STUDIES ON THE ENTEROCOCCI

By

Charles E. Winter

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STUDIES ON THE ENTEROCOCCI.

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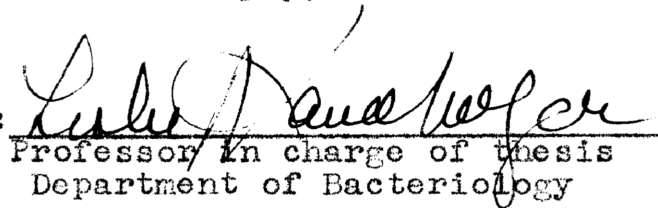

Professor in charge of thesis
Department of Bacteriology

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INTRODUCTION

It is generally recognized that there are three major groups of bacteria that might be used as indices of fecal pollution because of their presence in the intestine of normal warm blooded animals and man. The first, the coliform group, are characterized as the Gram-negative non-spore-forming, aerobic or facultative anaerobic, bacilli which ferment lactose with the formation of gas. At present this group is the one most commonly used for determining fecal pollution. The other two groups, namely the fecal streptococci (enterococci) and the spore forming lactose-fermenting anaerobes, have not been employed as fecal indices because of the difficulties involved in their cultivation.

In spite of the wide recognition of the coliform group as a fecal indicator, there are still times when it is not certain whether these organisms are of fecal origin. For example, when *Escherichia coli* is isolated consistently from sea water collected 50 miles from shore, it is hard to believe of its being of recent fecal origin. Occasionally a high coliform content is encountered in situations where a sanitary survey fails to show any source of fecal pollution. These observations cast considerable doubt upon the value of the coliform bacteria in sanitation under certain conditions. Whether or not the presence of these or-

ganisms in commercial food supplies is of public health significance cannot be determined by the present standard technic. The time has come to either replace the coliform group with a more suitable index of fecal pollution or to supplement the present tests by some technic which will indicate the most probable origin of the coliforms in any given situation. This is the purpose of the research presented in this report.

CHARACTERISTICS OF THE ENTEROCOCCI

In 1944 White and Sherman reported the use of a selective medium for the isolation of the enterococci. The medium utilized the inhibitory action of sodium azide to eliminate practically all bacteria except the staphylococci and streptococci. By adding penicillin, growth was restricted to the fecal streptococci, one of the few penicillin-resistant groups of streptococci.

This new medium offered possibilities for using the fecal streptococci as index organisms in the detection of fecal pollution. The characteristics of this group are distinct and for this reason confirmation of their presence can be easily accomplished. The one practical limitation that had to be overcome was the development of a rapid presumptive test. The clues to this were found in the nutrient requirements of these bacteria as indicated by Sherman (1937) in his monograph on the Streptococci.

The primary characteristics of the four main divisions of the genus Streptococcus, i.e., the pyogenic, the viridans, the lactic, and the enterococci, are shown in Table I. The enterococci consist of four species, *Streptococcus fecalis*, *Streptococcus liquefaciens*, *Streptococcus zymogenes*, and *Streptococcus durans*. These all belong to Lancefield's (1933) group D and are divided on the basis of hemolysis, two (*fecalis* and *liquefaciens*) being non-hemolytic. Of the

Table I

PRIMARY CHARACTERISTICS OF THE MEMBERS
OF THE GENUS STREPTOCOCCUS

DIVISION	GROUP OR SPECIES	Lancefield Group	Hemolysis	Growth at		Growth in presence of				Strong Reduction	Survival 60°C., 30 min.	NH_3 from peptone
				10°C.	45°C.	6.5 per cent NaCl	pH 9.6	650 Oxford units penicillin per liter	0.1 per cent methylene blue			
	S. pyogenes	A	+	−	−	−	−	−	−	−	−	+
	S. mastitidis	B	±	−	−	−	−	−	−	−	−	+
	S. equi	C	+	−	−	−	−	−	−	−	−	+
	"Animal pyogenes"	C	+	−	−	−	−	−	−	−	−	+
Pyogenic	The "human C"	C	+	−	−	−	−	−	−	−	−	+
	"Minute hemolytic"	F	+	−	−	−	−	−	−	−	−	+
	Group G streptococci	G	+	−	−	−	−	−	−	−	−	+
	Group E streptococci	E	+	−	−	−	−	−	−	−	−	+
	Group H streptococci	H	±	−	+	−	−	−	−	−	±	±
	S. salivarius		−	−	±	−	−	−	−	−	−	−
	S. equinus		−	−	+	−	−	−	−	−	±	−
Viridans	*S. bovis*		−	−	+	−	−	−	−	−	+	−
	Varieties of *S. bovis*		−	−	+	−	−	−	−	−	+	−
	S. thermophilus		−	−	+	−	−	−	−	−	±	−
Lactic	*S. lactis*		−	+	−	−	−	−	+	+	+	+
	S. cremoris		−	+	−	−	−	−	+	+	±	−
	S. fecalis	D	−	+	+	+	+	+	+	+	+	+
Enter-	*S. liquefaciens*	D	−	+	+	+	+	+	+	+	+	+
ococcus	*S. zymogenes*	D	+	+	+	+	+	+	+	+	+	+
	S. durans	D	+	+	+	+	+	+	+	±	+	+

four major divisions, the enterococci are the most active as far as cultural characteristics are concerned. These four species share with the lactic group the ability to grow at 10°C. In common with the viridans species and group H, they grow at 45°C. They are unique in their ability to tolerate and grow in 6.5 per cent sodium chloride and at pH 9.6 respectively, and are also unique in their resistance to penicillin. They share with all but the pyogenic group (group H excepted) the capacity to survive 60°C. for 30 minutes and share with all but the viridans group the ability to produce ammonia from peptone.

The cultural characteristics of the four species comprising the enterococci are shown in Table II. A primary separation can be made on the basis of hemolysis and the reaction in gelatin according to the following scheme:

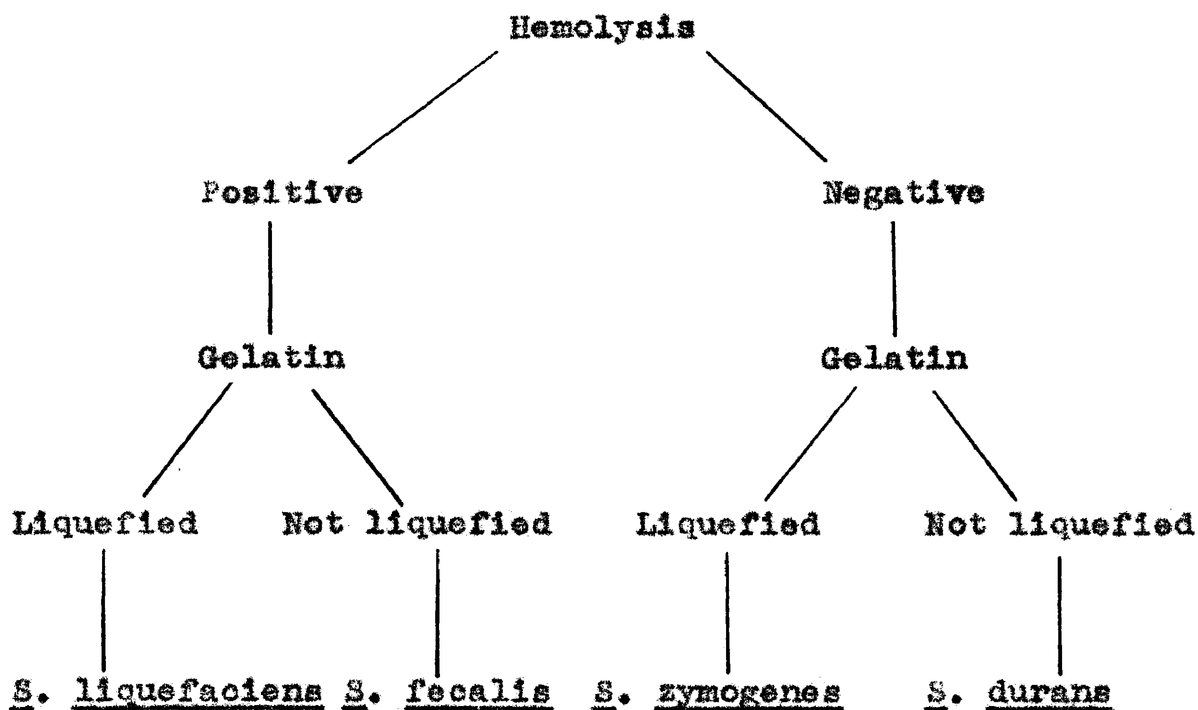


Table II

CULTURAL CHARACTERISTICS OF THE ENTEROCOCCI

SPECIES	Lancefield Group	Hemolysis	Gelatin Liquefaction	Strong Reduction	Active Fibrinolysis	Sodium Hippurate Hydrolysis	Starch Hydrolysis	Esculin Hydrolysis	Milk Curdled	Final pH in Glucose Broth	ACID PRODUCED FROM: Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin
S. fecalis...	D	−	−	+	−	±	−	+	+	4.5 - 4.0	±	+	±	+	+	±	−	±	+	±	+
S. liquefaciens.....	D	−	+	+	−	±	−	+	+	4.5 - 4.0	±	+	+	+	+	±	−	+	+	+	+
S. zymogenes.....	D	+	±	+	−	±	−	+	+	4.5 - 4.0	±	+	+	+	+	±	−	+	+	+	+
S. durans....	D	+	−	−	−	+	−	+	+	4.5 - 4.0	−	+	−	+	±	−	−	−	−	−	±

According to Sherman (1937) and Houston (1936), the four species, regardless of their hemolytic properties, all belong in Lancefield's group D when tested with antisera prepared using Streptococcus zymogenes as the antigen. The detailed taxonomic features of each respective species are listed below:

Streptococcus fecalis. Spheres, ovals, of various size, usually occurring in chains of two to eight cells. Gram-positive. Gelatin not liquified. Produces turbidity in broth, clearing later with abundant sediment. Reaction to litmus milk is acid, usually reduction of litmus before curdling; no digestion of clot. Acid from dextrose, maltose, lactose, trehalose, salicin, and mannitol; may or may not ferment arabinose, sucrose, raffinose, glycerol, sorbitol; inulin not fermented. Starch is not hydrolyzed; sodium hippurate may be and esculin is hydrolyzed. Ammonia is produced from 4 per cent peptone. Optimum temperature is 37°C.; will grow at 10°C. and 45°C.; survives 62.8°C. for 30 minutes. Tolerates 6.5 per cent NaCl. Final pH in dextrose broth is 4.5 to 4.0. Grows at pH 9.6. Tolerates 0.1 per cent methylene blue. Not hemolytic. Lancefield's group D. Facultative anaerobe. Habitat: Human and animal intestine.

Streptococcus liquefaciens. This species shows the same characteristics as Streptococcus fecalis except as given below.

Gelatin liquified with profuse growth. Litmus milk is acid, curdled and peptonized. Acid from dextrose, maltose,

sucrose, lactose, trehalose, mannitol, sorbitol, salicin and glycerol.

Streptococcus zymogenes. This species shows the same characteristics as *Streptococcus fecalis* except as given below.

Gelatin usually liquified. Litmus milk is acid, curdled and peptonized. Acid from dextrose, maltose, sucrose, lactose, trehalose, mannitol, sorbitol, salicin and glycerol. Beta hemolysis.

Streptococcus durans. This species shows the same characteristics as *Streptococcus fecalis* except as given below.

Litmus milk is acid; curdled followed by reduction of litmus. Acid from dextrose, maltose, lactose and usually salicin and trehalose. Raffinose, inulin, serbitol, arabinose, glycerol, mannitol and sucrose not fermented. Sodium hippurate is hydrolized. Beta hemolytic.

LITERATURE

The literature dealing with fecal pollution indicates that much of the early work was carried on by sanitarians in charge of water supplies. Following the cholera epidemic in London from the Broad street well in 1854, there evolved a growing consciousness of the role of polluted water in the spread of enteric diseases. During the 1890's, there were a number of outbreaks of typhoid fever in America, England and continental Europe. These epidemics stimulated research to find methods for determining the presence of human excrement in water. There were two methods of approach; chemical and bacteriological. The main interest centered about the determination of the normal bacterial flora of the intestine. Escherich in 1885, first described the Gram-negative bacilli which have served as an index of fecal pollution since the beginning of bacteriological sanitary science.

The first report of streptococci's being isolated from sewage was made by Roscoe and Lunt in 1891, who described what they called _Streptococcus ureae_. The description and photomicrographs of this organism indicate that it probably was one of the fecal streptococci. Three years later, Laws and Andrewes (1894) reported a small streptococcus as being the most common organism present in fresh sewage from the St. Bartholomew hospital. Thiercelin (1899) in his report on the morphology and method of reproduction of these organ-

isms, was the first to use the term "enterococci".

Houston (1899) was the first to call attention to the significance of this group of streptococci in water. He examined the rivers around London and found that streptococci could be isolated from 0.1 to 0.0001 ml. aliquots of water polluted with sewage. He was unable to isolate streptococci from 0.1 ml. quantities of water from those rivers "above suspicion of contamination". This led him in 1899 to state:

> In the present stage of our knowledge, the presence of streptococci in a substance, be it soil, or sewage, or water, suggests recent association of certain ingredients of that substance with an animal host. These microorganisms are readily demonstrable in waters recently polluted and they are seemingly altogether absent from waters above suspicion of contamination.

The method of isolation used by Houston at this time was to filter the water sample through sterile Pasteur filters and transfer the residue to an agar plate by means of a small sterile brush. The agar plates were incubated at 37°C. for 24 hours and then examined for minute colonies. These colonies were then picked for subculture and further study.

The following year Houston again stressed the fact that the streptococci were indicative of extremely recent pollution. He stated that if streptococci are absent, it does not imply "purity or safety" but their presence does indicate recent pollution. In his description of these organisms he mentions a fact that is recognized today, namely that many of the streptococci are almost rod shaped.

In 1901, Harrock published his book on the "Bacteriological Examination of Waters" in which he devotes one chapter to the sewage streptococci. In his experience, when dilutions of old sewage are kept in the dark on a veranda for a few days, the streptococci rapidly disappear, so he deducted "their presence in a water supply undoubtedly indicates a recent contamination but the contamination is not necessarily dangerous unless the streptococci are accompanied by B. coli".

The first mention of these organisms in America was in the two papers in 1902 by Winslow and Hunnewell. The first paper was primarily a review of the papers of Houston with a report of a few preliminary confirmatory experiments in which they found the occurrence of streptococci in polluted river water to be constant and significant. They suggested that these organisms be referred to as "the sewage streptococci of Houston". In the second paper, they report that they examined 157 samples of unpolluted water and found streptococci in only 3 samples, whereas they found these organisms present in 25 of the 50 samples of water known to be polluted. They note that they might have found the organisms in more of the polluted waters if a better method of isolation had been available. They concluded that "it is desirable that the occurrence of the streptococci, as well as the colon bacilli, should be noted in sanitary water analysis". Later Winslow and Nibecker (1903) found streptococci in only one sample of the 259 samples of unpolluted waters tested.

Prescott (1902) showed that in many cases, the strep-

tococci overgrew the colon bacilli in glucose broth after 24 hours of incubation. Prescott and Baker (1904) later reported that they had tested 50 samples of polluted water from the Charles river of Boston and found streptococci in all of the samples. From their studies, they agreed to the value of using tests for both the colon bacilli and the streptococci in water analysis.

Frankland (1911) working in Houston's laboratory and using his method of spreading 1 ml. of the water sample on Conradi-Drigalski's medium (appendix A), incubating at 37°C. for 24 hours and picking minute colonies, compared the waters of the Thames, Lea and New rivers. The first two were polluted while the last was a much cleaner river. The results showed that coliform bacteria are more often isolated from these waters than are the streptococci. He also found that human feces contains roughly 100,000 streptococci per gram, sometimes being more abundant than B. coli.

Clemesha (1912), in India, found the streptococci present in 0.0001 to 0.00001 grams of human feces which might be interpreted as 100,000 organisms per gram, as was previously reported by Frankland. However, he was able to isolate streptococci from 20 ml. of water on only six occasions out of 378 samples, 151 of which were grossly polluted. He concluded that streptococci were infinitely rarer in rivers and lakes in India than in England. In a series of bottle experiments in the laboratory and in the study of an artificially polluted tank outdoors he found that they

disappeared very rapidly in water, within 2 or 3 days at the most.

Savage and Read in 1917, examined 1314 samples of water (974 surface supplies and 340 deep well supplies) by adding the water to glucose neutral-red broth and making a direct microscopic examination for chains after incubation at 37°C. for 40 to 48 hours. Of these, 510 samples contained coliform bacteria in 1 ml. or less, while 425 (83% of the 510) contained streptococci in 10 ml. or less. These results indicate that only 17% of the samples containing coliform bacteria gave negative results for streptococci. This was the first indication that the streptococci might help to differentiate between the fecal and non-fecal coliform bacteria. In the summary of their report, Savage and Read stated:

> The absence of streptococci (at least with the methods used) is of less significance than their presence. The standards for permissable number of streptococci broadly correspond to similar numerical standards for B. coli but are of less significance and reliability. We are decidedly of the opinion that the streptococci estimation, carried out by the simple method described, is of undoubted value as evidence for or against excretal contamination. We think further that if the reliability of the method (of isolation) could be improved, the value of the streptococci enumeration would be enhanced and even more clearly demonstrable.

The following year, Savage and Wood (1918) found that when human feces, animal feces or sewage were added to sterile water blanks, in all cases the streptococci diminished or were practically eliminated after two weeks storage. This led to the conclusion:

> The finding of streptococci or *B. coli* in 1 or 10 cc. of sample can only indicate contamination considerable in amount and of recent origin. In particular, the finding of streptococci in any numbers can be accepted as indicating considerable and recent contamination. We consider that the streptococci determination is very valuable on its positive side as an indication of recent contamination. As a means of judging the recency of the contamination, it is even more valuable than the *B. coli* enumeration.

