AN EVALUATION OF A MODIFIED MEMBRANE FILTER PROCEDURE FOR ENUMERATING STRESSED FECAL COLIFORMS IN CHLORINATED SEWAGE EFFLUENTS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

I. INTRODUCTION

The health of mankind has been threatened by communicable diseases for many thousands of years. A communicable disease may be defined as a disease whose causative agent can be readily transferred from one organism to another. The transmittance may occur by direct contact with the disease, aerosols, water, or food (1). The usual entrance point for such diseases is the mouth.

Only within the last two centuries has it been established that many of the communicable diseases can be transmitted by water. Included are cholera, polio, dysentery, tuberculosis, typhoid, and hepatitis. The pathogens that cause these diseases are eliminated with the feces of ill persons and may eventually reach a natural water course or domestic water supply. To eliminate the possibility of such contamination, it is now common practice to disinfect the sewage at the wastewater treatment plant by chlorination (2). Such disinfection kills the pathogens or renders them incapable of causing disease.

Often it is desirable to perform an analysis of the treated wastewater to determine the efficiency of the chlorination step. Separate tests may be performed to determine if any of the pathogens that cause the previously mentioned diseases are present. Normally, only tests for coliform bacteria are performed because they are present in large numbers in domestic sewage and they can be detected easily. The presence of coliforms is taken as an indication that disease-causing pathogens may be present (3). Coliforms found in feces are termed "fecal coliforms" (4).

Presently, there are two methods for enumerating fecal coliforms from sewage mentioned in Standard Methods for the Examination of Water and Wastewater (4). The first is the multiple-tube fermentation procedure, more commonly known as the Most Probable Number (MPN) technique. The second is the membrane filter (MF) technique. The MPN method is based on a statistical analysis of the number of positive and negative results obtained when media are inoculated from a multiple set of dilutions of the original sample. The MF method is based on the direct colony count found on the membrane (4).

In recent years, much concern has arisen from the fact that coliform bacteria may not be totally indicated by present testing procedures when subjected to various environmental stresses. Such stresses include heat, low temperatures, irradiation, disinfectants, water pH, water chemistry, and microbial antagonists (5). Chlorination, one of the most frequent of these stresses in wastewater treatment, may accelerate dramatic changes in morphological and physiological properties of the bacteria. As a result, the bacteria may go undetected by the presently available tests. Thus, the results obtained by these tests in studies of fecal pollution may be questionable. An example of this problem is the higher numbers of coliforms that have been reported when the MPN method was used compared to the results obtained when the MF method was used to test chlorinated sewage (6,7,8,9). These lower results obtained by the MF method presents a definite disadvantage since the fecal MF method can be performed in 24 hours whereas the fecal MPN method requires 72 hours.

In an effort to obtain similar estimates of actual population densities by the MPN and MF methods for fecal coliform recovery, an improved MF procedure for stressed coliforms has been proposed (10). The objective of this study was to evaluate a modification of this proposed procedure, which will be discussed later, by comparing results obtained with it to results obtained by the standard fecal MPN and MF methods (4). The coliforms involved had been stressed by the wastewater treatment processes found within a trickling filter sewage treatment plant. The comparative study was conducted with samples taken from the effluents of the secondary clarifier and the chlorine contact basin at the Blacksburg, Virginia sewage treatment plant located on Stroubles Creek.

II. LITERATURE REVIEW

The use of coliforms as indicators of water quality has been discussed by a great many writers and has been shown to be of a great value. When the organisms are subjected to the environmental stresses alluded to in the introduction of this thesis, problems arise in accurately estimating their numbers. The literature related to the area of research reported in this thesis includes many topics that have a direct bearing on one's understanding of the recovery of fecal coliforms from chlorinated, domestic sewage. Topics that were reviewed in the literature, and which will be discussed in the following paragraphs, are: historical aspects of water-borne pathogens, significance and physiology of coliforms, chlorination of sewage and its bactericidal effect, and methods of testing for fecal coliforms.

Historical Aspects of Water-Borne Pathogens

Sawyer and McCarty (2), Holden (11), and Borchardt and Walton (12) presented a brief history of communicable diseases beginning with the fourteenth century when a plague known as the "Black Death" swept Europe, killing 25 percent of the population. In the winter of 1664-1665, 14 percent of London's population was killed by another outbreak of the plague. As the population in the urban areas grew, the frequency of communicable disease outbreaks increased. In 1854 an epidemic of Asiatic cholera broke out in London. Through investigations by John Snow and John York, it was demonstrated that the source of infection was a water pump station. The water from the pump station

was found to be contaminated with sewage that had entered through a damaged sewer, which had served the nearby home of an infected person. This discovery was an important event in public health engineering in that it established, without any doubt, that water was the major transmitter of Asiatic cholera.

Robert Koch provided the absolute proof of disease transmittance by water in 1875 when he successfully grew a pure culture of the bacterium that causes anthrax. Further proof was later established when he isolated cultures of the organisms causing cholera in 1883 and typhoid in 1884 (2).

Significance and Physiology of Coliforms

A bacterial species was first isolated by T. Escherich in 1885 (13,14) which he considered to be characteristic of human feces. He stated that the presence of this bacterial species in water was indicative of dangerous pollution because of its possible association with the enteric, disease-producing bacterial group found in the intestines of ill persons. The bacterial species isolated by Escherich was first referred to as *Bacterium coli* (*B. coli*), but in 1942 the term "coliform group" became common usage (14). *B. coli* is better known today as *Escherichia coli* (*E. coli*).

Many species of the coliform group were isolated from fecal matter by later investigators. Other investigators detected many species which resembled this coliform group biochemically in soils, plants, and similar locations that had not been subjected to any known

fecal pollution. As a result of the varied environments in which the bacteria were found, much confusion resulted as to their significance and their correlation with fecal pollution (14).

In 1895, Smith asserted that any member of the coliform group represented fecal pollution regardless of the locations in which the bacteria were found. He believed that all coliforms came from the intestines of warm-blooded animals and, thus, the presence of such bacteria indicated a possible danger to human health. In 1904, Eijkman disclaimed the then current theory that only coliforms of known fecal origin should be considered as indicators of dangerous pollution and that neglection of the natural habitat of the organisms slighted the public's interests in the area of health and safety (13,14,15,16). Eijkman recommended an elevated temperature for incubation when recovery testing was being performed to distinguish between fecal coliforms and non-fecal coliforms. He found that at a temperature of 46^{o}C, coliforms of fecal origin fermented glucose, while coliforms of non-fecal origin did not. This increased-temperature incubation procedure has become known as the "Eijkman Test" (14,15).

