A STUDY OF VARIOUS METHODS FOR THE ENUMERATION OF ENTEROCOCCI IN RIVER WATERS AND SEWAGE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

Karl Kereluk

1956

This is to certify that the

thesis entitled

A Study of Various Methods for the
Enumeration of Enterococci in River Waters and Sewage

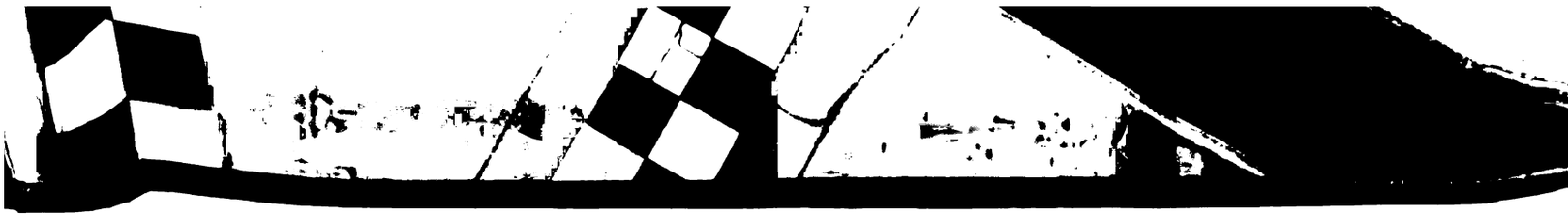
presented by

Karl Kereluk

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and Public Health

Date June 26, 1956



A STUDY OF VARIOUS METHODS FOR THE ENUMERATION OF ENTEROCOCCI IN RIVER WATERS AND SEWAGE

By

Karl Kereluk

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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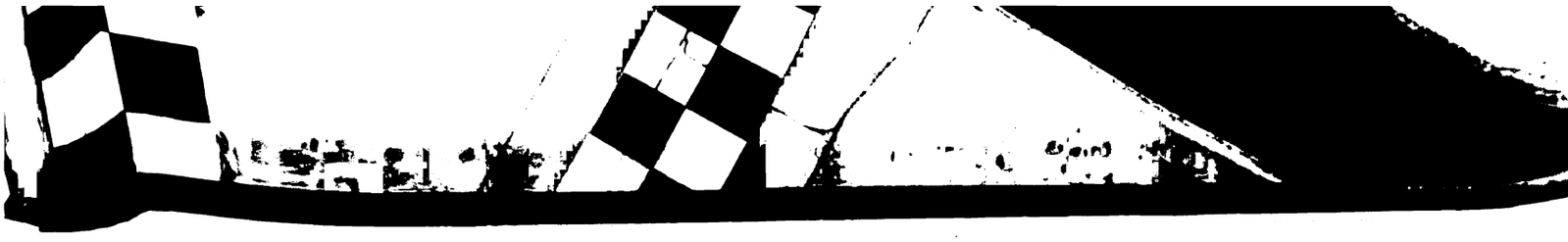


Karl Kereluk

A drop plate technic was introduced and a specific medium for growing typical and atypical enterococci was developed. The formula for this medium was as follows:

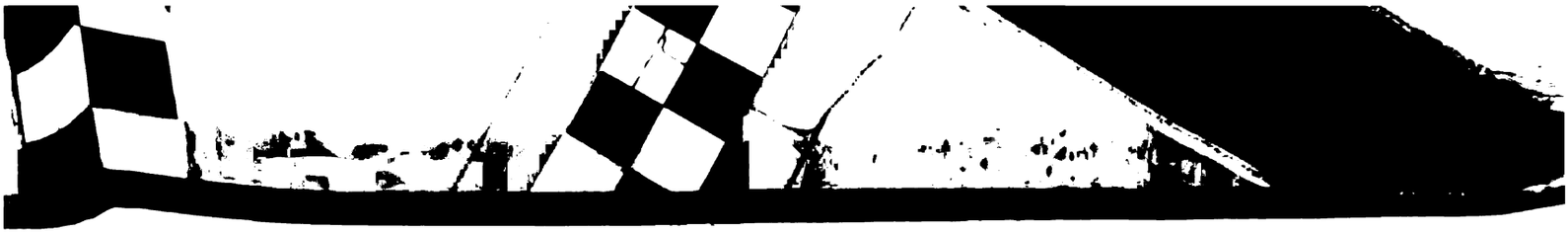
Ingredient	Grams per liter
Phytone	20.0
Lactose	5.0
Sodium chloride	5.0
Sodium azide	0.4
Yeast extract	5.0
Ethyl violet	0.00083
K_2HPO_4	2.7
KH_2PO_4	2.7
Agar	15.0

pH - 7.0

Sterilized at 121 C for 15 minutes

Samples of river and sewage waters were tested by a most probable number procedure, drop plate, and the membrane filter. The drop plate method detects more enterococci than by any method used, however, the method has its limitations in that it is effective only in examination of high population waters.

The membrane filter method serves amply where the limitations of the drop plate method begin. A modified ethyl violet azide medium for the enumeration of enterococci for use with the membrane filter technic was introduced.



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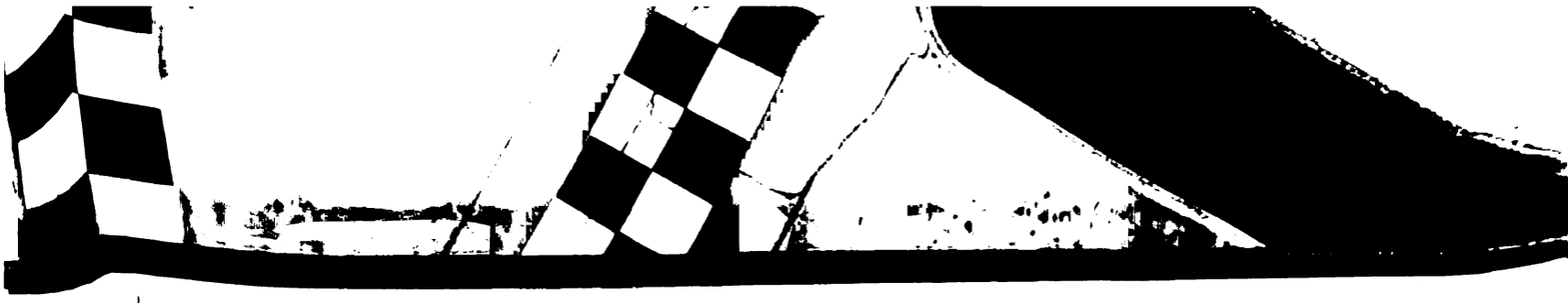

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VITA

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Doctor of Philosophy

Final examination, June 26, 1956, 10:00 A.M., Room 300, Giltner Hall.

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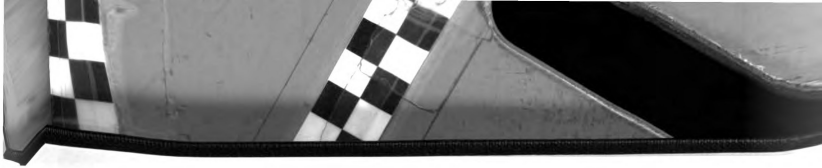

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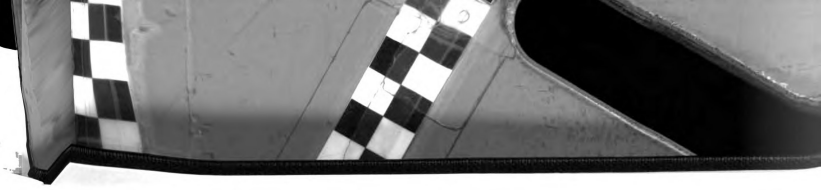

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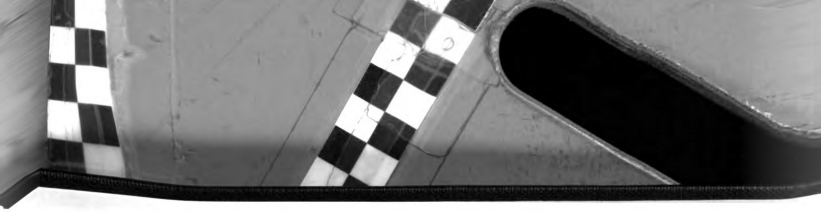
INTRODUCTION

Coliform organisms are the only recognized test organisms for the detection of sewage contamination of water and foods. The tests for these organisms are officially made by the United States Public Health Service and local health agencies. The official tests are prescribed in the Standard Procedure for the Analysis of Water and Sewage of the American Public Health Association.

Although the coliform group is the accepted test organisms, they do not always give a complete picture because these organisms persist in water and soil for extended periods of time and do not indicate recent pollution. These organisms are present in non-contaminated soil. Fecal and non-fecal strains of coliform bacteria cannot be differentiated.

Within the past 10 years, the enterococci group has been suggested as possible test organisms for the detection of fecal contamination in water supplies, foods, soil and other material. These organisms are used as indicators of pollution on much the same basis as the coliform organisms because they are present in feces, sewage, and known contaminated water but they are not present in non-polluted and virgin soil. They do not multiply outside of the human



or animal body except in the presence of a rich nutrient medium. The failure of these organisms to grow outside of the human or animal body makes them ideally suited for the detection of recent fecal or sewage contamination.

Mallmann and Litsky (39) demonstrated that in soil contaminated with sewage, the most probable numbers of enterococci were one to ten to that of the coliform organisms. It was also shown that the enterococci disappeared more rapidly from the soil while the coliform group persisted for long periods of time. When the typhoid bacillus was compared with the enterococci, the typhoid bacillus died out more rapidly. These workers believed that the enterococci were much more indicative of recent fecal pollution than the coliform group.

In 1950 Mallmann and Seligmann (41) reported a new medium, dextrose azide broth, for the detection of enterococci and other streptococci. However, growth in this medium must be confirmed for streptococci by microscopic examination.

Litsky, Mallmann, and Fifield (31) developed a confirmatory medium to determine the enterococci index in a manner similar to that used for the coliform group. They proposed a new test for the enterococci using azide dextrose broth as a presumptive medium and an ethyl violet azide broth as a confirmatory medium. The authors found that the dextrose azide and ethyl violet azide media confirmed 100 to 1,000

times as many enterococci as did the Hajna-Perry S. F. (18) method and the Winter-Sandholzer procedures (64).

With the introduction of the membrane filter method by Goetz (16) in 1947, numerous workers quickly adapted the new technic to the detection of the coliform group. Slanetz et al (55) applied the new technic to the enumeration of enterococci in river waters. They were able to demonstrate with this method that the counts for the enterococci were generally higher than those obtained by other procedures.

Mallmann, Peabody, and Broitman (40) applied the surface plating technic of Pomales-Lebrón and Fernandez (46) to the enumeration of high-population samples. Milk and polluted waters were used in the experiment. The interesting part of this research was that large numbers of coliform organisms were able to grow.

The purpose of this research was to adapt the surface plating technic for waters of gross pollution and the membrane filter method in slightly polluted water for the enumeration of enterococci. Present methods missed a large number of enterococci.

REVIEW OF LITERATURE

The Presence and Distribution of Streptococci

In 1894, Laws and Andrews (30) isolated streptococci from hospital sewage in England. Winslow and Hunnewell (60) in 1902 were the first in the United States to isolate streptococci from sewage. They also found them to be similar to those found on the hands of school children in association with Escherichia coli. Houston (24) reported in 1899 that streptococci, as well as staphylococci, were found in large numbers in sewage. Streptococci were found in 0.1 to 0.001 ml of water from six polluted rivers. Horrocks (23) 1901, found a high percentage of streptococci in sewage-polluted water but no E. coli were detected. Broadhurst (5) in 1915 stated that streptococci were less common in soil than in water.

In a study on the distribution of enteric streptococci, Ostrolenk and Hunter (43) examined soil and feces of man, dog, cat, mouse, guinea pig, rabbit, chicken, fly and monkey. Fifty-one fecal samples and two soil samples were examined using Perry's S. F. broth. The soil samples were negative for both E. coli and enterococci. Forty-nine of the samples from the 10 animals contained enterococci. Only 46 samples contained E. coli.

Andrews (2) examined 48 samples of hay, grass and leaves in 1906; only two samples showed streptococci. In another series of 18 samples, only one from a country roadside overflow showed a short-chained coccus.

Winslow and Palmer (63) in 1910 isolated and differentiated enterococci, using acid production in sugar, from the intestinal tract of horses, cows, and man.

In 1912, Clemesha (10) reported the presence of streptococci in polluted waters in India. Streptococci were present in 0.001 to 0.00001 of a gram of feces. They noted that these organisms were rare in waters which were not heavily polluted.

Broadhurst (5) in 1915 believed that the streptococci were neither indigenous to soil nor grains. She reported that the streptococci were difficult to obtain from feces of dogs, cats, and cattle; but that they were easily obtained from equine and human feces.