During the next decade little work was published on the fecal streptococci, possibly due to the increasing emphasis that was being put on the colon bacilli. Nevertheless, Houston was still using the streptococci and in 1930 his report to the Metropolitan Water Board of London contained a section on this subject. His suggested method was to put the water sample in Mac Conkey's broth (appendix A) and incubate at 37°C. for 24 hours. Two to four drops of this broth was then placed in the center of a Petri dish and spread with a needle. The open plates were then placed in a <u>dry</u> 37°C. incubator for 15 minutes and then transferred to a moist (85% humidity) 37°C. incubator for 45 minutes. 30-40 c.c. of melted Drigalski-Conradi's agar (appendix A) was then added to each plate and they were incubated at 37°C. for 24 hours. At the end of this period they were examined for minute colonies to be transferred to lactose-lemco-peptone-litmus medium (appendix A) and incubated at 37°C. for 24 hours. These tubes were then examined for the production of acid with no gas and then as a final check, a Gram stain was prepared. The drying procedure was an attempt to differentiate between the salivary and the fecal

streptococci. Lactose and raffinose fermentation were employed to differentiate between human and other animal streptococci. The human streptococci were always "lactose positive".

Two years later the same author (1932) had examined 14 human fecal samples and found the number of streptococci to range from 10,000 to 10,000,000 per gram. 32 samples of crude sewage tested contained from 10 to 1,000,000 streptococci per ml. with 1000 per ml. being the most common count. He continues to state that the corroborative finding of B. coli and streptococci are very valuable.

Harold (1936), one of Houstons colleagues, suggested using a 1:15000 concentration of potassium tellurite (appendix A) in the plating medium and the streptococci could then be recognized as small bluish black colonies about 1 mm. in diameter with a peripheral opalescence.

The influence of the recommendations of these British investigators is evidenced by the fact that the Committee of the British Ministry of Health (1939) continues to recommend the enumeration of streptococci as a routine procedure in sanitary water analysis. In this country, the 1912 Standard Methods was the last to mention this test and it was laid aside by stating, "the information afforded by the occurrence of these organisms seems to be of less value than in the case of B. coli and it is believed that for the present at least, the streptococcus test is of subordinate importance".

Mallmann (1928) and Mallmann and Gelpi (1930) began to

use the streptococci as an indicator of unsafe conditions in swimming pools and reported that the number of streptococci found in swimming pool waters parallels the amount of pollution as indicated by the number of bathers. In 1934, Mallmann extended the test to natural bathing places. He found that in Lake Lansing, a small inland lake of Michigan, the streptococci fluctuated with the bathing load, not being found at points free from bathing pollution, and disappeared from the water over night. It is not clear whether he is dealing solely with fecal streptococci, however, since his method is to use the lactose broth of the coliform test and examine for streptococci using a Gram stain. It is possible that his test is for the salivary streptococci alone or for a mixture of these with the fecal types. In 1937 Sherman presented a very complete report in which the Enterococci was perhaps the most clearly differentiated group.

Sherman and Stark (1931) made a study of 294 cultures of streptococci isolated from milk and other sources to determine species which grow at 45°C. All of the enterococci were in this group. Later the same two authors (1934) made clear the differentiation of _Streptococcus lactis_ and _Streptococcus fecalis_.

Sherman, Mauer and Stark (1937) pointed out that those organisms which had been referred to as _Streptococcus fecalis_, ever since Andrews and Horder reported this species in 1906 from the air and horse manure of London streets, are really a homogenous group of three species, _Streptococcus_

fecalis, *Streptococcus zymogenes*, and *Streptococcus liquefaciens*. Sherman and Wing (1937) later described a fourth species, *Streptococcus durans*, which belonged to this same group.

These and many other papers culminated in Sherman's Presidential Address, "The Enterococci and Related Streptococci" (1938) at the annual meeting of the Society of American Bacteriologists in 1937. He reported that antisera of Lancefield's group D streptococci (1933) had been used to test various non-hemolytic streptococci and that *Streptococcus lactis*, *Streptococcus bovis*, *Streptococcus equinus*, *Streptococcus salivarius*, *Streptococcus thermophilus* and *Streptococcus cremoris* all failed to give a positive precipitin test. *Streptococcus fecalis* and *Streptococcus liquefaciens*, which are the non-hemolytic members of the enterococci group, give positive precipitin tests.

Sherman and Niven (1938), reporting on the hemolytic streptococci of milk, found *Streptococcus mastitidis* (Group B) and animal pyogenes (Group C) to be the most common hemolytic streptococcus in raw milk while those in pasteurized milk are most commonly *Streptococcus durans* and *Streptococcus zymogenes*, both enterococci of Group D. They concluded that "the best method of identifying streptococci of sanitary significance from milk is Lancefield's serological technic". Smith and Sherman (1938) showed, by isolating it from normal human feces, that *Streptococcus durans* is a fecal organism of Group D. Heretofore this organism had been reported

only from milk products. Of 109 cultures of hemolytic streptococci from 45 fecal samples of normal individuals, 73 were Streptococcus zymogenes, 19 Streptococcus durans and 17 pyogenic streptococci.

In 1940, Snyder and Lichstein reported that sodium azide (0.01%) inhibited the Gram-negative bacilli of feces. Later, Lichstein and Soule (1944) showed that streptococci, pneumococci, anaerobes and lactobacilli were resistant to 0.03% sodium azide while Gram-negative rods, Gram-positive spore-forming aerobes and Staphylococcus citreus were very sensitive to the same concentration. Staphylococcus albus, Staphylococcus aurens and Corynebacterium diphtheriae were moderately resistant. Eight strains of streptococci tested were all tolerant to a 0.05% concentration.

As a result of the early studies on sodium azide, Mallmann (1940) suggested that sodium azide be added in a 1:5000 concentration to the coliform enrichment broth (appendix A) which Darby and Mallmann (1939) had just recommended and thus produce a selective broth for the streptococci. In 1943 Hajna and Perry reported a new medium for the isolation of fecal streptococci which they called "S. F. medium". The ingredients are:

Bacto tryptone	20	gm.
NaCl	5	gm.
Dextrose	5	gm.
K_2HPO_4	4	gm.
KH_2PO_4	1.5	gm.
Sodium azide (NaN_3)	0.5	gm.
Bromcresol purple (1.6% alc. sol.)	2	ml.
Distilled water	1000	ml.

The medium is sterilized at 15 pounds pressure for 15 minutes. After inoculation, it is incubated at 45.5°C. for 24-48 hours and then examined for turbidity due to growth and for acid as indicated by the bromcresol purple. Ostrolenk and Hunter (1946) using this S. F. medium, found that of 51 samples of human, cat, mouse, guinea pig, dog, rabbit, rat, chicken, fly, and monkey feces tested for enterococci and *Escherichia coli*, 49 contained enterococci in 0.1 to 0.000,001 gram, while 46 contained *Escherichia coli* in 0.01 to 0.000,000,1 gram. While only 46 contained *Escherichia coli*, all 51 contained coliforms of one type or another. Two soil samples were tested, giving negative results for both *Escherichia coli* and enterococci. A Gram stain preparation of each of the 71 tubes of S. F. medium in which an acid condition was produced showed that 60 tubes contained fecal streptococci alone or in combination with a Gram-positive, rod-shaped organism. Eleven tubes were regarded as false positives produced by this rod, for the rod-shaped organism was present without any streptococci. Seven tubes were re-

garded as false negatives because they contained streptococci yet an acid condition was not indicated. Ostrolenk, Kramer, and Cleverdon (1947) suggested following the initial broth enrichment by streaking the broth on the same medium solidified by adding 1.5% agar.

In 1932, Fleming found that the enterococci as a group were resistant to penicillin while all other streptococci, as well as staphylococci, pneumococci, B. diphtheriae and B. acidophilus were penicillin sensitive. This was noted again by Bornstein (1940) who found that 27 cultures of enterococci, some from his own collection and some from Sherman, were all resistant to penicillin while 13 cultures of viridans streptococci were all susceptible. Streptococcus lactis was also resistant. Helmholz (1944) obtained the opposite results when 30 cultures of Streptococcus fecalis were susceptible to 3 oxford units of penicillin per ml. in urine. Perhaps the difference may be due to the fact that here the penicillin is in a urine solution.

A new medium selective for the enterococci was suggested by White and Sherman in 1944. It takes advantage of the action of sodium azide to inhibit most all of the bacteria other than the streptococci, pneumococci, lactobacilli and the anaerobic spore formers. Penicillin is added to inhibit all of these organisms except the enterococci and the anaerobic spore formers. As the plates are incubated aerobically, only the enterococci should grow. The formula for this medium is:

0.5 %	Dextrose
0.5 %	Tryptone
0.5 %	Yeast Extract
0.03%	Sodium azide
1.5 %	Agar
325	Oxford units of penicillin per liter.

Sherman, Smiley and Niven (1943) reported in their paper that the cultures of the organism that Linden, Turner and Thom had isolated in 1926 from two samples of cheese causing "food poisoning" were really Streptococcus fecalis. The original authors had identified the organisms as Streptococcus lactis but they are typical enterococci in their physiological characteristics and belong to Lancefield's Group D. Buckbinder, Osler and Steffen (1946) have reported four outbreaks of natural food poisoning from canned milk, Charlotte Russe, barbecued beef and ham bologna, all of which contained unusually great numbers of enterococci. The toxicity of these cultures was then tested by feeding milk, custard or egg salad inoculated with the cultures to human volunteers. Of the 26 volunteers, 7 showed definite symptoms of food poisoning while none of the controls fed on uninoculated portions of the same foods were ill. These two reports indicate that the enterococci may have significance in outbreaks of food poisoning.

METHODS OF ISOLATION

In order that the cultural characteristics of the enterococci on the White and Sherman (1944) sodium azide-penicillin medium might be studied, two cultures of each of the four species of enterococci were obtained from Dr. Sherman. Fresh 4 hour cultures of each were grown in a dextrose-tryptone-yeast extract broth and 1 ml., 0.1 ml., 0.01 ml. and 0.001 ml. amounts inoculated into poured plates of the medium. These plates were incubated at 37°C. for 48 hours. Upon examination it was found that the Streptococcus durans seemed to be partially inhibited but that the other three species grew very well. These findings were in agreement with those of Sherman and White. Three types of colonies were commonly produced -- a small entire circular surface colony, a pin-point fusiform deep colony and a diffuse thin circular colony between the agar and the glass of the Petri dish.

After the typical colony structures of the enterococci on the medium were known, a number of samples of fresh and stored raw sewage was plated on the sodium azide-penicillin medium. Typical colonies were fished and these upon examination proved to be enterococci. Samples of raw and pasteurized milk were likewise tested with only enterococci being isolated from the plates.

In order to make certain that the coliform group of bacteria would not grow on the enterococci medium, four cul-

tures each of _Escherichia coli_ and _Aerobacter aerogenes_ were isolated from the raw sewage and inoculated by poured and streak plates on the medium. There was no evidence of growth on any of the poured plates and only one streaked plate of _Escherichia coli_ showed a very scanty growth along the line of inoculation. This plate had been inoculated very heavily with a loopful of organisms from a solid medium. Cultures of enterococci and coliforms were mixed and then inoculated into poured plates of the medium and only enterococci were found upon examination of the colonies.

Poured plates of parallel inoculations were then incubated at 20°C., 37°C. and 45°C. It was found at 48 hours of incubation that the colonies of both the 20°C. and 45°C. plates were extremely small and hard to count and that they always gave a lower count. Incubation at 37°C. produced larger colonies and higher counts.

At times during the early studies, the colonies of enterococci were extremely hard to differentiate from small specks of debris in the agar. It was extremely difficult, because of specks in the medium, to make counts on the medium prepared with shredded agar, even after the medium was filtered through cotton. It was decided therefore, to add an indicator which would permit the differentiation of colonies and debris in the medium. Brom thymol blue was tried first and after using several concentrations, it was found that 0.1 ml. of a 1.6% alcoholic solution in 100 ml. of medium produced faint yellow colonies on a bluish-green background.

By comparing with control plates without the indicator, it was found that the brom thymol blue had a very slight inhibitory action. Eosin Y was tried and although it did not inhibit the number of colonies, 0.1 ml. of a 0.4% aqueous solution in 100 ml. of medium produced pink colonies on a colorless background that did not facilitate the differentiation.

One of the characteristics of the enterococci is their ability to grow in a 0.1% methylene blue solution, so methylene blue was tried in the medium. 0.1 ml. of a 1% aqueous solution in 100 ml. of medium (0.001% in final concentration) did not produce any inhibition and the colonies were easily differentiated from the debris. The colonies have a deep blue center surrounded by a white zone where the methylene blue is reduced.

It was found that doubling the amount of penicillin did not inhibit the number of colonies, therefore it was felt advisable to double the amount of penicillin so as to increase the safety factor against penicillin resistant staphylococci and non-fecal streptococci.

This medium, with the modifications mentioned is recommended as a selective plating medium for isolation of the enterococci.

A number of samples of fresh and sea water were tested for the presence of enterococci by this plating method and it was soon evident that when the sample contained one or two enterococci per ml. or less, the plating technic was not

of much value. For example, a sample containing one enterococcus per ml., if plated in 1 ml. amounts should have one colony per plate. Actually, however, if duplicate plates were prepared, one might have two or three colonies while the second would not have any. This opened two questions: first, can a plate with one or two colonies be considered significant; and secondly, can a plate without any colonies be considered as evidence of the absence of enterococci? Both of these questions were answered in the negative. A better method of isolating the organisms from samples containing one or less per ml. would have to be developed if tests for these bacteria were to be made practical.

An attempt was made to concentrate the bacterial content of the sample by passing a given quantity of the sample through a Seitz filter and then to grind up the filter pad in a known amount of sterile phosphate buffer solution. This mixture was then plated on the selective medium. It was hoped that an accurate quantitative count could be obtained by this method but actual tests showed too much variation to warrant the inconvenience of the test.

A second method was to incubate a known amount of the sample in an enrichment broth and then determine the presence of the enterococci. A broth was prepared containing the dextrose, tryptone, yeast extract and sodium azide in the same proportions as the plating medium. 10 ml. portions of this could be inoculated with 1 ml. or less of the sample without serious dilution of the sodium azide. It is charac-

teristic of the enterococci to produce acid in the presence of dextrose, therefore the production of acid, as indicated by a dye, might be used as an index of the presence and growth of the organisms. Brom thymol blue was chosen because its pH range is from 7.6 to 6.0.

In order that an inoculum of 1.0 ml. or more might be used, a concentrated broth containing five times the amount of each ingredient was prepared and two ml. of this inoculated with 10 ml. of the sample. In a like manner, 20 ml. of the concentrate could be placed in a sterile bottle and inoculated with 100 ml. of the sample.

Since the enterococci grow in a medium adjusted to pH 9.6, the selectivity of the medium was enhanced by adjusting the reaction to at least pH 8.0. Since the pH would drop during sterilization, a buffer was added to stabilize the reaction, but it was found that the presence of a buffer increased the incubation time necessary for the organisms to produce sufficient acid to change the indicator. The results indicated that if the normal strength medium was adjusted to pH 8.5, with 0.1N NaOH, a drop to pH 8.0 - 8.2 could be expected after autoclaving. As would be expected, the change of pH was greater in the sterilization of the concentrated medium. However, since the Difco Laboratories have suggested that both the normal strength and concentrate be adjusted to pH 8.5 so that both may be prepared from one basic medium.

An incubation temperature of 45°C. was chosen to enhance

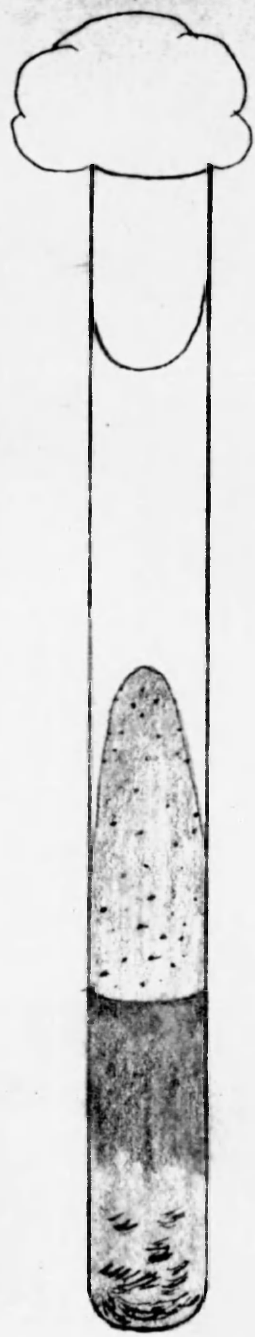
the selectivity of the enrichment test. The necessary incubation time was found to be as short as 7 hours in some cases, some tubes showing the presence of acid and turbidity after that time. Very seldom was it found that a tube with negative results after 16 hours would become positive upon further incubation. When time is an important factor, as in the analysis of certain food products, it is recommended that the tubes be checked periodically after 8 hours of incubation. A tube failing to show acid and turbidity after 24 hours usually can be regarded as negative.

This medium is similar to that of Hajna and Perry's S. F. medium in that the production of acid and turbidity is used to indicate the presence of enterococci. There are several differences however. This medium contains yeast extract in addition to tryptone as a source of nutrient and growth factors. No buffer is used, which hastens the test by not requiring the production of excessive acid. Brom thymol blue with a range of pH 6.0 - 7.6 is superior to brom cresol purple used in the S. F. medium which has a color range of pH 5.2 - 6.8. These differences eliminate a number of false negatives found when using S. F. medium, as found by Ostrolenk and Hunter (1946) and this laboratory.

