STUDIES ON THE MEMBRANE FILTER TECHNIQUE AND ITS APPLICATION TO THE DETECTION OF COLIFORM ORGANISMS FROM WATER

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STUDIES ON THE MEMBRANE FILTER TECHNIQUE AND ITS APPLICATION TO THE DETECTION OF COLIFORM ORGANISMS FROM WATER

By

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ABSTRACT

A detailed study was made of the membrane filter technique for the purpose of evaluating its usefulness in determining the sanitary quality of water supplies. During this investigation certain technical problems arose, the consideration of which was essential to the application of this technique. Among those considered, the problem of moisture control was given special attention.

Studies, using a pure culture of Escherichia coli, showed that a full recovery of these organisms could not be obtained with the membrane filter technique when EHC modified Endo medium was employed. The need for a more satisfactory selective medium was recognized and a search was undertaken in an attempt to formulate such a medium. Several different media formulations were compounded and tested. A medium was developed which was found superior to EHC modified Endo medium for the examination of certain types of waters. This medium derived its selective action primarily from bile salts and incorporated brom cresol purple which acted as an indicator to detect lactose fermentation.

Difficulties in the use of the membrane filter were encountered as a result of the precipitation of iron from some samples. Although iron precipitation could be prevented by the addition of Versene, the presence of this compound further complicated the interpretation of the results obtained.

It was found that the ability of an organism to ferment lactose with gas production could not be accurately predicted from its colonial appearance on the surface of the membrane filter.

The results of the study raised questions as to the adequacy of the membrane filter technique for evaluating the sanitary bacteriological quality of waters.

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INTRODUCTION

The membrane filter is a relatively new tool in the field of bacteriology. Since its release for general use by the U. S. government in 1951, the application of the membrane filter to the bacteriological analysis of water samples has been very widespread. In fact, the membrane filter has found its most extensive use in the detection of coliform bacteria from water.

The object of this investigation was to make a detailed study of the membrane filter technique and to attempt to evaluate its usefulness in the sanitary testing of water samples.

Many articles have been written recently which credit the membrane filter technique with having many advantages over the Standard Methods' MPN technique for determining the sanitary quality of a water supply. Most of these reports depict the merits of this new technique and few mention its faults. In the experience of the author with the membrane filter technique, however, many problems were encountered which had not been answered. This fact suggested the exigency for making a further study of the various aspects of the membrane filter technique for testing the sanitary quality of water.

The major difficulty which is encountered in the application of the membrane filter technique to the bacteriological analysis of water lay in the lack of a satisfactory selective medium which would permit the complete recovery of all coliform organisms present in water. Although the development of a successful medium formulation may be a highly perplexing task, the need for such a formulation was great. For this reason, studies were undertaken in an attempt to develop a culture medium which would support the growth of all coliform bacteria on the membrane filter.

REVIEW OF LITERATURE

Collodion membranes have been used in biological investigations for a number of years. Fick (1885) utilized such membranes in dialysis experiments. Sanarelli (1891) was the first to use collodion membranes for bacteriological work. His work included the ultrafiltration of blood plasma *in vivo*. He showed that these membranes were impervious to bacteria but not to their toxins. Ferry (1936) stated that Bechhold first produced collodion membranes of graded pore sizes in 1906. Disc membranes made of collodion were produced by Bigelow and Gemberling (1907). This process was further developed by Zsigmondy and Bachmann (1918). The latter were the first to develop a method whereby the membranes could be produced on a commercial scale and their method of preparation was patented. Elford (1931) used a similiar process to produce membranes on a smaller scale in England. According to Goetz and Tsuneishi (1951), Grabar also produced such membranes in France. Thus an interest in collodion membranes was again stimulated during the early 1930's.

Schutz and Kruse (1947) credit the Russians as being the first to use membrane filters for estimating the coliform content of water.[1] Apparently they used an Endo medium for this

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[1] The terms membrane filter, molecular filter, millipore filter, and ultrafilter are synonymous. The term millipore filter is a trade name used by the Millipore Filter Corporation, Watertown, Massachusetts.

purpose in 1933-1934. During World War II, the Germans used the membrane filter technique as an emergency procedure for the sanitary testing of water supplies. The fact is, they were forced to use it due to serious shortages of both agar and laboratory equipment. They seemingly had such success with the technique during the war that a widespread interest in it was developed. Goetz (1947) investigated the use of the membrane filter technique in Germany and introduced this technique into the United States. According to Goetz and Tsuneishi (1951), Goetz later developed a new method for the production of membrane filters in the United States. These membranes were described as an improvement over those which had previously been produced in Germany.

A procedure was described by Clark et al. (1951) for the estimation of coliform bacteria in water by the use of the membrane filter. They developed an Endo-type medium for this purpose. These authors found that a significant increase was obtained in the numbers of coliform organisms recovered when incubation on the Endo medium was preceeded by a two hour preliminary enrichment period on a complete medium. This procedure and the use of an Endo broth was also advocated by Goetz and Tsuneishi (1951), Clark and Kabler (1952), and Kabler and Clark (1952) for the detection of coliform bacteria in water. These authors considered EHC Endo broth to be a satisfactory medium for use with the membrane filter in the enumeration of coliform organisms. Clark and Kabler (1952)

stated that, "A few of the A. aerogenes and intermediates may be missed by this technique but the increased accuracy of the membrane procedure compensates for this loss when the data are compared with the MPN method". A complete description of the molecular filter technique for the bacterial examination of water is also given by McKee et al. (1953).

Goetz, Gilman, and Rawn (1952) demonstrated the use of the molecular filter in determining the coliform density of ocean water. This technique was compared with the MPN procedure as given in Standard Methods. These authors contended that the molecular filter technique showed greater accuracy than standard procedures and they pointed out the large deviations that can occur in coliform determinations which are made by the MPN procedure. Yet, in almost every case, their results showed the coliform estimation to be larger by the MPN procedure than by the molecular filter technique. It might also be mentioned that the above authors tried to convey the impression that the molecular filter technique is a very simple operation "as contrasted with the more delicate and time-consuming technique of the lactose broth fermentation tests". Goetz (1953) has also proposed the use of the molecular filter technique for the bacterial assay of sewage and waste waters.

Clark et al. (1952) compared domestic and European molecular filter membranes. They described both the total recoveries and the coliform densities obtained as being comparable on the two types of membranes. However, they found that

the domestic type membranes were somewhat easier and more convenient to handle.

A modification of an Endo medium to which was added 8-hydroxyquinoline, more commonly referred to as oxine, was employed by Yee, Krabek, and Schaufus (1953). They reported that the addition of oxine reduced the number of non-coliform colonies on the molecular filter membrane by approximately 25-50 per cent without causing any reduction in the number of coliform colonies present. These authors claimed that they generally obtained higher coliform counts with this medium and the molecular filter technique than were obtained by the MPN procedure. However, it should be pointed out that the above authors allowed the filters to dry before counting the colonies because this procedure resulted in higher coliform counts. This made it impossible to utilize confirmatory tests to determine whether or not the dried colonies which were counted were actually coliform colonies.

Hajna and Damon (1954) have developed a medium which contains sodium desoxycholate and which does not require a preliminary enrichment period. This medium is essentially EHC Endo broth to which sodium desoxycholate has been added.

In 1954, Jeter, Geldreich, and Clark introduced a brilliant green fuchsin medium (BGF medium) for use in examining waters which contained large numbers of non-coliform bacteria. With this medium, their coliform recovery was 8 per cent less

than that which was obtained with EHC Endo broth. In this study, both EHC Endo and BGF medium gave lower coliform estimates when used with the membrane filter than were obtained by means of the Standard Methods MPN procedure. The results of Geldreich et al. (1955) likewise showed that coliform counts which were made by the membrane technique tended to be appreciably lower than those which were obtained by the MPN procedure. The findings of Kabler (1954) are in agreement with these observations. Jeter, Geldreich, and Clark (1954) also pointed out that, in the case of the EHC Endo medium, 8 per cent of the colonies which were considered to be composed of coliform bacteria failed to produce gas when transfers were made to lactose broth fermentation tubes. On the other hand, 4 per cent of the colonies which were not considered to be composed of coliform bacteria were shown to produce gas from lactose broth. The same was true with the BGF medium. Here 5 per cent of the colonies which were counted as coliform colonies failed to produce gas when transferred to lactose broth fermentation tubes, while 7 per cent of those which were not considered to be coliform colonies on this medium produced gas when transferred to lactose broth. However, the authors justified this situation by stating that, "These two sources of error tended, in this investigation, to cancel one another in coliform enumeration".

Kabler (1954) compared the results which were obtained by

the membrane filter technique with those which were obtained by the Standard Methods 5-tube MPN procedure in the examination of water samples for coliform bacteria. He reported on the testing of 1,706 water samples by the two methods during which a comparison was made "on the basis of the 95 per cent confidence limits of the confirmed MPN". He found that, when compared on this basis, 1,260 samples or 73.8 per cent were in agreement. However, after making the statement that, "Sources selected were to show a coliform MPN index between 10 and 200 per 100 ml.", Dr. Kabler included in his results 457 samples in which no coliform organisms were demonstrated by either procedure. In addition to this, he included 114 samples which gave an MPN of less than 5.4 coliforms per 100 ml. Of these 114 samples, 85 showed the presence of coliform organisms on the MPN procedure but not by the membrane filter technique, while 29 gave coliform organisms by the membrane technique but not by the MPN procedure. The fact that Dr. Kabler had no vindication for including the samples which showed no coliform bacteria by either procedure in the determination of his final percentages should be considered. It might be pointed out that, in the case of these negative samples, an agreement of 100 per cent could have been obtained even if the membrane filters were not incubated on any nutrient material whatsoever. If these 457 negative samples are not included, then it can be shown that only 64.3 per cent of the

samples and not 73.8 per cent, as claimed by Kabler, are in agreement when compared on the basis of the 95 per cent confidence limits as indicated. It should also be mentioned that attempts to compare results on the basis of the 95 per cent confidence limits of the confirmed MPN do not really give any kind of a statistical comparison. Such an attempt is merely an endeavor to indicate an agreement in a case where evidently no good statistical comparison can be shown to exist. According to Bush (1955) it is on the basis of these results which have been reported by Kabler (1954) that the membrane filter technique has been included as a tentative procedure in the tenth edition of "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" (1955).