A coliform may be defined as any microorganism which is Gram-negative, non-spore forming, rod-shaped, and which will ferment lactose and form acid and gas within 48 hours when incubated at 35^{o}C (4,11,17). Coliforms are normally 2 to 3 μm in length and 0.5 μm in width and may be either motile with peritrichous flagella or non-motile (11). They are capable of growing in the presence of bile salts and can reduce nitrates to nitrites, give negative oxidase test results, and may

assimilate many carbohydrates under aerobic or anaerobic environments (11). The group includes the genera Citrobacter, Enterobacter (Aerobacter), Erwinia, Escherichia, Klebsiella, Pasteurella and Serratia (11,17).

There are two major fermentative patterns that are characteristic of the coliform bacteria. The first is mixed-acid fermentation in which the bacteria ferment glucose and form significant quantities of acetic acid, lactic acid, and succinic acid, and, in lesser amounts, ethanol, CO_2 and hydrogen gas. Equal amounts of CO_2 and hydrogen gas are formed because the bacteria can produce CO_2 only from formic acid through the enzyme system formic hydrogenlyase. The reaction proceeds as (17):

$$HCOOH \rightleftharpoons H_2 + CO_2$$

The second fermentation pattern exhibited by coliforms is termed 2,3-butanediol fermentation or butylene glycol fermentation. In this process, CO_2 and hydrogen gas are produced in similar process as mixed-acid fermentation; however, additional CO_2 is produced through the butylene glycol production to yield a CO_2 to H_2 ratio of 5:1. These two processes may be used to distinguish between members of the coliform group through the methyl red test, the Voges-Proskauer test, or by comparing the CO_2 to H_2 ratio (17).

Much research has been done on the use of coliforms, especially the use of fecal coliforms, as indicators of water quality. Contemporary investigators have been very concerned with the origins of the coliform and the temperatures at which recovery testing should be performed as

was first outlined by Eijkman in 1904 (14,15). Geldreich et al. (15) examined fecal samples from ten adult institutional residents who lived in a controlled environment, ate the same food, and worked at the same farm location. The samples were incubated at 44.5°C. The results from the human samples analyzed in this study were remarkably similar to 33 human samples taken from varied environments in another study. The human samples yielded 11 strains of the possible 16 types of coliforms when tested by the indole, methyl red, Voges-Proskauer, and citrate tests (IMViC). Fecal samples were collected from the farm livestock and poultry and analyzed at the same incubation temperature as the human samples. Escherichia coli appeared most often, and only five other strains occurred occasionally. This study led the authors to believe that 96 percent of the coliforms found in the feces of warm-blooded animals had the ability to ferment lactose and form gas at 44°C.

As stated previously, early investigators found coliform bacteria in locations, such as in soils and on plants, that had not been subjected to fecal pollution. Many studies have been instituted in an effort to distinguish between the possible variety of habitats of fecal and non-fecal coliforms. Geldreich et al. (18) analyzed 251 soil samples from 26 states and three foreign countries. The authors found that in unpolluted soils, fecal coliforms were either absent or, if present, were in very small numbers. Most of the samples contained less than two fecal coliforms per gram of soil while samples from polluted areas--such as animal feed lots, areas which had been subjected to recent

flooding by domestic sewage, or the banks of heavily polluted streams--contained fecal coliform densities of from 3,300 to 49,000 per gram.

The sanitary significance of the coliform group has been demonstrated by a great many researchers. Their objective was to show that Escherich's hypothesis that coliforms of fecal origin were indicative of the presence of enteric-disease causing pathogens was indeed valid. However, due to the great controversy that has existed through the years as to whether the habitat of the coliform should be considered when testing, there have been many inconsistencies in the use of coliforms as indicators. Many investigators have used the total coliform test in their analysis while others have analyzed only for fecal coliforms (14). The term "total coliforms" refers to the entire bacterial group which grows at 35°C, whereas the term "fecal coliforms" accounts for the group which will survive and replicate at 44.5°C. Both total and fecal coliforms must exhibit the signs of acid and gas formation, and total coliforms must ferment a lactose broth while fecal coliforms must ferment a tryptose-lactose broth (4). Some researchers have concluded that while the presence of any coliforms in potable water should not be tolerated, as this may indicate a deficiency in the treatment process, only fecal coliforms should be considered when analyzing for fecal pollution (4,14,16,19).

Kehr and Butterfield (20) examined samples of wastewater and found Samonella typhosa. They calculated that approximately six typhoid causing bacteria existed for each million coliforms present.

Chambers (21) reported that other investigators had shown a correlation between viruses and coliform bacteria found in sewage. He cited studies that showed a ratio of 700 viruses per 46 million coliforms. This is a fact of concern because the virus that causes infectious hepatitis is in sewage and is the most frequent water-borne disease (21).

The ratios presented indicate that testing for coliforms instead of pathogens would be easier simply because of their relative abundance. Also, the methods of testing for enteric-disease pathogens are more difficult; thus, making use of coliforms to indicate the possible presence of pathogens is even more attractive.

A number of studies have been performed that show that the coliform group may not be as good an indicator of water quality as was once thought. Some investigators have found that the coliforms surviving chlorination at a sewage treatment plant may increase in number when introduced into a clean water environment. This phenomenon has been termed "aftergrowth". Underhill (22) and Saunders (23) presented a review of the studies that had been performed in this area. Some of the studies have shown that aftergrowth is associated with the nonfecal coliforms (19,24) while other studies have shown aftergrowth by the fecal coliforms (23).

Schiemann et al. (25) performed studies on the effect of coliphage interference when recovering coliform bacteria. Their studies were done with E. coli as the bacterial host. They concluded that the presence of this bacteriophage could reduce the number of viable fecal

coliforms that could be recovered by testing. The authors stated that treatment processes such as chlorination could cause this problem to proliferate because the ratio of bacteria to virus would be decreased by such treatment, thus allowing more viral attack per fecal coliform.

The fecal coliform, nevertheless, is still used as an indicator of fecal pollution. The present State of Virginia standards use the fecal coliform for determining stream quality and sewage effluent quality (26,27).

Chlorination of Sewage and Its Bactericidal Effect

When coliform bacteria are subjected to environmental stresses such as heat, low temperatures, irradiation, disinfectants, water pH, water chemistry, and microbial antagonists much difficulty can arise in recovery tests for these organisms (5). Fecal coliforms may be subjected to such stresses. In the wastewater treatment plant, disinfection by chlorination is the major stress that fecal coliforms will encounter.

The history of chlorination has been discussed by a number of writers (22,23,28,29,30). Therefore, this thesis will not be concerned with a detailed historical discussion. Briefly, chlorination for disinfection began in 1859 and has continued until today. It is common practice in many portions of the United States. The State of Virginia requires continuous disinfection of all sewage by chlorination or ozonation. Chlorination, which is the most common process, requires 30-minutes chlorine contact time (27). The chlorine residual levels

must be maintained between 2.0 mg/l to 2.5 mg/l for dischargers to waters satisfactory for use as public water supplies and between 1.0 mg/l to 1.5 mg/l for all other discharges (31).