That enterococci however, were isolated from the normal digestive tract of calves by Orcutt (44) in 1926. He also showed that the streptococci were not a homogeneous group of organisms.

Winter and Sandholzer (64) reported that streptococci were not routinely found in virgin soil or in soils from heavily wooded areas. Streptococci were always present, however in samples of human and animal feces.

Steinhouse (56) in 1941 succeeded in isolating S. faecalis from seven orders of the class Hexapoda.

Walter and Weaver (57), Leiniger and McCleskey (31) and Ritter et al (50), showed the presence of enterococci in well water; they preferred the enterococci test to the coliform test.

Classification of the Enterococci

Escherich (14) in 1886 described the morphology of the streptococci in detail and found them to be normal inhabitants of the intestinal tract of infants. The lack of a definite classification system caused a mass of confusion in the literature. At present doubt exists as to what constitutes an enterococcus.

Hirsh and Libman (21) in 1897 described an organism, Streptococcus enteritis, which appeared to be identical with the enterococcus of Thiercelin (Micrococcus avalis of Escherich). He emphasized the pleomorphic morphology of the organism and stated that the organism was able to grow at 46 C, did not liquify gelatin, and inconsistently coagulated milk. The organism was killed by an exposure for 15 to 20 minutes at a temperature of 60 C and did not ferment sugars.

Winslow and Palmer (63) reported on the fermentative characteristics of 116 strains of Streptococcus isolated from

10 persons. The majority of the samples were from diarrheal stools.

Gordon in 1902 (17) started the important work of classifying streptococci. He introduced seven biochemical tests which are associated with his name. By means of these tests, he was able to distinguish 48 varieties among 300 streptococci isolated from normal saliva.

The first exhaustive studies on the human fecal streptococci were made by Houston (25) in 1904. He classified 300 strains by means of the Gordon fermentations, taken from 19 stool samples. Houston discovered that the organisms fell into 40 groups on the basis of their fermentative characteristics and proposed 10 classes into which the majority of his strains fell.

Andrews and Horder (3), using the results of Gordon and Houston, applied a series of tests to a large number of streptococci isolated from diseased individuals. Their results indicated that they were able to place the entire series of organisms into seven large groups, each having a definite type, indicated by its biological activity. The authors were the first to describe Streptococcus faecalis.

Donaldson (13) in 1917 summarized the characteristics of the enterococci. He reported that the enterococci grew in the form of pneumococcus-like diplococci, was non-hemolytic and produced acid from glucose, lactose, maltose, saccharose, raffinose, glycerol, mannitol, and inositol.

Weissenbach (58) in 1918 differentiated S. faecalis from S. pyogenes using a liquid medium containing 10 percent bile. The enterococci grew but the other streptococci did not.

Dible (12) in 1921 reported that the ability to withstand exposure to heat was not a consistent characteristic of all intestinal streptococci. By this differentiation he was able to divide the enterococci into two groups, one of which consisted of organisms having fermentative reactions corresponding to S. faecalis; the other group consisted of organisms which frequently occurred in saliva.

Bagger (4) in 1926 used one percent peptone plus one percent ox-bile for the classification of the enterococci.

Alston (1) confirmed the work of Dible. He clearly defined a group of organisms which could be classified as enterococci. The characteristics of this group are: (1) heat resistant at 60 C for 10 minutes, (2) cocci, oval in shape, occurring in pairs or short chains, (3) non-hemolytic, and (4) able to ferment mannitol.

Holman (22), using hemolysin production and the ability to ferment lactose, mannitol, and salicin, as the criterian, classified the streptococci into 16 types.

Welsh (59) in 1929 described six strains of streptococci common to human feces.

Sherman, Mauer and Stark (53) made an exhaustive study on 434 cultures of S. faecalis. They found that S. faecalis, S. durans, S. zymogenes, S. liquefaciens would meet the following requirements: (1) grow at 10 C and at 45 C, (2) complete reduction of litmus milk, (3) grow at pH 9.6, (4) grow in 6.5 percent sodium chloride, and (5) grow in 0.1 percent methylene blue in skimmed milk.

Sherman (52) in a later work indicated that the enterococci could grow only at a temperature of 45 C and in 6.5 percent sodium chloride. He used this as a basis for group classification of the enterococci.

Streptococci as Indicators of Pollution

Houston (24) in 1898 was the first to report on the significance of these organisms. He concluded that streptococci are of sanitary significance and indicate a more recent pollution than do the coliform organisms. This report lead to the name "sewage streptococci of Houston" which was given to these organisms.

In 1904, Houston (25) stressed the fact that streptococci as well as staphylococci, were indicative of recent pollution by human and animal wastes.

Later Horrocks (23) supported Houston's findings. He found these organisms in large numbers in sewage and polluted

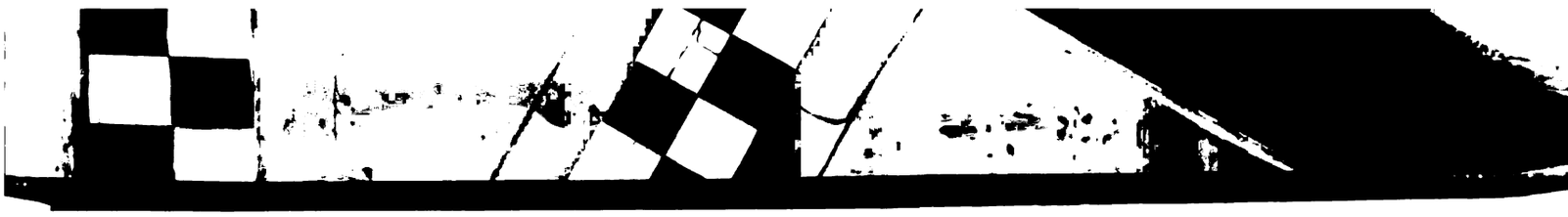
waters which contained no B. coli. He found that B. coli gradually disappeared from many specimens of sewage kept in the dark in an outside veranda. The organisms which persisted were varieties of streptococci and staphylococci.

Winslow and Hunnewell (61) were the first in the United States to report the presence of "sewage streptococci" in sewage in 1902. They isolated these organisms from the hands of school children in conjunction with E. coli and found them to be similar to those found in Boston sewage.

Prescott and Baker (45) found streptococci in 50 samples of polluted water. Winslow and Nibecher (62) reported streptococci in "unpolluted" water samples. The latter used a direct plating method and obtained one positive sample out of 259 water samples.

Mallmann (35) in 1928 reported that streptococci are constant indicators of intestinal pollution. In these studies the number of organisms present in a swimming pool paralleled the degree of pollution as indicated by the number of bathers. He also reported that B. coli grew slightly in water of the pool whereas the streptococci did not.

Savage and Wood (51) in 1918 reported that when both coliforms and enterococci were placed into a tank of water with small amounts of organic matter, the streptococci died out in about two weeks. The coliforms persisted for a longer period of time and in some cases actually increased in number.



They concluded that streptococci might be a better indicator of pollution than the coliform group.

Mallmann and Sypien (42) later made a comparison of the coliform and streptococci indexes. Samples were taken five feet from the shore of a bathing beach. The results showed that the coliform index and the total plate count did not always respond to changes in the bathing load. The streptococci indexes however, did show a change. They also reported that the streptococci disappeared overnight while the coliform organisms and total bacterial population as determined by a plate count, showed a slight decrease.

Winter and Sandholzer (64) confirmed the work of Mallmann and Sypien. They found that the coliform organism persisted in the water for a greater distance from the source of pollution than did the streptococci.

Mallmann and Litsky (39) reported a survival of enteric organisms in various types of soil. Using dextrose azide broth as an enrichment medium, they could not isolate enterococci from soils which were not treated with sewage. They found that the coliform organisms persisted for longer periods of time in sewage treated soils than the enterococci did. The latter die out rapidly but not as rapidly as virulent typhoid bacilli.

In 1951, Lattanzi and Wood (29) compared enterococci and coliform as indexes of water pollution. They found that the coliform test for water pollution subject to limitation although well established in the field of water bacteriology. Using the technic of Winter and Sandholzer, they examined samples weekly for 11 weeks during the winter months. The results indicated that enterococci densities followed the same pattern as that of coliform indexes.

Litsky et al (33) compared the most probable number of coliform bacteria and enterococci in raw sewage. They found a positive correlation of +0.9 between the number of coliform bacteria and enterococci in sewage from the settling tanks of the Amherst sewage treatment plant during the winter, spring and summer months. The investigators also reported that the density of coliform bacteria was approximately 13.3 times that of enterococci.

Litsky and Mallmann (32) compared the MPN of the coliforms and enterococci. They found a positive correlation of +0.84 existing between the number of coliforms and enterococci in samples taken from the Connecticut River during a two year period. Based upon median value of all the samples collected in this study, the density of enterococci was approximately 7.6 times that of the coliforms.

Leininger and McCleskey (31) examined various surface waters to determine bacterial indicators of pollution. In

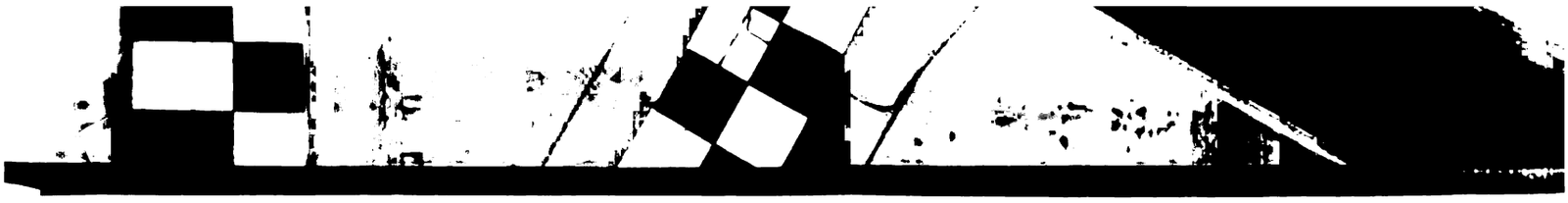
all the waters studied, high total bacterial counts were associated with relatively high coliform count, E. coli counts, and enterococci counts. They indicated that differences between relatively clean and recently polluted water was more strikingly shown by the enterococci test than by the coliform test.

Walter and Weaver (57) surveyed 52 wells to determine the value of streptococci as an index of pollution. The numbers of coliform and streptococci in well waters were identical. For the examination of stored samples, however the test for streptococci was slightly superior since they never increased in number whereas the coliform count varied.

Ritter et al (49) compared the coliform organisms with the enterococci in 595 well waters in Kansas. The data showed there was a positive association of the two groups of bacteria; the chi-square test indicated non-independence with a probability less than 0.001. The enterococci test was preferred to the coliform test. Atypical strains of enterococci were isolated from well water samples of good sanitary quality as determined by the coliform test.

Media Used for the Isolation of Enterococci

Prescott and Baker (45) reported in 1904 that when streptococci and E. coli were grown in mixed cultures, E.



coli reached a maximum growth before the streptococci. The E. coli however, were gradually displaced by the streptococci in 20 to 60 hours. In several trials the streptococci completely outgrew the E. coli.

Mallmann and Gelpi (38) observed a similar succession of growth in lactose broth. After the coliforms were confirmed, the tubes were reincubated for 48 hours, centrifuged and examined for streptococci by microscopic methods. They also noted that if the tubes were left at room temperature for three days after the initial incubation, a heavy sediment formed in the bottom of the test tube. This they believed was an indication of streptococci.

Houston (26) in 1930, described a method for the isolation of streptococci which is still used by the British Ministry of Health. The original samples were inoculated into lactose broth tubes. The tubes were incubated for 15 to 20 minutes in a 60 C water bath. The organisms were subcultured on MacConky agar. The red, pin-point colonies which appeared after 48 hours incubation at 37 C were transferred again to lactose broth. They were then streaked on a nitrate agar slant. Acid production without gas from lactose was used to indicate streptococci. A short-chain coccus in the water of condensation on the nitrate agar slant, and the absence of nitrate reduction confirmed the presence of streptococci.

The first selective medium for enterococci was described by Weissenbach (58) in 1918. He used sterile, filtered, ox-bile as an inhibitory agent.

Bagger (4) added a one percent peptone to ox-bile to promote the growth of the streptococci. Confirmation for the streptococci was made by a heat resistance test.

In 1932 Fleming (15) found that potassium tellurite was inhibitory to the coliforms in a concentration of 1:15,000 but permitted enterococci to grow. Harold (19) also used potassium tellurite in an agar medium. The streptococci appeared as small bluish-black colonies, with a peripheral opalescence.

Sodium azide was first used by Hartman (20) in 1937, as an inhibitory agent against gram-negative organisms. Since the introduction of sodium azide, many investigators have used it in media for the detection of enterococci.

Mallmann (36) reported in 1940 a medium using sodium azide for the estimation of enterococci. A broth medium made according to the formula of Darby and Mallmann (11) containing 1 to 5,000 concentration of sodium azide was found to support the growth of streptococci but inhibit growth of the coliform group.