Presnell, Arcisz, and Kelly (1954) compared the membrane filter and MPN methods in determining the coliform densities of samples of sea water. The 95 per cent confidence range of the MPN was used as a basis for comparison. Their results were 87.1 per cent in agreement when compared in this way.

Levin and Laubausch (1954) reported on the testing of 207 samples of drinking water which had been delivered to "hotel" trains under emergency conditions. Of these 207 samples, only three showed the presence of any coliform bacteria on the confirmed MPN test, while two (of these same three) also produced coliform-like colonies on the membrane filter. On the basis of these tests the authors concluded that, "It seems entirely feasible that, with the use of the membrane filter,

small compact laboratories that could be carried on a man's back would be capable of providing bacteriological control over the quality of drinking water provided by makeshift means".

Slanetz and Bartley (1955) reported a study of various selective media for the estimation of coliform bacteria in water. They found that their most satisfactory results were obtained by the use of M-Endo broth which was supplied by Difco and BBL Companies. They reported that these media gave higher coliform counts or more distinct coliform colonies than the other media tested.

Taylor, Burman, and Oliver (1953) compared the membrane filter technique with the methods which are standard procedure in Britain for the bacteriological analysis of water. They adapted a triple strength MacConkey broth which contained 12 times the usual amount of brom-cresol purple for use with the membrane filter. Their results were reported as promising and they appeared to have obtained a rather good agreement between the two methods which were used. However, it is possible that some bacteria may have failed to develop due to the high incubation temperature which is required in their standard procedures. These authors in a later report (1955) indicated further progress in this study. They used both Endo broth and a modified MacConkey broth with the membrane filter procedure. The results were compared with those obtained by the 5-tube method utilizing MacConkey broth and confirmation in brilliant-green bile broth which is the standard procedure that is used by

the British in testing the bacteriological quality of a water supply. They obtained fewer false positive results on the Endo medium, although a higher confirmed count was obtained with the MacConkey medium. Also, both media gave higher coliform estimations when used with the membrane filter procedure than were obtained by the multiple tube technique. However, as the authors point out, the possibility must be considered that the high initial incubation temperature (42 C) which is used in the multiple tube procedure may have prevented the subsequent growth of some of the coliform bacteria.

McCarthy (1955) used a two-hour incubation period on M-enrichment broth followed by M-Endo medium. He showed that when the results with the membrane filter were based on the appearance of a metallic sheen, the number of coliform bacteria obtained with this technique was considerably lower than that obtained by means of the MPN confirmed test. The author points out many of the difficulties and problems which are encountered in the use of the membrane filter technique. His work indicated the lack of a completely satisfactory medium which would fully permit the development and differentiation of coliform organisms from all waters. He emphasized that, "The most urgent need in connection with the membrane filter seems to be the formulation of a more satisfactory medium". McCarthy, however, did observe an excellent agreement between the membrane filter and the tube MPN test when sewage was examined, but he believed that this was due to the "freshness

of the coliforms in the sewage and the relatively low number of other organisms".

The fact has been acknowledged by Thomas, Woodward, and Kabler (1955) that coliform densities which are obtained by the MPN method "are generally somewhat larger than those obtained by the membrane filter technic". These authors stated that, "While such differences may be statistically significant, it does not follow logically that the differences are of much practical import". They believed that "the MPN tends to overestimate the true density" and referred to the MPN as "a biased estimator of the true density". In addition, they made the statements that, "The arithmetic means of replicate MPN's tend to be too high by a factor of 23 per cent in a 5-5-5-tube test", and that, "If a large number of replicate 5-5-5-tube MPN tests are made from water containing 100 coliform per 100 ml the arithmetic mean MPN will be 123 instead of 100".

Nevertheless, it has been admitted by Thomas and Woodward (1955) that, in the case of certain waters, estimates in coliform densities are greater with the MPN procedure than with the membrane filter technique and that the difference in densities is "larger than may be accounted for by the bias of the MPN". However, the authors were of the opinion that, "Some loss in recovery efficiency is acceptable and justifiable in view of the advantages gained by the new tool".

Thomas and Woodward (1955) have made it clear that, "The bias of the MPN derives only from mathematical considerations and is independent of the growth efficiency of the media used". Yet it is sometimes possible to isolate coliform bacteria from previously inoculated lactose broth fermentation tubes in which no gas production was observed after an incubation period of 48 hours as advocated in Standard Methods. The work of Chambers (1950) has indicated that when a high ratio of non-coliforms to coliforms is present in a water sample, failure to produce visible gas in lactose broth may occur even though considerable numbers of coliforms are present in the sample. According to Chambers, "A negative test, to say nothing of one in which a small quantity of gas is produced, certainly does not preclude the possibility that coliforms were present".

On the other hand, McCarthy (1955) took membrane filters which had no colonies with sheen, placed them in lactose broth fermentation tubes, and confirmed all tubes showing gas. By this procedure, he was able to obtain an average MPN from membrane filters which was more than twice the MPN that was obtained when the results were based on the appearance of sheen colonies only.

On the basis of results which have been reported thus far, the author was of the opinion that the membrane filter technique had been pushed too fast and too far in the field of water bacteriology. Those favoring the acceptance of this

technique seem to have used every means at their disposal to make their findings appear better than they really were. In addition, this new tool seems to have held much appeal to technicians who are engaged in water analysis. Recently, this technique has been included as a tentative procedure in Standard Methods and although some of the pitfalls are pointed out, Standard Methods states that, "When the limitations of the test are fully recognized and the difficulties of interpretation of the results are known, the technic may be used". However, Standard Methods does not intend that the membrane filter technique should be used as a substitute for the dilution MPN procedure in the examination of drinking water.

It was with these facts in mind that the experimental work was begun in this investigation.

MATERIALS AND METHODS

Membrane Filters

The membrane filters which were used in this investigation were of the hydrosol assay, white, grid marked type. They were circular discs 47 mm in diameter and 150 microns in thickness and were composed of a cellulose derivative. The membranes resembled paper in appearance but in appearance only. (Anon.,1951 and 1955)

These membranes were extremely porous and the pores were uniform in size. The pores comprised a volume of from 80 to 85 per cent of the total volume of the membrane. (Anon., 1955) The pore size was smaller on the upper surface of the membrane than on the lower surface. As a result of this property, filtration was achieved by means of a physical screening action. (Goetz and Tsuneishi, 1951) The pores in the membrane filter are tubular and roughly parallel to the direction of flow of a liquid during filtration. (Clark, et al. 1952) The number of pores per square centimeter have been calculated at 50 million. (Bush, 1955) The pore size can be controlled during the manufacturing process. Goetz has reported that, "The pore size is measured by the determination of a factor z (zeit = time) which is necessary to pass 100 cc of distilled water at room temperature through a filter area of 100 cm^2 at

a differential pressure of one atmosphere (15 lbs.)". (Goetz, 1947) The pore size has been calculated by the Millipore Filter Corporation to be 0.45 micron and the effective filtering area of these membranes is 9.6 cm^2. (Anon., 1955) The upper surface of the membrane filter has been imprinted with a grid to facilitate counting.

The membrane filters which were used in this study were manufactured by the Millipore Filter Corporation, Watertown, Massachusetts.

Filter Apparatus

The filter apparatus used in this study was constructed of pyrex glass (Fig. 1). It consisted of a 250 ml glass funnel (part number 4) which was joined to part number 2 by means of a clamp (part number 3). The membrane filter was supported on the fritted glass surface of part number 2. The filter apparatus was mounted on a filter flask and a vacuum was applied during the process of filtration (Fig. 2).

The funnel was coated with Desicote[1] so that no film of water would be left behind when the funnel was emptied. This allowed for a more complete rinsing of the funnel.

Sterilization Procedures

The membrane filters were packed in groups of ten with

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[1] A product of Beckman Instruments, Inc., South Pasadena, California.