It seems appropriate that the bacterial stress that occurs during chlorination should be discussed, as it is bactericidal in nature and has a direct influence on the recovery of fecal coliforms that were studied in this thesis. Before a discussion of the bactericidal effects can be made, it is desirable to review the chemistry and the dynamics of chlorination.

Chlorine chemistry. Disinfection of wastewater may be accomplished by adding chlorine in the form of liquid chlorine, sodium hypochlorite, or calcium hypochlorite. Upon addition of the chlorine to ammonia-free water, dissolution occurs instantaneously by the following hydrolytic reaction:

$$Cl_2 + HOH \rightleftharpoons HOCl + H^+ + Cl^-$$

At pH levels above 4 and in dilute concentrations, hypochlorous acid (HOCl) exists primarily with very low concentrations of free Cl_2 present. The hypochlorous acid formed is a very weak acid and will dissociate very poorly at pH levels below 6 as follows:

$$HOCl \rightleftharpoons H^+ + OCl^-$$

The relative quantities of HOCl or hypochlorite ions (OCl^-) present are a function of pH. The equilibrium expression may be shown as:

$$\frac{[H^+]\,[OCl^-]}{[HOCl]} = K,$$

where K is the ionization constant and is approximately equal to 2.7×10^{-8} at $20^{o}C$ (2,21). This relationship may be seen in Table I (32). The influence of pH is very important in the determination of whether HOCl or OCl^- will be formed. Many researchers have found that HOCl is a much more powerful disinfectant than OCl^- (2,21).

When hypochlorites are added to water, ionization occurs immediately with OCl^- being formed as shown in the following reaction:

$$Ca(OCl)_2 \longrightarrow Ca^{++} + 2OCl^-$$

The hypochlorite ion establishes an equilibrium with the hydrogen ions in the reaction:

$$H^+ + OCl^- \rightleftharpoons HOCl$$

Therefore, the same equilibrium conditions are established regardless of which form of chlorine is added. The major difference is the effect of pH on the formation of the hypochlorous acid or the hypochlorite ion at equilibrium. The addition of chlorine will decrease the pH, whereas, hypochlorite addition will increase the pH (2).

When chlorine is added to domestic wastewaters, the hypochlorous acid reacts with nitrogen compounds present in the sewage. The reaction occurs between ammonia, which is derived mainly from the bacterial breakdown of urea and albuminoid nitrogen, and the hypochlorous acid. Due to the high oxidative nature of the hypochlorous acid, the reactions occur immediately; and, therefore, hypochlorous acid is in existence a very short time. The reactions occur as follows and form monochloramine, dichloramine, and trichloramine respectively (2):

TABLE I

Percentages of Chlorine as HOCl and OCl^- at Different pH Values and Temperature - After Moore (32)

pH	Percentages of Total Chlorine as:			
	HOCl		OCl^-	
	0^oC	20^oC	0^oC	20^oC
4.0	100.00	100.00	0.00	0.00
5.0	100.00	99.70	0.00	0.30
6.0	98.20	96.80	0.80	3.20
7.0	83.30	75.20	16.70	24.80
8.0	32.20	23.20	67.80	76.80
9.0	4.50	2.90	95.50	97.10
10.0	0.50	0.30	99.50	99.70
11.0	0.50	0.03	99.95	99.97

$$NH_3 + HOCl \rightarrow NH_2Cl + H_2O,$$

$$NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O,$$

$$NHCl_2 + HOCl \rightarrow NCl_3 + H_2O$$

The reduced germicidal efficiency that results when chloramines are formed cannot be overemphasized. Some researchers have found that disinfection with hypochlorous acid is 25 to 100 times better than with monochloramine (33,34). In a secondary wastewater treatment plant where ammonia concentrations will be high (several mg/l), the existence of chlorine will be seen in the form of chloramines unless a free-chlorine residual (hypochlorous acid) is created through break-point chlorination (2).

Hypochlorous acid will react with other compounds including amino acids, proteinoceous materials, and organic compounds to form chlorine compounds which have low disinfecting powers (2,21). Due to the strong oxidative nature of chlorine, it will also react with substances such as ferrous iron, manganese, nitrate, and hydrogen sulfide. These possible reactions will create what is termed a chlorine demand, and it must be overcome to assure that enough chlorine is present for adequate disinfection (2).

The difference in the disinfection potentials of free chlorine (hypochlorous acid and hypochlorite ion) and combined chlorines (chloramines) was not recognized until 1940 (2). Since then, researchers have attempted to determine the amount of chlorine present in these

different forms. Standard Methods (4) outlines a number or procedures for determining free and combined chlorine residuals. Total chlorine, which is the sum of all chlorine residuals, may be analyzed for also.

The State of Virginia prefers that chlorine residuals be analyzed by the amperometric titration procedure (27). This procedure is considered to be the most accurate (2). The method consists of an oxidation-reduction reaction in which an electrode system is used to determine the reaction endpoint. A reducing agent is used to titrate the chlorine. The reducing agent is phenylarseneoxide (PAO), which will react with free chlorine at pH ranges 6.5 to 7.5. Combined chlorine may be analyzed with the PAO at pH ranges 3.5 to 4.5. Iodide is added to the sample and is oxidized by the chloramines to free iodine. The free iodine can then be reduced by the PAO to give a measure of the chloramines present (2,4).

Chlorination dynamics. Much research has been done on the use of chlorination in wastewater treatment. Studies have been performed to analyze the effects of such variables as dosage, chlorine contact time, mixing, and bactericidal effect.

Heukelekian and Smith (35) analyzed the effluents from both trickling filters and activated sludge units in municipal wastewater treatment facilities to determine if differing coliform residuals could be achieved by varying the chlorine dosage. They concluded that a standard coliform residual, based on a set chlorine dosage, could not be maintained due to great variations in the amount of chlorine required for differing samples of the same sewage. The

authors also found that at a given chlorine residual, a chlorine contact time of five minutes had only 50 percent the lethal efficiency as a chlorine contact time of 30 minutes. Heukelekian and Day (36) found that proper mixing of chlorine at the application point has a direct effect on producing a lower coliform population.

The bactericidal effect of chlorination has been investigated extensively. Chick was one of the first to describe the bacterial death rate caused by chlorination in a mathematical expression. The differential form may be written as:

$$\frac{dN}{dt} = -kN,$$

where N = number of organisms
t = time
k = constant

The equation may be integrated to:

$$\frac{N}{N_o} = e^{-kt},$$

where N = number of organisms remaining after time t
N_o = initial number of organisms
k = constant
t = time

It is common to find examples in the literature where this equation did not find observed experimental data. Such variables as differing susceptibilities of individual microbes, clumping by the organisms, temperature variations, chlorine concentration changes, and chemical variations in the wastewater will affect the survival rates of the organisms (3).