Hajna and Perry (18) used this medium and a 45 C incubation temperature for the selection of enterococci. Growth and acid production were regarded as indicative of fecal streptococci.

Chapman (7) in 1944 used a medium containing tripan blue, crystal violet, and sodium tellurite. The enterococci formed a dark brown or a smooth, black, slightly-raised colony. S. salivarius produced a pale blue, opaque colony and S. mitis produced a small blue colony.

Winter and Sandholzer (64) in 1946 reported a procedure for the isolation of enterococci. The method consisted of a sodium azide-presumptive broth and a penicillin-methylene blue sodium chloride agar medium. Confirmation of the streptococci is made by microscopic examination and a catalase test.

Later, Chapman (8) reported another medium for the isolation of enterococci. This was a modification of the former medium. It was called "mitis-salivarius agar". On this medium the enterococci produced a dark blue or a raised black colony.

In 1950, Mallmann and Seligmann (41) made a comparative study of several media used for the detection of enterococci. They reported that an azide dextrose broth gave a high MPN. Positive tubes should be checked microscopically because gram-positive rods also grew in this medium as well as the streptococci.

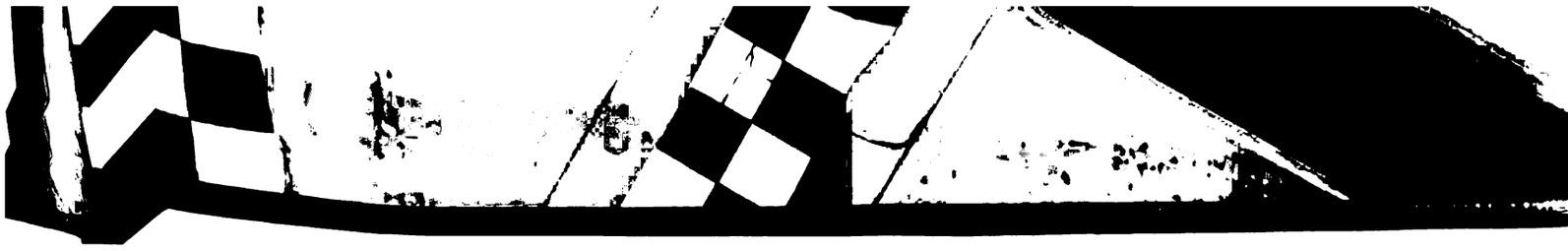
Reinbold, Swern and Hussong (48) described a plating medium for the isolation and enumeration of enterococci. The medium is based on the ability of the enterococci to

utilize sodium citrate as an available carbon source, to convert ditetrazolium chloride to a blue diformazan and to grow in the presence of 0.01 percent sodium azide. The medium was used to isolate and estimate the numbers of enterococci in raw milk.

Litsky, Mallmann and Fifield (31) reported a new medium for the detection of enterococci in water. They designed a selective medium, containing ethyl violet and sodium azide, which is specific for the growth of enterococci from pure cultures or from dextrose azide broth showing growth from sewage contaminated waters. They proposed a new test in which dextrose azide broth is used as a presumptive test medium and ethyl violet azide broth as a confirmatory medium.

Surface Plating Technic

In 1948, Reed and Reed (47) reported a "drop plate" method for counting viable bacteria. The method was relatively simple. A drop of a suitably diluted material delivered by a calibrated pipette was placed on a partially dried agar plate. Six such drops were made on an agar plate. They demonstrated that counts on pure cultures of bacteria made by the drop plate method are 7 percent higher than those made on the same culture by the pour plate method. They also stated that the technic is less laborious and it is a little more accurate with most of the species tested.



Campbell and Konowalchuk (6) using the method of Reed and Reed (47) showed that in parallel counts made by the pour plate method and drop plate method on raw milk samples, the drop plate method gave counts which were 27 percent higher than by the pour plate method. They suggested that this discrepancy resulted from the more efficient breaking up of clumps and chains of bacteria by the dilution procedure used in preparing the drop plate.

Pomales-Lebrón and Fernandez (46) in 1952 used a drop plate method which was similar to that of Reed and Reed. These workers used this method for the estimation of the number of various bacteria in liquids and tissues.

Mallmann, Peabody, and Broitman (40) applied the method of Pomales-Lebrón and Fernandez to the enumeration of high-population samples. Milk and polluted waters were used in the experiment.

Mallmann and Broitman (37), using the drop plate method of Pomales-Lebrón and Fernandez, made a comparison of the drop plate method and the pour plate (standard plate count) on retail milk samples. They concluded that the 36-hour drop plate count was comparable to the 48-hour standard plate count.

Membrane Filter Technic for the Enumeration of Enterococci

The report by Goetz (16) in 1947 on the nature and use of the membrane filters in Germany, stimulated a number of papers in this country on the membrane filter technic for the bacteriological analysis of water. A still greater interest occurred in the membrane filter after the publications of Clark et al (9) and Goetz (16). These investigators described a membrane filter procedure for the detection of coliform and other bacteria in water. They concluded that the membrane filter technic had distinct advantages over the MPN procedures of Standard Methods for the Examination of Water and Sewage.*

Slanetz, Bent, and Bartley (55) were the first to adapt the membrane filter technic to the enumeration of enterococci in water. They developed a selective medium for use with the membrane filter. With the membrane filter technic, the counts for enterococci were generally higher than those obtained by other procedures.

Litsky and Shaer (34) made a comparative study of the MPN and membrane filter technic for the enumeration of enterococci. The authors used the technic of Slanetz et al (53) and the MPN method of Mallmann, Litsky, and Fifield (31). In waters of low populations, 75 percent of the samples showed a higher count using the MPN technic than with the membrane filter.

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*American Public Health Association, 10th. edition, 1955.

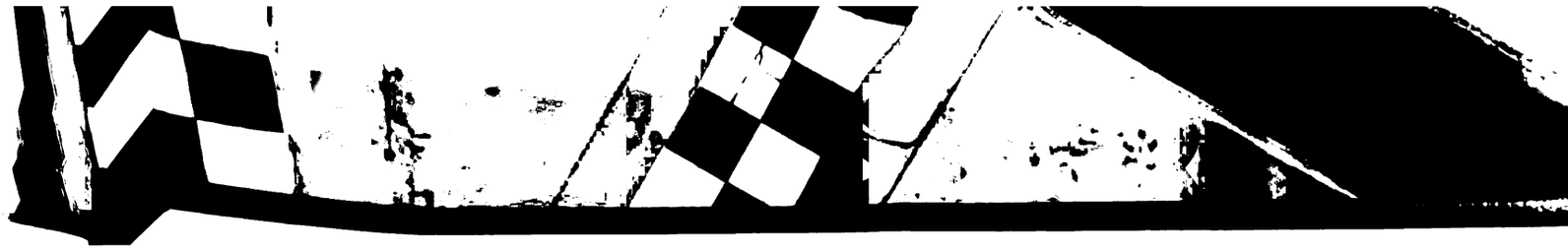
Slanetz, Bartley and Ray (54) made further studies on the membrane filter procedures for the determination of the enterococci count in water and sewage. They reported that by incubating the membranes on agar instead of the usual procedure of incubating the membranes on broth pads, the count and the size of the colony increased. In 70 percent of the samples tested, the membrane filter technic gave a higher count than did the MPN method of Litsky *et al* (31).

EXPERIMENTAL

Part I: Drop Plate Method

Sherman and Albus, Walker and Winslow, demonstrated that the lag phase is the most critical stage in the bacterial growth cycle. In order to formulate a medium for a drop plate method, a base medium first had to be selected which would support the growth of a minimal inoculum of organisms as described by Darby and Mallmann (11). The greater the number of bacteria that survive the critical lag phase, the better the chance that these organisms have to multiply. The following experiments were carried out on the assumption that the shorter the lag phase of a bacterial growth curve, the better the medium is for a particular species.

For a base medium, the Mallmann Litsky (31) ethyl violet confirmatory medium was investigated. The authors have demonstrated that the concentration of ethyl violet dye (0.00083 grams per liter) inhibits the gram-positive bacilli. The concentration of sodium azide (0.4 grams per liter) was sufficient to inhibit the growth of the gram-negative bacteria and still permit the growth of S. faecalis. The objective of these growth curve studies was to formulate a medium not only for S. faecalis, but also for S. durans, S. zymogenes and S. liquefaciens.



The Mallmann Litsky medium, minus the peptone and carbohydrate was the base medium used in a series of growth curve studies. The first growth curve experiments were carried out to determine a suitable peptone source. Phytone, tryptic-case, polypeptone, and phytone plus yeast extract were used. The concentration of the peptones was two percent.*

The growth experiments were executed by using the drop plate method of Pomales-Lebrón and Fernandez (46). This seemed to be the proper step, since the medium was being designed for drop plate technic. The method lent itself to the conservation of media and glassware.

The method used is as follows. An 18-hour culture of S. faecalis, which was transferred daily for three days, was used as the test organism. The size of the inoculum of S. faecalis into the experimental medium was critical, and a preliminary count on the culture was made to determine the number of organisms present. The Petroff-Hauser counting chamber was used to make this determination. After the total count was determined, serial dilutions of the culture was made in 99 ml saline dilution blanks to give from five to 25 organisms per 0.04 ml. The flasks containing 100 ml of the experimental base medium were inoculated with the appropriate dilution of the culture. The flasks were shaken on a Boerner shaker for five minutes and a sample withdrawn at the end

*The peptones were procured from Baltimore Biological Laboratory, Incorporated, Baltimore, Maryland.

of the shaking period. A 0.2 ml pipette, graduated in 0.01 ml was used to remove the sample. Duplicate aliquots of 0.01 ml, 0.02 ml, and 0.04 ml were placed on the agar plate at six equally spaced positions. See Plate I. The counts were made on the duplicate drops from the three different amounts; thus the final count shown in the tables is an average of the six counts. Counts were made at the end of 0, three, six, nine, 12 and 15 hours. The agar plates (drop plates) and the base medium were incubated at 37 C.

The medium used in the agar drop plates was recommended by Mallmann and Seligmann (Azide Dextrose Broth) but modified by omitting the sodium azide and adding 1.5 percent agar. Before the agar plates were used, they were dried in an inverted position with the top lid ajar for four hours in a 37 C incubator. This drying of the agar allowed the drop-sample to penetrate into the agar medium within several minutes. The inoculated agar drop plates were incubated at 37 C for 48 hours and counted. The drop plates were counted by viewing under a stereoscopic microscope. See Plate I for a typical drop plate.

This method for growth curve studies was repeated, using S. durans, S. zymogenes, S. liquefaciens. See Figure 1 for a schematic drawing of the technic used.

Comparison growth experiments were conducted using the drop plate method and a colorimeter. A larger inoculum was

