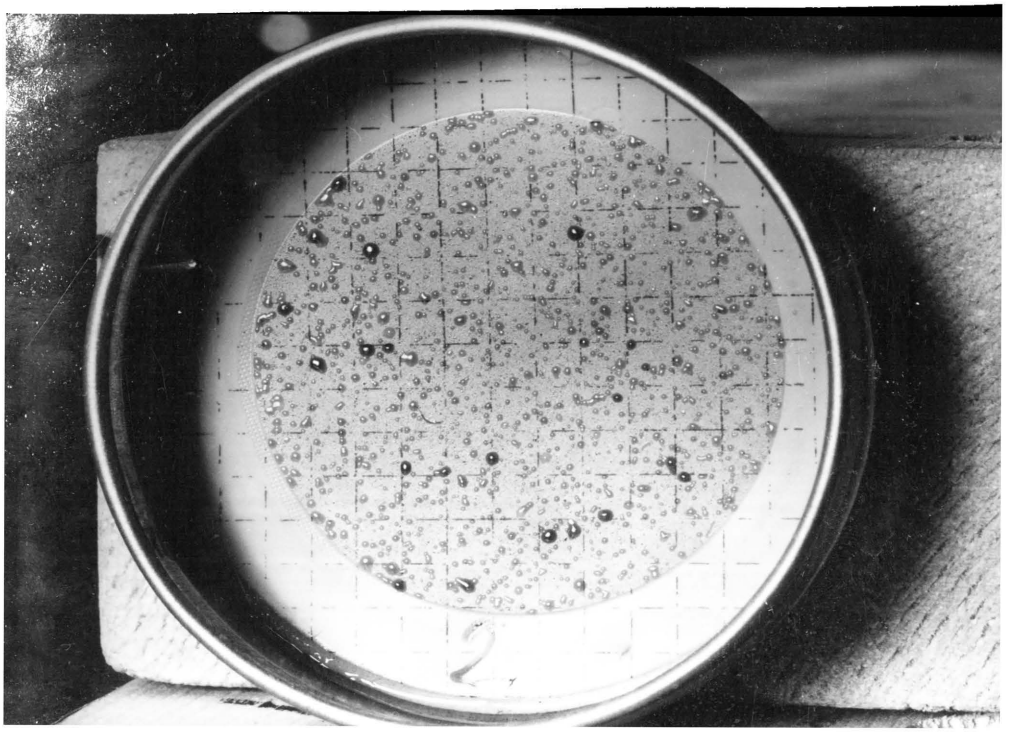


FIGURE 11

MEMBRANE FILTER PLATED WITH A WATER SAMPLE TAKEN FROM BIG PINEY RIVER, MARCH 4, 1959. COLIFORM COLONIES APPEARING ON FILTER ARE INDICATED IN DRAWING