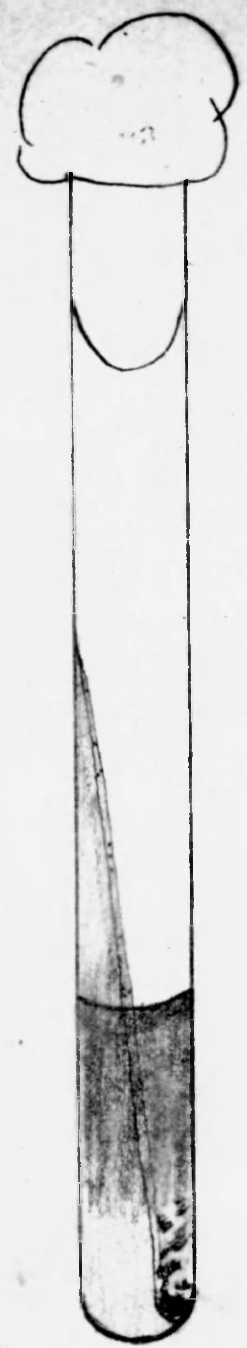
The enrichment medium described above can only be employed as a presumptive test for the enterococci and a confirmatory medium is essential if the tests are to be significant. To this end it was desirable to develop a simple, selective, practical procedure. An agar slant, the lower

half of which is covered with a broth (Figure 1), provides a gel surface for development of colonies as well as a liquid culture. Characteristic growths can be observed in a single tube inoculated in one operation. A basic medium can then be prepared, using 1.5 per cent agar in the slants. After inoculation and incubation, characteristic colonies with blue centers appear on the slant and a dark blue sediment is seen in the broth. Sodium azide is added to both broth and agar. Sodium chloride (6.5%) was at first added to the basic medium but it was found that the salt inhibited the colony formation on the agar. The salt should be added only to the broth. It would be desirable to add penicillin to the basic medium but it would be inconvenient to incorporate into the agar medium and as it is felt that autoclaving might destroy it, 650 Oxford units per liter are added aseptically only to the broth. The salt and penicillin become equally distributed between the broth and agar but in the final tube, since the concentration of the penicillin is double that suggested by White and Sherman (1944), the resulting concentration, after equalization, is still effective. Although the sodium chloride is not present in a 6.5 per cent solution, it remains in an inhibitory concentration. An incubation temperature of 37°C. yields more rapid and characteristic colony formations. Therefore this temperature is recommended. A Gram stain should be prepared from the broth culture. As a final test, 5 ml. of a 3% H_2O_2 solution may be added to the slant-broth preparation to determine the

FIGURE 1



Front View

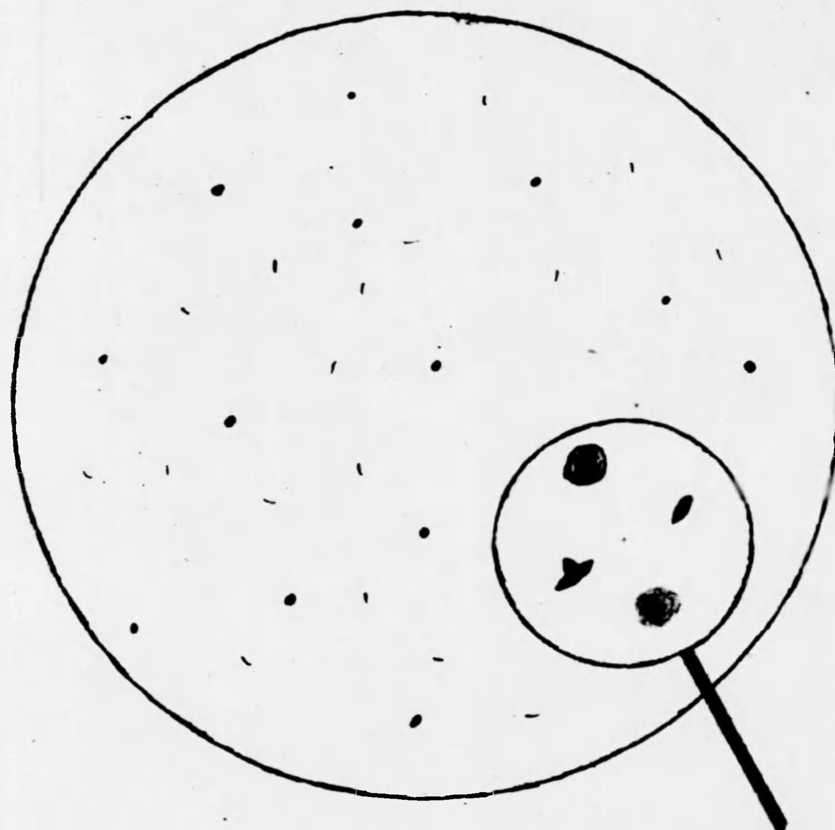


Side View

ENTEROCOCCI CONFIRMATORY TUBE

Actual Size

FIGURE 2



ENTEROCOCCI AGAR PLATE

Actual Size

production of catalase.

On the basis of the above, the following methods have been proved most satisfactory for the isolation and identification of the enterococci:

A. Direct Plating Method

The modification of White and Sherman's sodium azide-penicillin medium recommended for isolation of the enterococci by direct plating technic has the following composition:

Dextrose	5	grams
Tryptone (Bacto)	5	grams
Yeast extract (Bacto)	5	grams
Sodium azide	0.4	grams
Methylene blue	0.01	grams
Agar	15	grams
Penicillin	650	Oxford units
Water	1	liter

For convenience, it is recommended that a 1 per cent aqueous solution of sodium azide be prepared and then 40 ml. of this added per liter of medium. A 1 per cent aqueous solution of methylene blue may be prepared and one ml. of this used per liter of medium. The ingredients, with the exception of the penicillin, are put into solution and the pH adjusted to 7.4 - 7.6. After being autoclaved at 15 pounds pressure for 15 minutes, the medium is cooled to 45°C. and the penicillin added just before the plates are poured. It is conven-

ient to prepare a sterile distilled water dilution of the penicillin containing 100 Oxford units per ml. and then add the proper amount of this dilution to the medium. The plates should be incubated at 37°C. for 48 - 72 hours.

Plates should be poured with a dilution of the sample that will result in not less than 10 and not more than 100 colonies per plate. When more than 100 colonies grow on a plate, they are extremely small and atypical. All colonies of enterococci have a blue center with a white edge and may have any one of the four characteristic shapes (Figure 2) -- (a) fusiform deep colony, (b) triangular fusiform deep colony, (c) entire circular surface colony or (d) filmy diffuse colony lying between the agar and the glass of the dish.

Since the colonies formed are usually small and deep in the agar, it is suggested that the colony be removed with one stroke of a needle and transferred to a tube of broth containing 0.5 per cent dextrose, 0.5 per cent tryptone and 0.5 per cent yeast extract adjusted to pH 7.4. After 24 hours incubation at 37°C., a sediment should appear in the broth. A Gram stain is made from the broth culture. If a typical sediment is found in the broth, and if large, Gram-positive, ovoid cocci in chains of two to seven cells are found microscopically, the presence of characteristic streptococci is confirmed.

For complete identification, inoculations may be made from the broth culture into (a) a sterile tube of tryptone-glucose-yeast extract broth, incubated at 45°C., (b) a sim-

ilar tube of broth to be incubated at 10°C., and (c) a third tube of the same broth containing 6.5 per cent NaCl for incubation at 37°C. After these inoculations have been made, 5-10 ml. of 3 per cent H_2O_2 is added to the original broth culture to determine the production of catalase. Typical enterococci should grow at 45°C., at 10°C., in the presence of 6.5 per cent NaCl and be catalase negative.

In routine work, it is felt that the complete identification is not necessary but that the presence of colonies on the selective plating medium and the characteristic findings of the Gram stain are sufficient.

B. Enrichment and Confirmation Method

The recommended presumptive enrichment medium for the isolation of the enterococci has the following composition:

Dextrose	5	grams
Tryptone (Bacto)	5	grams
Yeast extract (Bacto)	5	grams
Sodium azide	0.4	grams
Brom thymol blue	0.032	grams
Water	1	liter

For convenience, it is recommended that a 1 per cent aqueous solution of sodium azide be prepared and then 40 ml. of this used per liter of medium. For the same reason a 1.6 per cent alcohol solution of brom thymol blue may be prepared and 2 ml. of this used in each liter of medium.

The medium is adjusted with sodium hydroxide to a

pH 8.5, dispensed in 8 ml. aliquots and autoclaved at 15 pounds pressure for 15 minutes.

The concentrated presumptive enrichment medium is prepared by using the same ingredients as those of the normal strength medium, but the concentration of each is increased five fold. The pH is adjusted in the same manner and is dispensed in 18-20 mm. tubes in 2 ml. aliquots and autoclaved at 15 pounds pressure for 15 minutes.

To determine only the presence or absence of the enterococci, it is recommended that one tube of the concentrated medium be inoculated with 10 ml. of the sample and that tubes of the normal strength medium be inoculated with 1 ml. and 0.1 ml. amounts. If a quantitative determination of the enterococci is desired, it is recommended that a series of three to five tubes be inoculated with each of the three decimal dilutions and that the dilutions be made to a degree where the last series will yield negative results. These tubes are then incubated in a water bath at 45°C. and observed periodically after 8 hours for the production of acid as shown by the indicator and for growth as indicated by turbidity. The production of acid and turbidity is interpreted as a positive presumptive test. All positive presumptives should be further confirmed.

The confirmation procedure requires the slant-broth preparation previously described. The slants are prepared as follows:

Dextrose	5	grams
Tryptone (Bacto)	5	grams
Yeast extract (Bacto)	5	grams
Sodium azide	0.4	grams
Methylene blue	0.01	grams
Agar	15	grams
Water	1	liter

The sodium azide is added in the same manner as previously described and the methylene blue is added by using 1 ml. of a 1 per cent aqueous solution. The medium is adjusted with sodium hydroxide to pH 8.5, tubed in 6 ml. aliquots, autoclaved at 15 pounds pressure for 15 minutes and slanted with long surface slopes.

The broth has the following composition:

Dextrose	5	grams
Tryptone (Bacto)	5	grams
Yeast extract (Bacto)	5	grams
Sodium azide	0.4	grams
Methylene blue	0.01	grams
NaCl	65	grams
Penicillin	650	Oxford units
Water	1	liter

All ingredients except the penicillin are put into solution, adjusted with sodium hydroxide to pH 8.5 and autoclaved in flasks in measured amounts at 15 pounds pressure for 15 minutes. After cooling to room temperature, the proper amount of a sterile dilution of penicillin is added. Enough of

this broth is then added aseptically to each slant to cover approximately one half of the gel surface when the tube is vertical.

One loopfull of the positive presumptive broth should be transferred to the broth and one loopfull to the slant of the slant-broth preparation. These tubes are then incubated at 37°C. and observed after 12-24 hours for pin-point colonies on the slant surface and for growth sediment in the broth (Figure 2). A Gram stain is made from the broth and, if desired, 5 ml. of 3 per cent H_2O_2 may be added to the slant-broth preparation for a catalase test. Pin-point colonies on the slant, a growth sediment in the broth, large Gram-positive ovoid streptococci in chains of 2-5 cells, and a negative catalase test is interpreted as confirmatory evidence for the presence of enterococci.

If a quantitative estimate is sought and a series of each dilution has been inoculated, the Most Probable Number may be obtained from Hoskins' Tables (1934).

Sample

Inoculate enrichment medium

Incubate 45°C. for 8-24 hrs.

Acid and turbidity

POSITIVE PRESUMPTIVE TEST

No acidity or turbidity

NEGATIVE TEST

Inoculate confirmation slant-broth

Pinpoint colonies

Sediment growth

Gram-positive streptococci

Catalase negative

POSITIVE CONFIRMED TEST

ENTEROCOCCI PRESENT

No growth or colonies

ENTEROCOCCI ABSENT

At first difficulty may be encountered in interpreting the production of acid in the 10 ml. inoculum concentrated presumptive tube but, if it is observed by reflected light rather than by transmitted light, little difficulty will be encountered in clearly differentiating positive and negative tubes.

When the sample is of such material as crabmeat, oysters or frozen foods, weighed samples are ground with phosphate buffer solution (see section on Materials) in sterile Waring Blendors for 1 minute. This mixture is used as the source of inoculum. The test material sometimes interferes with the interpretation of the presumptive test. It is recommended that the presumptive tubes be inoculated as stated and after 18-24 hours of incubation at 45°C., and without attempting to differentiate between positive and negative tubes, the confirmation slant-broth preparation be inoculated from each presumptive tube to determine the presence or absence of enterococci.

MATERIALS AND METHODS

All of the tests for the presence of enterococci were carried out by either the direct plating method or the enrichment presumptive method. Colonies from plates were confirmed by determining cultural characteristics. The positive presumptive tubes of the second method were confirmed by using the slant-broth tubes. All materials and procedure for preparation of media were the same as those recommended in the previous section. During the early part of this study, these media were prepared from the individual ingredients but later dehydrated media obtained through the courtesy of Difco Laboratories was used. The dehydrated media are now available on an experimental basis.

The materials and methods used in the coliform bacteria tests were those recommended in the Standard Methods for the Examination of Water and Sewage (1936), i.e., lactose broth fermentation tubes for the presumptive test, confirmed by colony characteristics on Levine's eosin methylene blue agar. Double strength lactose broth was used when the inoculum was greater than 2 ml. For confirmation, the two lactose broth tubes having the highest dilution of inoculum and showing gas were streaked on individual eosin methylene blue agar plates and observed for colony types after incubation at 37°C. for 24 hours. In some of the early studies, in addition to the Standard Methods procedure, poured plates of

the abdomen was opened and the large intestine removed. The intestinal content was then ejected into the sample bottle. The specimens from birds were collected as fresh droppings from under the pens. Ten grams of the fecal specimen was then shaken with 90 ml. of buffer solution in a bottle with sterile beads. This emulsion was then diluted by decimal amounts and inoculated into the proper media. The fish fecal specimens were obtained by aseptically opening the abdomen, removing the gut and grinding the whole gut in the Waring Blendor with 90 ml. of buffer solution.

Soil samples. The soil samples were collected aseptically in sterile, 8 oz., glass stoppered, wide mouth bottles. Ten gms. of the sample was then emulsified by shaking with 90 ml. of buffer solution. Decimal dilutions were made and inoculated into proper media.

Sewage and Water samples. All sewage and water samples were collected in sterile, 8 oz., glass stoppered, wide mouth bottles, diluted according to Standard Methods and then cultured as described above.

Sludge and Mud samples. The sewage sludge and mud samples were collected and prepared for inoculation in the same manner as soil samples.

Frozen food and Crabmeat samples. The frozen food packages were opened in the laboratory. The crabmeat was collected in sterile, 8 oz., glass stoppered, wide mouth bottles. Ten gms. of either type of sample was then ground in the Waring Blendor with 90 ml. of buffer solution and

decimal dilutions of this used as the inoculum.

Oyster samples. The standard procedure of oyster examination was used. One hundred gm. of drained oyster meat was ground in the Waring Blendor with 100 ml. of buffer solution. Two ml. of this was used as the first inoculum to obtain 1 gm. of oyster. Twenty ml. of the blendor mix is then placed in 80 ml. of buffer solution to obtain a 1:10 dilution. From here, regular decimal dilutions were made to obtain the desired inoculum.

Mouth washings as samples. For the determination of the presence of the enterococci in the buccal cavity, 10 ml. of sterile buffer solution was taken from a test tube into the mouth. After swishing in the mouth, the solution was put into a concentrated enterococci presumptive tube and sufficient sterile buffer added to bring the volume to 10 ml. This tube was then incubated, observed and confirmed in the usual manner.

Tolerance of enterococci to skatole and indole. One gram of the skatole or indole was dissolved in 10 ml. of 95 per cent ethyl alcohol. Distilled water was then added to bring the volume to 100 ml. to produce a 1:100 dilution. Proper amounts of this dilution were then added to sterile 2 per cent peptone water that had been adjusted to pH 7.2. The various dilutions of skatole or indole were inoculated from broth cultures, 24 hours old, by means of a "standard" 4 mm. loop. Incubation was carried out at 37°C. and the tubes observed at 24, 48 and 96 hours for the presence or

absence of turbidity to determine whether the skatole or indole had exerted a bacteriostatic effect.

RESULTS

A. The Coliform Bacteria and Enterococci Content of Feces.

A study of the coliform bacteria and the enterococci was made on 63 fecal specimens from 9 humans, 18 domestic animals, 12 wild animals, 4 chickens, 14 wild ducks and gulls, and 6 fish. The domestic animal specimens were obtained from the University of Maryland farm and the Beltsville Research Center farm. The wild animal specimens were taken from animals trapped on the Patuxent Wildlife Refuge, and the ducks and gulls were wild birds that were being kept in pens at the refuge. The fish were from commercial catches. All samples were obtained as freshly as possible and tests were made immediately so that any changes in the flora and moisture content would be kept at a minimum.

The enterococci content of the fecal material from mammals was determined by the direct plating method, whereas the dilution method using the enrichment broth was used in testing bird and fish feces. All results were expressed as the number of bacteria per gram of feces. A summary of the results is given in Table III. With one exception, enterococci and coliform bacteria were present in the intestinal tracts of all warm blooded animals and humans tested. The exception was one of the three specimens from muskrats which contained no enterococci and a relatively few coliform bacteria.

TABLE III

THE COLIFORM BACTERIA AND ENTEROCOCCI CONTENT OF ANIMAL AND HUMAN FECES

Source	No. of Specimens	Average Count Per Gram	
		Coliform bacteria	Enterococci
Human	9	11,000,000	140,000
Cow	5	110,000	5,600
Horse	6	110,000	201,000
Sheep	4	110,000	101,000
Pig	3	11,000,000	1,880,000
Raccoon	3	110,000,000	3,230,000
Skunk	2	11,000,000	2,100,000
Opossum	2	11,000,000	1,100,000
Muskrat	2	9,300,000	870,000
Muskrat	1	120,000	0
Squirrel	2	430,000	1,000,000
*Duck	8	15,950,000	88,700,000
*Gull	6	1,770	633,000
*Chicken	4	9,300,000	2,460,000
*Mullet	2	0	0
*Herring	2	0	0
*Red snapper	2	0	0

* These specimens were tested for enterococci by the enrichment method; all others were done by direct plating. Confirmatory tests were run in both instances.

In most cases the enterococci are outnumbered by the coliform organisms by a ratio varying from 1:10 to 1:100. The average enterococci count is greater than the average coliform bacteria count in the specimens from gull, ducks and squirrel where the enterococci-coliform ratios are 300:1, 6:1 and 2:1 respectively. In the gull, this finding might be expected because of the high acid condition of the digestive system, the enterococci being more resistant to such conditions. The 6:1 ratio of the duck specimens is not significant because three of the eight duck specimens tested contained more coliform than enterococci. The largest number of enterococci encountered was from the duck specimens, being 88,700,000 per gram. The lowest enterococci count encountered was that of cow feces, being 5,600 per gram. In general it was found that the bacterial counts were higher for those animals which void the feces as small dehydrated pellets and lower for those whose excreta are of a loose, moist nature. The intestinal contents of six fish of three different species contained no enterococci or coliform bacteria.