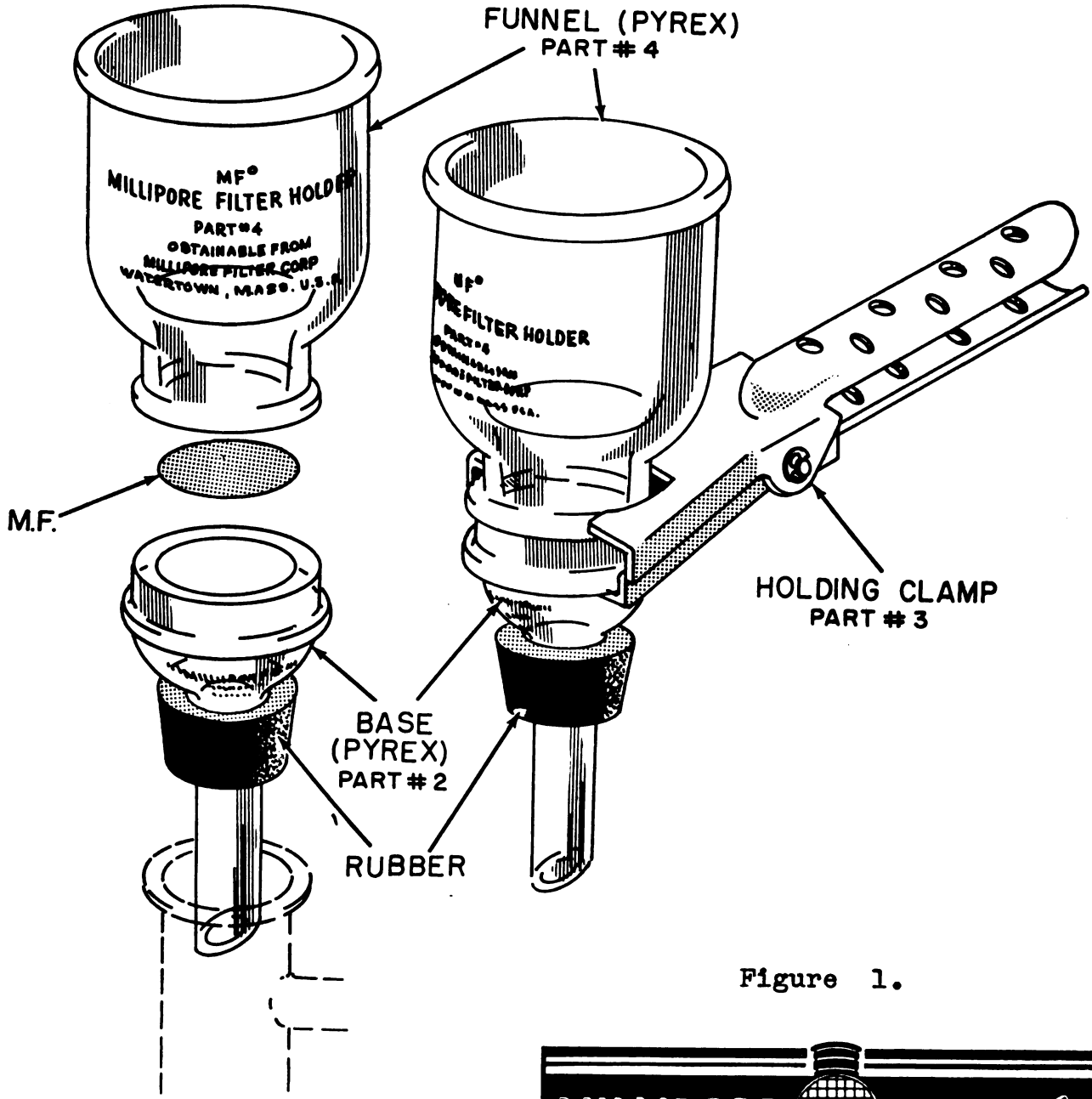

Figure 1.

MILLIPORE MF FILTER Corp.

WATERTOWN 72, MASSACHUSETTS

PYREX FILTER HOLDER

CAT XX 10 047 00

FOR ANAYSIS OF HYDROSOLS

USES 47 MILIMETER TYPE HA FILTERS

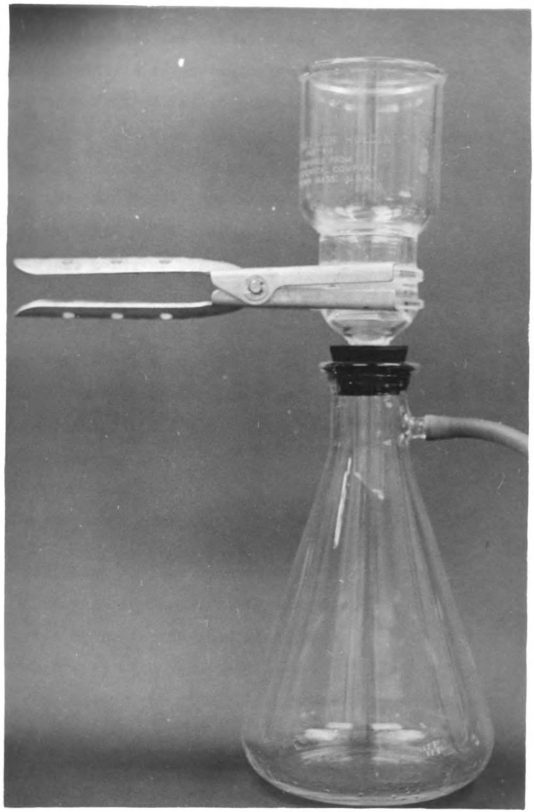

Figure 2. Assembled Filter Apparatus

absorbent pads between the filter discs, wrapped in Kraft paper, and sterilized in the autoclave at 15 lbs. pressure per square inch (121 C) for 10 minutes. At the end of this 10 minute period the steam pressure was rapidly released to prevent condensate from collecting on the membranes.

Extra absorbent pads were wrapped in Kraft paper and sterilized as mentioned above.

Both glass parts of the filter apparatus were wrapped separately in Kraft paper and sterilized in the autoclave at 121 C for 15 minutes. The filter apparatus was sterilized after each sample was examined.

Forceps were sterilized prior to each use by dipping in 95 per cent ethyl alcohol and passing them through the flame of a Bunsen burner.

Filtration Procedure

The following filtration procedure was employed:

1. A sterile membrane filter was placed with its grid side up on the fritted glass surface of the filter apparatus with the use of sterile forceps. The funnel was then attached by means of the holding clamp.
2. The desired volume of a water sample or of an appropriate dilution of a water sample was passed through the membrane filter. The funnel was rinsed three times using at least 50 ml of sterile distilled

water each time. A vacuum of 30-40 cm of mercury which was provided by a central source in the building, was used to draw the water through the filter.

3. The membrane filter was removed from the filter apparatus with sterile forceps and placed on an absorbent pad which had previously been saturated with culture media. Care was taken so that no air bubbles would be trapped between the membrane filter and the pad. The absorbent pads were contained in sterile 60 x 15 mm glass petri dishes.
4. The petri dishes were incubated in an inverted position at a temperature of 35 C for whatever length of time was desired. The surface tension exerted kept the absorbent pads in the bottoms of the petri dishes. In order to maintain an atmosphere of saturated humidity during the incubation period, the petri dishes were placed in a plastic refrigerator tray the bottom of which had been lined with moist cheese cloth. This tray had a tight-fitting lid.
5. During incubation the nutrient solution penetrated through the pores by means of capillary action to the bacteria which were deposited on the surface of the filter. After a given period of incubation, colonies were observed on the membrane filter as shown in Fig. 3 and 4.
6. A stereoscopic microscope which had a magnification of 10 diameters was used in counting the colonies. A light

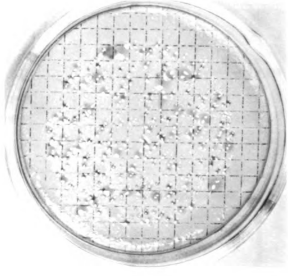

Figure 3. Bacterial Colonies on the Membrane Filter (Magnification 1.4X)

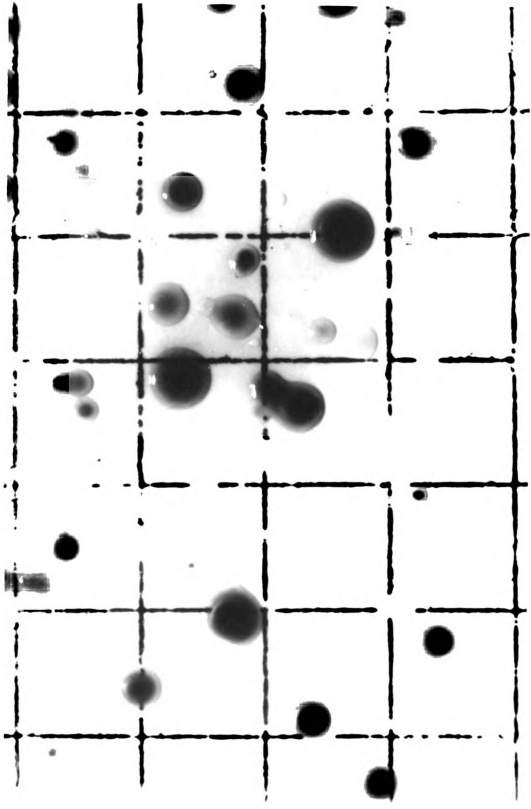

Figure 4. Bacterial Colonies on the Membrane Filter (Magnification 10.7X)

source which was nearly perpendicular to the membrane surface was used in making the counts.

Standard of Comparison

The results which were obtained in the estimation of coliform bacteria by the membrane filter technique were compared with estimations which were obtained simultaneously by means of the 5-tube, 3-decimal dilution MPN confirmed test as given in the tenth edition of "Standard Methods for the Examination of Water, Sewage and Industrial Wastes" (1955). In the Standard Methods' procedures lauryl tryptose broth was used in the presumptive test and all tubes which showed any amount of gas within 48 hours were confirmed in brilliant-green lactose bile broth. Difco dehydrated media were used throughout in the Standard Methods' tests. The completed test was not carried out. The numbers of coliform organisms which were recovered by means of the two techniques will be given as numbers per 100 ml of water sample.