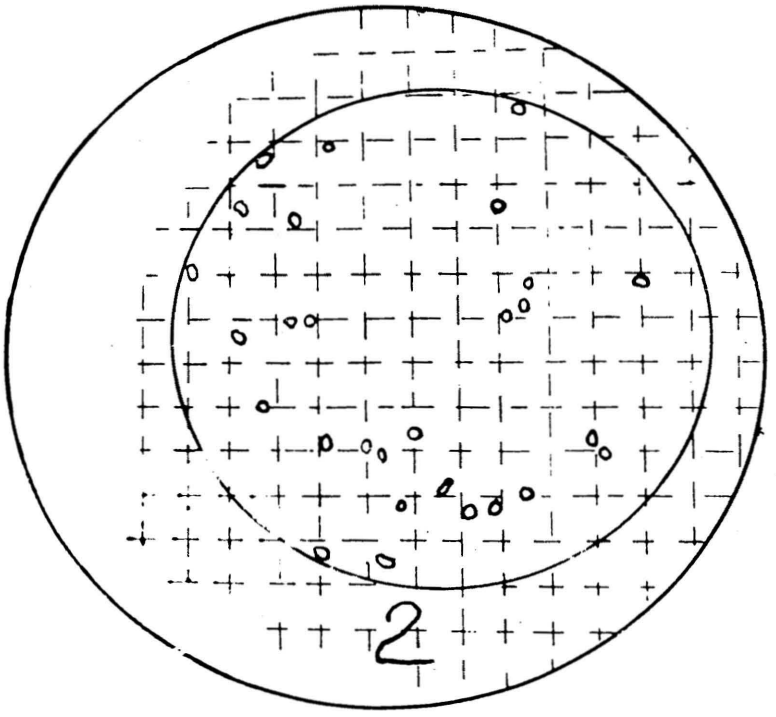
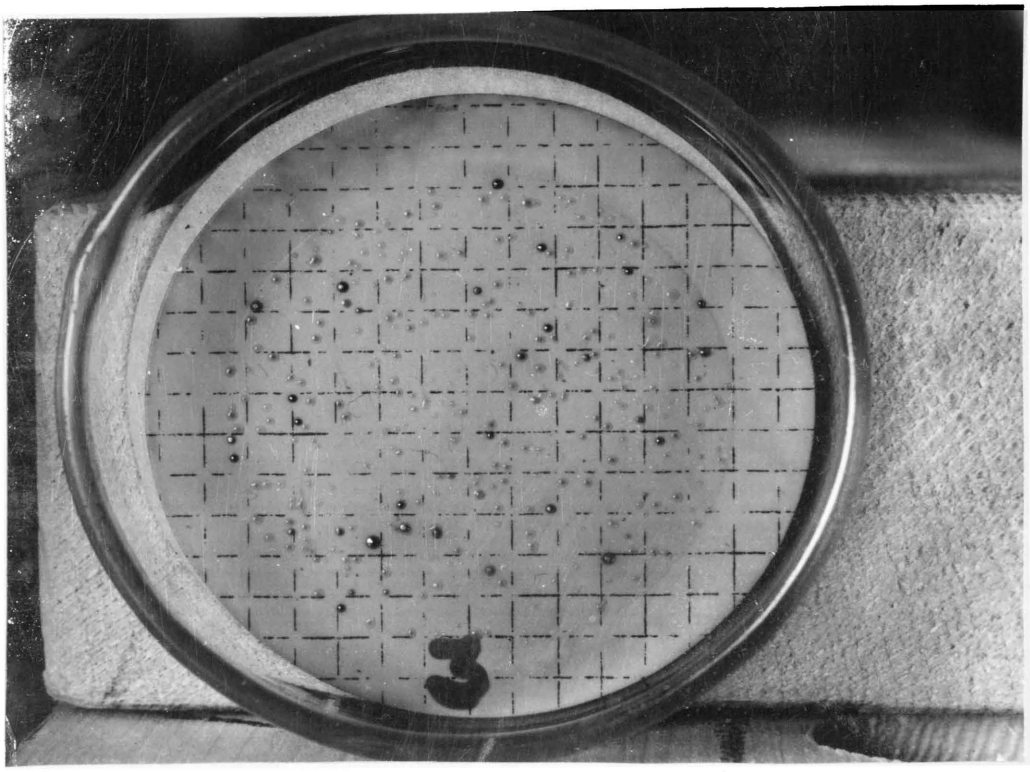


FIGURE 12

MEMBRANE FILTER PLATED WITH A WATER SAMPLE TAKEN FROM BIG PINEY RIVER, MARCH 4, 1959. COLIFORM COLONIES APPEARING ON FILTER ARE INDICATED IN DRAWING