B. Coliform Bacteria and Enterococci Content of Sewage, Water and Soil.

Table IV shows the relative content of enterococci and coliform bacteria obtained from sewage, water and soil. Fecal specimens from 9 humans yielded average counts per gram of 140,000 enterococci and 11,000,000 coliform bacteria with *Escherichia* type predominating in the latter group.

TABLE IV

COLIFORM BACTERIA AND ENTEROCOCCI CONTENT OF SEWAGE, WATER AND SOIL

Source	Enterococci		Coliform	
	M.P.N. per gm.	Method	M.P.N. per gm.	Type#
Human feces (9)	140,000	Plate	11,000,000	Esch.
Raw sewage (18)	8,070	"	568,000	Esch.
Raw sewage July-Sept. (9)	10,500	"	650,000	Esch.
Raw sewage Oct.-Dec. (9)	6,400	"	494,000	Esch.
*Sea water polluted (5)	2,470	"	240	Aero.
**Sea water polluted (25)	17	Enrichment	165	Esch. & Aero.
Sea water non-polluted	0		0	
Sewage sludge #1	450	Plate	74	Esch. & Aero.
Sewage sludge #2	13,000	"	1,330	Esch.
Fresh water polluted (14)	1	Enrichment	11	Esch.
Fresh water non-polluted	0	"	0	
Polluted river water (6)	3	"	24	Aero.
Soil river edge (6)	0.3	"	2.3	Aero.
***Soil cultivated (6)	2.4	"	0	Aero.
Soil virgin (12)	0		0	

* Samples of sea water collected where human feces enters water.
** Samples of sea water collected at least 50 yds. off shore.
*** Samples collected from field fertilized with cow manure 3 mo. previously.
\# Colony type observed on eosin methylene blue agar.
M.P.N. — Most Probable Number.
Esch. — *Escherichia*.
Aero. — *Aerobacter*.

Raw sewage collected from the Greenbelt, Maryland treatment plant, contained an average of 8070 enterococci and 568,000 coliform bacteria per ml. The population of this small community is unusually stable. These samples, collected over a period from July to January, show that during the warmer months both groups of bacteria are more abundant. The average count per ml. for the months of July, August and September was 10,500 enterococci and 650,000 coliform bacteria. The average for the October, November and December period was 6,400 enterococci and 494,000 coliform bacteria.

Two samples of sewage sludge were examined, the first from a large disposal plant in Rochester, New York where the sludge from the Imhoff tanks is drawn off once every three months, and the other from a small plant in Cambridge, Maryland where the sludge from the sedimentation tank is drawn off daily. Both of these sludges were being dried and used for farm fertilizer. The average enterococci and coliform bacteria counts per gram were 450 and 74 for the first sludge, and 13,000 and 1330 for the second sludge.

Of a large number of sea water samples tested, a few typical samples have been selected to demonstrate the ratio between the enterococci and coliform bacteria. At a point where raw, untreated domestic sewage was being dumped into a small cove of the Little Annemessex River of Somerset county, Maryland, at the rate of 150 gallons per day, the enterococci outnumbered the coliform group 10 to 1. The respective average counts per ml. were 2470 and 240. At this point

there was a predominance of the Aerobacter type. Several of the negative lactose presumptive tubes contained a blue-green pigment characteristic of members of the genus Pseudomonas.

The bacteriological results of 25 sea water samples, all of which contained both groups of fecal bacteria, were averaged to obtain the enterococci-coliform bacteria ratio in polluted waters. All of these samples were collected at stations at least 50 yds. from shore. Some of the samples were grossly polluted while others contained minimum numbers of bacteria. The average enterococci counts for these samples was 17 per ml. as compared to the average coliform count of 165 per ml., a ratio of 1 to 10. Both Escherichia and Aerobacter types were found. There were many cases where the waters further off shore gave no evidence of either enterococci or coliform organisms and this was taken as a definite indication of no pollution.

In studies of fresh water, 14 samples which gave evidence of both enterococci and coliform bacteria contained an average of one enterococcus and 11 coliform bacteria per ml., a ratio of 1 to 10. Many fresh waters gave no evidence of either group of bacteria. The Patuxent River, at the Patuxent Wildlife Refuge, Maryland, known to be polluted, contained an average of 3 enterococci and 24 coliform bacteria per ml., a ratio of 1 to 8.

Soil samples collected from the banks of the Patuxent River and from fields that are often flooded by the river contained both groups of bacteria in the usual 1 to 10 ratio.

Several samples of soil taken from a corn field of the Beltsville Research Center, which had been fertilized with cow manure about 3 months previously, yielded enterococci but no coliform bacteria. Twelve samples of soil taken from woods known to have had no cultivation or fertilization for at least 50 years gave no evidence of either groups of fecal bacteria.

C. Survival of Fecal Organisms in Sewage Stored at 5°C.

Samples of sewage were collected periodically from the Greenbelt, Maryland sewage treatment plant and stored in stoppered bottles in the refrigerator at 5°C. Enterococci plate counts, with confirmation, and coliform bacteria counts by the standard method, using eosin methylene blue confirmation, were made at intervals of from one to two weeks. The results are shown in Tables V and VI. The average enterococci count of fresh sewage samples taken over the six months period was 8,070 per ml. as compared to an average coliform bacteria count of 568,000 per ml. Both groups of bacteria were more abundant during the summer months than during the winter. Greenbelt is a community with an unusually stable population. In addition, a minimum of storm water is allowed to enter the sewer system. As a result there are only slight fluctuations in the volume of sewage and the data on the bacterial counts probably represents intrinsic variation in the intestinal flora.

After 3 or 4 weeks of storage at 5°C., the enterococci and coliform bacteria numbers both had dropped to 10 per

TABLE

ENTEROCOCCI PER ML. OF

No. of weeks stored	Date								
	7/16	7/20	7/30	8/6	8/13	8/20	8/27	9/4	9/17
0	9500	2500	9500	17700	12300	13300	9600	8200	14900
1	2200	700	7900	16600	7600	16700	2800		4950
2	1000	2100	1000	3500	3200	2500		1120	7200
3	2000	150	920	2100	1100		660	550	2000
4	300	61	230	450		580	340	398	
5	143	38	27		210	260	307	200	200
6	50	4		93	75	275	90		
7	13		9	19	95	44		28	140
8		2	2	25	10		25		76
9	4	1	24	3		4		13	
10	1	2	1		3		19	5	41
11	3	0		1		2	13		
12	1		2		3	1		3	28
13		0		1	2		9		
14	1		2	0		0		2	10
15		0	3		1		5		
16	0	0		1		1		1	4
17	0		1		0		0		
18		0		0		0		1	0
19	0		0		0		2		
20		0		1		1		1	
21	0		0		0		3		
22		0		0		4			
23	0		0		1				
24		1		0					
25	0		0						
26		0							
27	0								

V

SEWAGE STORED AT 5°C.

collected										No. of weeks stored
9/24	10/2	10/9	10/23	11/6	11/13	12/6	12/24	1/7	1/21	
7170	24200	5000	3000	2950	4500	7000	2300	5600	3100	0
10700	15800			1000						1
800		450	2100		1100	7200	620	350		2
	200		1100	450						3
110		300			100	1200	110			4
	150	10	210	120						5
53	70				71	350				6
20		18	70	61						7
	43				30					8
18		9	49	21						9
	14				21					10
19		2	38	12						11
	5									12
5		3	56							13
	1									14
0		1								15
	0									16
1										17
										18
										19
										20
										21
										22
										23
										24
										25
										26
										27

TABLE

COLIFORM PER ML. OF

No. of weeks stored	Date 7/16	7/20	7/30	8/6	8/13	8/20	8/27	9/4	9/17
0	240M	90M	80M	240M	2400M	1500M	460M	110M	930M
1	200M	160M	24M	240M	1200M	1500M	1100M		46M
2	130M	9300	4300	93M	240M	110000		15M	240M
3	9300	9300	9300	75M	46M		240M	7500	9300
4	9300	4300	9300	46M		9300	2400	4300	
5	4300	73	230		4300	1500	2400	4600	930
6	430	91		2400	110	2400	2400		
7	91		150	24	110	11000		930	930
8		5	4	1	460		14		930
9	9	.3	46	110		93		28	
10	1	0	3		24		14	21	110
11	.4	2		4		21	9		
12	9		2		2	15		9	24
13		2		1	1		0		
14	9		1	.4		9		14	46
15		1	2		4		3		
16	4	1		0		9		14	21
17	9		1		1		4		
18		2		4		14		9	43
19	1		3		2		1		
20		2		0		15		43	
21	4		2		0		3		
22		4		4		21			
23	3		4		4				
24		1		1					
25	4		1						
26		0							
27	1								

M = Roman numeral representing 1000

VI

SEWAGE STORED AT 5°C.

collected										No. of weeks stored
9/24	10/2	10/9	10/23	11/6	11/13	12/6	12/24	1/7	1/21	
39M	1500M	230M	240M	930M	460M	750M	46M	150M	140M	0
240M	930M			240M						1
4300		21M	15M		240M	460M	24M	93M		2
	9300		4300	46M						3
930		930			15M	11M	930			4
	430	900	4300	15M						5
150	930				4600	46M				6
75		460	430	9300						7
	93				4600					8
110		150	930	2400						9
	14				1100					10
21		110	930	1100						11
	46									12
75		93	240							13
	46									14
46		24								15
	24									16
93										17
										18
										19
										20
										21
										22
										23
										24
										25
										26
										27

cent of the original numbers. The average time required for a 10 per cent survival was 3½ weeks for both organisms.

The enterococci count decreased to 5 per ml. or less within 10 weeks on the average; the coliform count decreased to 10 per ml. or less in 9½ weeks. Throughout the 9½ - 10 week period, the reduction in numbers of each of the organisms corresponds rather closely. However, beyond this point, the enterococci seem to disappear almost entirely by the 15th. week, whereas the coliform group survive in small numbers for the entire period of storage (27 weeks) (see Table VI).

D. Tolerance of Enterococci to Skatole and Indole.

The tolerance of the enterococci to the intestinal protein putrefactive substances, indole and skatole, was determined by observing the ability of the organism to grow in varying concentrations of these substances. Sixteen cultures of enterococci were used in all, four of each of the four species. A series of three tubes of each concentration were inoculated with each culture. The results obtained with the various species in indole are given in Table VII. All grew in a concentration of 1:3500 and all except one strain each of *Streptococcus fecalis* and *Streptococcus durans* grew in 1:3000. The data further show that 12 of the 16 strains grew very well in the 1:2500 concentration while only two strains grew in the 1:2000.

Table VIII gives the results obtained using varying concentrations of skatole. All four strains of *Streptococcus fecalis* were able to grow in a 1:3000 concentration. Most

TABLE VII

TOLERANCE OF ENTEROCOCCI TO INDOLE

Species	Strain	Concentration of Indole Number of tubes with growth						
		1/1000	1/1500	1/2000	1/2500	1/3000	1/3500	1/4000
S. fecalis	1	0	0	0	3	3	3	3
" "	2	0	0	0	3	3	3	3
" "	3	0	0	0	3	3	3	3
" "	4	0	0	0	1	0	3	3
S. durans	1	0	0	0	3	3	3	3
" "	2	0	0	1	3	3	3	3
" "	3	0	0	0	0	0	3	3
" "	4	0	0	0	1	3	3	3
S. liquefaciens	1	0	0	3	3	3	3	3
" "	2	0	0	0	3	3	3	3
" "	3	0	0	0	3	3	3	3
" "	4	0	0	0	0	3	3	3
S. zymogenes	1	0	0	0	3	3	3	3
" "	2	0	0	0	3	3	3	3
" "	3	0	0	0	3	3	3	3
" "	4	0	0	0	3	3	3	3

TABLE VIII

TOLERANCE OF ENTEROCOCCI TO SKATOLE

Species	Strain	Concentration of Skatole Number of tubes with growth						
		1/1000	1/1500	1/2000	1/2500	1/3000	1/3500	1/4000
S. fecalis	1	0	0	0	0	3	3	3
" "	2	0	0	0	0	3	3	3
" "	3	0	0	0	1	3	3	3
" "	4	0	0	0	0	3	3	3
S. durans	1	0	0	0	1	3	3	3
" "	2	0	0	0	3	3	3	3
" "	3	0	0	0	3	3	3	3
" "	4	0	0	0	3	3	3	3
S. liquefaciens	1	0	0	0	1	3	3	3
" "	2	0	0	0	2	3	3	3
" "	3	0	0	1	3	3	3	3
" "	4	0	0	3	3	3	3	3
S. zymogenes	1	0	0	0	3	3	3	3
" "	2	0	0	2	3	3	3	3
" "	3	0	0	3	3	3	3	3
" "	4	0	0	3	3	3	3	3

strains of the other species grew well in a 1:2500 concentration. Three of the four Streptococcus zymogenes strains were able to grow in a 1:2000 concentration.

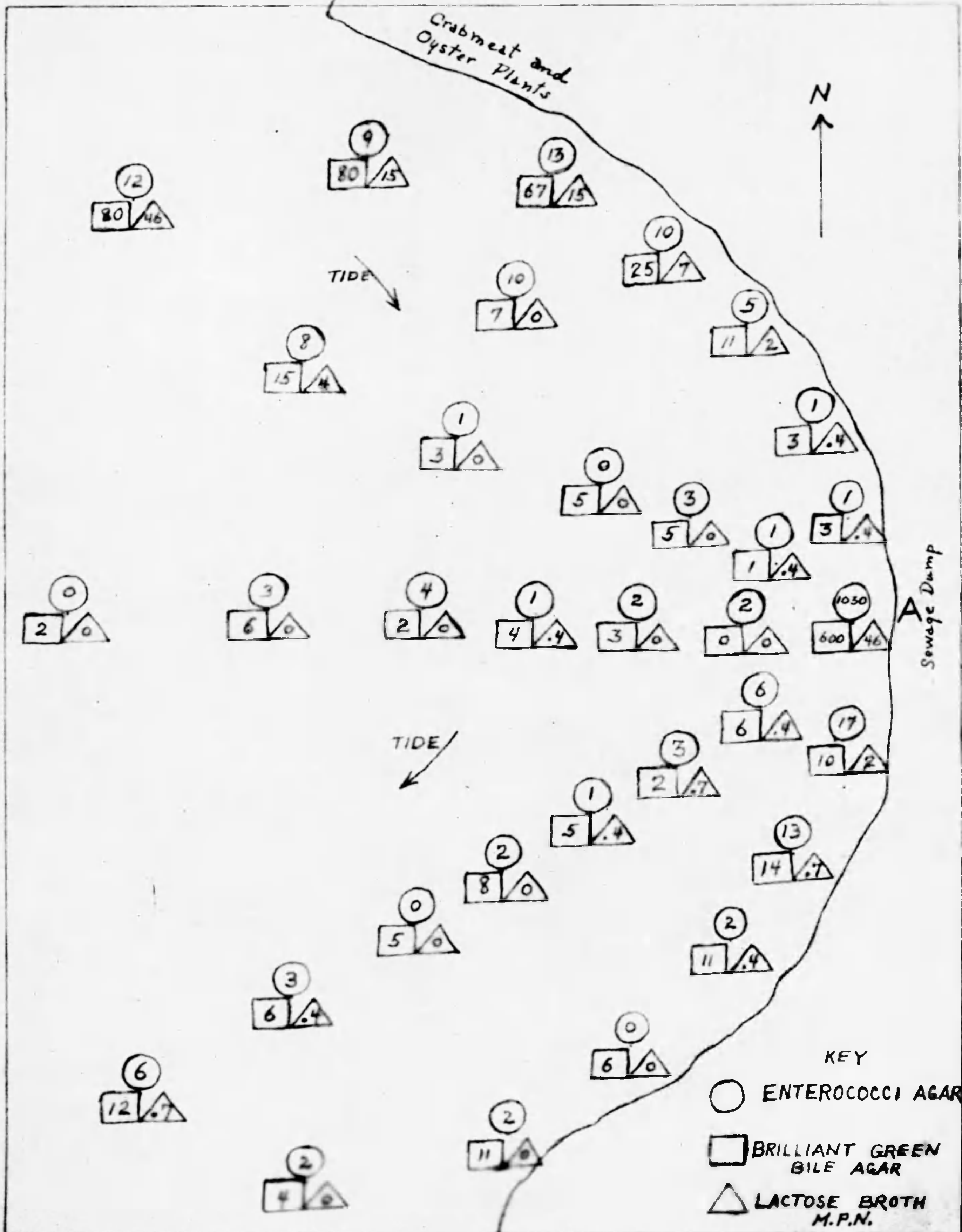
E. Enterococci-Coliform Bacteria Survey of a Polluted Cove in Chesapeake Bay.

The results of a survey to determine the enterococci and coliform content of the waters of a known polluted sea water cove are given in Figure 3. At each sampling station three values are given representing, (1) the enterococci plate count, (2) the coliform bacteria presumptive tube Most Probable Number and (3) the brilliant green lactose bile agar plate count. At point A, raw human excrement was being dumped from a wagon at the rate of 150 gallons of drained fecal material per day. An abundance of algae was growing in the water at this point. The temperature of the water was 10°C. and the tide was flowing to the south.

Figure 3 reveals several interesting facts. The coliform bacteria counts are extremely low, even at the point where raw sewage is introduced. At this point the Most Probable Number of coliform bacteria was 46 per ml. while 100 feet from this point it had diminished to an average Most Probable Number of less than 1 per ml. In contrast, the enterococci appeared in greater numbers at nearly every station, being 20 times more abundant at the point of pollution and approximately 10 times more abundant at most other stations. At point A, the enterococci count was 1030 per ml. Both counts were relatively large near the crab and oyster

FIGURE 3

BACTERIAL SURVEY OF CHESAPEAKE BAY COVE



houses at the north end of the cove, indicating a second source of pollution which was verified by sanitary survey.