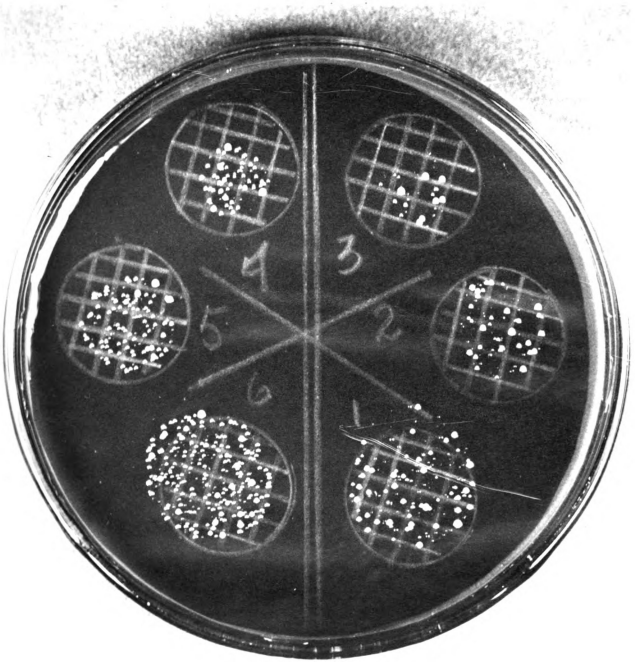
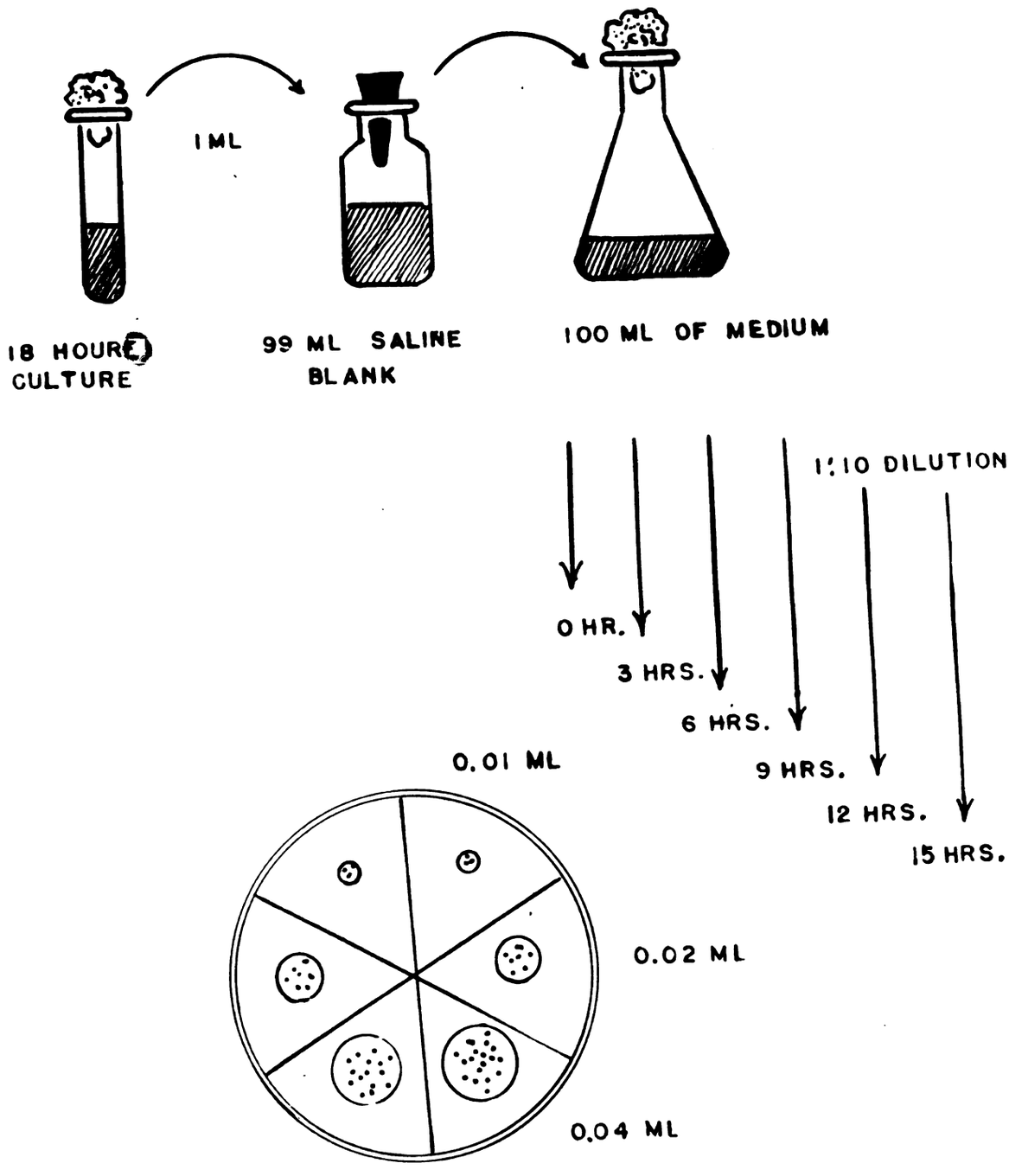


PLATE I

FIGURE 1

DROP PLATE GROWTH CURVE METHOD



used in the colorimeter method. The colorimeter used was the Bausch and Lomb "Spectronic 20", set at 525 millimicrons. The percent light transmission was recorded at zero hour incubation and at the end of every three-hour period for a total of 15 hours incubation. The results are tabulated in Tables 1 and 2.

The media used in the experiments had the following formulations:

Medium No. 1

K_2HPO_4	2.7 grams/liter	Base
KH_2PO_4	2.7	
Ethyl violet dye	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Phytone (BBL)	20.0	
Lactose	5.0	pH 7.0

Medium No. 2

Base plus		
Phytone	20.0 grams/liter	
Lactose	5.0	
Yeast extract	5.0	pH 7.0

Medium No. 3

Base plus	
Lactose	5.0 grams/liter

TABLE 1

DROP PLATE COUNTS OF S. FAECALIS, S. ZYMOGENES, S. LIQUEFACIENS, S. DURANS GROWN IN MEDIUMS NO. 1 AND NO. 2

Incubation Time	S. faecalis		S. zymogenes		S. liquefaciens		S. durans	
	Medium No.1	Medium No.2	Medium No.1	Medium No.2	Medium No.1	Medium No.2	Medium No.1	Medium No.2
0 (hours)	30*	40	20	30	30	30	30	40
3	40	50	-	30	40	40	40	50
6	60	290	-	110	50	120	50	140
9	-	1,600	20	590	60	580	60	520
12	1,110	13,050	-	3,650	190	5,600	100	5,400
15	5,000	65,000	-	15,000	500	20,000	250	17,500

*Organisms/0.04 ml.

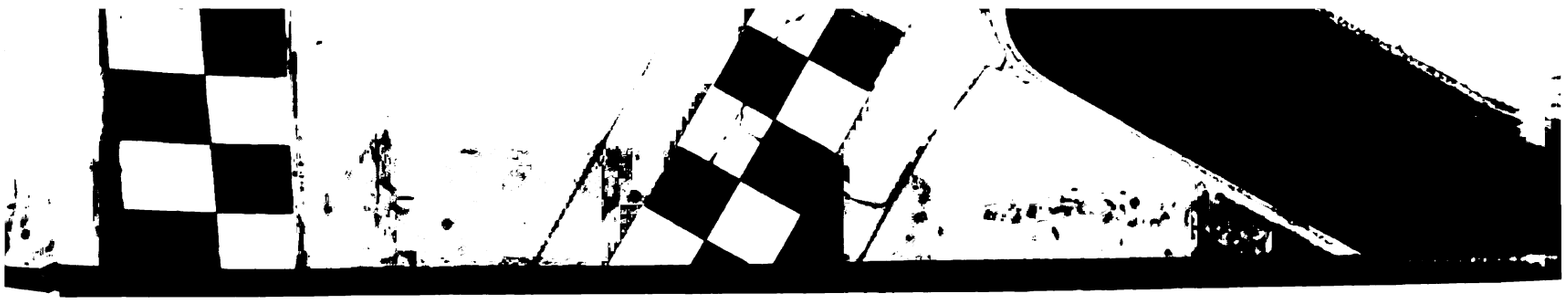


TABLE 2

PERCENT LIGHT TRANSMISSION AT 525 mμ BY ORGANISMS GROWN IN VARIOUS EXPERIMENTAL BASE MEDIA

Incubation Time	Medium No.1	Medium No.2	Medium No.3	Medium No.4
		S. faecalis		
0 (hours)	100	100	100	100
3	97	96	96	98
6	95	95	91	98
9	93	90	88	98
12	84	55	87	91
15	40	18	75	65
		S. zymogenes		
0 (hours)	100	100	100	100
3	96	93	97	98
6	95	91	94	98
9	91	88	93	98
12	82	82	93	96
15	44	40	90	87
		S. liquefaciens		
0 (hours)	100	100	100	100
3	94	96	96	97
6	94	91	91	96
9	92	89	90	96
12	84	82	87	92
15	65	40	64	81
		S. durans		
0 (hours)	100	100	100	100
3	98	96	96	100
6	97	91	91	98
9	94	90	90	97
12	82	75	88	94
15	44	35	85	83

Polypeptone (BBL)	20.0	pH 7.0

Medium No. 4

Base plus		
Lactose	5.0 grams/liter	
Trypticase (BBL)	20.0	pH 7.0

Sterilized at 121 C for 15 minutes

An examination of the data revealed that there was close relationship between the results of two methods in the growth studies.

Medium No. 2 showed the shortest lag phase of the four base media tested for all four organisms used. Medium No. 2, containing 0.5 percent yeast extract, showed a shorter lag phase than medium No. 1 which did not contain any yeast extract. Medium No. 1 and No. 2 contained phytone as the peptone source. The phytone demonstrated a shorter lag phase than the other peptone sources, trypticase (Medium No. 3) and polypeptone (Medium No. 4).

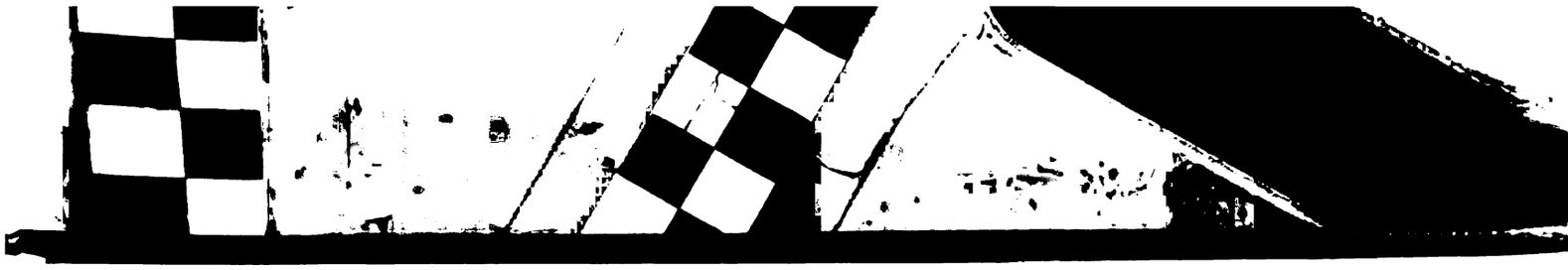
A second set of growth experiments was run to determine the effect of lactose and to make a comparison with the Mallmann Litsky medium. Medium No. 1 contained 0.5 percent lactose and Medium No. 5 was devoid of lactose. The drop plate method was used as previously described. The data of the second growth experiments are tabulated in Table 3. The

TABLE 3

DROP PLATE COUNTS OF ORGANISMS GROWN IN MEDIUM NO. 1, NO. 5, AND MALLMANN LITSKY FORMULATION

Incubation Time	Medium No. 1	Medium No. 5	Mallmann Litsky
	S. faecalis		
0 (hours)	-	10	15
3	20*	10	15
6	45	25	20
9	125	35	25
12	175	125	25
15	400	162	62
	S. zymogenes		
0 (hours)	20	20	25
3	20	-	30
6	-	30	-
9	32	35	50
12	1200	50	75
15	2000	75	75
	S. liquefaciens		
0 (hours)	30	15	40
3	35	25	50
6	55	35	45
9	75	125	75
12	200	175	75
15	400	125	75
	S. durans		
0 (hours)	15	5	20
3	20	15	20
6	60	25	20
9	125	75	50
12	-	-	-
15	-	-	-

*Organisms/0.04 ml.



results indicated that the lactose aids in the reduction of a lag phase. This held for the four organisms. The phytone-lactose medium (Medium No. 1) and the phytone medium minus the lactose (Medium No. 5) showed a marked reduction in the lag phase as compared to the Mallmann Litsky medium.

A third series of growth experiments was run to determine the amount of phytone which could be used in a medium for the enterococci. The data are recorded in Table 4. The results indicated that an increasing phytone concentration caused a decreasing lag phase period. A two percent concentration of phytone permitted the greatest number of organisms to grow.

To determine the amount of lactose which could be used with the base medium containing two percent phytone, the lactose concentration was varied. The amounts of lactose used were: 0.5, 1.0, and 2.0 percent. The results of the experiment are in Table 5. The highest count was observed with S. faecalis at 0.5 percent concentration of lactose, while S. zymogenes was the opposite. S. zymogenes gave a higher count at the two percent lactose concentration.