The effect of chlorine on bacteria is not fully understood. The actions are generally considered to include (37):

1. The cell wall may be removed or damaged, thus exposing the fragile cytoplasmic membrane to the environment.
2. The cytoplasmic membrane may be disrupted, allowing loss of essential metabolic materials.
3. Essential enzyme activities may be interfered with.

Many investigators have reported that chlorine places a non-lethal stress on the coliform bacteria, a stress that affects the bacteria in such a way that they are not recognized in the standard recovery testing procedures (6,8,10). There may be a need for a recovery period in an enriched environment that would allow the coliform to recover from the effects of the bactericidal or other stressing agent before testing procedures can begin.

Methods of Testing for Fecal Coliforms

Since the time when Eijkman discovered fecal coliforms would ferment glucose at an incubation temperature of 46^{o}C and nonfecal coliforms would not, many improvements have been made in the fecal coliform recovery testing procedures. Other researchers found inconsistencies in his original proposal. Some investigators found that certain strains of *E. coli* would not ferment glucose at 46^{o}C, while others found that only a small portion of human fecal coliforms fermented glucose at that temperature. These inconsistent results led researchers to develop better testing media and temperature control. In 1933, the concentration of glucose was reduced and, in 1936 glucose was replaced by lactose (13).

There are presently two procedures outlined by Standard Methods (4) for enumerating fecal coliforms in wastewater. These procedures are the fecal Most Probable Number (MPN) method and the fecal membrane filter (MF) method. Each method employs lactose as a portion of the testing medium (4).

The MPN procedure was first suggested by McCrady in 1915 when he proposed that inoculating aliquots of serial dilutions into replicate samples of test medium would enable one to determine coliform concentrations. The concentrations are calculated with probability formulas based on the number of replicate inoculations that show signs of growth. The procedure was adopted by Standard Methods of Water Analysis in 1936 (38).

In 1943, a medium for isolating fecal coliforms was developed. The medium was named EC and was to isolate E. coli at 44.5°C. However, later investigations showed that the medium was not adequate as a presumptive test medium (13).

In 1957, interest in the usage of lactose was renewed as a presumptive test medium, and EC was incorporated as the confirming test medium for fecal coliforms (13). Presently, lactose or lauryl tryptose broth may be used in the presumptive test (4). However, the fifteenth edition of Standard Methods will permit only lauryl tryptose broth.

The present fecal MPN procedure consists of four steps: the presumptive test, the confirmed test, the completed test, and the

Gram strain (4). Most researchers use only the first two steps of the fecal MPN procedure for some research purposes (7,9,39).

The presumptive test consists of inoculating three to five tubes of the medium with at least three decimal dilutions of the original sample. The culture medium may be either lactose broth or lauryl tryptose broth. Some investigators have found that lauryl tryptose broth will yield fewer false positives in the presumptive test (1). At the end of an incubation period of 48 hours at 35$\pm$ 0.5^{o}C, all tubes exhibiting signs of growth and gas production are accepted as a positive test (4).

Samples of the positive presumptive test tubes are then transferred to the confirmation EC medium, which contains bile salts and other inhibitory substances that allows fecal coliform growth but restricts growth of other organisms. Samples must be incubated at 44.5 $\pm$ 0.2^{o}C as opposed to 46^{o}C as stated by Eijkman (4). Geldreich (40) reported that the temperature should be controlled within $\pm$ 0.2^{o} of 44.5^{o}C. He stated that the inhibition of fecal coliforms to form gas could occur at temperatures in excess of 45.6^{o}C. After 24 hours, the tubes exhibiting signs of growth and gas formation are accepted as positive.

Geldreich (1) stated that the probability formulas first outlined by Hoskins in 1934 may be used to determine the concentration of fecal coliforms in the sample based on the number of positive tubes in the confirmed test (1,4).

The MF procedure was used in Europe for years before it was introduced in this country. The use of the MF procedure was not seen

in the public health field until 1951. Originally, the MF procedure was used for enumerating total coliforms from treated water supplies, but later it was discovered that the MF procedure could be utilized also in wastewater bacteriological studies (41).

Much study has been done to find an adequate medium for enumeration of fecal coliforms. Early investigators tried many approaches including both one and two-step procedures.

According to Geldreich et al. (42), Taylor, Burman, and Oliver suggested a two-step procedure. The filter was placed on a nutrient broth solution and incubated at 37°C for two hours after which it was transferred to a selective medium and incubated at 44°C for 16 hours. Fecal coliforms appeared yellow in color.

Delaney et al. (13) suggested a medium called Tryptone Bile Agar (TBA). Inoculated membrane filters were incubated in the medium for 20-24 hours at 44.5°C. The membrane filter was then transferred to a medium where the fecal coliform produced indole. Fecal coliforms could be distinguished by a dark-red color. The authors stated that adequate growth could be achieved in 24 hours.

Geldreich et al. (42) introduced a medium known as MFC in 1965 which could recover fecal coliforms when samples were incubated at 44.5°C. This method had advantages over earlier methods in that no transfer of the filter or change in incubation temperature was required. The method was introduced in the 13th Edition of Standard Methods (43) in 1971.

The fecal MF procedure (4) consists of filtering samples of wastewater through a cellulose membrane which is supported by a filter-funnel apparatus. After filtration the membrane is placed in a culture dish containing MFC medium that may be in the form of a broth, which is poured on an absorbent pad, or in a semi-solid form which is achieved by adding 1.5 percent agar to the broth. The culture dish is then sealed and incubated at 44.5 $\pm$ 0.2°C for 24 hours.

After incubation, the membrane filter is examined with a microscope. Colonies appearing blue are considered to be fecal coliforms. The blue colonies are counted and an estimation of the number of fecal coliforms may be made based upon the actual colony count and the dilution of the sample (4).

Researchers have found many advantages and disadvantages for each of the standard fecal coliform tests. The fecal MPN procedure, as stated earlier, requires a minimum of 3 days before results are obtained, while the fecal MF procedure will yield results in 24 hours. This is an obvious advantage to water and wastewater treatment personnel.

The fecal MPN procedure uses probability formulas for calculating the bacterial density of the sample based on the number of tubes showing growth. The MPN procedure tends to overestimate the true concentration of fecal coliforms. The fecal MF procedure is based on a direct colony count, and, therefore, no probability formulas are needed. Many investigators have found differences in the results of the two procedures (44), and many believe that the MF procedure may allow greater precision than the MPN procedure (38,45).

The MF procedure has been estimated by some investigators to be the most economical of the standard coliform tests. This is attributed to the savings in labor time and apparatus of the test (7,46).