The brilliant green lactose bile agar plate counts were conspicuously high, in most cases being larger than the combined count of the other two tests. Possible reasons for this were the failure of the coliform bacteria to produce gas in lactose broth and the ability of the enterococci to produce typical _Escherichia_-_Aerobacter_-like colonies on brilliant green lactose bile agar. Because of the latter possibility, six plates of B.G.L. B. agar were poured using as an inoculum in each, a 1 ml. aliquot taken from a tube of enterococci presumptive medium known to contain enterococci. Coliform bacteria type were isolated and streaked on the enterococci plating medium. These cultures were then confirmed. The results are shown in Table IX. Six colonies were selected from each B.G.L.B. agar plate. A total of 26 of the 36 isolates grew on the enterococci medium, all of which were confirmed as typical enterococci.

F. Bacteriological Survey of Little Annemessex River.

Twelve sampling stations were set up in the Little Annemessex River at Crisfield, Maryland. These stations are shown on Figure 4. The point marked as station No. 1 was originally the end of the river but a channel six feet deep and approximately 25 ft. wide has been dug from this point to the Great Annemessex River. The tides now flow through this channel producing a flushing action that did not exist before. The stations are in mid-channel, approximately one-

TABLE IX

GROWTH OF ENTEROCOCCI ON BRILLIANT GREEN LACTOSE BILE AGAR*

Colonies on B.G.L.B. agar	Colonies Isolated	Isolates growing on Enterococci Medium	Confirmed as Enterococci
840	6	6	6
6	6	2	2
6	6	0	0
120	6	6	6
19	6	6	6
65	6	6	6
Total	36	26	26

* Formula in Appendix A.

FIGURE [illegible]

LOCATION OF SAMPLING STATIONS ON THE LITTLE ANNEMESSEX RIVER

half mile apart. Station No. 12 is at the mouth of the river as it enters Chesapeake Bay.

One sample of the surface water and one sample of the water from the bottom, taken with the bottom sampler described in Appendix B, were collected at each station. This was repeated four times at intervals of 3 hours so that one complete set of samples was taken at mean flood tide, flood tide, mean ebb tide and ebb tide. The presence of enterococci was determined by the enrichment-confirmation method and the presence of coliform bacteria by the usual methods.

The bacteriological results obtained under these four tide conditions are given in Table X. The data show a marked shift of the fecal bacteria with the tides. This is especially true of the surface samples. As the tide comes in, the enterococci and coliform bacteria are carried back into the river and in turn are carried out with the tide. The bacterial content of the bottom water shifts more slowly than that of the surface. Observations of Table X will show that the bottom water samples taken from stations 5, 6, and 7, which are directly off-shore from the industrial section of the city, show the presence of enterococci and coliform bacteria under most of the tide conditions. This is particularly true of the enterococci. A sanitary survey has shown many sources of pollution in this section of the water front. It will also be noted that the shift of pollution, as indicated by the surface waters, is from stations 2, 3, and 4 to stations 9 and 10. The region of constant fecal

TABLE X

BACTERIOLOGICAL SURVEY OF LITTLE ANNEMESSEX RIVER

Station	Surface Water			Bottom Water		
	Enterococci	Coliform		Enterococci	Coliform	
	presence	presence	type*	presence	presence	type*
	Mean Flood Tide, Oct. 24, 1946, 7-9 A.M.					
1	-	P	A	-	P	A
2	-	-		-	P	A
3	P	P	E & A	-	P	A
4	P	P	E & A	P	P	E
5	P	P	E & A	P	P	A
6	-	P	E	P	P	E
7	P	P	E	-	-	
8	-	-		P	P	E & A
9	-	-		-	P	A
10	-	-		-	-	
11	-	-		-	-	
12	-	-		-	-	
	Flood Tide, Oct. 24, 1946, 10-12 A. M.					
1	-	P	A	P	P	E
2	P	P	E	P	P	A
3	P	P	E	-	-	
4	P	-		P	P	E
5	-	-		P	P	A
6	-	-		P	P	E
7	-	-		P	-	
8	-	-		-	-	
9	-	-		-	-	
10	-	-		-	-	
11	-	-		-	-	
12	-	-		-	-	
	Mean Ebb Tide, Oct. 24, 1946, 2-3 P.M.					
1	-	-		P	P	E
2	-	-		P	P	E
3	-	-		-	P	E
4	-	-		P	-	
5	P	P	E	P	P	A
6	P	P	E	P	P	E & A
7	-	P	A	P	P	E
8	P	-		P	-	
9	-	-		-	-	
10	-	-		-	-	
11	-	-		-	-	
12	-	-		-	-	

TABLE X (continued)

BACTERIOLOGICAL SURVEY OF LITTLE ANNEMESSEX RIVER

Station	Surface Water			Bottom Water		
	Enterococci	Coliform		Enterococci	Coliform	
	presence	presence	type*	presence	presence	type*
	Ebb Tide, Oct. 24, 1947, 5-7 P.M.					
1	-	-		-	P	A
2	P	P	E & A	-	P	A
3	-	P	E	-	P	E
4	-	-		-	-	
5	-	-		-	P	A
6	-	-		-	-	
7	-	-		P	P	A
8	P	-		P	P	E & A
9	P	P	E & A	P	-	
10	P	P	E & A	P	P	E
11	-	-		-	-	
12	-	-		-	-	

* Colony type on eosin methylene blue agar
\- Organism not present
P Organism present
E *Escherichia*
A *Aerobacter*

flora in the bottom waters lays between these two extremes.

G. Bacteriological Survey of Fresh Waters of Chatauqua County, N. Y.

An enterococci and coliform bacteria analysis was made of the waters of Lake Erie and four streams in Chatauqua County, N. Y. A map of the sampling stations is given in Figure 5. Most Probable Numbers per ml. of enterococci and coliform bacteria were determined. Confirmation of each was carried out in the usual manner. The bacteriological results are given in Table XI.

Practically all of these waters contained both enterococci and coliform bacteria. Although these streams flow through country where very few human dwellings are found, the bacteriological results were confirmed when a sanitary survey showed that one stream passed through a pig farm and the other three through cattle pasture lands where fecal material was much in evidence. It is of interest that of the three reservoirs, each fed by one of these streams and used as storage reservoirs for the Westfield, N. Y. water system, two (samples 16 and 22) were free of fecal organisms while the third (sample 24) contained an *Aerobacter* type of coliform and no enterococci. More vegetation was observed in the last reservoir.

H. An Analysis of the Occurrence of Enterococci With *Escherichia* and *Aerobacter* in Various Water Samples.

The presence of the enterococci and the *Escherichia-Aerobacter* group have been determined in 226 samples of

FIGURE 5

DIAGRAMATIC MAP OF FRESH WATER SAMPLING
STATIONS OF CHATAUQUA COUNTY, N. Y.

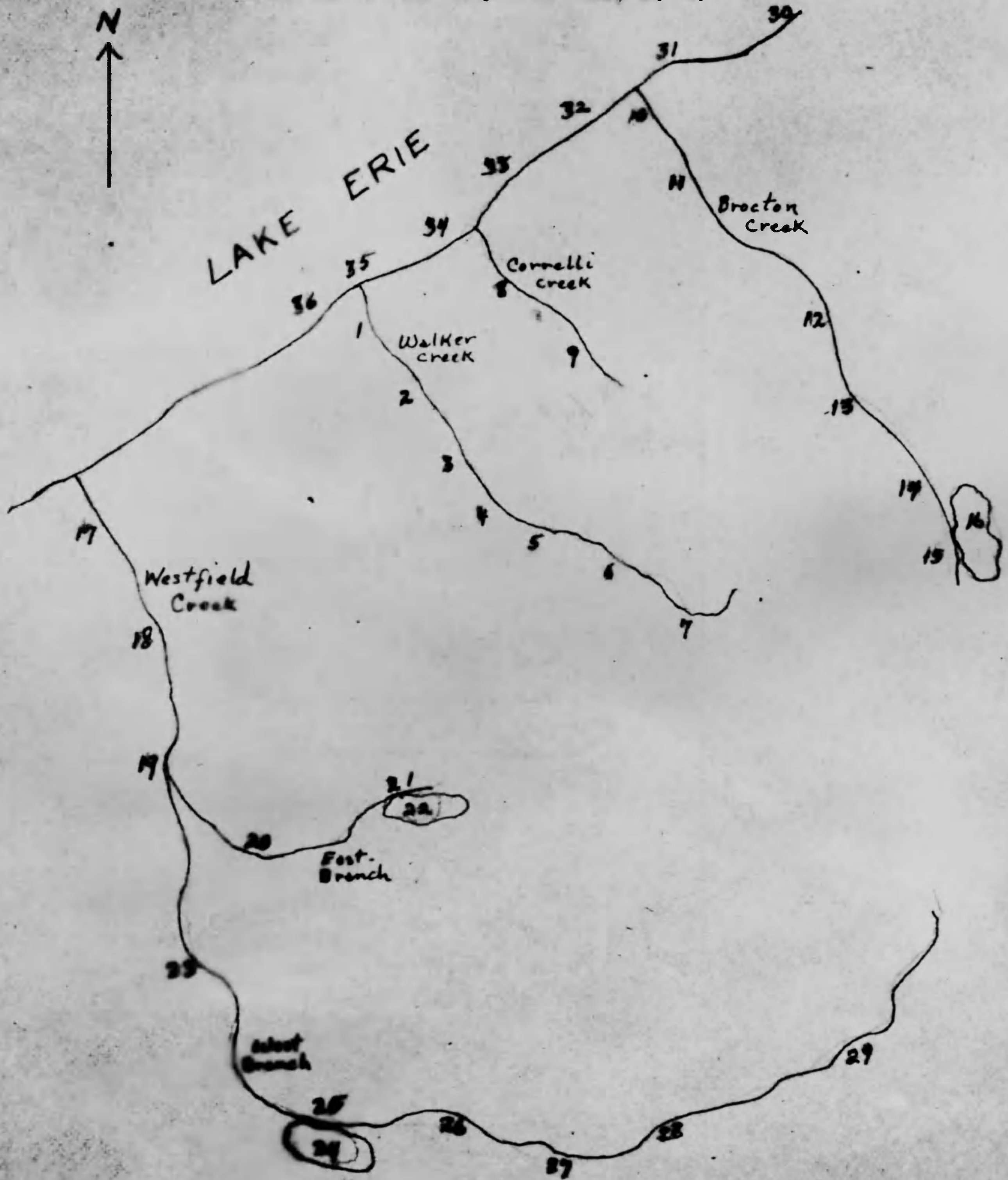


TABLE XI

BACTERIOLOGICAL SURVEY OF FRESH WATERS OF CHATAUQUA COUNTY, N. Y.

Sample No.	Source	Coliform M.P.N./ml.	Coliform Type*	Enterococci M.P.N./ml.
1	Walker Creek	11	Esch	2.4
2	Walker Creek	11	Esch	2.4
3	Walker Creek	2.4	Esch	0.2
4	Walker Creek	4.6	Esch	2.4
5	Walker Creek	11	Esch	2.4
6	Walker Creek	11	Aero	2.4
7	Walker Creek	2.4	Aero	0
8	Correlli Creek	11	Esch	2.4
9	Correlli Creek	0.9	Esch	0
10	Brocton Creek	11	Esch	2.4
11	Brocton Creek	2.4	Aero	0
12	Brocton Creek	11	Aero	0
13	Brocton Creek	11	Esch	2.4
14	Brocton Creek	4.6	Esch	0.2
15	Brocton Creek	4.6	Aero	2.4
16	Brocton Reservoir	0		0
17	Westfield Creek	11	Aero	2.4
18	Westfield Creek	11	Esch	2.4
19	Westfield Creek	1.5	Esch	2.4
20	E. Westfield Creek	11	Esch	2.4
21	E. Westfield Creek	11	Esch	2.4
22	Reservoir	0	E	0
23	W. Westfield Creek	11	Esch	0.2
24	W. Westfield Creek	11	Aero	0
25	W. Westfield Creek	11	Esch	2.4
26	W. Westfield Creek	11	Aero	0.2
27	W. Westfield Creek	11	Aero	2.4
28	W. Westfield Creek	11	Aero	2.4
29	W. Westfield Creek	11	Aero	2.4
30	Lake Erie	0.7	Esch	0.2
31	Lake Erie	2.4	Esch	2.4
32	Lake Erie	2.4	Esch	2.4
33	Lake Erie	4.6	Aero	0.2
34	Lake Erie	4.6	Esch	0.2
35	Lake Erie	4.6	Aero	2.4
36	Lake Erie	0		0
37	Tap water	0		0
38	Tap water	0		0

* Colony type on eosin methylene blue agar
M.P.N. Most Probable Numbers
Esch *Escherichia*
Aero *Aerobacter*

fresh and sea water from various sources. These waters include those from Section F and G above, as well as another group of miscellaneous waters. An analysis has been made of the results of these tests determining the number of samples in which the enterococci were found together with either member of the coliform group and in turn the number of samples in which either member of the coliform group was found without the enterococci. If both members of the coliform group were present, it was considered that the Escherichia was the most significant and the Aerobacter was therefore disregarded. The results of this analysis is given in Table XII. The enrichment-confirmation test was used for the enterococci and the usual standard method procedure for the coliform organisms.

Forty-seven sea water samples were taken along the shore where the water was usually about 18 inches deep. Eighty-seven other sea water samples were taken at the surface from stations at least 50 feet from shore and 48 were taken from the bottom of the mud from stations at least 50 feet from shore. Forty-four fresh water samples were tested, 38 from the Chatauqua county survey and six from sources near College Park, Md.

Of all samples, 28 per cent contained both Escherichia type of coliform bacteria and enterococci, 20 per cent contained Aerobacter and enterococci, and 28 per cent contained neither of the fecal groups. There was agreement between the enterococci and coliform bacteria in 76 per cent of the

TABLE XII

OCCURRENCE OF ENTEROCOCCI WITH *ESCHERICHIA* AND *AEROBACTER* IN VARIOUS WATERS

Enterococci Occurrence	Coliform Occurrence	Coliform Type*	Sea Water Sample: Shore	Sea Water Sample: 50 ft. off shore Surface	Sea Water Sample: 50 ft. off shore Bottom	Fresh Water Sample	Total	%
Present	Present	Esch	15	16	12	20	63	28
Present	Present	Aero	17	14	4	10	45	20
Absent	Absent		6	32	18	7	63	28
Absent	Present	Esch	2	6	3	2	13	6
Absent	Present	Aero	6	16	7	5	34	15
Present	Absent		1	3	4	0	8	4
Total			47	87	48	44	226	100

* Colony type on eosin methylene blue agar.

In the 26 per cent where the two tests did not agree, 7 out of 11 samples, or 15 per cent, showed the presence of Aerobacter members in the absence of enterococci. In 6 per cent of the samples, Escherichia was present alone and in 4 per cent, the enterococci were present alone. These ratios were consistent in the different types of waters.

I. Enterococci and Coliform Bacteria in Frozen Foods, Oysters and Crabmeat.

A quantitative enterococci and coliform bacteria count was made of 32 commercially packed frozen foods, 24 samples of shucked oysters, and 32 samples of fresh crabmeat. The enrichment-confirmation method was used for enterococci and the standard method with eosin methylene blue confirmation was used for the coliform group. The frozen food samples consisted of 5 frozen fruits, 13 frozen vegetables, 1 frozen hamburger, and 13 frozen sea foods. The results obtained are shown in Table XIII.

Nine samples of the frozen foods (raspberry, 2 plum, mixed vegetables, 2 cauliflower, brussels sprouts, cooked soft-shell crab, and cooked shrimp creole) gave negative results for both groups of bacteria. Five samples, all frozen sea foods, gave positive tests for both Escherichia and enterococci, while 3 frozen vegetables yielded both Aerobacter members and enterococci. The hamburger sample contained Escherichia with no enterococci. Three frozen vegetables contained Aerobacter with no enterococci. The most prominent group consisted of 11 samples which contained enterococci and

TABLE XIII

ENTEROCOCCI AND COLIFORM BACTERIA IN FROZEN FOODS

Source	Coliform bacteria		Enterococci
	M.P.N. / gm.	Type*	M.P.N. / gm.
Mixed fruit cocktail	11	Aero.	0
Sliced strawberry	0		0.2
Black raspberry (2)	0		0
Plum (2)	0		0
Broccoli	0		2.4
Mixed vegetables	1.6	Aero.	0
Mixed vegetables	0		0
Succotash	11	Aero.	0
Cut corn (2)	0		0.1
Pea	0.4	Aero.	2.4
Spinach (2)	0.3	Aero.	0.9
French green bean	0		0.1
Cauliflower (2)	0		0
Brussel sprout	0		0
Hamburger	11	Esch.	0
Fried scallop	1.5	Esch.	2.4
Cooked soft shell crab	0		0
Deviled crab	0		2.4
Cooked peeled shrimp	0		0.2
Oyster (2)	0		2.4
Oyster newburg	0		2.4
Scallops (2)	4.6	Esch.	2.4
Clam (quohog) (2)	0.9	Esch.	2.4
Cooked shrimp creole	0		0
Cod fish cake	0		2.4

* Colony type on eosin methylene blue agar.
Esch. - Escherichia
Aero. - Aerobacter
M.P.N. - Most Probable Numbers

no coliform bacteria. All types of frozen food were represented in this last group.

Of the 24 oyster samples, 20 were meat and liquor mixtures and 4 were liquors. The results of these tests are shown in Table XIV.

Six oyster-meat samples gave negative results for the coliform group. Eight produced Most Probable Numbers between 30 and 160 per 50 grams and 6 produced Most Probable Numbers greater than 1100 per 50 grams. _Escherichia-coli_ was the predominant type of coliform found. All samples contained 240 or more enterococci per 50 grams. All of the liquor samples yielded high counts of coliform bacteria as well as enterococci.

Eight of the crabmeat samples, Table XV, contained no coliform bacteria while the remaining twenty-four contained between 31 and 100 per gram. _Escherichia_ and _Aerobacter_ members were found in 13 and 11 samples, respectively. Only two samples gave negative results for enterococci, one of which was also negative for coliform organisms and the other containing 210 _Aerobacter_ per gram. All the remaining 30 samples contained enterococci with Most Probable Numbers between 23 and 240 per gram.