In some of the investigations, laboratory stock strains of Escherichia coli were employed in testing various media before these media were used in the cultivation of coliform bacteria from raw waters. When this was done the standard plate count was used as the standard for comparison. The cultures were transferred daily in Brain Heart Infusion broth (Difco) for at least three days prior to the date of testing.

Appropriate dilutions were made using 90 ml sterile saline dilution blanks. Five replicate plates were poured for each determination and an average of the five plates was taken as the standard plate count. Brain Heart Infusion Agar was used as the plating medium. An incubation period of 24 hours at 35 C was also used.

Collection of Samples

The samples were collected in sterile, wide-mouth bottles with ground glass stoppers, according to the procedure outlined in Standard Methods. Prior to sterilization the tops of the sampling bottles were covered with Kraft paper and tied securely.

All samples which failed to show the presence of coliform bacteria by either the membrane filter or the MPN procedure were eliminated and these samples were not considered in the tabulation of results.

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Where deviations from the above procedures occurred, mention will be made in the proper places.

EXPERIMENTAL STUDIES AND RESULTS

Preliminary Experimental Problems

Certain technical problems were encountered during early studies with the membrane filter technique. The consideration of these problems was essential to the successful application of this technique. Therefore, it was necessary to deal with each of these problems, separately, as they arose and to reach an immediate solution or conclusion with regard to each before further progress could be made in the subsequent studies to be undertaken. The nature of each of these problems is described below.

I. Regulation of Humidity During Incubation

In the early studies which were made by the author, an attempt to maintain an atmosphere of saturated humidity during incubation by inverting the bottoms of petri dishes (which contained the membrane filters) on a moist towel placed in a glass tray was found to be most unsatisfactory. This method was suggested by McKee et al. (1950) and by Goetz and Tsuneishi (1951). When the membrane filters were incubated in this manner, the towel was found to be completely dried after a period of 18 hours in the incubator at 35C and the

absorbent pads were also found to be in a partially dried state. This resulted in extremely low counts on the membrane filter when a pure culture of Escherichia coli was used. Such a result was to be expected since the nutrient was not supplied to the bacteria during the full incubation period. During this study, 10 replicate plate counts were made utilizing Brain Heart Infusion Agar (Difco) as a plating medium. At the same time, 10 replicate membrane filters were also run. EHC modified Endo medium was employed for use with the membrane filters. This was preceded by a 2-hour preliminary enrichment period on Albimi M broth (Clark et al., 1951). The average count per membrane filter was found to be 51, whereas the average plate count was 170. Thus, the percentage of organisms recovered on the membrane filter, as compared to the plate count, was 30 per cent.

The above experiment was repeated with the following changes. The petri dish bottoms, containing the membrane filters, were inverted on a moist towel as previously mentioned. However, this time the petri dishes were covered with a second moist towel. A metal lid was then placed on top of the tray which contained the petri dishes and incubation was allowed to proceed in the usual manner. In this case, the humidity produced was apparently too great and, as a result, such extreme spreading occurred that it was impossible to make colony counts.

It was then discovered that a constant amount of moisture could be insured by the following procedure. The membrane filter was placed on an absorbent pad in the bottom of a petri dish. The petri dish was inverted and a sheet of moist filter paper was placed inside the cover. The dish was sealed with Scotch brand masking tape and placed in the incubator at 35C. This method gave satisfactory results, but it became too laborious when one had many filters to run.

Another procedure which gave reliable results and proved to be more practical is described below. In this procedure, incubation was carried out in a plastic refrigerator tray lined with moist cheese cloth and with a tight-fitting cover. Petri dishes, which contained membrane filters, were placed inside these trays in an inverted position. The trays were set in an incubator at 35C. In the experience of the author, the most dependable results were obtained by this method. Henceforth, in this study all membrane filters, when used with absorbent pads, were incubated in this manner.

II. Difficulties Encountered in the Preparation of EHC Modified Endo Medium

The EHC modified Endo medium was prepared as directed in the tenth edition of "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes"(1955).

In the preparation of the basal medium, it was observed that Neopeptone (Difco) formed a precipitate in distilled water.

The amount of precipitate increased on boiling. It was possible to dissolve this peptone completely by the addition of acid, but, as soon as the pH of the solution approached neutrality, a precipitate immediately formed.

Preliminary studies were made in which Bacto-Peptone, Bacto-Tryptose, Bacto-Tryptone, Proteose Peptone (Difco), Proteose Peptone No. 3 (Difco), and Peptone M (Albimi) were individually substituted for Neopeptone. When a pure culture of E. coli was used, Bacto-Peptone, Bacto-Tryptose, and Bacto-Tryptone gave results which were similiar to those obtained with Neopeptone both in total recovery and in production of metallic sheen. However, the total number of tests run was not sufficient and no significant conclusions could be drawn.

In preparing the complete medium from liquid indicator solutions, some of the basic fuchsin crystallized when this dye was added to the sodium sulfite solution. The amount of crystallization varied depending upon the manner in which these two solutions were combined. Rapid mixing produced less crystallization, but some crystals were always formed. Thus the question arose in the mind of the author as to whether or not this condition could result in a uniform dye solution in all batches of finished medium.

Powdered indicators also presented difficulties in preparation. Basic fuchsin, sodium sulfite, and lactose were ground to as fine a powder as was possible with a motar and

pestle. The homogeniety of this mixture, however, was highly questionable. When the complete medium was prepared from powdered indicators, the amount of dye which failed to go into solution was greater than could be accounted for by the amount of inert material initially present in the dye powder.

III. Recovery of *Escherichia coli* with EHC Endo Medium and Comparison of the Membrane Filter Count with the Standard Plate Count

Early investigations with a pure culture of *E. coli* indicated that all of these organisms were not recovered when EHC modified Endo medium was employed with the membrane filter technique. Therefore, the following study was undertaken to determine the recovery of *E. coli* in the use of this technique when the above mentioned medium was utilized.

In this study a series of 29 suspensions of a pure culture of *E. coli* were prepared in sterile saline dilution blanks. The number of organisms present in these suspensions was determined by both the membrane filter technique and the standard plate count. EHC modified Endo medium, prepared as directed in Standard Methods, was used with the membrane filter technique. A 20-hour incubation period on this medium was preceded by a 2-hour preliminary enrichment period on M-enrichment broth (Difco). (Note: The criterion for the recognition of coliform colonies on EHC Endo medium is the development of a metallic sheen.) Brain Heart Infusion agar

(Difco) was used as the plating medium in determining the plate counts. Five replicate membrane filters and five replicate plate counts were prepared for each series.

The results which are given in Table I are reported as averages of the five determinations made in each series. The average membrane filter count was 174 colonies, whereas the average plate count was 229 colonies. This gave a recovery of 76.0 per cent on the membrane filter as compared with the recovery on the standard plate count. The question arose as to whether this loss in recovery was due to inhibitory effects of the medium used or whether some of the organisms had been lost during the filtration process. Another question was also introduced here as to the possibility of obtaining a total recovery of E. coli with the membrane filter technique. In an attempt to answer these questions the following experimental study was conducted.

IV. Recovery of Escherichia coli with M-Enrichment Broth and Comparison of the Membrane Filter Count with the Standard Plate Count

In this investigation M-enrichment broth, a non-selective medium, was used with the membrane filter technique to eliminate the introduction of any inhibitory substances. Otherwise the procedure was the same as that which was employed in the preceding section. A series of 23 suspensions of a pure culture of

TABLE I

MEMBRANE FILTER RESULTS WITH EHC ENDO BROTH
COMPARED TO THE STANDARD PLATE COUNT

Series	Colony Count Per Filter	Plate Count	MF* Count as Percentage of Plate Count
1	161	210	76.7
2	96	153	62.7
3	115	165	69.7
4	209	296	70.6
5	175	217	80.6
6	193	265	72.8
7	177	233	76.0
8	256	293	87.4
9	140	173	80.9
10	165	235	70.2
11	201	265	75.8
12	254	347	73.2
13	108	149	72.5
14	267	325	82.2
15	173	253	68.4
16	232	313	74.1
17	154	211	73.0
18	219	309	70.9
19	158	193	81.9
20	186	293	63.5
21	165	217	76.0
22	157	205	76.6
23	105	124	84.7
24	132	169	78.1
25	152	227	67.0
26	176	203	86.7
27	146	189	77.2
28	198	229	86.5
29	170	167	101.8
Total	5040	6628	
Average	174	229	76.0

* Membrane Filter

E. coli was made and the recovery, which was obtained with the use of M-enrichment broth and the membrane filter technique, was compared with that obtained by the standard plate count utilizing Brain Heart Infusion agar.

The results are summarized in Table II and they are reported as averages of the five determinations which were made in each series. The average membrane filter count was 197. The average plate count was 203. The result was a recovery of 97.4 per cent on the membrane filter as compared to the recovery with the standard plate count. This indicated that a good agreement between these two procedures was possible. It also indicated that a satisfactory recovery can be obtained with the membrane filter technique if the medium which is used will allow the full development of the organisms desired. This verified the fact that the loss of 24 per cent in the recovery of E. coli obtained with the membrane filter in section III was due to the inhibitory effect which the EHC modified Endo broth had upon these organisms.

During this study, several attempts were made to recover E. coli from the filtrates which were passed through the filters. At no time were these attempts ever successful. This substantiated the finding that the loss in recovery was not due to the fact that E. coli was passing through the filters.