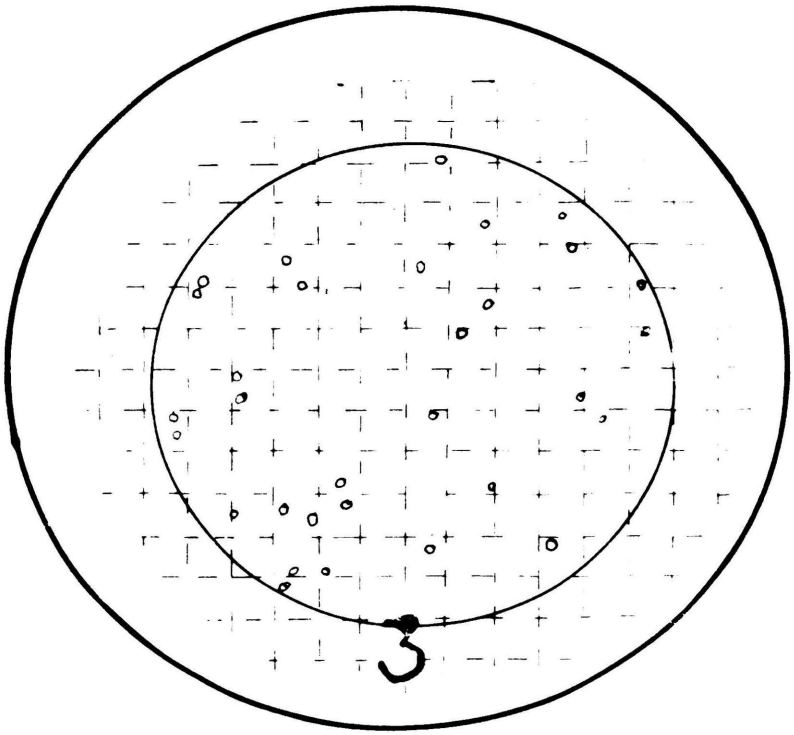


FIGURE 13

MEMBRANE FILTER PLATED WITH A WATER SAMPLE TAKEN FROM BIG PINEY RIVER, MARCH 4, 1959. COLIFORM COLONIES APPEARING ON FILTER ARE INDICATED IN DRAWING

V. EQUIPMENT FOR FIELD USE

During the period the experiments were being conducted the author devised several different types of field kits. An ideal field kit would contain the essential materials and equipment required to perform, in the field, a complete biological examination of a water. While meeting this requirement the kit should also be compact and small enough to permit ease in handling. The final kit devised is shown in Figures 14, 15 and 16. Figure 14 shows the kit completely assembled and ready for movement in the field. The funnel element is carried in an inverted position in the kit. When performing filtrations in the field the funnel element can be placed in this holding device during changes of membrane filters.

In Figure 15 the equipment carried in the kit has been removed and assembled. Table VI is an itemized list of all the equipment contained in the portable field kit. To provide a supply of fresh liquid culture media when required, the proper amount of dehydrated media is placed in a 30 ml bottle at a central laboratory. The distilled water containing ethanol is not added to the bottle containing the dehydrated media until it is to be used in the field. 14 bottles are included in the kit, thus providing 30 ml of fresh liquid culture media each day for 14 days or a sufficient amount for approximately 196 individual filtrations.

A one liter bottle is provided for carrying the distilled water mixed with ethanol. An additional one liter bottle has been included to provide a supply of rinse or dilution water. The supply of this water

FIGURE 14

MEMBRANE FILTER KIT PREPARED FOR USE IN THE FIELD

FIGURE 15

SUFFICIENT MATERIAL FOR 196 INDIVIDUAL FILTRATIONS OR APPROXIMATELY 14 DAYS OPERATION IS CONTAINED IN THE CHEST. THE NUMBER OF DAYS IS CONTROLLED BY THE NUMBER OF INDIVIDUAL BOTTLES CONTAINING DEHYDRATED MEDIUM.