A final series of growth experiments was conducted to establish the combination concentrations of phytone and lactose that would yield the shortest lag phase. The phytone concentrations used were 0.5, 1.0, and 2.0 percent and the

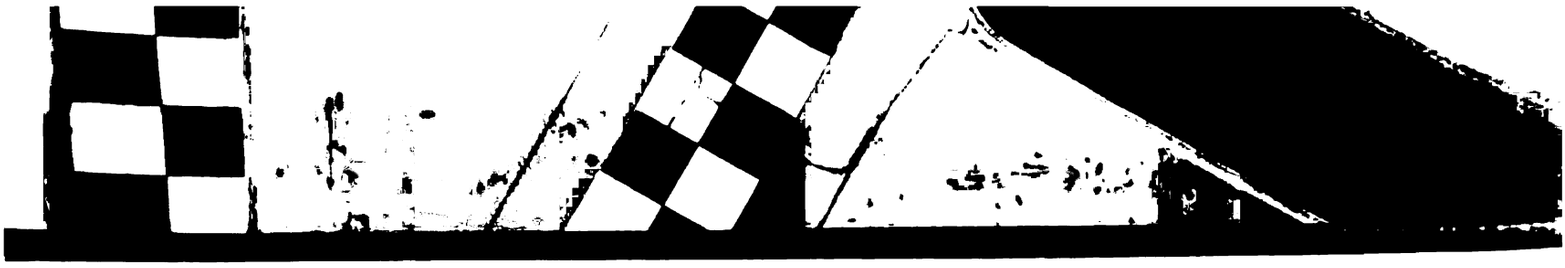


TABLE 4

DROP PLATE COUNTS OF ORGANISMS GROWN IN A BASE MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF PHYTONE

Hours of Incubation	Concentration of Phytone in Percent			
	0	0.5	1.0	2.0
	S. faecalis			
0	10*	15	15	10
3	10	10	20	5
6	35	20	40	20
9	50	50	-	42
12	50	35	100	275
15	100	125	450	950
	S. zymogenes			
0	30	30	25	20
3	40	20	25	50
6	50	75	50	35
9	75	50	100	175
12	150	300	425	850

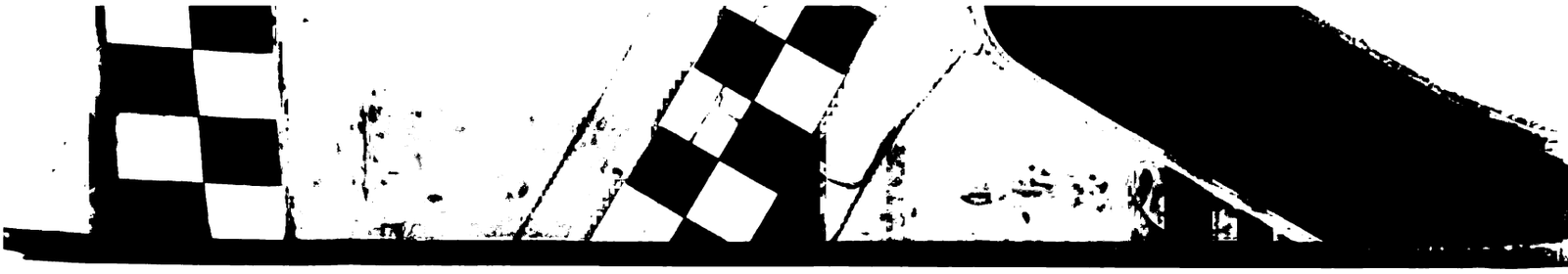
*Organisms/0.04 ml.

TABLE 5

DROP PLATE COUNTS OF S. FAECALIS AND S. ZYMOGENES GROWN IN A BASE MEDIUM CONTAINING VARIOUS AMOUNTS OF LACTOSE

Hours of Incubation	Concentration of Lactose in Percent		
	0.5	1.0	2.0
	S. faecalis		
0	15*	15	5
3	10	10	15
6	110	50	-
9	325	250	225
12	1550	675	775
15	8200	4100	3000
15			950**
	S. zymogenes		
0	30	25	10
3	30	30	20
6	50	125	50
9	100	280	250
12	350	925	1125
12			850**

*Organisms/0.04 ml.
**Medium containing 1.5 percent dextrose and no lactose.



same concentrations of lactose were also used. The results are compiled in Table 6. The results showed that the 2.0 percent concentration of phytone increased the amounts of growth. This was demonstrated in the previous experiments. S. faecalis exhibited the shortest lag phase when the concentration of phytone was two percent in combination with a 0.5 percent concentration of lactose. However, S. zymogenes grew best in a 2.0 percent concentration of phytone plus a 2.0 percent concentration of lactose.

Previous experiments indicated that the yeast extract shortened the lag phase for all four test organisms. The results of these series of growth experiments indicated a possible medium for use in a drop plate technic for the detection of enterococci in various waters. The following is the medium to be used in further investigations:

K_2HPO_4	2.7 grams per liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Phytone (BBL)	20.0	
Yeast extract	5.0	
Agar	15.0	pH 7.0

Sterilized at 121 C for 15 minutes

*National Aniline Division, Allied Chemical and Dye Corporation. Lot No. 12552. Dye content 57.5 percent.

TABLE 6

DROP PLATE COUNTS OF S. FAECALIS AND S. ZYMOGENES GROWN IN VARIOUS COMBINATIONS OF PHYTONE AND LACTOSE

Hours of Incubation	Phytone 0.5% Lactose %			Phytone 1.0% Lactose %			Phytone 2.0% Lactose %		
	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%
				S. faecalis					
0	10*	10	10	5	10	20	15	15	5
3	20	15	-	10	10	30	10	10	15
6	70	40	-	30	30	-	110	50	-
9	62	25	50	200	75	62	325	250	225
12	200	225	75	175	200	175	1550	675	775
15	500	275	75	1250	350	560	8200	4100	3000
				S. zymogenes					
0	30	30	25	30	20	10	30	25	10
3	-	25	50	30	20	25	30	30	20
6	35	25	50	50	125	75	50	125	50
9	75	25	125	125	175	100	100	280	250
12	325	100	175	450	300	500	350	925	1125

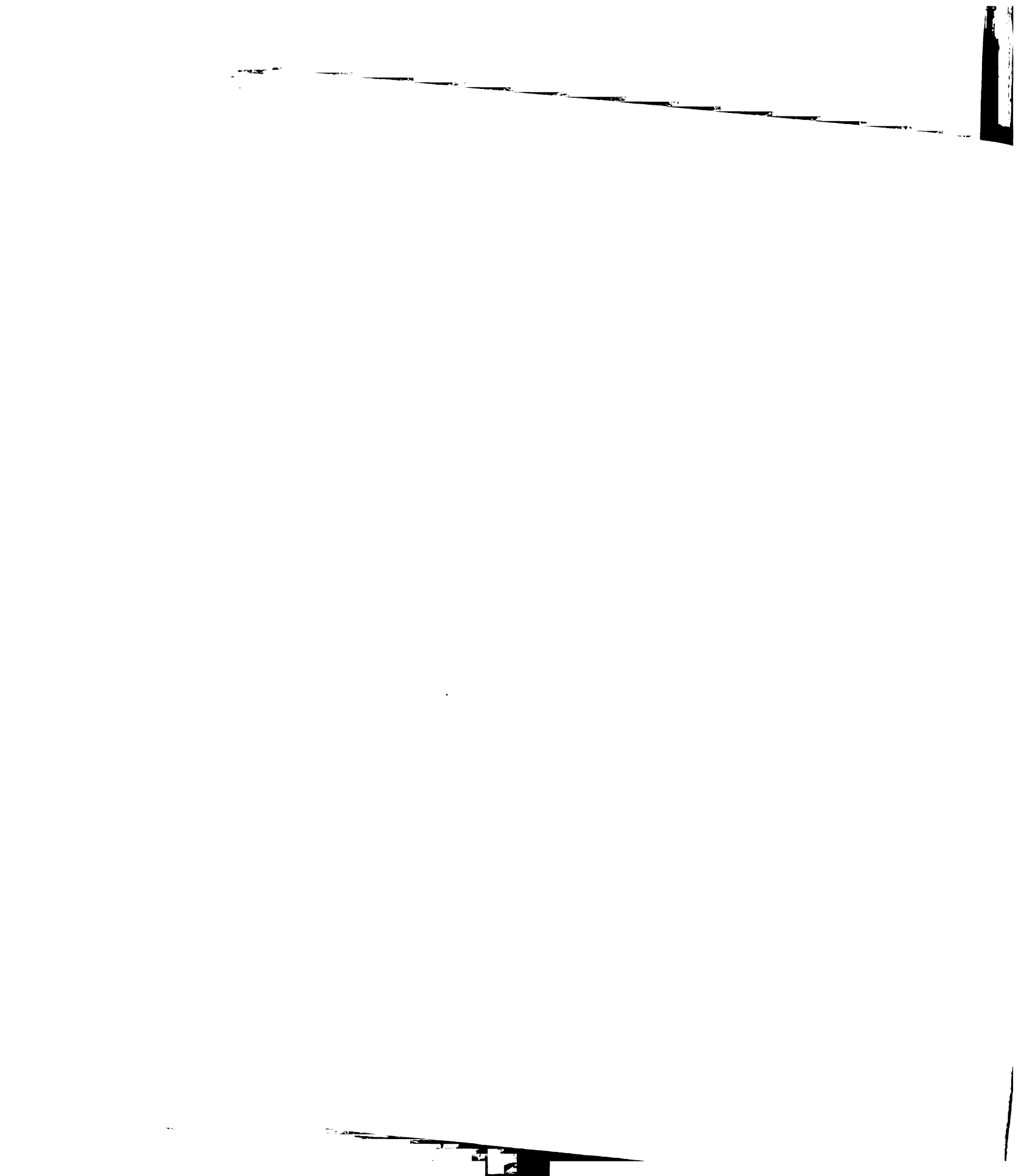
*Organisms/0.04 ml.

The medium above shall be designated as Ethyl Violet Azide Modified or EVA modified.

In order to determine the specificity of the medium, a number of selected test organisms were used. The EVA modified medium was made up minus the agar and dispensed into test tubes. The organisms were inoculated into the broth medium. The tubes were read after 48 hours incubation at 37 C. A plus or minus sign designated growth or absence of growth. The results are tabulated in Table 7. None of the organisms grew except the enterococci (S. faecalis, S. durans, S. liquefaciens, and S. zymogenes). The phytone did not interfere with the activity of the sodium azide or the ethyl violet dye. The concentration of sodium azide inhibited the growth of gram-negative organisms and the concentration of ethyl violet dye inhibited the growth of gram-positive bacilli.

Five Red Cedar River samples and 22 sewage samples from the East Lansing Sewage Plant were analyzed to determine the number of enterococci. The drop plate method using EVA modified medium, the MPN method of Mallmann Litsky and the EVA modified broth medium (used in place of the Mallmann Litsky EVA confirmatory medium) were used to enumerate the enterococci. Azide dextrose broth was used as the presumptive medium in the last two cases.

The results (Table 8) showed that the EVA modified broth medium was 100 percent more effective than the Mallmann



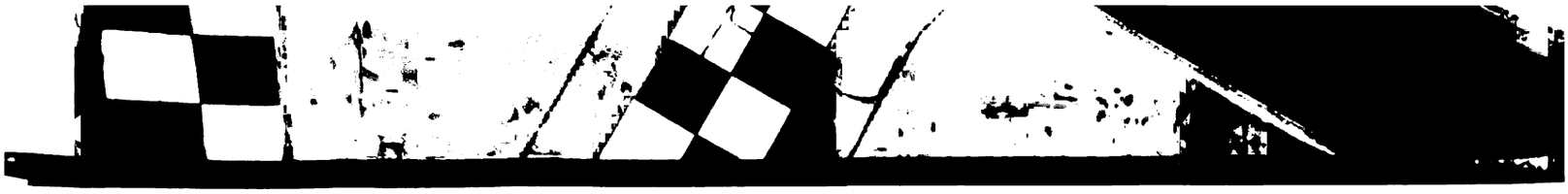


TABLE 7

GROWTH OF VARIOUS STRAINS OF ORGANISMS IN THE MODIFIED ETHYL VIOLET AZIDE-PHYTONE MEDIUM

Species	Source	Growth After 48 Hours Incubation
Aerobacter aerogenes	MSU (Neu)	-*
Pseudomonas aeruginosa	MSU	-
Sarcina citreus	MSU	-
Proteus vulgaris	MSU	-
Sarcina lutea	MSU	-
Chromobacter violaceum	MSU	-
Bacillus cereus	MSU (Neu)	-
Bacillus megatherium	MSU "	-
Bacillus circulans	Wayne U.	-
Bacillus mesentericus	Wayne U.	-
Bacillus subtilis var. aterrimus	Wayne U.	-
Bacillus ruber (of Kiel)	Wayne U.	-
Bacillus rubidum	Wayne U.	-
E. coli var. mutabilis	Wayne U.	-
E. coli strain B	Wayne U.	-
E. coli	MSU	-
Micrococcus pyogenes var. aureus Brail strain	MSU (Neu)	-
Micrococcus pyogenes var. aureus	Wayne U.	-
Micrococcus pyogenes var. albus	Wayne U.	-

TABLE 7 (Cont.)