The conclusion reached by the author was that the main difficulty encountered in the recovery of E. coli with the membrane filter technique was due to the lack of a satisfactory

TABLE II

MEMBRANE FILTER RESULTS WITH M-ENRICHMENT BROTH COMPARED TO THE STANDARD PLATE COUNT

Series	Colony Count Per Filter	Plate Count	MF* Count as Percentage of Plate Count
1	95	120	79.1
2	274	340	80.6
3	204	202	101.0
4	164	154	106.5
5	243	234	103.8
6	207	186	111.3
7	198	217	91.2
8	183	192	95.3
9	257	273	94.1
10	214	211	101.4
11	133	134	99.3
12	114	106	107.5
13	120	131	91.6
14	233	212	109.9
15	125	142	88.0
16	254	240	105.8
17	236	242	97.5
18	252	249	101.2
19	195	207	94.2
20	310	343	90.3
21	215	193	111.4
22	155	165	93.9
23	142	151	94.0
Total	4523	4644	
Average	196.7	201.9	97.4

* Membrane Filter

selective medium. For this reason, the search for the formulation of such a medium was undertaken.

Media Formulation Studies

I. Formulation Number One (F-1)

As a result of exploratory studies which were carried out, the F-1 medium formulation was developed.

F-1

Bacto-Tryptose	40.0 g
Bacto-Lactose	20.0
Yeast Autolysate (Albimi)	6.0
Dipotassium Phosphate	2.75
Monopotassium Phosphate	2.75
Sodium Chloride	5.0
Sodium Lauryl Sulfate	0.2
Brom Thymol Blue	0.1
Distilled Water	1000 ml
KOH to give a pH of 7.0	

Suspensions of *E. coli* were prepared and the recovery which was obtained by the use of this medium and the membrane filter technique was compared with that obtained by the standard plate count. The results are given in Table III. Each figure recorded represents the average of five determinations.

In the study of the F-1 medium, the average colony count per membrane filter was 197 and the average plate count was 204. Thus, a recovery of 96.7 per cent was obtained on the membrane

filter as compared with the plate count. A satisfactory recovery was observed with the F-1 medium, but the colonies of E. coli were colorless and did not exhibit any differentiating characteristics which would make it possible to differentiate between these colonies and colonies of non-coliform bacteria. For this reason, the F-1 medium was not used in any future study.

TABLE III

MEMBRANE FILTER RESULTS WITH F-1 MEDIUM COMPARED TO THE STANDARD PLATE COUNT

Series	Colony Count Per Filter	Plate Count	MF* Count as Percentage of Plate Count
1	197	211	93.4
2	179	184	97.3
3	251	263	95.4
4	162	158	102.5
Total	789	816	
Average	197	204	96.7

* Membrane Filter

II. Formulation Number Two (F-2)

The possibility of using neutral red as an indicator for the detection of lactose fermenters from non-lactose fermenters growing on the membrane filter was considered. The F-2 medium was developed in an attempt to determine whether or not this indicator would give a satisfactory performance for use with the membrane filter. The F-2 medium differed in the following way from the F-1 medium which was used in the preceding section. The sodium lauryl sulfate was reduced to 0.1 g and the brom thymol blue was replaced with 0.08 g of neutral red and 0.001 g of crystal violet.

A suspension of E. coli was made in sterile saline solution. A total of ten replicate counts was made from this suspension utilizing the membrane filter technique and the F-2 medium. An average of 179 colonies was obtained for the ten filters. A total of ten replicate standard plate counts, made simultaneously, yielded an average of 201 colonies. This gave a recovery of 89.1 per cent on the membrane filter as compared with the standard plate count.

The F-2 medium produced a pink coloration on the entire membrane filter as was expected. The colonies, however, became only very slightly colored and no differentiating characteristics were observed. When acid was produced by the colonies, whole areas of the filter surface changed color. In other words, any color changes produced were not confined to

the colonies. Because of this, neutral red was not an adequate indicator for use with the membrane filter.

Other studies showed that Andrade's indicator was also unsatisfactory. When this indicator was incorporated into the F-1 medium, no hint of differentitation could be detected. The membrane filter and all colonies were white or colorless.

III. Formulation Number Three (F-3 EMB)

The following investigation was undertaken in an effort to develop an eosin methylene-blue medium which could be used successfully in the enumeration of coliform bacteria with the membrane filter technique. It has been stated that, "Eosin-methylene-blue broth to date has not been satisfactory for membrane filter work because the eosinate of methylene blue apparently does not diffuse through the membrane." (Kabler and Clark, 1952) These authors had found it feasible to use this combination of dyes in broth "only after the membrane has been saturated with methylene blue and washed free of the excess dye before filtration". (Clark, et al., 1952)

Several different media formulations were developed and investigated as this study was pursued. Numerous trials were made with various combinations of ingredients. Changes were also made in the composition of each medium under development by changing the concentration of each individual ingredient. The recovery of E. coli which was obtained by the use of each

formulation developed was compared with the recovery obtained by means of the standard plate count. These studies resulted in the development of F-3 EMB medium.

F-3 EMB

Base Medium

Bacto-Peptone ..... 20.0 g
Bacto-Lactose 20.0
Bacto-Agar 6.0
Eosin Y (93% dye content) ..... 24 ml
(an 8% aqueous solution)
Distilled Water ..... 900 ml

The base medium was dispensed in 90 ml quantities and sterilized in the autoclave at 121 C (15 lbs. pressure) for 15 minutes.

Dipotassium Phosphate Solution

Dipotassium Phosphate ..... 2.0 g
Distilled Water ..... 133 ml

This solution was dispensed in convenient quantities and sterilized in the autoclave at 121 C for 15 minutes.

Methylene Blue Solution

Methylene Blue (88% dye content) ... 2.0 g
Make up to 100 ml with distilled water

Preparation of Finished Medium

The finished medium was prepared by adding the following substances, in the amounts indicated, to 90 ml quantities of the base medium:

Dipotassium phosphate solution .....6.6 ml
Methylene blue solution .....3.6 ml

The concentration of dipotassium phosphate in the finished medium was found to be very critical with regard to sheen development in the colonies. The concentration which will give optimum sheen production must be determined for each lot of dipotassium phosphate used.

The F-3 EMB medium was poured into standard size petri dishes in approximately 25 ml quantities. The petri dishes were allowed to stand undisturbed at room temperature for at least 30 minutes. A sheet of sterile Whatman No. 1 filter paper was then placed on the surface of the medium in each petri dish. After filtration the membrane filters were laid on the surface of the filter papers. It was necessary to remove the membranes from the filter papers and re-lay them in order to liberate the air bubbles which became trapped beneath the membranes. Because the F-3 EMB was a semi-solid medium, the petri dishes were not inverted during incubation. For this reason, the use of clay top petri dishes was preferable. The petri dishes were incubated at 35 C for 22 hours. No attempt was made to control the humidity during the incubation period.

When F-3 EMB was used in this way, no difficulty was experienced and both dyes appeared to diffuse evenly through the membrane filters. However, when the agar was not included and attempts were made to use the medium as a broth, many difficulties occurred and the dyes failed to diffuse through the filters evenly. The results which are reported below were obtained with the agar medium. Coliform colonies on F-3 EMB medium were characterized by the presence of a metallic sheen. No preliminary enrichment period was used with this medium.

1. Recovery of *Escherichia coli* on F-3 EMB Medium and Comparison of the Membrane Filter Count with the Standard Plate Count

Thirty-four separate suspensions of a pure culture of *E. coli* were prepared using sterile saline solution. The recovery of *E. coli* from these suspensions by the use of the membrane filter technique utilizing F-3 EMB was compared with the recovery which was obtained by means of the standard plate count. The results are summarized in Table IV. Each series represents the average of five replicate membrane filter counts and of five replicate plate counts. The average filter count was found to be 148, while the average plate count was 163. This represents a recovery of 90.7 per cent on the membrane filter as compared with the standard plate count. These results with a pure culture of *E. coli* indicated a loss in recovery of approximately 10 per cent with F-3 EMB medium.

2. Recovery of Coliform Bacteria from Water on F-3 EMB and Comparison of the Membrane Filter Count with the Multiple-tube MPN

The Red Cedar River was selected as the source of water which was to be used in this investigation. The stream, which flows through the campus of Michigan State University, is heavily polluted. A series of dilutions was made of this water using 90 ml sterile saline dilution blanks. The MPN's were determined on the basis of the 5-tube, 3-decimal dilution confirmed test by the procedure as given in Standard Methods.

TABLE IV

MEMBRANE FILTER RESULTS WITH F-3 EMB
COMPARED TO THE STANDARD PLATE COUNT

Series	Colony Count Per Filter	Plate Count	MF* Count as Percentage of Plate Count
1	104	163	63.8
2	179	204	87.7
3	146	178	82.0
4	192	198	97.0
5	126	183	68.9
6	208	235	88.5
7	134	148	90.5
8	148	145	102.1
9	135	136	99.3
10	135	132	102.3
11	200	219	91.3
12	197	206	95.6
13	121	144	84.0
14	150	162	92.6
15	177	173	102.3
16	108	94	114.9
17	143	165	86.7
18	139	106	112.1
19	111	126	88.0
20	166	197	84.3
21	94	103	91.3
22	202	188	107.4
23	134	132	101.5
24	163	174	93.7
25	138	140	98.6
26	131	146	89.7
27	142	150	94.7
28	126	167	74.6
29	134	174	77.0
30	129	192	67.2
31	159	171	93.0
32	157	157	100.0
33	119	128	93.0
34	173	194	89.2
Total	5020	5532	
Average	148	163	90.7

* Membrane Filter

In an attempt to evaluate the F-3 EMB medium, a comparison was made between the recovery of coliform organisms obtained with this medium and the membrane filter technique and the recovery obtained by the MPN confirmed test. In this study, 20 membrane filters and 20 MPN's were run and estimations of the number of coliform bacteria present in the water were obtained by each procedure. The average MPN per 100 ml of sample computed from the membrane filter counts was 9,580. The average 5-tube MPN was 18,300. Therefore, the MPN calculated from the Standard Methods' confirmed test was found to be approximately twice that calculated from the membrane filter test utilizing F-3 EMB medium.