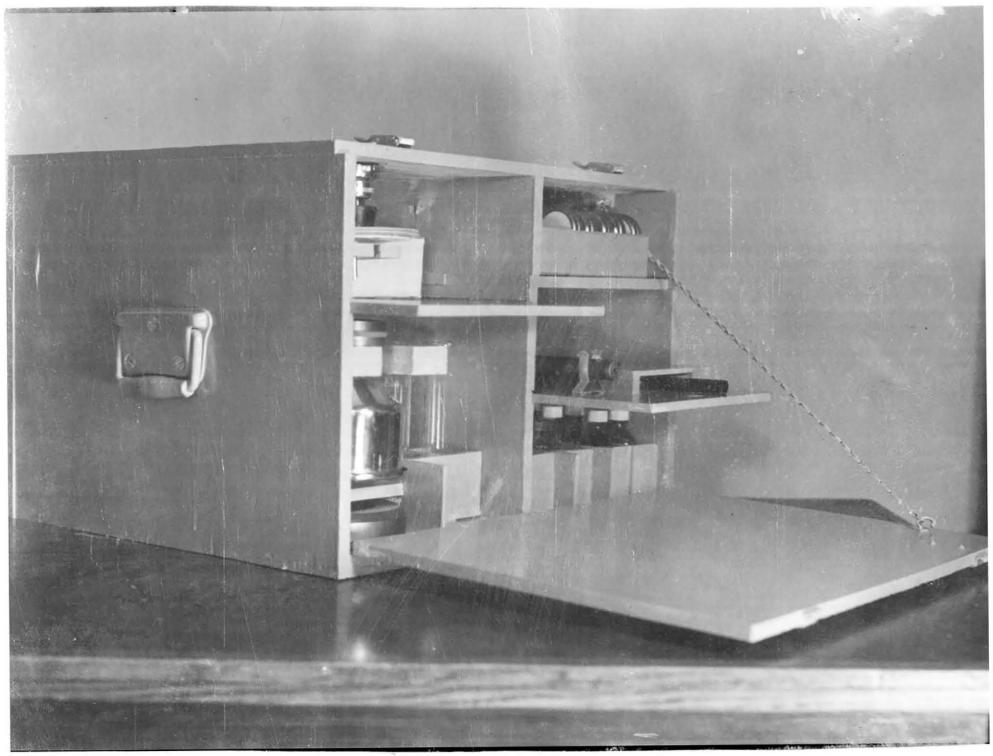


FIGURE 16

PORTABLE MEMBRANE FILTER KIT
DIMENSIONS OF CHEST ARE:

HEIGHT	16 inches
WIDTH	20 inches
DEPTH	18 inches
WT. (EMPTY)	33 pounds
WT. (PACKED)	53 pounds

TABLE VI

EQUIPMENT RECOMMENDED FOR INCLUSION IN A PORTABLE MEMBRANE FILTER FIELD TESTING KIT

ITEM	TYPE	QUANTITY
Filter Unit	Stainless steel	1 each
Suction Flask	One liter size	1 each
Water Sample Bottles	Wide mouth, screw cap	10 each
Media Bottles	30 ml size, screw cap	14 each
Reading Glass		1 each
Hand Pump		1 each
Rubber Hose	3/16" I.D.	4 ft.
Distilled Rinse Water Bottle	One liter size	1 each
Prepared Media Bottle	One liter size	1 each
Rubber stopper	To fit suction flask	1 each
Enamel Pitcher	Interior graduated, 1000 ml	1 each
Membrane Filters	Millipore, 47 mm, HA grid, white	300 each
Absorbent Pads	Millipore, 47 mm	300 each
Metal Ointment Cans	2 oz.	48 each
Metal Ointment Cans	3 oz.	48 each
Pipette, graduated	2 ml	2 each
Eye Dropper	1 ml	6 each
Pipette, graduated	10 ml	2 each
Forceps		1 each
Cylinders, graduated	50 ml	2 each
Pencil, wax, paper coated		2 each
Incubation belts		4 each

may be replenished in the field as required by taking from the suction flask filtrate that has passed through a membrane filter. When this is to be done the suction flask should be sterilized at the beginning of the filtration series. All incubation is to be performed by wearing a waist belt. Figure 16 is a side view of the kit. To retain the equipment in place during movement of the kit to different sites in the field two sliding trays are used as shown in Figure 16. Access to the equipment during a filtration series is accomplished by removing the sliding trays.