Species	Source	Growth After 48 Hours Incubation
Micrococcus pyogenes var. roseus	Wayne U.	-
Streptococcus sp. B. hemolytic	MSU (Neu)	-
S. sp. B. hemolytic	MSU "	-
S. sp. Lancefield E	MSU "	-
S. equisimilis	MSU "	-
S. pyogenes human A	MSU "	-
S. faecalis	MSU "	+**
S. durans	MSU "	+
S. liquefaciens	MSU "	+
S. zymogenes	MSU "	+

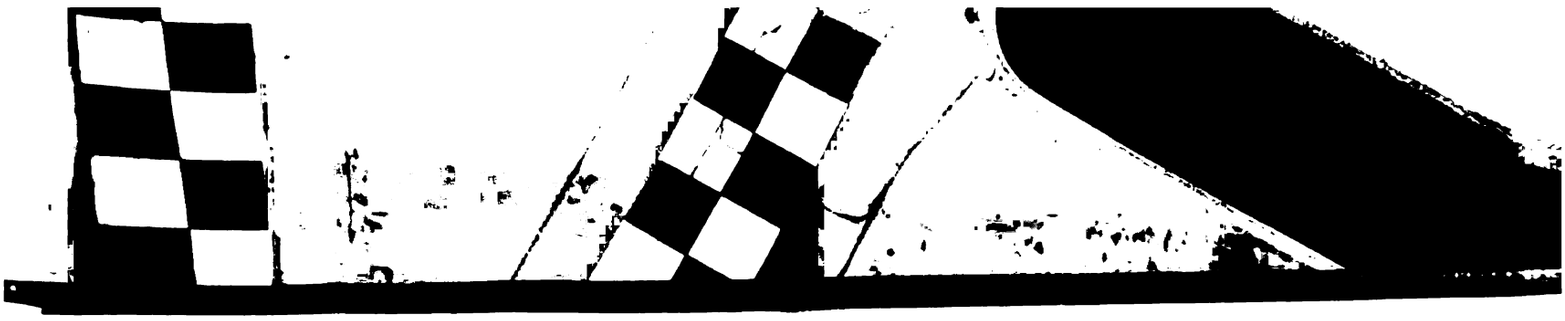
*Negative, no growth

**Positive, growth

TABLE 8

M.P.N. OF ENTEROCOCCI USING AZIDE DEXTROSE BROTH AS A PRESUMPTIVE MEDIUM WITH MALLMANN LITSKY ETHYL VIOLET AZIDE CONFIRMATORY BROTH AND ETHYL VIOLET AZIDE MODIFIED BROTH AS CONFIRMATORY MEDIA. COMPARISON WITH THE DROP PLATE TECHNIC USING ETHYL VIOLET AZIDE MODIFIED AGAR MEDIUM

Sample No.	Type of Sample	Mallmann Litsky EVA	Modified EVA	Drop Plate
1	River	410	17,000	26,300
2	Sewage	130,000	350,000	347,000
3	River	2,400	7,000	4,000
4	Sewage	79,000	540,000	550,000
5	Sewage	170,000	350,000	270,000
6	Sewage	170,000	170,000	420,000
7	River	2,400	2,400	6,000
8	Sewage	1,800,000	2,800,000	1,110,000
9	Sewage	36,000	140,000	402,000
10	River	1,100	1,400	5,000
11	Sewage	240,000	240,000	820,000
12	Sewage	1,800	1,800	15,000
13	Sewage	79,000	79,000	410,000
14	River	130	700	2,000
15	Sewage	240,000	240,000	920,000
16	Sewage	330,000	490,000	560,000
17	Sewage	330,000	330,000	640,000
18	Sewage	49,000	79,000	250,000
19	Sewage	130,000	240,000	1,280,000
20	Sewage	170,000	350,000	350,000
21	Sewage	1,100,000	1,100,000	1,015,000
22	Sewage	35,000	100,000	182,000
	Log Averages	40,400	81,500	158,000



Litsky EVA medium in the MPN method. The drop plate utilizing the EVA modified medium was 98 percent higher than the MPN of the EVA modified confirmatory medium. The results are compiled in Table 8.

From drop plates 259 colonies were isolated and transferred into brain heart infusion broth. The isolations were made under a stereoscopic microscope with 10X magnification. The tubes were incubated at 37 C for 48 hours. From the brain heart infusion broth, transfers were made into the following media: brain heart infusion broth for incubation at 45 C in a water bath; 0.1 percent methylene blue in skim milk, brain heart infusion broth with 6.5 percent sodium chloride, and brain heart infusion broth at a pH of 9.6, incubated at 37 C. If the isolates grew in all four media, the organism was considered to be a member of the enterococci group based on the Sherman classification (52). Microscopic examinations were made on the isolated organism. The results are compiled in Table 9. Of the 259 organisms isolated, 95.4 percent were classified as enterococci.

Two types of colonies were present on the drop plate. One was a round, raised white colony and the other a round, flat, grey colony. The 95.4 percent of the colonies which confirmed as enterococci by the Sherman criteria were white colonies. Forty-one additional isolations were made taking only the flat grey colonies, and tested by the Sherman criteria

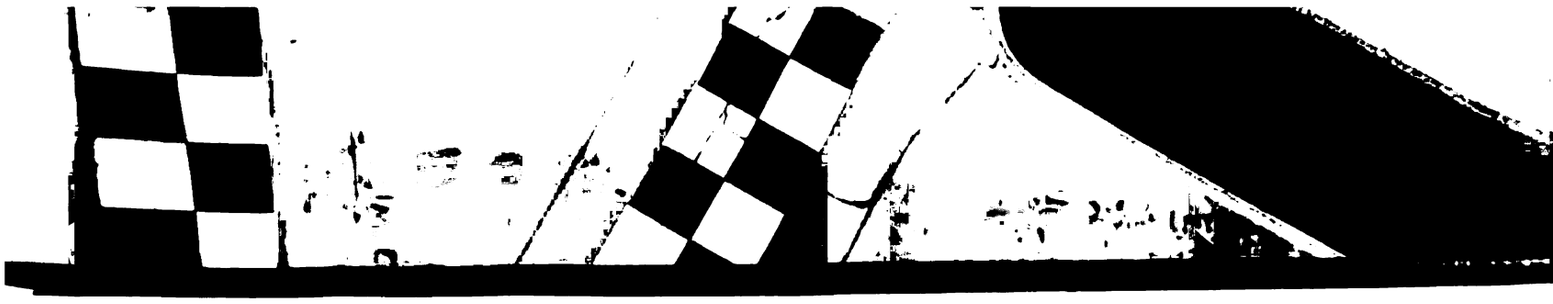


TABLE 9

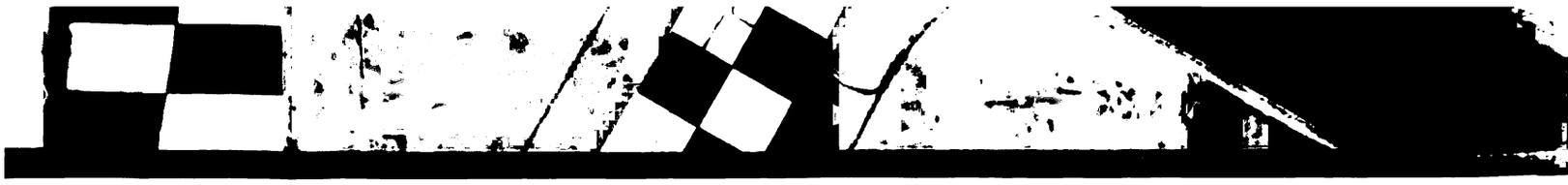
PERCENTAGE OF CONFIRMATION OF 259 COLONIES ISOLATED FROM THE DROP PLATES BY THE SHERMAN STANDARDS FOR ENTEROCOCCI

	45 C Incubation	0.1% Methylene Blue in Skim Milk	Broth with 6.5% Sod. Chloride	Broth at pH 9.6	Microscopic Examination - Gram positive cocci in pair or short chains
Number of positives	258	241	242	247	259
Percentage	99.8	95.4	95.6	96.9	100

for enterococci. These colonies grew at 45 C but did not meet the other requirements. The microscopic examination of these isolates revealed gram-positive cocci in pairs or short chains. Their morphology was typically that of the enterococci. The 41 isolates were transferred every 12 hours for five days in brain heart infusion broth. After the tenth transfer, the organisms were again tested by the Sherman requirements. Four of the 41 isolates were confirmed as enterococci. Based on these data the flat grey colonies were atypical or attenuated enterococci.

Discussion

Many investigators have demonstrated that the enterococci can be used as indicators of fecal pollution. The investigation indicates that the numbers of enterococci recovered is small as compared to the coliforms recovered. These workers considered the enterococci poor indicators of pollution. This may explain why for the past 50 years the enterococci have not been used extensively as indicators of pollution. Lack of research in this may have been due to the fact that there was no satisfactory medium. Mallmann and Litsky (31) improved the medium for determining the number of streptococci by adapting it to a MPN method similar to that used for coliforms. The method used azide dextrose



broth as a presumptive medium and confirmed all the positive azide dextrose tubes in the EVA medium for confirmation. In the development of the Mallmann Litsky EVA confirmatory medium, no attempt was made to study types of peptone used in the formulation. Three peptone sources were chosen: phytone, polypeptone, trypticase, and a combination of phytone and yeast extract. The phytone produced the highest number of organisms in the shortest period of time.

Phytone is a papaic digestion of soya meal, containing a considerable quantity of carbohydrate derived from plant tissue. With this in mind, the selection of the amount of carbohydrate to be used in the medium had to be given some thought. In these studies, dextrose and lactose were used. Dextrose has been used in many formulations for the enterococci. Mallmann and Litsky (31) found that lactose was equally as good as dextrose for the carbohydrate source.

Two percent phytone was found to be a sufficient amount of peptone in the formulation. Varying amounts of lactose and dextrose were used with two percent phytone to find which carbohydrate and the amount that would yield the greatest number of organisms in the shortest period of time. At the end of 12 hours of incubation the growth indicated that S. faecalis multiplied best in a 0.5 percent concentration of lactose. The count was 1,550 in this concentration while the 1.5 percent concentration of dextrose gave

a count of 275 organisms. S. zymogenes showed a different growth response. At the end of 12 hours of growth, the 2.0 percent concentration of lactose gave a count of 1,125 organisms per 0.04 ml, while the 1.5 percent concentration of dextrose gave a count of 850 organisms per 0.04 ml. S. liquefaciens and S. durans failed to grow during this particular series of experiments and the trials were not repeated.

The enterococci in waters are of fecal origin and too little is known of the length of survival or comparative numbers of different groups. From the isolation of 300 colonies made from EVA modified agar drop plates, 41 were found to be atypical enterococci. When selecting the colonies to be isolated for confirmation by the Sherman method, one can usually differentiate between the typical and atypical enterococci. The typical enterococci are raised white colonies and the atypical are flat grey colonies. The white colonies were confirmed as enterococci by the Sherman method. The atypical colonies do not confirm, but 99 percent grew at 45 C and have the morphology of enterococci, a gram-positive cocci in pairs or in short chains. The number of typical and atypical enterococci are recorded in Table 10. It can be noted from the table that the number of typical enterococci are in the majority. However, four of the 41 original atypical isolates confirmed after being transferred twice daily for five days. The indication is that the atypical

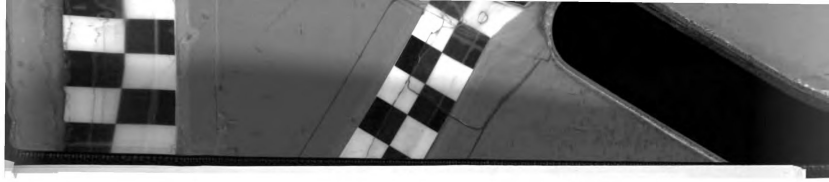
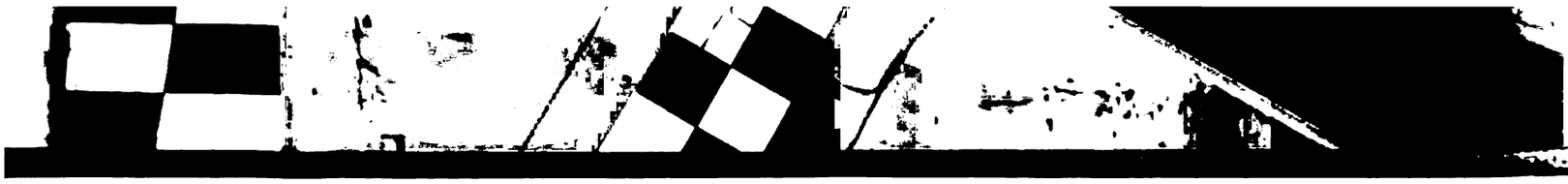


TABLE 10

NUMBER OF TYPICAL AND ATYPICAL COLONIES PRESENT ON A SINGLE DROP PLATE DETERMINATION

Sample No.	Typical	Atypical	Total
1	7	4	11
2	59	27	86
3	9	5	14
4	10	9	19
5	26	14	40
6	20	10	30
7	20	8	28
8	26	15	41
9	6	4	10
10	18	4	22
11	203	10	213
12	314	15	329
13	9	0	9
Average	56.0	10.0	65.5



enterococci are attenuated typical enterococci and need only to be restored.

From several positive tubes of Mallmann Litsky EVA confirmatory broth, streaks were made on EVA modified agar medium. After 24 hours incubation at 37 C atypical and typical enterococci colonies were present. The ratio of the atypical colonies to the typical colonies was one to five. This may support the indication that the Mallmann Litsky medium does not support the growth of the atypical organisms as well as the EVA modified medium. A number of atypical colonies were seeded into the Mallmann Litsky EVA medium. The atypical organism formed a round, light purple button on the bottom of the test tube. When compared to a pure culture of S. faecalis, which forms a compacted round, purple button on the bottom of the tube, the atypical organisms have a much lighter color and the round button is not as compacted. The same results were demonstrated when EVA modified broth medium was used in the place of Mallmann Litsky medium.