Random isolations were made from 496 of the colonies, grown on the membrane filters, which exhibited a metallic sheen. Of these, only 85 or 17.1 per cent produced gas in lauryl tryptose broth. The high dye concentrations which were necessary for the production of sheen apparently had an adverse effect upon the coliform organisms. For this reason, no later studies were carried out in an attempt to further develop an EMB medium.

It was discovered, however, that coliform bacteria could be grown on the membrane filter in the absence of dyes and that a metallic sheen could then be produced by placing the filters on F-3 EMB medium for four hours at room temperature. The F-4 medium was developed for use with the membrane filter in the study of this phenomenon.

IV. Formulation Number Four (F-4)

F-4

Bacto-Tryptose	40.0	g
Bacto-Lactose	20.0	
Dipotassium Phosphate	2.75	
Monopotassium Phosphate	2.75	
Sodium Chloride	5.0	
Sodium Lauryl Sulfate	0.15	
Bacto-Agar	6.0	
Distilled Water	1000	ml

The medium was dispensed in 100 ml quantities and sterilized in the autoclave at 121 C for 15 minutes. Because F-4 was a semi-solid medium, the method of preparing the petri dishes for incubation with the membrane filters was the same as that which has been described in the preceding section for the F-3 EMB medium.

A series of four suspensions of a pure culture of E. coli was prepared. For each series, five replicate membrane filter counts using F-4 medium, without a preliminary enrichment period, were obtained. Five replicate standard plate counts were also simultaneously obtained for each series. The membrane filters were incubated at 35 C on the F-4 medium for 18 hours. They were then removed and placed on F-3 EMB at room temperature for four hours. A metallic sheen developed on the colonies.

The recovery of E. coli, which was obtained by the two procedures, is given in Table V. An average of 209 colonies

was counted on the membrane filters, while the average plate count was 205. This represented a recovery of 101.8 per cent on the membrane filter as compared with the plate count.

TABLE V

MEMBRANE FILTER RESULTS WITH F-4 MEDIUM COMPARED TO THE STANDARD PLATE COUNT

Series	Colony Count Per Filter	Plate Count	MF* Count as Percentage of Plate Count
1	234	220	106.4
2	181	187	96.8
3	158	144	109.7
4	261	268	97.4
Total	834	819	
Average	209	205	101.8

* Membrane Filter

The results which were obtained with a pure culture of E. coli appeared to be satisfactory. However, when water samples were tested, very excessive overgrowth occurred on the membrane filters with F-4 medium. This overgrowth was, for the most part, caused by non-coliform organisms. Because of this, F-4 medium was not used in further studies.

V. Formulation Number Five (F-5)

Studies were carried out in search of a selective agent which could be incorporated into a medium designed for the

enumeration of coliform bacteria in water. Several different media formulations were investigated in the study of the following selective agents:

Dowicide K-7643[1]
Sodium Lauryl Sulfate
Oxgall
Sodium Azide
Brilliant Green
Di Sodium Versenate
Acid Fuchsin
Ethyl Violet
Sodium Desoxycholate
Bile Salts

None of the substances tested gave results which were completely satisfactory. The most promising results, however, were obtained with the use of Di Sodium Versenate (Versene). The F-5 medium was developed as a result of this investigation.

F-5

Base Medium

Bacto-Tryptose	40.0	g
Bacto-Lactose	20.0	
Sodium Chloride	5.0	
Bacto-Agar	6.0	
Distilled Water	930	ml

The base medium was tubed in 28 ml quantities in 50 ml test tubes and sterilized in the autoclave at 121 C for 15 minutes.

Dipotassium Phosphate Solution

Dipotassium Phosphate	2.0	g
Distilled Water	133	ml

Versene Solution

Di Sodium Versenate	3.0	g
Distilled Water	60	ml

[1] A product of Dow Chemical Company, Midland, Michigan.

Both the dipotassium phosphate and the Versene solutions were tubed in convenient quantities and sterilized in the autoclave at 121 C for 15 minutes.

Preparation of Finished Medium

The finished medium was prepared by adding 2.0 ml of dipotassium phosphate solution and 0.4 ml of Versene solution to 28 ml quantities of the base medium.

The F-5 medium, being semi-solid, was prepared for incubation in the same manner as described in section III for the F-3 EMB medium.

1. Recovery of *Escherichia coli* on F-5 Medium and Comparison of the Membrane Filter Count with the Standard Plate Count

A suspension was made of a pure culture of *E. coli*. Estimations of the number of *E. coli* present in the suspension were made by the use of the membrane filter technique employing F-5 medium. Simultaneous plate counts were made from the same suspension. A total of 20 counts was made on this suspension with each method. The average membrane filter count was 143. The average plate count was 145. This indicated a recovery of 98.6 per cent on the filter as compared with the plate count. Thus, a highly satisfactory recovery was obtained when a pure culture of *E. coli* was used.

2. Recovery of Coliform Bacteria from Water on F-5 Medium and Comparison of the Membrane Filter Count with the Multiple-tube MPN

When F-5 medium was employed with the membrane filter, coliform colonies growing on the filter did not exhibit any

characteristics by which they could be differentiated from other organisms. However, this medium did inhibit the growth of almost all non-coliform bacteria. Since a need existed for a study to determine whether or not it was possible to obtain a comparable recovery between the membrane filter technique and the 5-tube decimal dilution confirmed MPN with a raw water, the following investigation was undertaken.

A total of 31 samples was collected from the Red Cedar River. The recovery of coliform bacteria was determined by the membrane filter technique and by the MPN confirmed test. Since coliforms could not be differentiated from non-coliforms on the F-5 medium, transfers to lauryl tryptose broth were made from all colonies which developed on the membrane filters. During this study, isolations were made from 2,102 colonies. Of these, 1,724 or 82.0 per cent produced gas within 48 hours at 35 C in lauryl tryptose broth. In the case of the membrane filters, the MPN's per 100 ml of sample were computed on the basis of gas production of the isolates. The results are given in Table VI. The average MPN with the membrane filter technique was 13,600 coliforms per 100 ml of sample, and an average of 14,200 was obtained by means of the 5-tube, 3-decimal dilution MPN.

The results of this investigation showed that it was possible to obtain a satisfactory agreement between these two procedures with the river samples which were examined. The F-5 medium, however, was not practical for the routine

TABLE VI

MEMBRANE FILTER RESULTS ON WATER SAMPLES WITH F-5 MEDIUM COMPARED TO THE STANDARD METHODS' CONFIRMED MPN

Sample	Membrane Filter MPN Per 100 ml of Sample	Multiple Tube Confirmed MPN per 100 ml of Sample
1	10,000	13,000
2	31,000	49,000
3	28,000	33,000
4	36,000	23,000
5	25,000	44,000
6	31,000	17,000
7	29,000	12,000
8	22,000	31,000
9	26,000	15,000
10	16,000	11,000
11	19,000	17,000
12	14,000	17,000
13	6,000	24,000
14	8,000	13,000
15	6,000	13,000
16	8,750	7,900
17	5,500	3,300
18	7,250	4,000
19	4,750	7,000
20	4,500	7,900
21	8,500	6,200
22	5,750	4,600
23	6,500	7,000
24	9,500	7,900
25	8,500	11,000
26	3,750	4,900
27	7,750	8,400
28	10,400	7,900
29	9,000	5,400
30	5,600	7,000
31	8,800	7,900
Total	421,800	440,300
Average	13,600	14,200

examination of water samples because it was impossible to differentiate between coliform bacteria and non-coliform bacteria by colony appearance on the membranes. Therefore, the search for a satisfactory medium was continued.

VI. Formulation Number Six (F-6)

Since the results which were obtained with the F-5 medium showed a satisfactory recovery of coliform bacteria from water samples by the use of the membrane filter technique, the search for an adequate medium was pursued further. In this investigation, 145 different medium formulations or variations in medium formulations were compounded and tested. The F-6 medium was the result of this investigation.

F-6

Bacto-Tryptose	15.0	g
Sodium Chloride	5.0	
Bacto-Lactose	10.0	
Bacto-Bile Salts	5.0	
Brom-Cresol Purple	0.15	
Methylene Blue (88% dye content)	0.01	
Ethyl Violet[1]	0.0175	
Distilled Water	1000	ml

The medium was tubed in 25 ml quantities and sterilized in the autoclave at 121 C for 15 minutes. The pH was

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[1] The amount indicated is on the basis of 100 per cent dye content.

adjusted with KOH so that a final pH of 6.8 resulted after autoclaving. Before use, 0.25 ml of a sterile one per cent solution of sodium lauryl sulfate was added to each 25 ml quantity of medium.

The medium was added in 2.2 ml amounts to sterile absorbent pads contained in 60 x 15 mm glass petri dishes. Plastic refrigerator trays, lined with moist cheese cloth, were used to maintain an atmosphere of saturated humidity during incubation. An incubation period of 14 hours at 35 C was found to yield the best results. If incubation is allowed to continue for more than 15 hours, the results obtained cannot be considered reliable. No preliminary enrichment period was necessary with the F-6 medium.