Investigators have stated other advantages of the MF procedure. The ability to separate the bacteria from soluble materials or particles too small to be retained on the filter is a definite advantage if such substances are hazardous to bacterial growth. Another advantage is that the filter may be kept for further observation at a later time (41).

When the MPN and MF procedures are used to determine total and fecal coliform densities in chlorinated secondary sewage effluent, the MPN procedure has been found to yield higher results. The difference has been attributed to the differences in testing procedures and the statistics used to enumerate the bacterial concentrations (6,7,8,9). As a result, many investigators have proposed many modifications to the MF procedure in order to make results between it and the MPN procedure more comparable. In some cases, completely new methods have been proposed that are supposed to yield more accurate fecal coliform counts in chlorinated sewage.

In 1958, McKee et al. (6) found differences in the results between the MPN and MF procedure when coliforms were recovered from chlorinated sewage. The authors stated that the reason for these differences could be that the bacteria were able to free themselves of the monochloramine in the aqueous solution, whereas on the membrane filter they could not. As a result the bacteria were able to reverse the inactivation of the

enzyme system by the monochloramine when they were tested by the MPN procedure.

McCarthy et al. (45) found that when weak coliforms were subjected to a highly selective medium, growth would not occur. They suggested an enrichment step that used lauryl tryptose broth. This step allowed non-selective growth, thus, allowing the bacteria a chance to recover from the effects of a toxic substance. Maxcy (47) found similar results when E. coli were subjected to chlorine. He found that bacteria that were sub-lethally injured grew poorly when cultured on selective media.

With the advent of the fecal MF procedure in 1965 (42) and its adoption by Standard Methods (43) in 1971, much study has been instituted to determine its efficiency for recovering fecal coliforms from chlorinated sewage. Studies were performed to determine if the medium or filter needed improvement or if additional steps in the method were needed.

Taylor et al. (48) proposed a delayed incubation fecal MF procedure for fecal coliforms when samples could not be analyzed within the recommended six-hour period after collection. The procedure allowed holding the MF for 3 days on a holding medium called M-VFC. It contained vitamin-free casitone, sodium benzoate, sulfanilamide, and ethanol. The filter can be placed on this medium in the field and then sent to the laboratory. There it is transferred to the MFC broth for normal fecal coliform testing. The procedure, as is outlined in Standard Methods (4), is a tentative procedure.

Lin (8) tested chlorinated secondary-treated sewage for fecal coliforms using both the fecal MPN and fecal MF procedures and found that the MPN procedure yielded higher results. He concluded that an enrichment step similar to the one outlined by McCarthy et al. (45) could possibly increase the MF procedure results. He stated that until a more precise method is developed for enumerating fecal coliforms, a mathematical expression similar to the following may be useful:

$$\log MF = 0.012 + 0.942 \log MPN$$

The correlation coefficient for his regression was 0.987.

Braswell and Hoadley (39) evaluated the MFC medium for recovery of E. coli that had been subjected to the stress of chlorination. He found that the ability of the bacteria to grow on the MFC broth decreased as the chlorine contact time increased. Mowat (9) found similar results.

Bissonnette et al. (49) examined E. coli from various aquatic environments and found that selective media restricted growth when the bacteria had been injured sub-lethally. They found that the bacteria could recover when a non-selective medium was used for enumerating the organisms.

Presswood and Brown (50) examined two membrane filters made by different manufacturers. They found that higher results could be achieved with the Gelman GN-6 filter than with the Millipore HAWG 047SO filter. This led other investigators to study membrane filters more closely. Sladek et al. (51) found that higher fecal coliform counts could be achieved with a membrane filter composed of a mixture of

cellulose esters and having a pore size of 0.7 μm and surface opening of 2.4 μm. Green et al. (52) tested the recovery of fecal coliforms with six different filters. They also found that a filter (Millipore Type HC) with a pore size of 0.7 μm and surface opening of 2.4 μm gave the highest recoveries. Lin (53) compared two Millipore filters, types HA and HC, and found the type HC superior for fecal coliform enumerations.

Lin (54) proposed a modification to the MF procedure for stressed fecal coliform recovery consisting of two steps, the first being an enrichment step. The membrane filter was placed initially on a phenol red lactose broth and incubated for four hours at 35°C. The membrane was then transferred to MFC agar and incubated for 18 hours at 44.5°C. Lin compared his results with the standard MPN procedure and found good agreement between results obtained with the two.

Rose et al. (10) proposed an enrichment procedure for stressed fecal coliforms in which no transfer of the membrane filter was required. Lauryl tryptose agar (2 ml) was placed in a culture dish containing 5 ml of MFC agar. The membrane filter was placed on the two media and incubated at 35°C for two hours and then at 44.5°C for 22 hours. The authors theorized that the initial incubation period would allow the bacteria to recover from the effect of the chlorine while in contact with the non-selective lauryl tryptose agar. Upon transfer to the 44.5°C incubation, only fecal coliforms were presumably allow to grow, and the selective medium would have diffused into the lauryl tryptose agar by

this time. The highly selective MFC agar would further restrict growth of nonfecal coliforms.

Green et al. (55) used a preincubation period of five hours at 35°C followed by incubation at 44.5°C for 19 hours. The authors found that incubation periods in excess of five hours allowed nonfecal organisms to proliferate and interfere with identification of typical coliform colonies. This five hour preincubation period may be accepted for inclusion in the 15th Edition of Standard Methods for recovery of stressed organisms (56,57).

Presswood and Strong (58) eliminated rosolic acid from MFC medium and found that recovery of fecal coliforms from chlorinated sewage could be improved. The enumerations without rosolic acid were higher 77 percent of the time. The elimination of the rosolic acid has been suggested as a modification to the fecal MF procedure when the fecal coliforms are stressed (57).

III. METHOD AND MATERIALS

The basic experimental plan of this study involved analyzing secondary-treated, settled sewage before and after chlorination for fecal coliforms by several methods. The tests performed, as stated in the introduction, included the fecal MPN and the fecal MF procedures as outlined by Standard Methods (4) and a modified fecal MF procedure designed for improved recoveries of stressed fecal coliforms. Fifteen sets of samples were obtained beginning on June 30, 1977 and ending on August 18, 1977. Samples were immediately returned to the Sanitary Engineering Laboratory at Virginia Polytechnic Institute and State University where fecal coliform testing began. The period between sample collection and coliform testing never exceeded six hours. Standard Methods (4) procedures were followed at all times unless otherwise indicated.