An analysis, similar to that on water samples (section H) of the occurrence of enterococci with _Escherichia_ and _Aerobacter_, is presented in Table XVI. In all, 88 samples of food were tested. Both groups of organisms were present in 56 per cent of the samples and both were absent in 11 per

TABLE XIV

ENTEROCOCCI AND COLIFORM BACTERIA CONTENT OF FRESHLY SHUCKED OYSTERS

Sample No.	Coliform bacteria		Enterococci M.P.N. / 50 gm.
	M.P.N. / 50 gm.	Type*	
1	1100	Esch.	240
2	1100	Aero.	240
3	0		240
4	0		240
5	0		240
6	0		240
7	50	Esch.	240
8	0		240
9	50	Esch.	240
10	1100	Esch.	240
11	1100	Esch.	240
12	120	Esch.	240
13	120	Esch.	240
14	120	Aero.	240
15	120	Esch.	240
16	1100	Esch.	240
17	0		240
18	1100	Aero.	240
19	30	Esch.	240
20	160	Aero.	240
**21	1100	Aero.	240
**22	1100	Aero.	240
**23	1100	Esch.	240
**24	240	Aero.	240

* Colony type on eosin methylene blue agar.
** Oyster liquor only.
Esch. - *Escherichia*
Aero. - *Aerobacter*
M.P.N. - Most Probable Numbers

TABLE XV

ENTEROCOCCI AND COLIFORM BACTERIA CONTENT OF CRABMEAT

Sample No.	Coliform bacteria		Enterococci M.P.N. / gm.
	M.P.N. / gm.	Type*	
1	93	Esch.	240
2	36	Esch.	240
3	460	Aero.	240
4	0		240
5	120	Aero.	240
6	93	Aero.	240
7	240	Esch.	240
8	460	Esch.	240
9	93	Aero.	240
10	93	Aero.	240
11	36	Esch.	240
12	36	Esch.	23
13	31	Aero.	23
14	36	Aero.	240
15	0		240
16	0		240
17	210	Aero.	0
18	240	Esch.	240
19	36	Aero.	240
20	0		240
21	36	Esch.	240
22	36	Esch.	240
23	1100	Esch.	240
24	0		240
25	93	Aero.	23
26	120	Esch.	240
27	0		0
28	93	Esch.	23
29	120	Aero.	240
30	36	Esch.	23
31	0		240
32	0		23

* Colony type on eosin methylene blue agar.
Esch. - Escherichia
Aero. - Aerobacter
M.P.N. - Most Probable Numbers

TABLE XVI

OCCURRENCE OF ENTEROCOCCI WITH *ESCHERICHIA* AND *AEROBACTER* IN SEAFOOD AND FROZEN FOOD

Enterococci Occurrence	Coliform bacteria Occurrence	Type*	Seafood Oyster	Seafood Crabmeat	Frozen Food	Total	%
Present	Present	Esch.	11	13	5	29	33
Present	Present	Aero.	7	10	3	20	23
Absent	Absent		0	1	9	10	11
Absent	Present	Esch.	0	0	1	1	1
Absent	Present	Aero.	0	1	3	4	5
Present	Absent		6	7	11	24	27
		Total	24	32	32	88	100

* Colony type on eosin methylene blue agar.
Esch. - *Escherichia*
Aero. - *Aerobacter*

cent, so that the two tests agree in 67 per cent of the samples. In only one case was Escherichia present when the enterococci were absent and in 4 cases, Aerobacter present and enterococci absent. In 27 per cent of the samples, enterococci were present while no coliform bacteria were detected.

J. Prevalence of Enterococci in the Oral Cavity.

Sterile phosphate buffer solution (See Materials and Methods) mouth washings of 107 individuals were tested by the enrichment-confirmatory method for the presence of enterococci. The washings from only two individuals gave positive results. Fresh samples of these two individuals were recultured again five days later and negative results were obtained.

K. Specificity of Enterococci Enrichment-confirmation Method.

The specificity of the enterococci enrichment-confirmation test was checked by determining the cultural characteristics of 156 cultures which had given confirmed positive tests. The characteristics checked were morphology, Gram stain reaction, growth at 10°C. and 45°C., growth in 6.5 per cent NaCl and catalase production.

The results were: 148 or 95 per cent, gave typical enterococci reactions; 4 or 2½ per cent would not grow at 45°C.; 2 or 1¼ per cent would not grow at 10°C. nor in 6.5 per cent sodium chloride; and 2 or 1¼ per cent, would not grow at 45°C., 10°C., nor in 6.5 per cent sodium chloride. All cultures gave characteristic morphology and Gram reaction,

as well as being catalase negative. Those failing to grow at 45°C. would still be considered as enterococci, therefore the specificity of the test would be 97½ per cent.

L. Bacteriological Survey of Sea Waters of Mt. Hope Bay, R. I.

A bacteriological survey was carried out on the Rhode Island waters of Mt. Hope Bay. The 24 sampling stations selected were distributed as evenly as possible over the area and were marked either by anchored buoys or by drawing lines between prominent land marks on shore. The northern part of this area ends in a closed tide-water bay with several towns along the shore. There are two outlets at the south end -- one into Narragansett Bay and the other into Sakonnet River, both in turn opening into the ocean.

At each station, two samples were collected, one of surface water and the other of bottom water, collected with the bottom sampling device described in Appendix B. Each complete set of samples was collected in as short a period as possible (approximately 2 hrs.) so that the tide condition would not vary between the first and last station any more than necessary. Four such complete sets were collected, two at mean ebb tide, one at full ebb tide and one at mean flood tide. Using the mud dredge described in Appendix B, mud samples were collected at 12 of the stations at the same time that the second and third set of water samples were being taken. All water and mud samples were examined using the enrichment-confirmation method for enterococci and the standard methods for coliform bacteria with confirmation on

eosin methylene blue agar. The results are given in Table XVII.

In order to present the results graphically, a map has been prepared for each of the four sets of data with a square representing each station. The bacteriological findings are indicated by the same method as is used with the water samples.

Figure 6 represents the first set of samples taken at mean ebb tide with a calm sea, an average water temperature of 6°C. and an air temperature of 8°C. The data on stations 2, 7, 8, and 17 indicate the presence of both groups of organisms in surface and bottom waters. Eleven stations, evenly distributed over the area, all showed evidences of pollution. The overall picture indicates that the area was polluted.

Figure 7 represents the second set of samples taken at mean ebb tide with a calm sea, an average water temperature of 7°C. and an air temperature of 12°C. None of the squares are completely black, neither are any completely clear. Enterococci were present at only 6 scattered stations, once in surface water, once in bottom water and 4 times in mud. Coliform bacteria were present in both the surface and bottom water of nearly all stations except the two most southerly in the Sakonnet River. Of the 45 waters containing coliform organisms, only one surface sample contained *Aerobacter* alone. All of the other 44 contained *Escherichia* alone or with *Aerobacter*.

TABLE XVII

ENTEROCOCCI AND COLIFORM BACTERIA IN THE WATERS OF MT. HOPE BAY, R. I.

Mean Ebb Tide

Station No.	Surface water			Bottom water		
	Coliform bacteria		Enterococci	Coliform bacteria		Enterococci
	M.P.N. /100	Type*	M.P.N. /100	M.P.N. /100	Type*	M.P.N. /100
1	150	E.&A.	0	93	E.	0
2	93	E.&A.	23	240	E.&A.	240
3	93	E.&A.	0	240	E.&A.	0
4	0		0	23	E.	0
5	150	E.&A.	240	150	E.	0
6	150	E.&A.	240	460	A.	0
7	75	A.	240	93	E.&A.	9
8	93	E.&A.	240	93	E.&A.	240
9	460	E.&A.	0	93	E.&A.	9
10	93	A.	23	43	E.&A.	0
11	93	A.	0	93	E.&A.	0
12	93	E.&A.	23	93	E.&A.	0
13	240	E.&A.	0	240	E.&A.	0
14	460	E.&A.	240	93	E.&A.	0
15	460	E.&A.	0	240	E.&A.	9
16	1100	E.&A.	0	75	E.&A.	23
17	1000	E.&A.	240	93	E.&A.	23
18	460	E.	0	43	E.&A.	0
19	310	A.	0	240	A.	0
20	43	E.&A.	0	93	E.	240
21	240	E.	23	23	E.	0
22	150	E.	0	43	A.	0
23	0		0	93	E.	0
24	43	E.	23	93	E.	0

* Colony type on eosin methylene blue agar.
E. - Escherichia
A. - Aerobacter
M.P.N. /100 - Most Probable Number per 100 ml.

TABLE XVII (continued)

ENTEROCOCCI AND COLIFORM BACTERIA IN THE WATERS
OF MT. HOPE BAY, R. I.

Mean Ebb Tide

Station No.	Surface water			Bottom water		
	Coliform bacteria		Enterococci	Coliform bacteria		Enterococci
	M.P.N. /100	Type*	M.P.N. /100	M.P.N. /100	Type*	M.P.N. /100
1	23	E.&A.	0	43	E.	0
2	93	E.&A.	0	43	E.&A.	0
3	43	E.&A.	0	43	E.&A.	0
4	460	E.	23	93	E.&A.	0
5	23	E.&A.	0	21	E.&A.	0
6	23	E.&A.	0	23	E.&A.	23
7	23	A.	0	23	E.&A.	0
8	43	E.	0	43	E.&A.	0
9	93	E.&A.	0	23	E.&A.	0
10	43	E.	0	43	E.&A.	0
11	23	E.	0	9	E.&A.	0
12	23	E.&A.	0	23	E.&A.	0
13	23	E.&A.	0	23	E.	0
14	43	E.&A.	0	23	E.&A.	0
15	23	E.&A.	0	23	E.&A.	0
16	43	E.&A.	0	23	E.&A.	0
17	1100	E.&A.	0	290	E.&A.	0
18	75	E.&A.	0	460	E.&A.	0
19	14	E.&A.	0	9	E.&a.	0
20	0		0	9	E.&A.	0
21	23	E.&A.	0	23	E.&A.	0
22	39	E.&A.	0	43	E.&A.	0
23	0		0	9	E.&A.	0
24	0		0	9	E.	0

* Colony type on eosin methylene blue agar.
E. - *Escherichia*
A. = *Aerobacter*
M.P.N. /100 - Most Probable Number per 100 ml.

TABLE XVII (continued)

ENTEROCOCCI AND COLIFORM BACTERIA IN THE WATERS
OF MT. HOPE BAY, R. I.

Ebb Tide

Station No.	Surface water			Bottom water		
	Coliform bacteria		Enterococci	Coliform bacteria		Enterococci
	M.P.N. /100	Type*	M.P.N. /100	M.P.N. /100	Type*	M.P.N. /100
1	43	E.&A.	0	9	E.&A.	0
2	75	E.	0	43	E.&A.	0
3	43	E.&A.	0	23	E.	0
4	43	E.	23	43	E.	0
5	14	E.	9	14	E.&A.	0
6	43	E.&A.	0	0		0
7	23	E.&A.	23	23	E.&A.	0
8	23	E.	23	3	E.&A.	0
9	43	E.&A.	0	23	E.&A.	0
10	93	A.	23	23	A.	0
11	43	E.&A.	0	39	E.	0
12	23	E.&A.	23	23	E.&A.	0
13	43	E.	23	9	E.	0
14	0		0	9	E.	0
15	1100	E.&A.	23	23	E.&A.	23
16	23	E.	240	23	E.&A.	23
17	23	E.&A.	0	43	E.&A.	0
18	23	E.&A.	0	43	E.&A.	0
19	23	A.	23	23	E.&A.	0
20	43	E.&A.	0	23	E.&A.	0
21	93	E.&A.	0	43	A.	0
22	23	E.&A.	23	23	E.	0
23	23	E.&A.	0	9	E.&A.	0
24	0		0	23	E.	0

* Colony type on eosin methylene blue agar.
E. - *Escherichia*
A. - *Aerobacter*
M.P.N. /100 - Most Probable Number per 100 ml.

TABLE XVII (continued)

ENTEROCOCCI AND COLIFORM BACTERIA IN THE WATERS OF MT. HOPE BAY, R. I.

Mean Flood Tide

Station No.	Surface water			Bottom water		
	Coliform bacteria		Enterococci	Coliform bacteria		Enterococci
	M.P.N. /100	Type*	M.P.N. /100	M.P.N. /100	Type*	M.P.N. /100
1	23	E.&A.	0	9	E.	0
2	3	E.	0	9	E.	0
3	20	E.	0	23	E.&A.	23
4	0		0	23	E	0
5	3	E.	0	21	E.&A.	23
6	0		0	0		0
7	0		0	0		0
8	43	E.&A.	0	3	E.	0
9	0		0	14	E.	0
10	0		0	3	A.	0
11	3	E.	0	0		0
12	0		0	0		0
13	3	E.	0	3	E.	0
14	43	A.	0	0		0
15	43	E.&A.	240	43	E.	23
16	29	E.	23	11	E.&A.	23
17	39	E.	0	93	E.&A.	23
18	93	E.	23	150	E.	240
19	150	E.&A.	0	75	A.	0
20	75	A.	0	43	A.	0
21	14	A.	23	150	E.	0
22	0		0	39	E.&A.	0
23	9	E.	0	43	E.	0
24	0		0	0		0

* Colony type on eosin methylene blue agar.
E. - *Escherichia*
A. - *Aerobacter*
M.P.N. /100 - Most Probable Number per 100 ml.

TABLE XVII (continued)

ENTEROCOCCI AND COLIFORM BACTERIA IN THE WATERS
OF MT. HOPE BAY, R. I.

Station .No.	Bottom Mud Samples		
	Coliform bacteria		Enterococci
	M.P.N. /100	Type*	M.P.N. /100
	Mean Ebb Tide		
1	0		0
2	230	A.	0
5	230	A.	0
7	430	E.	0
9	0		0
10	230	E.	240
14	0		240
16	0		0
18	1200	A.	0
19	1200	E.&A.	240
21	11000	E.	240
24	430	A.	0
	Ebb Tide		
1	4600	A.	0
2	0		0
5	0		0
7	0		0
9	0		0
10	1500	E.	23
14	0		23
16	0		0
18	91	E.&A.	0
19	930	E.	0
21	0		0
24	0		240

* Colony type on eosin methylene blue agar.
E. - Escherichia
A. - Aerobacter
M.P.N. /100 - Most Probable Number per 100 gm.