After an incubation period of 14 hours, coliform bacteria produced yellow or pinkish-yellow colonies while colonies of non-coliform bacteria were blue or colorless. The F-6 medium, like the MacConkey broth developed by Taylor, Burman, and Oliver (1953 and 1955), incorporated bile salts as a selective agent and high concentrations of brom cresol purple as an indicator to detect lactose fermentation.

1. Results Obtained with F-6 Medium in the Determination of the Number of Coliform Bacteria in Water Samples

Three types of water samples were used in testing the F-6 medium. The first group of samples was taken from the Red

Cedar River which represents a very heavily polluted stream. The second group was collected from Lake Lansing. This lake was selected because it contained a very high non-coliform population in relation to the coliform population. The third group of samples was taken from various contaminated wells.

Coliform determinations were made on each sample by means of the membrane filter technique. Both F-6 and EHC modified Endo medium were used in making these determinations. The results were compared with coliform determinations which were simultaneously obtained by means of the Standard Methods' 5-tube, 3-decimal dilution confirmed test. The results, which were obtained with the Red Cedar River and Lake Lansing, are given in Tables VII and VIII. Each membrane filter count is based on an average of two filters.

The average of the membrane filter counts for a total of 26 Red Cedar River samples was 12,223 with the F-6 medium and 7,845 with EHC modified Endo medium. The average of the 5-tube confirmed MPN's was 13,754 for these same samples. Thus, F-6 medium gave a better agreement with the MPN determination than that obtained with the Endo medium.

In the case of the Lake Lansing samples, the average membrane filter count for 20 samples was 21 with F-6 and 17 with Endo medium. The average for the tube MPN's was 29. Because, all three coliform determinations were fairly close, no conclusion could be drawn from these results. However,

TABLE VII

NUMBERS OF COLIFORMS ESTIMATED
PER 100 ML OF SAMPLE

RED CEDAR RIVER

Sample Number	F-6 Broth	EHC Modified Endo Broth	Multiple Tube MPN
1	10,600	8,800	23,000
2	22,400	16,900	40,000
3	18,400	14,800	21,000
4	20,400	10,200	17,000
5	11,700	7,700	13,000
6	14,200	5,200	2,100
7	4,700	3,400	1,700
8	12,200	6,100	11,000
9	9,100	7,200	7,900
10	4,300	3,300	3,300
11	8,500	6,900	7,900
12	7,900	4,300	2,300
13	4,300	3,200	5,200
14	14,400	14,400	13,000
15	28,300	19,300	31,000
16	17,500	14,300	35,000
17	18,400	13,100	19,000
18	12,600	3,700	17,000
19	17,300	3,160	17,000
20	3,700	1,700	2,300
21	13,300	6,100	17,000
22	11,500	9,200	23,000
23	6,900	3,800	4,900
24	11,400	6,400	11,000
25	8,300	6,100	7,900
26	5,400	4,700	4,100
Total	317,800	203,960	357,600
Average	12,223	7,845	13,754

TABLE VIII

NUMBERS OF COLIFORMS ESTIMATED
PER 100 ML OF SAMPLE

LAKE LANSING

Sample Number	F-6 Broth	EHC Modified Endo Broth	Multiple Tube MPN
1	20	14	22
2	12	18	33
3	10	4	23
4	4	4	13
5	8	0	7.8
6	0	8	23
7	4	2	17
8	61	20	49
9	35	45	43
10	57	41	64
11	16	19	23
12	18	12	17
13	13	8	31
14	0	4	17
15	4	12	14
16	8	8	33
17	8	8	13
18	55	15	33
19	45	52	46
20	51	38	54
Total	429	332	576
Average	21	17	29

excessive overgrowth of non-coliform bacteria was observed with both F-6 and EHC modified Endo medium. Because of this, neither medium was completely satisfactory with the Lake Lansing samples.

The well samples were collected on the day prior to the test date. They were then stored in the refrigerator at 8 C for 24 hours. The samples were clear at the time of collection, but, on standing, iron precipitated from many of them and during filtration this iron was deposited on the surface of the membrane filters. These iron deposits caused extreme spreading among the colonies on the filters which often resulted in confluent colonies. This was especially true when EHC modified Endo medium was used. It also resulted in the failure of colonies to produce a metallic sheen on this medium. With F-6 medium, however, discrete, isolated colonies were obtained regardless of whether or not iron deposits were present on the filters. Because of the difficulty with iron precipitates, all well samples were collected in duplicate 16-oz sampling bottles. One sampling bottle contained approximately 0.04 g of sodium thiosulfate while the second bottle contained 0.05 g of di sodium Versenate (Versene). Otherwise the procedure was the same as was used with the two water sources previously described.

The results which were obtained with the well samples are summarized in Table IX. When Versene was present in the sampling bottles, a five-fold increase was observed in the coliform counts on the membrane filters and a three to four-fold increase was observed in the tube MPN determinations.

2. Gas Production in Lauryl Tryptose Broth of Isolates from F-6 Medium and from EHC Modified Endo Medium

During the previous study on water samples from the Red Cedar River, Lake Lansing, and various contaminated wells, isolations were made from many colonies growing on the membrane filters. These isolations were made from both F-6 and from EHC modified Endo medium. The isolates were tested for their ability to produce gas in lauryl tryptose broth within 48 hours at 35 C.

In the study of the F-6 medium, isolations were made from 1,028 yellow colonies. Of these, 941 or 91.5 per cent produced gas from lactose. Of 155 isolations which were made from pinkish-yellow colonies, 114 or 73.5 per cent produced gas. There were also 253 isolations made from other colony types and of these only 3 or 0.01 per cent produced gas.

From 313 isolations which were made from sheen colonies on EHC modified Endo medium, 298 or 95.2 per cent produced gas. Of 300 isolations which were made of non-sheen colonies, 42 or 14 per cent produced gas.

TABLE IX

COLIFORM ESTIMATIONS ON CONTAMINATED WELLS

Numbers of Coliforms per 100 ml of Sample

	Sodium Thiosulfate in Sampling Bottle			Di Sodium Versenate in Sampling Bottle		
Sample Number	F-6 Broth	EHC Endo Broth	Multiple tube MPN	F-6 Broth	EHC Modified Endo Broth	Multiple tube MPN
1	12	8	17	12	6	27
2	2	0	7.8	10	2	2
3	890	970	330	3500	3100	3300
4	10	78	230	430	310	350
5	142	81	230	102	84	140
6	83	122	170	90	154	270
7	940	960	390	4200	3500	3200
8	61	156	790	314	164	330
9	4	0	33	92	16	130
10	420	411	460	4700	4200	3300
11	0	4	6.8	10	4	9.3
12	16	10	23	58	26	22
13	480	360	540	4400	3420	3500
14	80	197	460	450	580	490
15	51	190	490	720	490	490
16	14	12	31	80	36	170
Total	3205	3559	4209	19168	16092	15730
Average	200	222	263	1198	1006	983

DISCUSSION

The membrane filter, a relatively new tool in the field of bacteriology, has found its widest application in the bacteriological analysis of water. Satisfactory results in detecting coliform organisms in water by the use of the membrane filter technique have been reported by many authors. The technique has received rather wide acceptance among technicians who are engaged in water analysis. Kenyon (1955) intimates that the membrane filter has "displaced methods for the examination of contaminants in potable waters which have remained standard for nearly fifty years." He also refers to the Standard Methods' dilution tube technique as "the old M.P.N. procedures". Such assumptions are premature, of course, for Standard Methods states, "It must be understood that this is in no way a standard technique, and it cannot be considered an acceptable substitute for the dilution tube method."

In the experience of the author with the membrane filter technique, several difficulties were encountered which cannot be ignored. When membrane filters were incubated on absorbent pads which contained nutrient media, problems arose concerning the regulation of humidity during incubation. Taylor, Burman, and Oliver (1953 and 1955) have experienced difficulties similar to those of the author with regard to moisture control.

On the other hand, Slanetz and Bartley (1955) have indicated that incubation in an atmosphere of saturated humidity did not seem to be necessary and they reported that comparable counts were obtained when incubation was carried out under normal incubator conditions. However, it was the experience of the writer that humidity control could not be disregarded in the application of the membrane filter technique if valid results were to be obtained.

Certain difficulties were also encountered in the preparation of EHC modified Endo medium. As a result, the writer considered it highly doubtful that all batches of complete medium, prepared from either liquid or powdered indicators, contained uniform amounts of dye in solution.

With a pure culture of E. coli, a recovery of only 76.0 per cent was obtained when EHC modified Endo medium was employed with the membrane filter technique as compared with the recovery obtained by the standard plate count. Thus, if it is impossible to obtain complete recovery on EHC modified Endo medium with a strong laboratory culture of E. coli, then it is certainly unwarranted to expect a complete recovery when dealing with attenuated coliforms which are commonly encountered in the examination of a water supply.

It was later shown that a full recovery could be obtained on the membrane filter with a pure culture of E. coli when a non-selective medium was employed. This observation, together with the finding that all attempts to recover E. coli from the

filtrates were unsuccessful, indicated that the loss in recovery was not due to the fact that E. coli was passing through the filters. It also showed that the loss in recovery was not due either to a toxicity of the filters for the organisms or to any effect resulting from the mechanical process of filtration. The only other factor, then, which could account for the loss in recovery was that a toxicity for E. coli was exhibited by the EHC modified Endo medium. Thus, it was concluded that this medium was toxic to E. coli and that the main difficulty in the application of the membrane filter technique to the bacteriological analysis of water lay in the lack of a satisfactory selective medium.