Sample Collection

Sewage used in this study was obtained from the Stroubles Creek Sewage Treatment Plant of the Blacksburg-VPI Sanitary Authority, Blacksburg, Virginia. The treatment scheme at the plant consisted of grit removal, primary settling, trickling filtration with recycle, secondary settling, and chlorination. The unchlorinated samples were taken from the secondary settling tank near the effluent weir; thus, the wastewater had been subjected to the full settling period. Chlorinated samples were taken from the chlorine contact tank near

the effluent weir, thus permitting the maximum chlorine contact time for the particular flow at the time of collection. Flow measurements of the wastewater passing through the plant were recorded. However, because the flow recorder was located at the influent of the plant, flow adjustments were made to estimate the actual flow at the time of sampling. It was estimated by plant operators there was a period of 1 1/2 hours between the time the flow entered the plant and the time it reached the chlorine contact tank at high flow periods. Therefore, flow rates used to calculate the chlorination time at the time of sample collection were those for sewage that had entered the plant one and one-half hours earlier.

Samples for fecal coliform determinations were collected in 160-ml wide-mouth, glass, milk-dilution bottles that had previously been autoclave sterilized. Before sterilization, approximately 0.1 ml. of a 10 percent solution of sodium thiosulfate solution was added to each bottle. All sample bottles contained the dechlorinating agent because the sewage applied to the trickling filters was chlorinated on occasions to control filter flys and other insects. Additional samples were collected and analyzed to characterize the physical and chemical nature of the wastewater at each time and location where fecal coliform samples were collected. The following were determined: pH, dissolved oxygen (DO), temperature, total chlorine residual (in the chlorine contact tank), suspended solids, and turbidity. Measurements of pH, DO, temperatrue, and total chlorine residual were made at the treatment plant. Measurements of

pH were made at the treatment plant laboratory with a pH meter (Model 7, Corning Scientific Instruments). Measurements of DO and temperature were made in stream with a YSI meter (Model 54ARC, Yellow Springs Instrument Co.). Samples for total chlorine residual determinations were collected in 1000 ml. beakers and then measured with an amperometric titrator (Model 17T1010, Fischer & Porter Co.).

Samples for the suspended solids and turbidity determinations were collected in 500-ml., wide-mouth, polyethylene bottles that had been thoroughly washed. For suspended solids determinations, the wastewater samples were filtered through 0.45 μm filters (Type HA, Millipore Corporation). Weight determinations were made with a precision balance (Model H10, Mettler Instrument Corporation). Turbidity measurements were made with a turbidimeter (Model 2100A, Hach Chemical Corporation).

Fecal Coliform Enumerations

As stated previously three procedures were used for enumeration of fecal coliforms. Each of these methods required serial dilutions for each sample tested. Dilutions were made in sterile,glass, milk-dilution bottles containing 99 ml. of dilution water that had been autoclave sterilized at 121°C (15 psi pressure) for 15 minutes. The dilution water consisted of distilled water plus 0.1 percent peptone (4, 59, 60). Dilutions ranged from 1:1,000 (10^{-3}) to 1:1,000,000 (10^{-6}) for samples from the secondary settling tank and from 1:1 (10^{0}) to 1:10,000 (10^{-4}) for samples from the chlorine contact tank. A diagram

showing the processes for the fecal coliform enumeration tests used in this study is shown in Figure 1.

Fecal MPN procedure. The fecal MPN procedure (4) employed in this study consisted of a five-tube, serial-dilution analysis for each of the samples. The test included both the presumptive and the confirmed tests. The presumptive test was performed by making inoculations of 1.0 ml. of the serial dilutions of the original sample into tubes containing an inverted Durham tube and 10 ml. of lauryl tryptose broth (Baltimore Biological Laboratories). The tubes were incubated at 35° ± 0.5°C for 48 hours in an incubator (Model 330, Napco). At the end of this period, those tubes exhibiting growth and gas formation were recorded as positive. The confirmed test was performed by placing three loopsful (3mm loop) from each positive presumptive tube into tubes containing an inverted Durham tube and 5 ml. of EC broth. Much variation was seen between the the positive results of the presumptive and confirmed tests. In an effort to eliminate these variations, larger volumes of inoculum were transferred to the confirming broth on several occasions. Both 0.1 ml. and the standard three loopsful of the presumptive broth were transferred on several occasions to determine if the larger inoculum might produce more confirmed samples. No differences were seen so the standard procedure (three, 3-mm loopsful) was followed for all other tests. The tubes were then incubated at 44.5° ± 0.2°C for 24 hours in a water bath (Coliform Incubator Bath, GCA-Precision Scientific