FIGURE 6

BACTERIOLOGICAL SURVEY OF MT. HOPE BAY, R. I.
Mean Ebb Tide

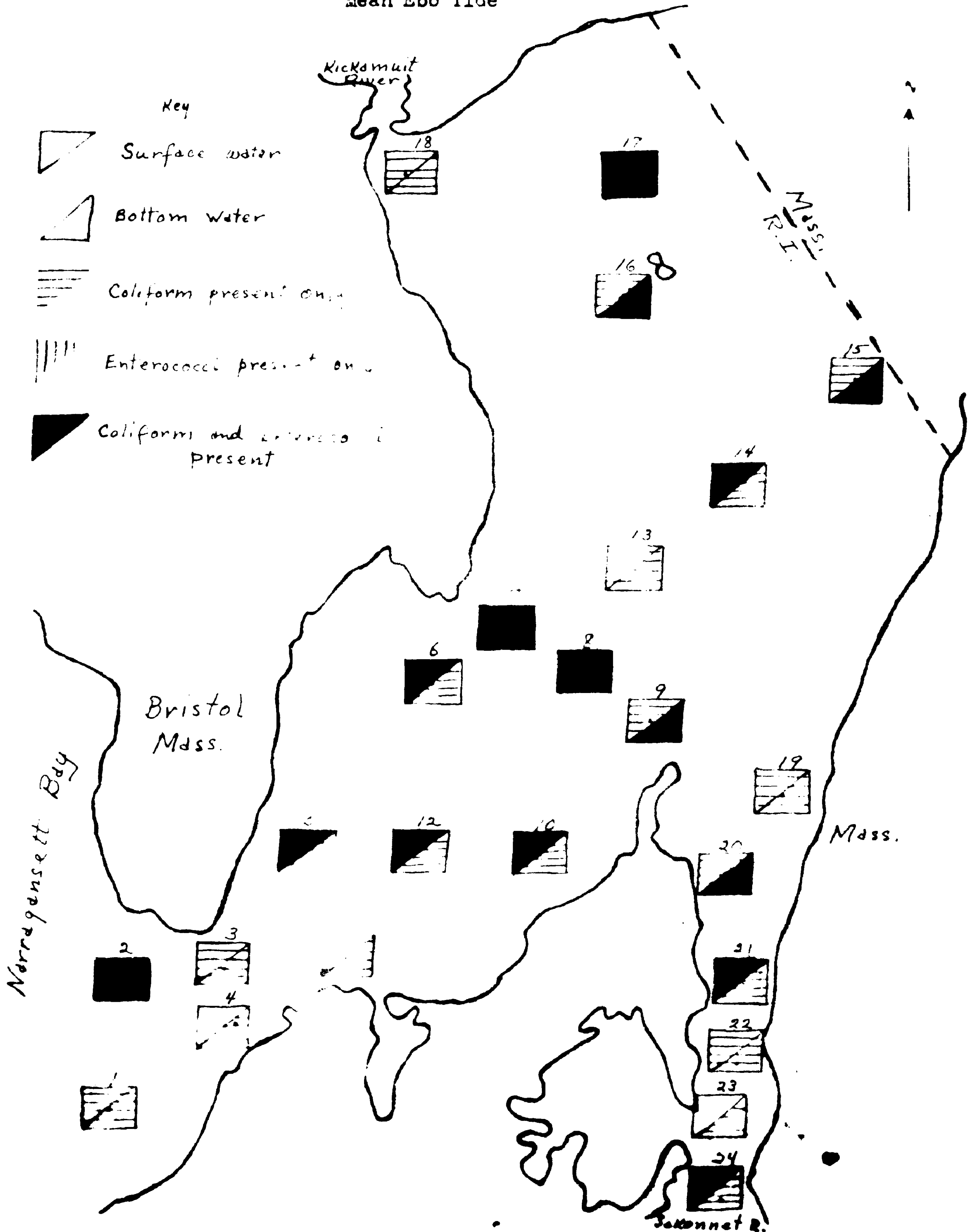


FIGURE 7
BACTERIOLOGICAL SURVEY OF MT. HOPE BAY, R. I.
Mean Ebb Tide

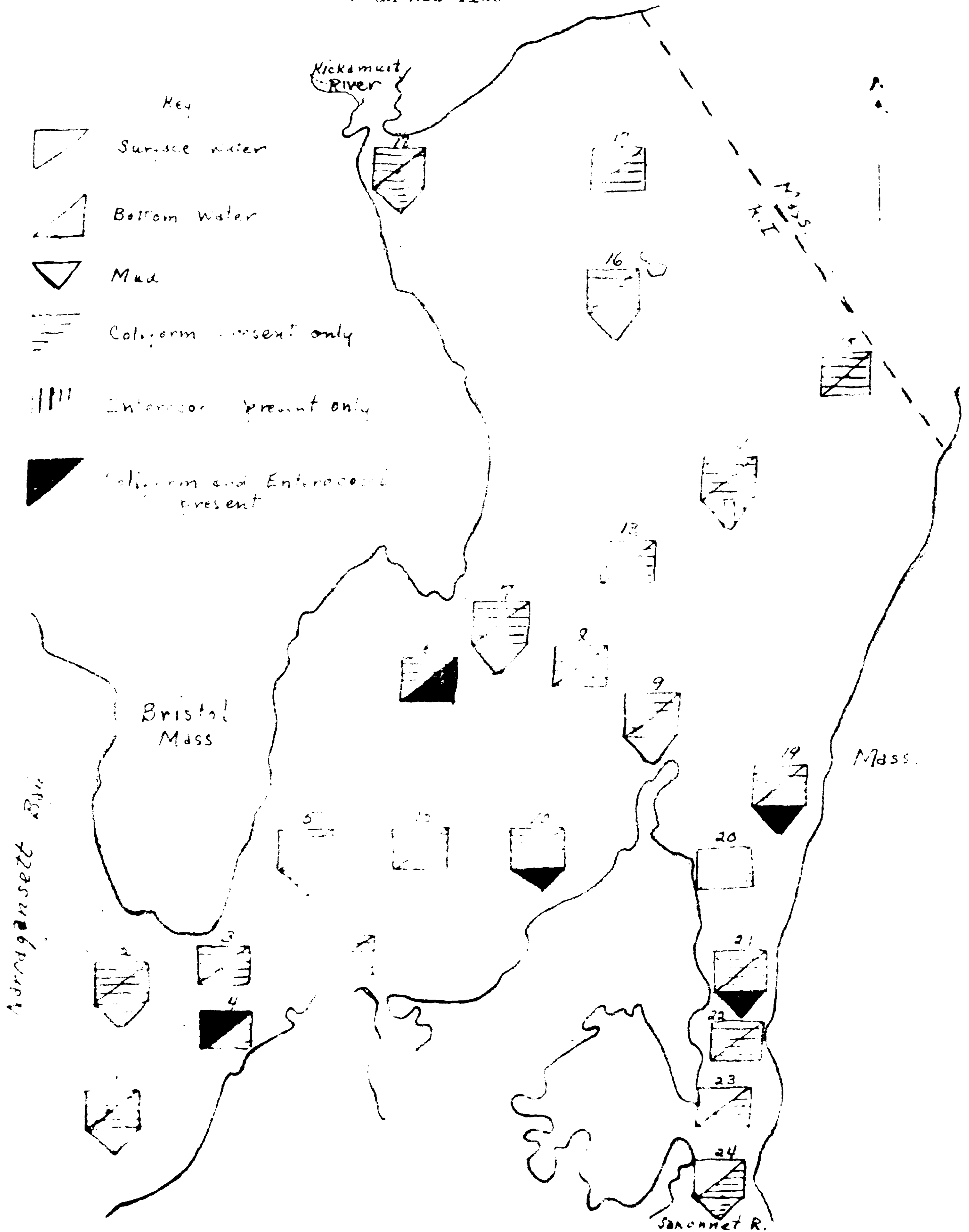


FIGURE 8

BACTERIOLOGICAL SURVEY OF MT. HOPE BAY, R.I.
Ebb Tide

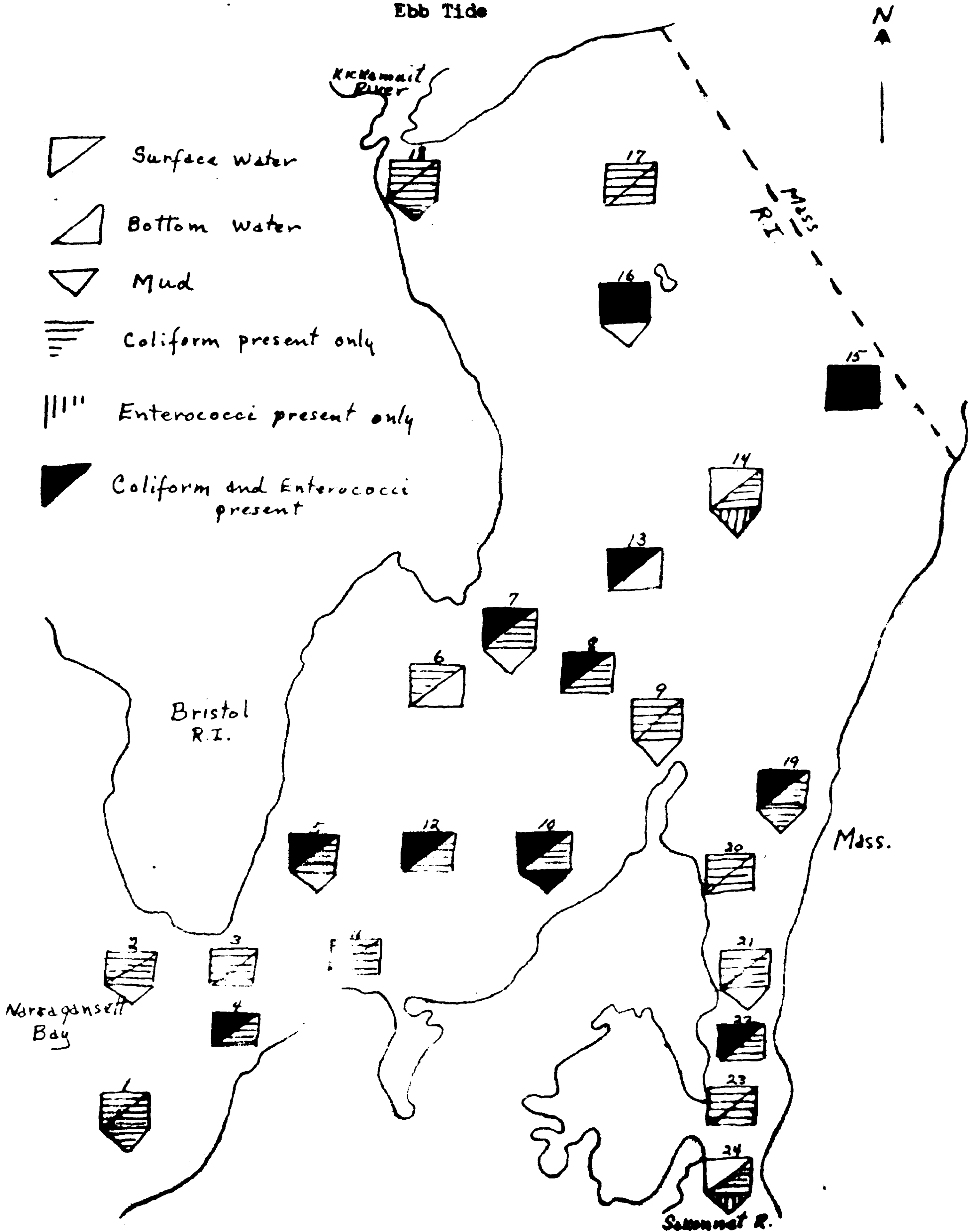


FIGURE 9

BACTERIOLOGICAL SURVEY OF MT. HOPE BAY, R. I.
Mean Flood Tide

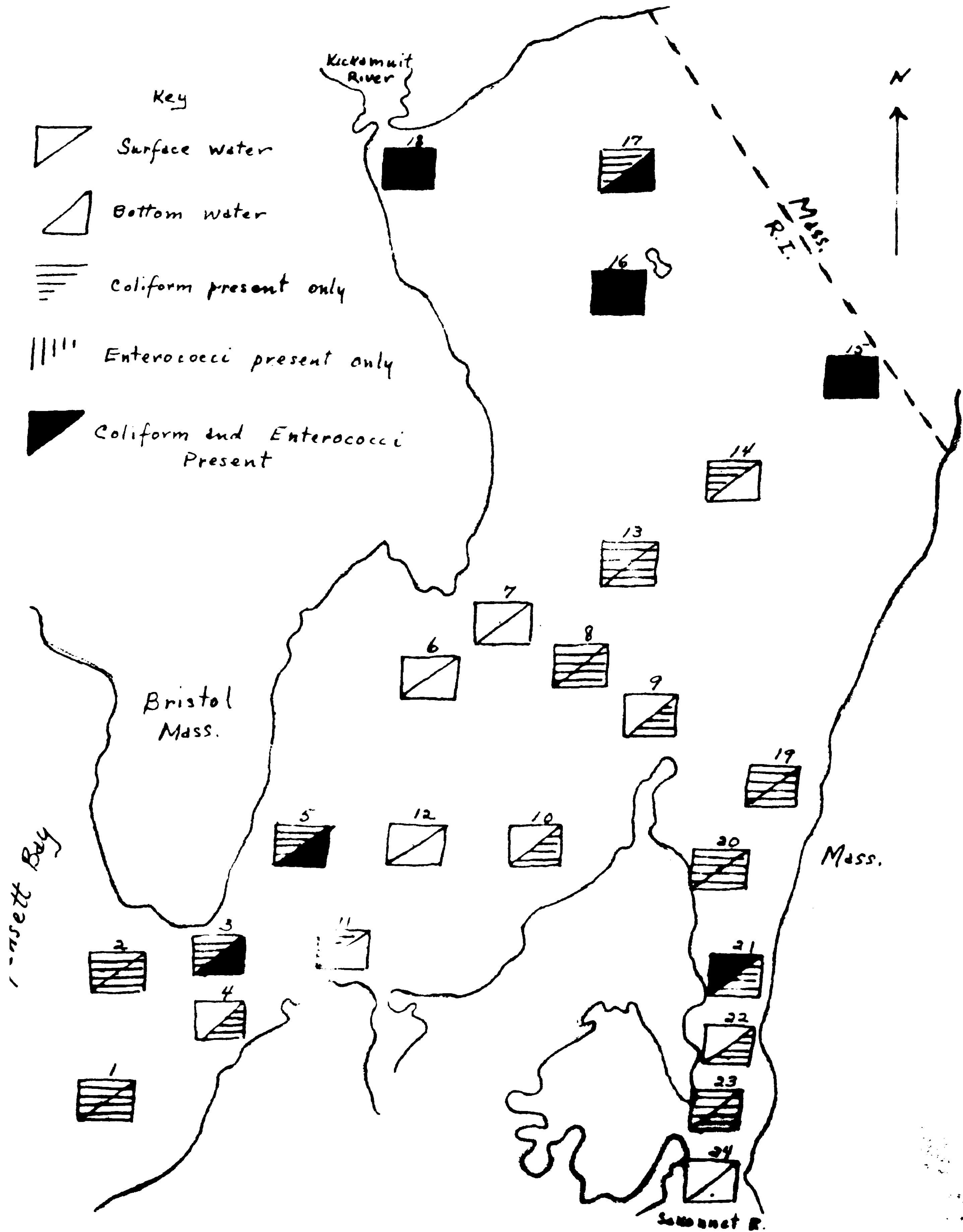


Figure 8 represents the third sampling run taken at ebb tide with a choppy sea, a strong westerly wind, an average water temperature of 6.5°C. and an air temperature of 10°C. All stations show evidence of pollution on a bacteriological basis. All of the bottom water samples contain coliform organisms while only two contain enterococci. This evidence indicates that a polluted condition has shifted south with the outgoing tide.

Figure 9 represents the last set of samples taken at mean flood tide with a choppy sea, an average water temperature of 3°C. and an air temperature of -6.5°C. The conspicuous thing here is the lack of fecal bacteria in the central area. Three stations are entirely free of enterococci and coliform bacteria and the others show a marked reduction. The northern stations 15, 16, 17, and 18 showed the presence of both types of organisms indicating that the pollution is shifting north.

DISCUSSION

The enrichment-confirmation test provides a very rapid and simple test for the isolation of the enterococci. The advantage of this test over the plating method is that a larger inoculum can be used and the enrichment broth will detect enterococci when they are present in very small numbers. A quantitative estimation can be made by using the "Most Probable Numbers" tables of Hoskins (1934). The presumptive test requires between 12 and 20 hours and the confirmation test a similar period so that a completed test may be run in 24 to 40 hours. A negative test safely may be recorded as such in 20 hours. The test is simple and easy to carry out, requiring no special apparatus or equipment. Dehydrated media are being prepared by the Difco Laboratories, making the preparation of the media very simple.

The specificity of the enterococci enrichment-confirmation test has been tested by checking the cultural characteristics of 156 organisms. All were characteristic in their morphology and Gram reaction and 97½ per cent were characteristic as indicated by growth at 45°C., 10°C. and in the presence of 6.5 per cent sodium chloride. The selectivity of the confirmation medium is based upon the presence of 0.04 per cent sodium azide, 6.5 per cent sodium chloride, 650 Oxford units penicillin per liter and 0.001 per cent methylene blue. The streptococci, staphylococci,

lactobacilli and the anaerobic spore forming rods are the only organisms capable of growing in the presence of sodium azide. An incubation temperature of 45°C. retards many of these. In the confirmation medium, the sodium chloride, penicillin and methylene blue will inhibit the staphylococci, lactobacilli, the anaerobes and all of the streptococci except the enterococci. Occasionally, falsely positive presumptive tests are caused by a Gram-positive, non-spore-forming, rod shaped organism, but these do not confirm. Reports of similar results have been received from other laboratories using this test.

It is evident from the results of this study that the intestinal material of man and warm blooded animals, including birds, contains enterococci in numbers ranging from 5,000 per gram to 88,700,000 per gram. This is in agreement with the findings of Ostrolenk and Hunter (1946) who found that rodents, dogs, cats, monkeys and flies, as well as humans, all contain enterococci in the intestinal content. An occasional fecal specimen may fail to show the presence of enterococci, but this might be expected, since it is not uncommon to find an occasional specimen from a human or warm blooded animal which contains no coliform bacteria. The feces of ducks and chickens contain large numbers of both enterococci and coliform bacteria. The gull, possibly due to the high acid condition in the gut, contains fewer coliform bacteria than enterococci. The intestine of fish does not contain either coliform bacteria or enterococci. This

is in agreement with the findings of Griffiths (1937) who concluded that "the bacteria of the intestine of fish appear to depend largely on the type of food being ingested and that it appears unlikely that there exists in fish any typical, commensal bacterial flora similar to that which occurs in the intestine of mammals". Fish do not act as a source of enterococci in water and therefore, do not interfere with the sanitary significance of the organisms.

Sewage and polluted waters contain both the enterococci and coliform bacteria in a usual ratio of 1 to 10. Studies have shown that the number of fecal organisms in sewage increases during the summer months and decreases during the winter, probably reflecting the seasonal variation in the intestinal tract. Although both types of bacteria are capable of surviving in sewage sludge, the enterococci survive in larger numbers than the coliform group. This would be expected because the enterococci are usually considered to be very resistant organisms.

A survey of a small salt water cove indicated that when the usual tests are employed, it is possible to have a very low "Most Probable Number" of coliform bacteria in water taken from a spot where raw human excrement is entering. At this point, the enterococci outnumber the coliform group 10 to 1. As the distance from this point increased, the ratio decreased and then reversed, so that at a point 50 yards or more away, the ratio became 1 to 10. With the usual ratio of 1 enterococcus to 10 coliform bacteria in

both human feces and raw sewage, it is difficult to explain this reversed ratio unless the coliforms are being suppressed by some factor. The blue-green pigment producing organisms found were suspicioned, resulting in studies by Bertullo and Sandholzer (1947) which have demonstrated that the presence of Pseudomonas aeruginosa in lactose presumptive tubes will inhibit gas formation by the coliform organisms. If, however, brilliant green lactose bile agar is used, a higher count of coliform bacteria will result. This was true at practically all stations regardless of the distance from the source of pollution. The higher counts may be due partially to the lack of antagonistic effects by other microorganisms on a solid medium and also because enterococci produce coliform-like colonies which are indistinguishable from colonies of true coliform bacteria.

In sewage stored at 5°C., the decrease in the number of enterococci and coliform bacteria seem to correspond rather closely for the first 10 weeks. At the end of this period both have decreased to 5 per ml. and 10 per ml. respectively. By the 15th. week the enterococci have disappeared completely but the coliform bacteria survive in small numbers for a long period. This survival of the enterococci does not agree with the observations of Clemesha, (1912) who found that in India, enterococci survived for only 2 or 3 days in stored river water. His results may have been due to bacteriophage versus streptococci being present in the water resulting in a situation similar to that of cholera in cer-

tain oriental waters. Studies of anti-streptococci bacteriophages which might be found in the rivers of China are being planned by the author. Other investigators (Savage and Wood, 1918) also have found a short survival time for enterococci. These findings, as well as those of Clemesha, may have been due in part to inadequate methods of isolation.

Enterococci are not normal inhabitants of soil which has not been recently contaminated with animal or human excrement. This is in agreement with the findings of Ostrolenk and Hunter (1946).

It is evident from the results of this study that small amounts of indole and skatole will inhibit the growth of the enterococci. However, all strains tested were able to grow in a 1:3500 concentration of indole and in a 1:3000 concentration of skatole. Since the concentration of indole in feces is approximately 1:10,000 (Pierce, 1932) and the concentration of skatole is between 1:10,000 and 1:20,000 (Herter, 1897) it is not probable that the concentration of either of these substances will be great enough in any natural condition outside of the intestinal tract to inhibit the enterococci. It is impossible to state what the concentrations are in the intestinal tract but they are undoubtedly greater than the concentrations in feces, for it is known that both substances are absorbed from the intestine and some of each is destroyed by oxidation. In comparison, the coliform bacteria are inhibited by concentrations of 1:2000 of indole (Tittsler and Sandholzer 1935) and 1:4000 of ska-

tole (Tittsler, Sandholzer and Callahan, 1935).