CHAPTER VI

SUMMARY AND RECOMMENDATIONS

The results of this investigation indicate the membrane filter method for determination of the quality of a water has many advantages when compared to the Presumptive, Confirmed and Completed Tests or the Most Probable Number Method. Some of these advantages are:

a. The equipment for performing the tests is small and require very little maintenance.

b. Less media, glassware, washing and sterilization of equipment is required for this method than for the Most Probable Number Method.

c. The techniques of performing filter tests and recording their results is not so difficult as to require a lengthy training program.

d. Filtration and incubation of samples is carried out at the site, thus eliminating the difficulty, expense, and time required to transport water samples to a central laboratory.

e. Results of a test are obtained in 20 hours while the standard test tube method requires a minimum of 48 hours and may require as much as 5 days for a completed test.

f. Permanent records of the actual coliform present in a filtration sample can be preserved.

g. The technique permits the concentration of a small number of bacteria from a relatively large quantity of water.

h. It gives a direct count instead of a statistical most probable number in the determination of coliform organisms present in any given volume.

i. Using the portable field kit recommended, it is possible to test as many as 14 samples daily for a 14 day period without returning to a central laboratory or supply point for resupply of materials.

j. Incubation of cultures by wearing the culture container around the waist is definitely possible and produces satisfactory results.

k. Ointment cans are satisfactory culture containers and at the same time control of humidity is not as great a problem as it is when using petri dishes as containers.

The incubation belt work by the author was made of cotton cloth and insulated with a thin layer of cotton. A thorough investigation of different types of material and insulation would perhaps result in a lighter weight belt and at the same time provide satisfactory incubation.

During all the tests conducted on the raw water there was not sufficient algae or turbidity present in the sample volumes to clog the filter pores. Sample volumes from sources containing large amounts of algae or having a high turbidity may cause clogging of the membrane filter pores before a sample volume large enough to provide an accurate coliform determination can be filtrated.

The author did not perform any comparison tests between the membrane filter method and the Most Probable Number Method. This phase of the problem is currently being studied by several agencies and any work in this field would be repetitious. The Public Health Service has reported in detail a comparison study that was made on 1,706 water samples (16). Several municipal water treatment plants throughout the United States are currently performing daily comparison tests between the two methods. Kansas City and St. Louis, Missouri have reported portions of their results (17) (18).

I. RECOMMENDATIONS

In conclusion it is recommended that:

a. Appropriate civil defense and military agencies procure an adequate number of membrane filter field kits identical or similar to the field kit devised as a result of this study.

b. That minimum training in the technique and operation of membrane filter equipment be given to the appropriate personnel in military and civilian agencies.

c. In emergency instances, including those of a military nature, the membrane filter method be utilized because of the rapid time in which an accurate determination of the quality of a water can be determined.

d. At the time the membrane filter method is accepted as an approved field method by the United States Public Health Service that the military, as well as other civil agencies, immediately use the method as a standard one. This acceptance could be forthcoming in the new issue of Standard Methods (11th Edition) to be published in 1960.

e. A series of experiments be conducted to determine the most suitable material to use in making an insulated, light weight, incubation belt.

f. Tests on a large scale be continued by appropriate agencies, using ointment cans as culture containers and body incubation as a source of heat.

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VITA

Charles Liston Gambill was born on January 19, 1923 at Pryor, Oklahoma, the son of Charles S. and Georgia Gambill. He attended Browning Springs Grade School and Pryor High School, graduating from high school in 1940.

He entered Oklahoma A & M College in September 1941 and attended college until entering the United States Army in June 1943. He was commissioned a Second Lieutenant in July 1945, and was integrated into the Regular Army, Corps of Engineers, in 1947.

His services in the United States include troop duty, attendance at the Engineer Officers' Advanced Course, Fort Belvoir, Virginia, and a construction assignment with the Tulsa District, Corps of Engineers. Overseas service includes duty in Europe, Korea and Okinawa.

While assigned as Assistant Professor of Military Science and Tactics at Marquette University he completed his studies and was graduated with a Bachelor of Civil Engineering Degree in June 1958. In 1958 he was selected to participate in the Army Civilian School Program and in June of that year enrolled as a graduate student at the Missouri School of Mines and Metallurgy, for the purpose of studying for a Master of Science Degree in Civil Engineering.

On 1 February 1944 he was united in marriage to Elizabeth Anne Jaynes of Muskogee, Oklahoma. A son, Charles Richard, was born on May 22, 1947.