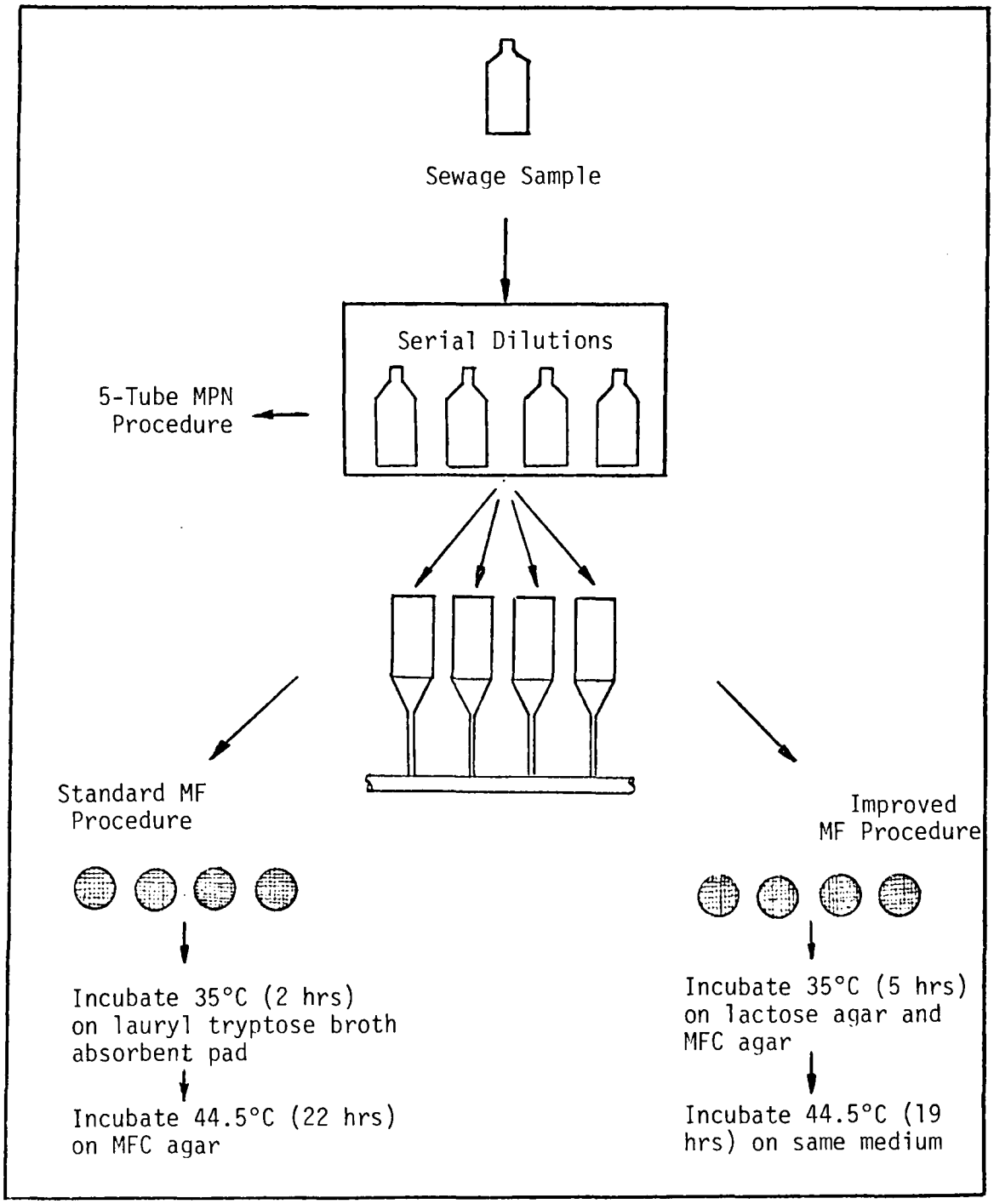

FIGURE 1. Processes for Fecal Coliform Enumerations Using the Standard MF, Improved MF, and MPN Procedures.

Company). Tubes exhibiting growth and gas formation were recorded as positive. At the completion of the confirmed test the statistical tables of Standard Methods (4) were consulted to determine the fecal coliform concentrations of the samples.

Fecal MF procedures. The standard fecal MF procedure (4) (SF-MF) and the improved fecal MF procedure (10) (IF-MF) were performed by filtering four, 10 ml. replicates of at least two serial dilutions for each sample analyzed. The highest dilutions were filtered first.

Filtrations were performed through filter funnels (Model E30, Gelman Instrument Co.). Six funnels were arranged so six replicate samples could be filtered simultaneously. The configuration of the filter apparatus is shown in Figure 2. Valves were installed so that any funnel not in use could be sealed off from the manifold while samples were being filtered through the others. A valve was also installed at the end of the tubing to permit release of the vacuum after filtration was completed. The filtration apparatus was connected to a vacuum pump (Model 13152, Gelman Instument Co.).

The filter funnels were disinfected between sample analyses by an ultraviolet lamp (Model G8T5, General Electric Company). The sides of the funnels were washed with sterile water between filtrations because this practice had been demonstrated to be adequte in preventing contamination of the sterile membrane filters by the previously filtered sample (7). Occasionally, membrane filteres were used to filter only sterile water and were then incubated to ensure that the method of

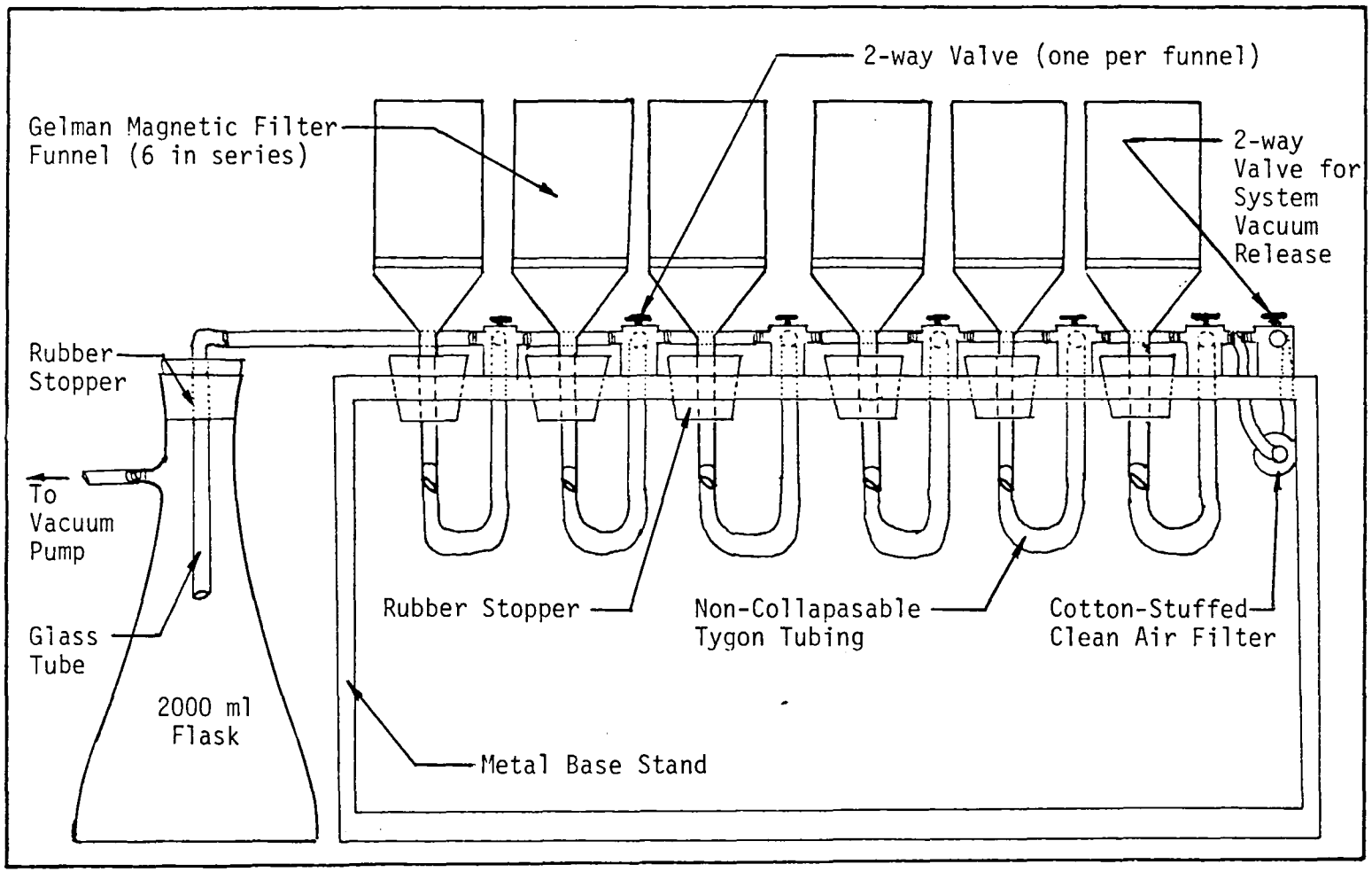

FIGURE 2. Membrane Filter Apparatus - After Brown (59)

sterilization was acceptable. The "blank filter" was eliminated from later analyses after it was demonstrated that there was no contamination by previously filtered samples.