Experimental studies were carried out in an attempt to develop a more satisfactory selective medium for the enumeration of coliform bacteria from water by the use of the membrane filter technique. During these studies six main media formulations were developed. Five of these formulations were eventually discarded and eliminated from further studies. This was due to either a lack in development of differentiating characteristics of the coliform bacteria growing on the media or as in the case of the F-3 EMB, to an adverse effect which the medium had upon these bacteria.

The most satisfactory results in this investigation were obtained with the F-6 medium. Although small amounts of methylene blue and ethyl violet were present, this medium derived its selective action primarily from bile salts and it

incorporated brom cresol purple which acted as an indicator to detect lactose fermentation. The MacConkey broth of Taylor, Burman, and Oliver (1953 and 1955) and the F-6 medium are the only media which have been developed thus far for the detection of coliform bacteria from water which do not employ high concentrations of a toxic dye as a selective agent and which do not rely upon the production of a metallic sheen as the criterion for the indication of coliform colonies. To date, all media which have received wide acceptance for use with the membrane filter in the detection and enumeration of coliform bacteria have been modifications of an Endo-type broth and coliform production on these media is determined by the presence or absence of a metallic sheen. The amount of basic fuchsin which is required for the production of this metallic sheen is large and such a quantity apparently exhibits a toxic effect upon some of the coliform bacteria as well as upon non-coliform bacteria.

Three types of water samples were selected for the testing of the F-6 medium. The first group of samples were taken from a heavily polluted river, the second group from a lake which contained a very high non-coliform population in relation to the coliform population and the third group from various contaminated wells. Coliform determinations were made on each sample by means of the membrane filter technique. Both F-6 and EHC modified Endo medium were employed in the use of this technique. Simultaneous coliform

determinations were also made by means of the Standard Methods' 5-tube, 3-decimal dilution confirmed test.

F-6 medium was found to be superior to EHC modified Endo medium in the coliform determinations which were made on the river samples. In the testing of 26 samples, an average coliform estimation of 12,223 per 100 ml of sample was obtained with F-6 medium. An average estimation of 7,845 was obtained by the use of EHC modified Endo medium, whereas the 5-tube confirmed MPN gave an average of 13,754 for the same 26 samples.

In the case of the lake samples, neither F-6 medium or EHC modified Endo medium was completely satisfactory. Difficulties were experienced with both media due to excessive overgrowth of the surface of the membrane filters by non-coliform bacteria.

With the well samples, difficulties were experienced due to the precipitation of iron. Filtration resulted in the concentration of iron deposits on the surface of the membrane filters. This condition caused both extreme spreading which resulted in confluent colonies and failure to produce a metallic sheen when EHC modified Endo medium was employed. When F-6 medium was used, however, discrete, isolated colonies were obtained regardless of whether or not iron deposits were present. Since the results obtained with F-6 medium are not dependent upon the presence or absence of a metallic sheen,

any difficulties which were encountered in the use of this medium were greatly minimized.

It was learned that the precipitation of iron in the well samples could be prevented by the addition of di sodium Versenate (Versene) to the sampling bottles. However, the presence of Versene in the sampling bottles resulted in a five-fold increase in the average coliform counts obtained with the membrane filters and a three to four-fold increase in the 5-tube MPN determinations. Therefore, if Versene is used to prevent the precipitation of iron, this would necessitate a revision in the standards to be met for such waters. This would be a definite disadvantage of the membrane filter technique since it would be far from desirable to have two sets of standards, one for well waters or those which contained iron and one for waters of other types.

As far as the F-6 and the EHC modified Endo media were concerned, comparable results were obtained with both media on the well samples and the results were in agreement with those obtained by means of the Standard Methods' multiple-tube MPN confirmed test.

During this study, numerous isolates from both F-6 medium and EHC modified Endo medium were tested for their ability to ferment lactose with gas production. It was found that it was impossible by means of colony appearance on the surface of the membrane filter to differentiate accurately between an organism which was capable of fermenting

lactose with the production of both acid and gas and one which was capable of fermenting lactose with the production of acid only. This finding was in agreement with observations which have also been made by Taylor, Burman, and Oliver (1953 and 1955).

The author is of the opinion that many of the advantages which are claimed to make the membrane filter technique superior to standard procedures are more apparent than real. It is true that the short incubation period used with the resulting saving of time and the fact that two or more different media can be applied in succession are factors which favor the adoption of this technique. The increased size of the sample which may be examined has often been stated as an advantage of this technique since this made possible the examination of waters which have a low bacterial density. However, it is unnecessary to examine larger volumes of water than is permitted by the use of present standard techniques since these techniques have proven to be more than adequate for the detection of a safe water supply. In fact, these techniques might be criticized on the basis that their use may condemn a safe water supply, but certainly not for their inability to detect a supply which is unsafe.

Claims have been made that the membrane filter technique is simpler in operation than present standard techniques. (Goetz, 1947) (Goetz and Tsuneishi, 1951) The opinion of the writer is in disagreement with this viewpoint.

It is also to be questioned as to whether the membrane filter technique is more economical than standard procedures especially when the high cost of the membranes is considered. And although small volumes of media are employed in this technique, the fact that the media has to be used in high concentrations must be taken into account.

Field applications of the membrane filter technique have been suggested by Geldreich et al. (1955), Levin and Laubausch (1954), and others. However, in the bacteriological testing of water it is necessary that the quantity of water which is passed through the membrane filter be adjusted according to its coliform density. Small quantities of heavily polluted waters must be diluted with sterile water so that errors will not occur due to the use of too small a sample. For this reason, all proposed field tests can be used only with low density waters since it would be unfeasible to carry sterile dilution bottles out into the field to prepare dilutions.

In addition, the membrane filter technique cannot be used satisfactorily in the examination of waters which contain suspended solids as this would result in the clogging of the filters. In order to examine such waters by means of the membrane filter technique, it would be necessary to utilize the "Concentrometer" as devised by Goetz (1953a) which would further add to the cost of the examination.

SUMMARY AND CONCLUSIONS

1. A detailed investigation of the membrane filter technique was made in order to evaluate its usefulness for determining the sanitary quality of water supplies. Studies were conducted on various aspects of this technique and its application to the bacteriological analysis of water. During early studies that were carried out, certain technical problems arose. The solution of these problems was necessary before subsequent studies could be undertaken. The nature of each of these problems has been described.

2. Studies, using a pure culture of E. coli, showed that a recovery of only 76.0 per cent of the organisms was obtained by the use of the membrane filter technique when EHC modified Endo medium was employed. However, when a non-selective medium was used, a recovery of 97.4 per cent was obtained with this technique. These results showed that EHC modified Endo medium had an inhibitory effect upon E. coli. Since there was a loss of 24 per cent in the recovery of E. coli, it was realized that this Endo medium would not permit the complete recovery of all coliform bacteria from water samples. Therefore, the need for a more satisfactory selective medium was recognized.

3. A search was undertaken in an attempt to formulate a culture medium for use with the membrane filter which would

permit the complete recovery of all coliform organisms from water samples. During this study six main media formulations were developed and tested. Five of these formulations were eventually discarded. However, it was shown that a satisfactory agreement in results could be obtained between the membrane filter technique and Standard Methods' 5-tube, 3-decimal dilution confirmed test.

4. The F-6 medium resulted from the compounding and testing of 145 different formulations or variations in formulations. This medium was the most satisfactory of those developed. In the examination of the river samples, it was found superior to EHC modified Endo medium in that it gave a better agreement with the Standard Methods' MPN confirmed test. With lake samples, which contained a very high non-coliform population in relation to the coliform population, neither F-6 or the Endo medium was completely satisfactory. In the case of well samples, comparable results were obtained with both F-6 and the Endo medium as far as coliform determinations were concerned. However, when iron deposits were concentrated on the filters extreme spreading took place which resulted in confluent colonies. In addition these colonies failed to produce a metallic sheen on the Endo medium. With the F-6 medium spreading among the colonies was greatly reduced and, since the criterion for coliform detection did not depend upon the production of a metallic sheen, less difficulty was experienced in the use of this medium.

5. It was discovered that the precipitation of iron from the well samples could be prevented by the addition of Versene to the sampling bottles. This, however, resulted in a substantial increase in the coliform estimations which were obtained with both the membrane filter technique and the Standard Methods' MPN confirmed test. The use of Versene in the sampling bottles would therefore necessitate a revision in the standards which are to be met.

6. Many isolates from both F-6 medium and EHC modified Endo medium were tested for their ability to ferment lactose with gas production. It was found that gas production could not be accurately predicted from the colonial appearance of organisms growing on the surface of the membrane filter.

7. Several advantages have been reported in favor of the membrane filter technique which seemingly make this technique superior to the Standard Methods' MPN procedure. The writer is in disagreement with many of these so-called advantages.

8. The results of this study indicate the inadequacy of the membrane filter technique for evaluating the sanitary bacteriological quality of waters. The main difficulty encountered in the use of the membrane filter technique results from the lack of a satisfactory selective medium. Although the F-6 medium was found to be more satisfactory than EHC modified Endo medium in the examination of certain waters, it was not

found to be completely satisfactory with all types of waters. Before the membrane filter technique can be accepted as a standard procedure for the examination of waters, a selective medium must be developed which will permit the complete recovery of all coliform bacteria from all types of waters. Otherwise, the use of this technique can produce a distorted view of the condition of the water by giving misleading results.

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