Ritter et al (49) has isolated atypical enterococci from good quality well water samples as shown by the coliform test. The atypical enterococci have a definite place of importance in sanitary water supplies and further investigations should be carried out along this line.

A new medium has been described in the thesis which is a modification of the Mallmann Litsky EVA medium. The EVA

modified medium used with the drop plate method gives a higher count of enterococci than by any of the methods used today. The drop plate method has some distinct advantages over the time consuming MPN method used by Mallmann and Litsky. The method can be used in the smallest water laboratories. The drop plate method gives accurate counts and reproducible results. In addition to higher bacteria counts it conserves media and glassware. With the drop plate method two water samples at three different dilutions can be made on one petri dish. However, the method is not as successful in low population waters. It is, therefore, recommended for high population waters, such as grossly polluted rivers and sewage. Some river water samples have been successfully examined for enterococci by using a larger amount of sample than the 0.04 ml. A 0.1 ml sample can be used. Four such samples can be made on one petri dish.

Part II: Membrane Filter Method

The membrane (molecular) filter, hereinafter designated as MF, was first described by Goetz (16) in 1947. The MF procedure has been extensively applied to the bacteriological analysis of water. Several investigators have described the MF procedures for the determination of coliform and other bacteria in water and concluded that the MF might have distinct advantages over the MPN procedure of Standard Methods for the Examination of Water and Sewage. Slanetz et al (55) reported on the use of the MF technic to enumerate enterococci in water.

The filter apparatus used in this study is shown in Plate II and Figure 2. The apparatus consisted of a glass funnel, a fritted glass base on which the membrane rests, and a rubber stopper by which a vacuum flask may be attached by use of a special clamp. The membranes* are 150 microns, or 0.005 inch thick, and composed of cellulose esters. The membranes are white with an imprinted grid with each square representing 1/100th of the effective filtering area. The pore size of the membrane is such that it will retain particles of 0.5 microns or greater. They were sterilized by placing them between absorbent pads, wrapped in desired numbers, in paper, and autoclaved at 121 C. (15 pounds pressure) for 15 minutes. When ready for use, the membranes and pads

*Type HA, 47 mm, Millipore Filter Corporation, Watertown, 72, Mass.

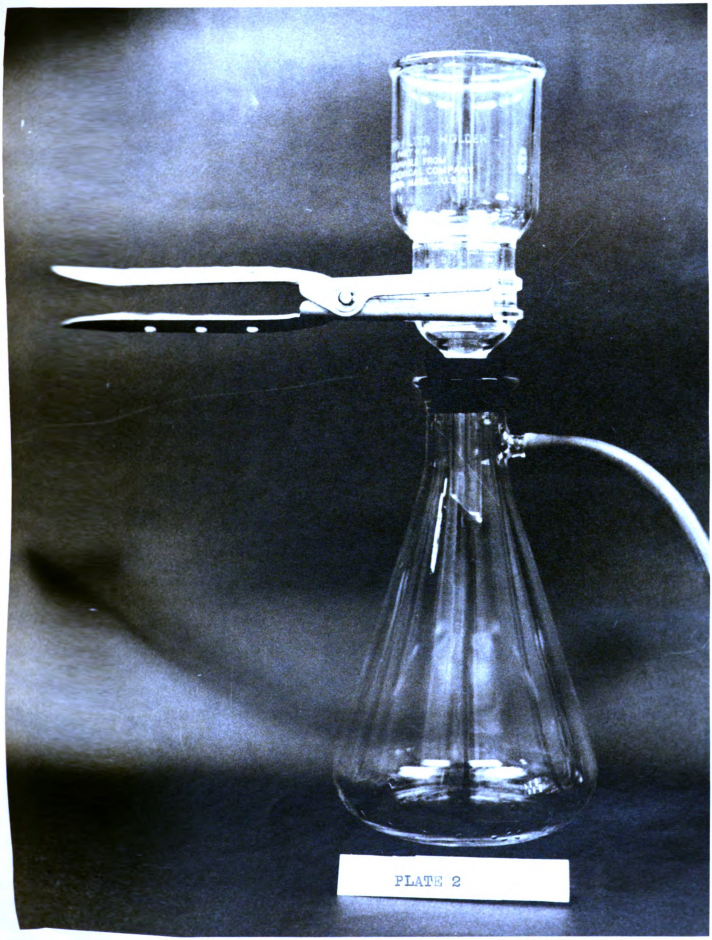
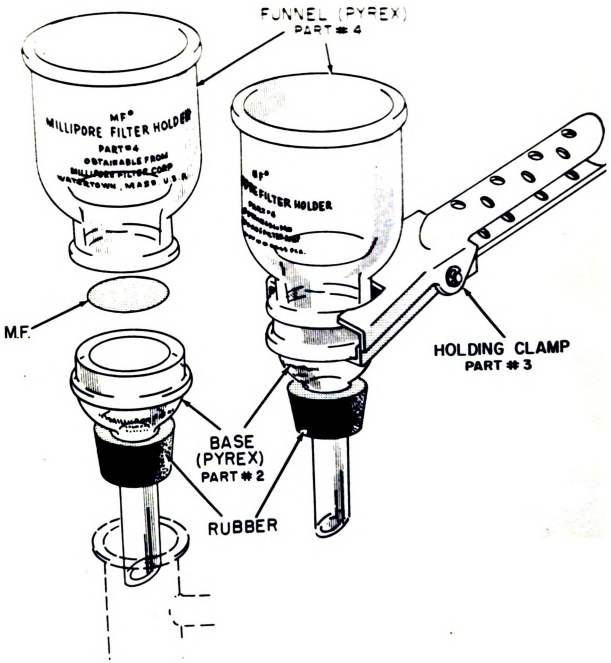


PLATE 2

FIGURE 2


FUNNEL (PYREX)
PART # 4
MF°
MILLIPORE FILTER HOLDER
PART #4
OBTAINABLE FROM
MILLIPORE FILTER CORP
WATERTOWN, MASS U.S.A.
M.F.
HOLDING CLAMP
PART # 3
BASE
(PYREX)
PART # 2
RUBBER

may be easily handled by placing them in sterile petri dishes. The glass apparatus was covered with freezer foil and sterilized by autoclaving at 121 C for 15 minutes.

The culture media used in the studies are the enterococci medium of Slanetz (55) (see Appendix) and the EVA agar medium to which 0.01 percent of 2,3,5 triphenyltetrazolium chloride (TTC) was added. The TTC was made into a one percent stock solution, placed in a brown bottle and steamed for two hours. The TTC solution was added aseptically to the above medium.

The filtration and cultivation procedures used during these studies were similar to those used for the determination of coliform bacteria in water by various investigators. The various broth culture media tested were added in two ml amounts to the absorbent pad when a broth base was used and five ml were added when an agar base was used. The desired quantity of water sample was filtered through the membrane using a vacuum. The filters were then transferred directly to the petri dishes containing the media. The petri dishes were placed into a plastic vegetable crisper ($13\frac{1}{8}$" x $10\frac{1}{2}$" x $4\frac{1}{2}$") that closed tightly. A piece of moistened cheese cloth was placed on the bottom of the vegetable crisper to maintain a high relative humidity. The container of petri dishes was incubated at 37 C. After 48 hours incubation the colony counts were made, using a stereoscopic microscope magnifying 10 times.

All previous work with the MF has been done with broth media soaked into an absorbent pad. It was thought that the membrane could be incubated directly on an agar medium. The initial studies demonstrated that the membrane could be incubated directly on top of an agar medium. It should be noted here that with this procedure the membrane must be rolled on to the agar surface with care so as not to leave air-pockets between the membrane and the agar. In Table 11 the results of the colony counts are recorded when the membranes are incubated on the agar surface as compared to that incubated on the nutrient broth pads. The results indicate that the membranes incubated on the former gave a slightly higher enterococci count than did the membranes incubated on the nutrient broth pads.

The studies on the membrane filter used a white (type HA) membrane which were difficult to count because the enterococci appear as white colonies. A black MF was tried.

The black HA membrane filter was first sterilized by autoclaving at 121 C for five minutes but it became brittle and very difficult to handle. Some membranes were so brittle they could not be touched without breaking. A personal communication (28) regarding the sterilization of the black membranes was as follows: "The filter is, of course, composed of cellulosic-esters. By nature, the chemical bonds are not strong.....The addition of the non-