The survey of the waters of the Little Annemessex River yielded many interesting facts. As the tide moves in and out of the river, the fecal bacterial flora of the surface water shifts correspondingly. This shift is not so marked in the bottom water. Bottom waters from stations directly off shore from the city of Crisfield, Md. show a relatively constant number of both coliform bacteria and enterococci. This region of polluted bottom water lies midway between two extremes of pollution in the surface waters. This suggests that the bottom water may give a more uniform and more accurate index of the degree of fecal pollution. For this reason it is suggested that in surveys of this type, samples of the bottom water should always be studied. (A special sampling device for taking samples of water from the bottom has been used with very satisfactory results (Appendix B).

A study of the waters of four streams of Chatauqua County, N. Y. demonstrated that the enrichment-confirmation enterococci test provides a very rapid and simple test for fecal pollution in fresh waters. It was thought, before these tests were done, that these streams were non-polluted but after the positive bacteriological results were found, a sanitary survey was conducted which showed that these streams were passing through pig farms and cattle pasture lands.

An analysis of the results of tests on 226 sea water

and fresh water samples shows that the enterococci and coliform tests agree in 75 per cent of the samples tested. When the two tests do not agree, there is usually an absence of enterococci with the presence of Aerobacter members. It is felt that the presence of enterococci differentiates between fecal and non-fecal coliform organisms since the former have been shown to be primarily of intestinal origin. If enterococci and members of the coliform group are present in any given sample, the sample has been fecally polluted. However, if the enterococci are consistently absent in waters giving positive tests for coliform bacteria and a sanitary survey reveals no source of pollution, the latter probably have no sanitary significance.

From a bacteriological study of frozen foods, oysters and crabmeat, it is evident that the enterococci may often be found in the absence of coliform organisms. Just what the significance of this may be, is not clear. The enterococci are resistant to heat shock such as is used in blanching foods before freezing. Enterococci have been found in all oyster and all except two of the crabmeat samples examined in the study. However, many of these sea food samples contained Escherichia members, indicating probable pollution. More study is needed before any statement can be made as to the significance of enterococci in food products.

The enterococci were found to be present in the buccal cavity of less than two per cent of the normal persons examined. The second test on these positive individuals yield-

ed negative results. It is possible that the food eaten at the last meal might have been the source of these organisms and they were merely part of the transient flora of the mouth. Cheese would be an excellent source of large numbers of enterococci. The enterococci probably cannot be considered as a normal inhabitant of the buccal cavity under ordinary circumstances.

The enterococci are of a definite aid in determining the limits of fecal pollution in natural sea water areas. In surveys like that of the Mt. Hope Bay, R. I., the coliform organisms may represent an indigenous flora and are not necessarily indices of recent fecal pollution. When the enterococci are found in conjunction with the coliform members, the evidence of recent pollution is evident. It has been shown that the waters taken from the bottom are less influenced by currents caused by the wind and tide. A few mud samples have been examined and the results indicate that these samples may be influenced even less than the bottom water by shifting currents and tides. In examinations of shell fish beds, tests on mud are particularly significant because the shell fish grow in the mud. It is recommended that the enterococci be included in the examination of fresh and sea waters for fecal pollution and that where possible, bottom water and mud samples be included to give a more complete picture of the degree of fecal pollution.

SUMMARY

1. An improvement of the White-Sherman agar plating medium for the isolation of the enterococci is suggested.

2. A new, highly specific, enrichment-confirmation method for the isolation of the enterococci is suggested as a supplement to present bacteriological methods for detection of fecal pollution. The presumptive test requires 12-18 hours and the confirmation 12-24 hours. A completed test may require only 24 hours.

3. Enterococci have been isolated from all specimens of human feces examined and from all except one of the fecal specimens of the domestic and wild animals and birds tested. Enterococci were not present in the intestinal tract of fish.

4. Enterococci are present in most sewage and polluted water and soil samples in numbers approximately one-tenth as great as the coliform organisms.

5. Soil samples from areas that have not been cultivated for at least 50 years and where there is no pollution other than that from wild animals and birds, do not contain enterococci.

6. The enterococci are capable of surviving storage at 5°C. for from 10 to 15 weeks.

7. The enterococci are tolerant to a 1:3500 concentration of indole and a 1:3000 concentration of skatole.

8. The enterococci are of value in differentiating the

fecal and non-fecal members of the coliform group.

9. Enterococcci are often present in frozen food, shucked oysters and fresh crabmeat when the coliform group are absent.

10. The enterococci are not present as normal inhabitants of the buccal cavity.

11. It is recommended that in the examination of waters for fecal pollution, the enterococci test be carried out in conjunction with the standard methods for the coliform group and that in surveys of large bodies of water, surface water, bottom water and mud be examined from each station.

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APPENDIX A

CULTURE MEDIA

Lactose Broth.

Beef extract	3 g.
Peptone	5 g.
Lactose	5 g.
Distilled water	1 l.

Mix and heat slowly on a water bath to 65°C., stirring until dissolved. Make up the lost weight with distilled water. Adjust reaction to pH 7.2. Place in fermentation tubes and sterilize in autoclave at 15 lbs. pressure for 15 minutes.

Levine's Eosin Methylene Blue Agar.

Peptone	10	g.
Lactose	10	g.
Dipotassium phosphate	2	g.
Agar	15	g.
Eosin Y	0.4	g.
Methylene blue	0.065	g.
Distilled water	1	l.

Boil until all ingredients are dissolved and make up loss due to evaporation with distilled water. Adjustment of reaction is not necessary. Place in bottles and sterilize in autoclave at 15 lbs. pressure for 15 minutes.

Brilliant Green Lactose Bile Agar.

Peptone	16.5	g.
Lactose	3.8	g.
Bacto-Oxgall (0.05%)	11.8	ml.
Sodium sulfite (10%)	4.1	ml.
Ferric chloride (1%)	5.9	ml.
Potassium dihydrogen phosphate (3.4%)	0.9	ml.
Erioglaucine (2.2%)	5.9	ml.
Basic fuchsin (4.25%)	3.65	ml.
Brilliant green (0.001%)	5.9	ml.
Special agar (Noble)	20.3	g.
Distilled water	1	l.

Melt the agar in the water by autoclaving at 15 lbs. pressure for 45 minutes. Make up loss due to evaporization with distilled water. Add the other ingredients and after all are dissolved, place in bottles and sterilize in autoclave at 15 lbs. pressure for 15 minutes.

White and Sherman Enterococci Agar.

Glucose	5	g.
Bacto tryptone	5	g.
Yeast extract	5	g.
Agar	15	g.
Sodium azide	.3	g.
Penicillin	325	Oxford units / liter.
Distilled water	1	l.

Mix all ingredients except penicillin and dissolve by boiling. Adjust reaction to pH 7.4. Place in flask in

known quantity and sterilize in autoclave at 15 lbs. pressure for 15 minutes. Cool to 45°C. and add penicillin just before pouring.

Hajna and Perry's "S.F." medium.

Bacto tryptone	20	g.
Sodium chloride	5	g.
Glucose	5	g.
Dipotassium phosphate	4	g.
Monopotassium phosphate	1.5	g.
Sodium azide	0.5	g.
Brom cresol purple (1.6%)	2	ml.
Distilled water	1	l.

Dissolve in warm water, place in tubes and sterilize in autoclave at 15 lbs. pressure for 15 minutes. Incubate at 45.5°C.

Mallmann's Enterococci Broth

Bacto tryptose	1.5	g.
Lactose	5	g.
Dipotassium phosphate	4	g.
Monopotassium phosphate	1.5	g.
Sodium chloride	5	g.
Distilled water	1	l.

Dissolve in warm water, place in tubes and sterilize in autoclave at 15 lbs. pressure for 15 minutes.

<u>Lactose Lemco Peptone Litmus Broth.</u>

Lactose	5 g.
Lemco (meat extract)	5 g.
Peptone	5 g.
Distilled water	1 l.

Dissolve by heating; adjust reaction to pH 7.5. Now add 10 per cent litmus solution until medium attains a purplish color. Distribute in test tubes and sterilize in autoclave at 15 lbs. pressure for 15 minutes.

<u>Houston and Harold's Potassium Tellurite Enterococci Medium.</u>

Peptone	10 g.
Lactose	5 g.
Dipotassium phosphate	2 g.
Sodium chloride	5 g.
Agar	20 g.

Dissolve by heating; place in bottles and sterilize in autoclave at 15 lbs. pressure for 15 minutes. Cool to 45°C. and add potassium tellurite to make a 1:15000 concentration. Pour into plates.

Conradi-Drigalski's Medium.

Sodium chloride	5 g.
Peptone	20 g.
*Nutrose	10 g.
Beef extract	4 g.
Sodium hydroxide N/1	50 ml.
Agar	20 g.
Distilled water	1 l.

Dissolve by heating; place in bottles and sterilize.

*A sodium caseinate used as a nutrient.

APPENDIX B

A. Bottom Water Sampler.

The bottom water sampler used in these studies is a modification of that used by the Rhode Island State Public Health Laboratory and was made according to the specifications of this laboratory by the Engineering Associates Company of Chevy Chase, Maryland. It is made entirely of brass and is devised to use a 8 oz., wide mouth, ground glass stopper sampling bottle with a mushroom type stopper. The overall height is 19 inches.

By holding the sampler by the hook to which the rope is attached (Figure 10) and raising the disk, the sterile bottle can be inserted without being opened. It is then held in place by letting the disk drop over the mushroom of the stopper and by fastening the spring around the body of the bottle. The apparatus is then lowered into the water without allowing the bottle to open. The bottle will remain closed as long as the weight of the sampler is carried by the rope. When it comes to rest on the bottom, the weight is then brought to bear on the sliding rods holding the stopper. This causes the bottle to open (Figure 11). After allowing a few seconds for the bottle to fill, the apparatus is raised by the rope which immediately closes the bottle. When the apparatus is brought to the surface, the closed bottle can be removed.

FIGURE 10

BOTTOM WATER SAMPLER

Closed

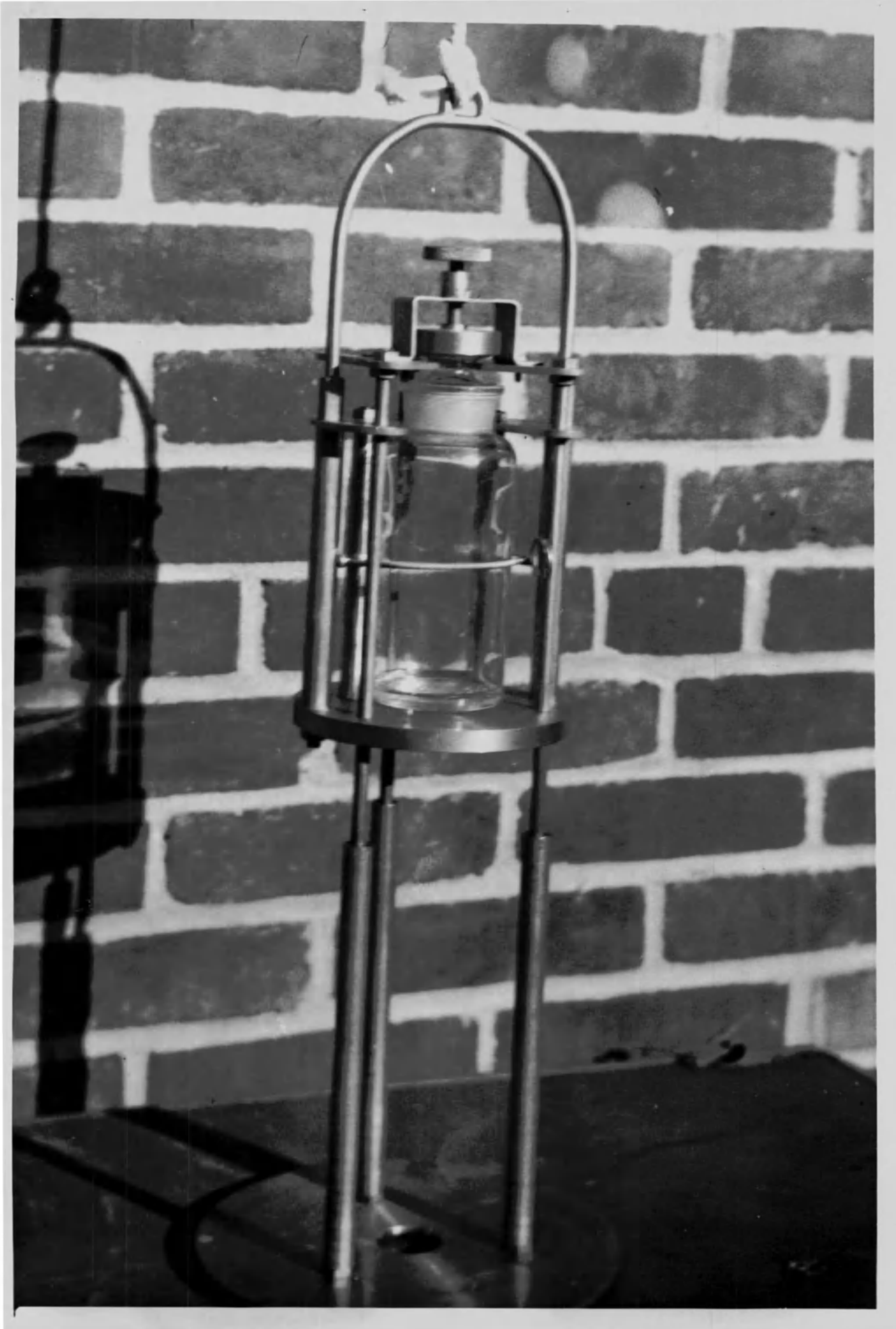

FIGURE 11

BOTTOM WATER SAMPLER

Open

B. Mud Sampling Dredge.

The mud sampling device is a small iron clam-shell dredge. The mud chamber is cylindrical with an inside diameter of 6 inches and a length of 10 inches. Iron weights are provided which can be screwed to the cylinder if additional weight is needed to dig into the mud.

The dredge is opened and the "trip bar" set to keep the chamber open (Figure 12). It is lowered into the water in this position. When it comes to rest on the bottom, the "trip bar" drops and as the rope is pulled, the clam-shell chamber digs into the mud, as it closes, encasing the mud sample. When the dredge is brought to the surface, the chamber is opened and a bacteriological mud sample removed from the center of the mud mass, using a sterile, wooden tongue blade. The samples are carried to the laboratory in 8 oz., wide mouth, ground glass stoppered bottles. The dredge must be thoroughly washed before each sample is taken.

FIGURE 12

MUD SAMPLING DREDGE

Open

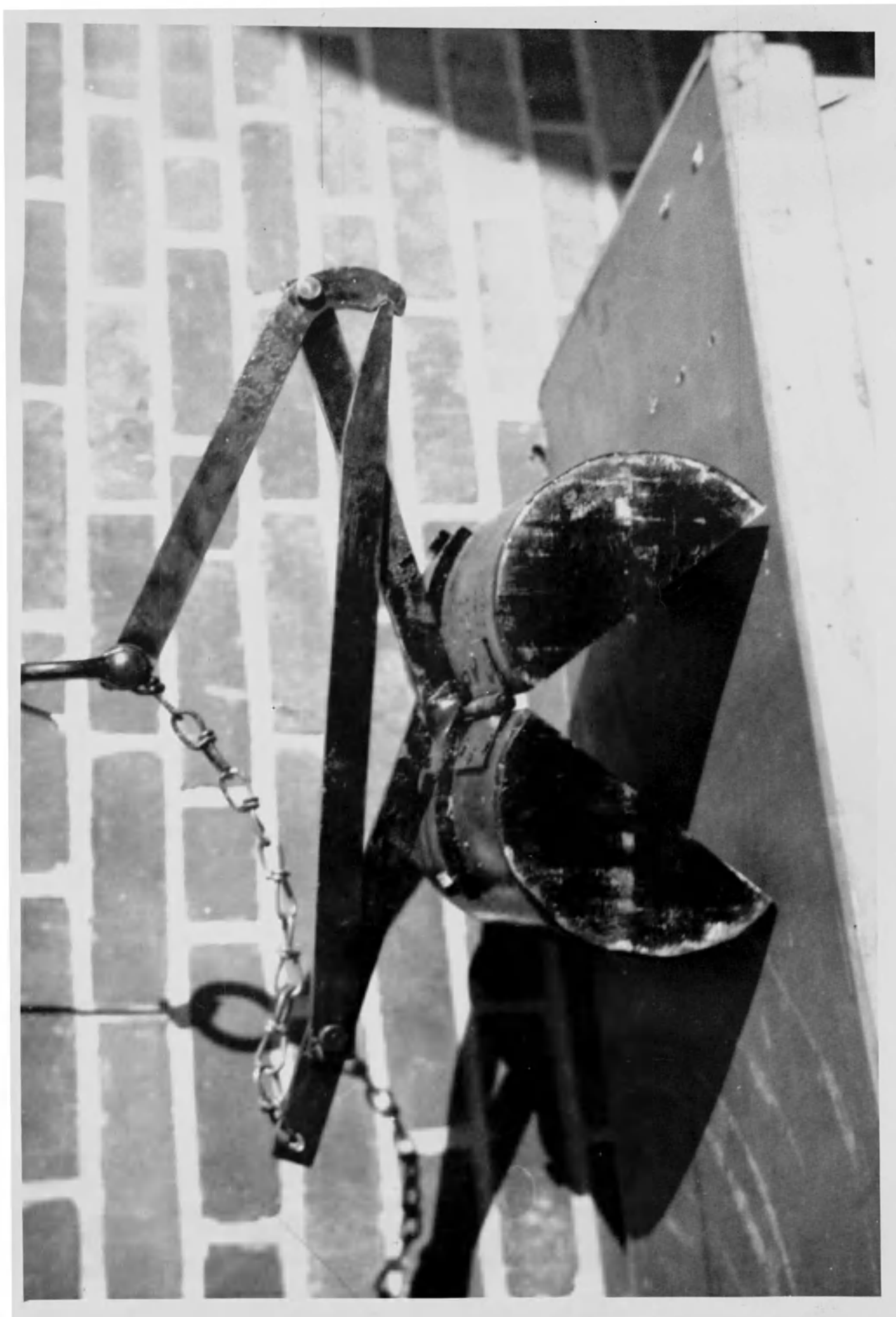

FIGURE 13

MUD SAMPLING DREDGE

Closed