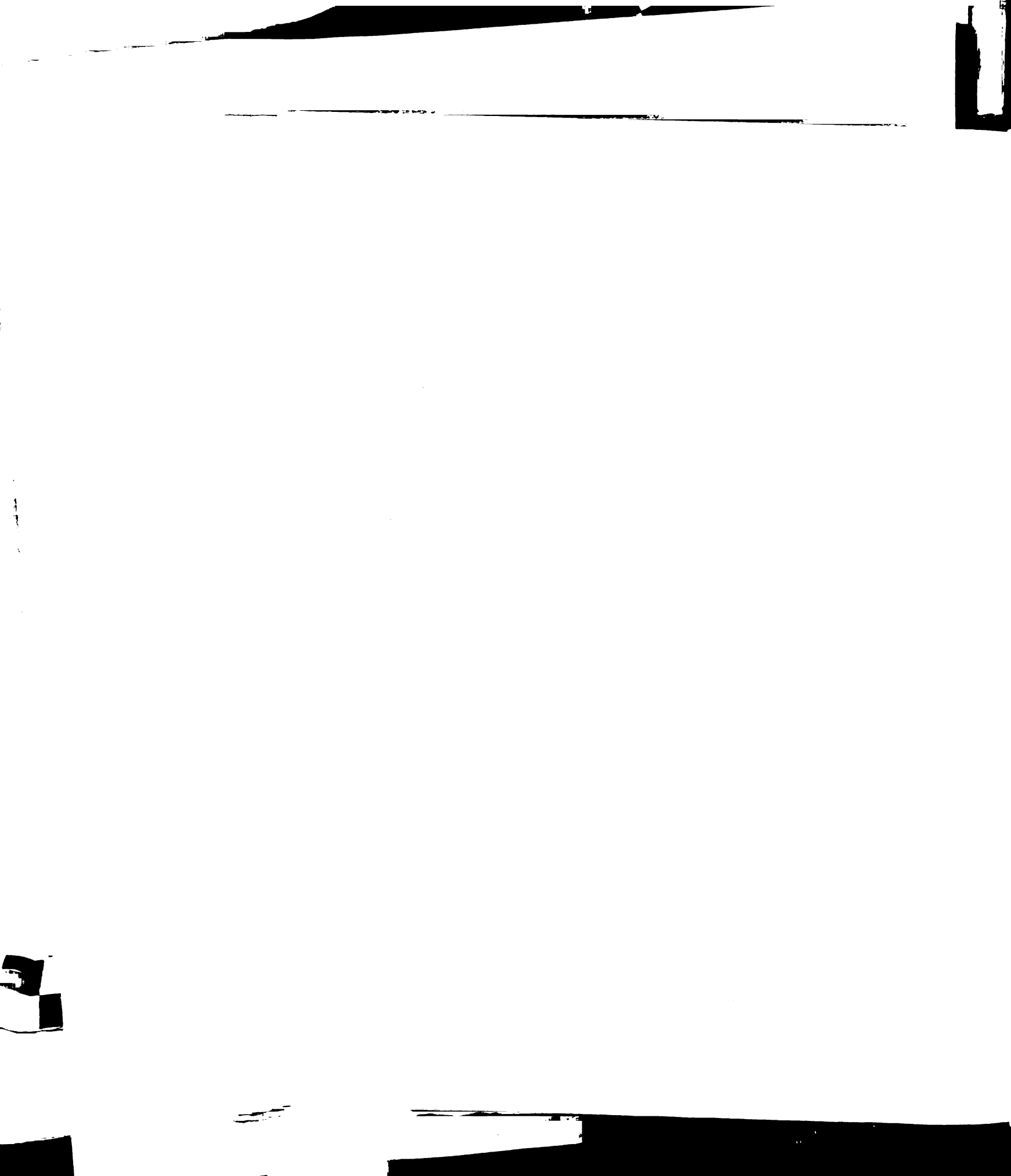


TABLE 11

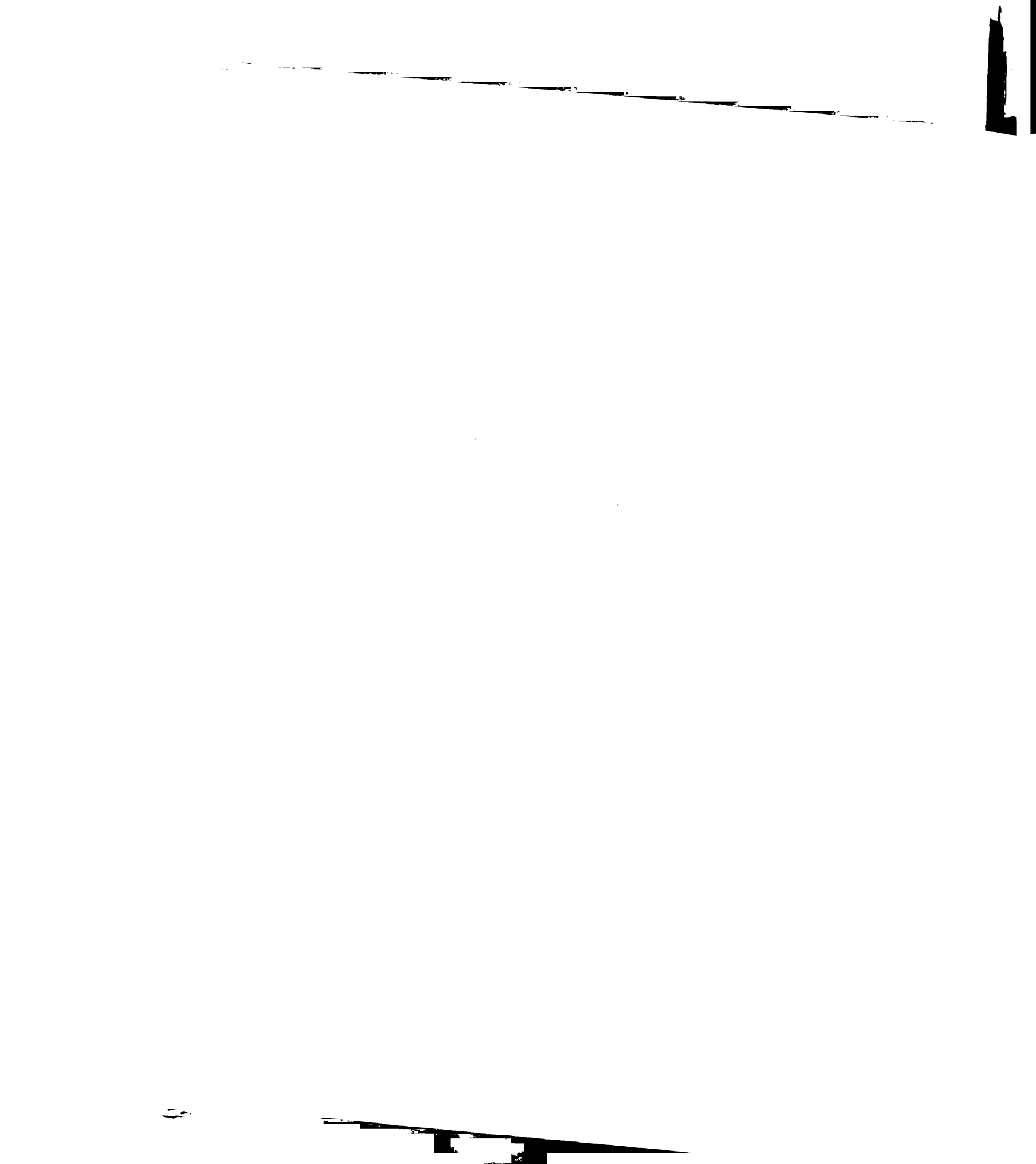
ENTEROCOCCI GROWTH ON EVA AGAR MEDIUM AND ON BROTH MEDIUM USING THE MEMBRANE FILTER

Sample	MF on Agar Base	MF on Broth Base
	(No./10 ml)	(No./10 ml)
River Water 1	494	391
" " 2	307	191
" " 3	163	54
" " 4	100	93
" " 5	34	27
" " 6	89	70
Average	196	141

TABLE 12

COMPARISON OF THE NUMBER OF ENTEROCOCCI GROWN ON EVA MEDIUM USING A WHITE MILLIPORE MEMBRANE AND A BLACK MEMBRANE

Sample No.	Agar Base		Broth Base	
	White Membrane	Black Membrane	White Membrane	Black Membrane
	(No./5 ml)	(No./5 ml)	(No./5 ml)	(No./5 ml)
1	26	17	34	13
2	34	17	8	20
3	34	26	27	-
4	93	43	70	43
5	93	36	-	45
6	81	54	70	42
Average	60.1	32	51.8	32.6



flourescent dye, which for this purpose must be done in the process of manufacture rather than afterward, seems in some manner to alter the internal bonds of the constituent molecules in the filter so that they can not be sterilized by steam. They may, however, be sterilized either by high voltage as in an electron accelerator Van de Graff machine, or more commonly by ethylene oxide followed by a suitable period of aeration in their container to obviate any inhibitory action of residual ethylene oxide in the pores of the filter."

Since there was no Van de Graff machine available at the time, or ethylene oxide, the membranes were sterilized by using ultraviolet light.

The white colonies on a black background were easier to count. The results of the experiment (Table 12) indicated that the black membrane had an effect on the number of colonies that would grow. In every case the white membrane gave higher counts than the black membrane.

Slanetz (55) reported the use of the Bac-T-Flex* membrane filter. He stated that the membrane was more flexible and durable than other membranes used. He conducted all his enterococci determinations using the Bac-T-Flex membranes; however, he did not make any comparison of numbers of enterococci which would grow on both membranes. This was the

*Bac-T-Flex filters (8 mm square grid markings), and S and S absorbent pads No. 407. Supplied by Carl Schleicher and Schuell Company, Keene, N. H.

next experiment which was carried out and the results tabulated in Table 13. A river water sample was used for the comparison. EVA agar, EVA broth and Slanetz's medium were tested on both the membranes. There was no significant difference in the number of enterococci recovered from the two membranes.

A comparison of the number of enterococci in river water was made by the membrane filter using an EVA medium and Slanetz's medium on Bac-T-Flex membranes. The results of 12 river samples are tabulated in Table 14. The EVA medium showed a greater recovery of enterococci than the Slanetz medium.

Discussion

The introduction of MF technic offers a new method by which organisms in slightly polluted waters could be concentrated on a membrane. This membrane retains the organisms on its surface and allows nutrients to pass through its microscopic pores, permitting growth. The method is advantageous when the sample of water that is to be tested needs to be concentrated to detect the microorganisms present.

The membranes which were incubated directly on agar medium showed a higher colony count and larger colonies than did the membranes impregnated with broth medium. This was

TABLE 13

ENTEROCOCCI GROWTH ON A MILLIPORE MEMBRANE AND Bac-T-Flex MEMBRANE

EVA Agar		EVA Broth		Slanetz Medium	
MF	BTF	MF	BTF	MF	BTF
357	348	326	251	268	227
315	225	309	262	215	231
289	321	276	246	262	277
Average:					
327	296	303	253	248	245

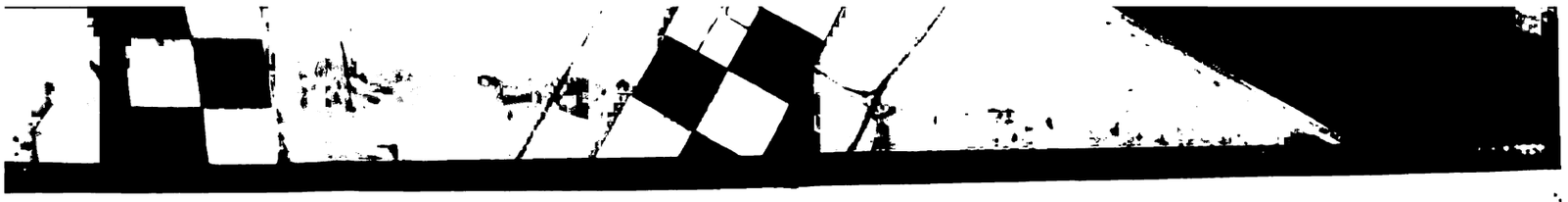


TABLE 14

COMPARISON OF NUMBERS OF ENTEROCOCCI IN RIVER WATER BY MEMBRANE FILTER TECHNIC USING SLANETZ MEDIUM AND EVA MEDIUM

Sample No.	EVA Medium	Slanetz Medium
1	222	174
2	240	152
3	95	52
4	92	54
5	25	12
6	24	24
7	357	268
8	315	215
9	289	262
10	284	227
11	255	231
12	321	277
Average	201.6	162.1

noted by Slanetz et al (54), but no explanation for this occurrence has been given.

Red and pink colonies were isolated from the membranes grown with Slanetz medium and the EVA medium. A total of 407 colonies were isolated from the membranes on EVA medium and 168 colonies were isolated from membranes on Slanetz medium. All the isolated colonies were inoculated in brain heart infusion broth and incubated for 24 hours at 37 C. From the brain heart infusion broth transfers were made into: 1) brain heart infusion broth and incubated at 45 C, 2) 0.1 percent methylene blue in skim milk, 3) brain heart infusion broth with 6.5 percent sodium chloride, 4) brain heart infusion broth at a pH of 9.6. If the organisms grew on all four media, they were considered to be enterococci according to Sherman. A total of 13 colonies from the EVA medium and 48 colonies from Slanetz medium did not confirm as enterococci. The 48 colonies from Slanetz medium were white colonies which were present on the membrane. Thus the EVA medium had a higher percentage of organisms confirmed as enterococci than the isolates from Slanetz medium.

SUMMARY

A drop plate technic and a specific medium for growing typical and atypical enterococci have been introduced. After a series of transfers the atypical enterococci proved to be typical enterococci. The atypical enterococci must, therefore, be given consideration in the evaluation of the sanitary quality of water. However, the drop plate method has its limitations in that it is effective only in examination of high population waters. This method detects more enterococci than by the Most Probable Number method described by Mallmann and Litsky.

The membrane filter method can serve amply where the limitations of the drop plate technic begin. An Ethyl Violet Azide medium was introduced which gives slightly higher colony counts of enterococci than the method and medium of Slanetz.

The drop plate method detects the greatest number of enterococci in high population waters, whereas the membrane filter method can detect enterococci in low population waters.

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APPENDIX

Composition of the Various Experimental Media

Medium No. 1

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Phytone (BBL)	20.0	
Lactose	5.0	pH 7.0

Medium No. 2

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Phytone (BBL)	20.0	
Lactose	5.0	
Yeast extract	5.0	pH 7.0

*Ethyl violet dye -- National Aniline Division, Allied Chemical and Dye Corporation. Lot No. 12552. Dye Content 57.5 percent.

Mediu

Med

M

Medium No. 3

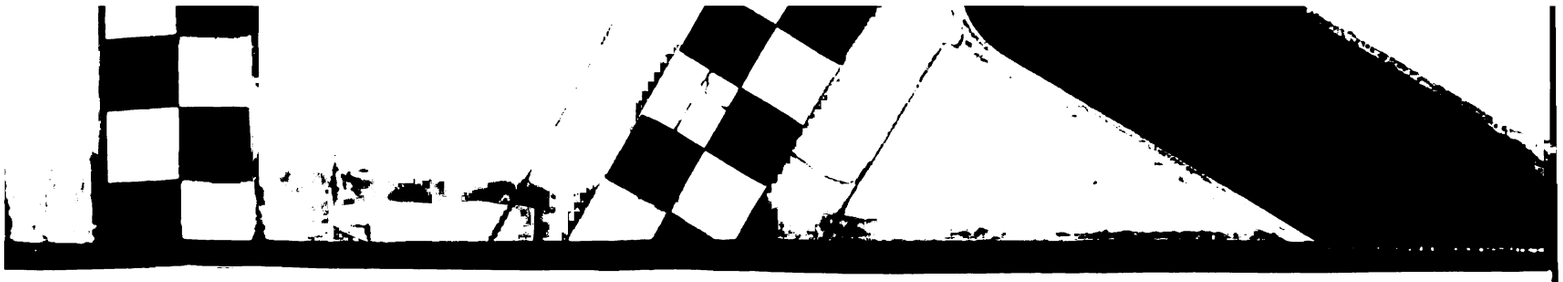
K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Lactose	5.0	
Polypeptone (BBL)	20.0	pH 7.0

Medium No. 4

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Lactose	5.0	
Trypticase (BBL)	20.0	pH 7.0

Medium No. 5

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Phytone (BBL)	20.0	pH 7.0



Mallmann Litsky Medium

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Ethyl violet dye*	0.00083	
Sodium azide	0.4	
Sodium chloride	5.0	
Dextrose	15.0	
Tryptose (Difco)	20.0	pH 7.0

EVA Medium for the MF Technic

K_2HPO_4	2.7 grams/liter	
KH_2PO_4	2.7	
Sodium azide	0.4	
Sodium chloride	5.0	
Ethyl violet dye*	0.00083	
Lactose	15.0	
Tryptose	20.0	pH 7.0

Slantez Medium

Tryptose	4 percent	
Yeast extract	1	
Dextrose	0.2	
Sucrose	10.0	
K_2PO_4	0.4	
Sodium azide	0.04	pH 7.0-7.2

Prepare a one percent 2,3,5 triphenyl tetrazolium chloride (TTC) solution, sterilize, and add aseptically to the above two media just before use to give 0.01 percent final concentration.