The membrane filters (Millipore Corporation) were Type HC with a pore size of 0.7 μm, a surface opening diameter of 2.4 μm, and an overall diameter of 47 mm. The medium was M-FC broth (BBL) prepared from the dehydrated powder. To solidify the medium 1.5 percent purified agar (Difco Laboratories) was added. The medium was prepared according to instructions outlined in Standard Methods (4).

The SF-MF was performed by first placing the membrane filters (after filtration of the sample) on sterile, cellulosic absorbent pads (Millipore Corporation) contained in 50-mm sterile glass culture dishes. The pads had been moistened with from 1.8 to 2.0 ml. of lauryltryptose broth (BBL). The culture dishes were then placed in sealable plastic bags (Whirl-Pak type, Nasco). Wet paper towels were placed in the bags to assure that the dishes did not dehydrate during incubation. The dishes were then incubated in the 35°C incubator for two hours. At the end of this period the membrane filters were removed from the absorbent pads and placed in 50 mm, sterile, plastic culture dishes (Millipore Corporation) that contained 5.0 ml. of MFC media. The culture dishes were resealed in the plastic bags and immersed in the 44.5°C water bath for 22 hours. The plates then were removed and the blue colonies appearing upon the plates were counted by examination under a microscope at 19.5x magnification. Only blue colonies greater than 0.3 mm were considered to be fecal coliform. Colonies less than 0.3 mm

were considered atypical and, hence, were not considered to be fecal coliforms.

The IF-MF technique consisted of placing 2.0 ml of lactose agar (lactose broth containing 1.5 percent agar) on 5.0 ml of solidified MFC agar contained in 50-mm, plastic, culture dishes. The lactose agar overlay was placed on the MFC agar within one hour of placement of the membrane filter to ensure that diffusion of the selective medium into the lactose agar could be held to a minimum (10). The dishes were sealed in the plastic bags and placed in the 35°C incubator for 5 hours and then transferred to the 44.5°C water bath and incubated for an additional 19 hours. At the end of this period, the blue colonies on the membrane filter were counted.

Confirmations of fecal coliforms were performed occasionally by randomly selecting 10 colonies from membrane filters used in the IF-MF analysis of samples from both sampling locations. The colonies were inoculated into test tubes containing 5 ml of EC broth as in the procedure for confirmation in the MPN test.

Non-Fecal Coliform Investigations

The small, blue-tinted, colonies, which were found in samples from both the secondary settling tank and the chlorine contact tank, were observed with both the SF-MF and IF-MF techniques. Some of these colonies were transferred to an enriched agar medium (1.5 percent) containing trypticase-soy broth and yeast extract (0.3 percent) and incubated until well-defined, isolated colonies had developed. The

colonies were examined after staining by the Gram-stain technique and were transferred into lauryl tryptose broth and EC broth to determine if gas would be produced. Colony color and morphology also were noted.

IV. RESULTS OF STUDY

General

During a two month period, a total of fifteen samples were collected from both the secondary settling and the chlorine contact tanks and analyzed for fecal coliforms using three testing procedures. Other physical parameters were also measured in an effort to characterize the sewage. These data will be presented in this portion. A statistical analysis was also performed to determine which testing procedure gave significantly different results. These data will be included also. The following comparisons of recoveries were made between:

1. The IF-MF and the SF-MF procedures.
2. The IF-MF and the MPN procedures.
3. The SF-MF and the MPN procedures.

The bacterial recoveries were examined to determine if any variations could be related to the physical and chemical characteristics of the sewage samples.

Fecal Coliform Determinations

Enumerations. Fecal coliform concentrations, expressed as microorganisms per 100 ml, were determined by each testing procedure. The mean, fecal coliform concentrations for both the secondary settling and chlorine contact tanks, as were determined by each testing procedure, are presented in Tables II and III. The actual MF plate counts are presented in Appendix Tables A-I and A-II for the secondary settling

TABLE II

Fecal Coliform Concentrations
in Secondary Settling Tank[a]

Trial Number	Date	Fecal Coliforms per 100 ml*		
		SF-MF[b]	IF-MF[c]	MPN[d]
1	June 30	6.60×10^7	5.80×10^7	2.40×10^6
2	July 2	1.70×10^7	2.30×10^7	4.60×10^5
3	July 5	3.40×10^5	1.20×10^6	3.30×10^5
4	July 8	1.02×10^6	1.66×10^6	7.00×10^5
5	July 12	9.70×10^5	4.40×10^6	1.30×10^6
6	July 14	7.20×10^5	2.60×10^6	4.90×10^5
7	July 19	5.30×10^5	2.70×10^6	3.30×10^5
8	July 23	3.70×10^5	1.60×10^6	4.90×10^5
9	July 25	4.60×10^5	2.50×10^6	3.30×10^5
10	August 1	4.30×10^5	6.40×10^5	7.90×10^5
11	August 3	2.90×10^5	5.70×10^5	3.30×10^5
12	August 5	4.80×10^5	7.50×10^5	4.60×10^5
13	August 8	7.80×10^5	1.11×10^6	2.40×10^6
14	August 10	8.40×10^5	1.03×10^6	7.00×10^5
15	August 18	1.02×10^6	1.28×10^6	1.10×10^6

a. Sewage from secondary settling tank of Blacksburg-VPI Sewage Authority Treatment Plant, Blacksburg, Va.

b. Standard fecal coliform membrane filter technique (4).

c. Improved fecal coliform membrane filter technique (10).

d. Most probable number technique (4).

* All MF counts based on the mean of four replicate plates. MPN based on 5-tube serial dilution.

TABLE III

Fecal Coliform Concentrations in Chlorine Contact Tank[a]

Trial Number	Date	Fecal Coliforms per 100 ml*		
		SF-MF[b]	IF-MF[c]	MPN[d]
1	June 30	2.00×10^5	7.90×10^6	2.20×10^3
2	July 2	4.00×10^2	4.00×10^2	$<2.00 \times 10^2$ e
3	July 5	1.00×10^4	3.30×10^4	7.90×10^3
4	July 8	2.00×10^3	3.10×10^3	$<2.00 \times 10^2$ e
5	July 12	2.90×10^3	7.90×10^3	3.30×10^3
6	July 14	2.40×10^3	6.00×10^3	$<2.00 \times 10^2$ e
7	July 19	1.80×10^3	5.90×10^3	$<2.00 \times 10^2$ e
8	July 23	3.90×10^3	3.00×10^3	1.3×10^3
9	July 25	9.50×10^4	2.61×10^5	1.60×10^5
10	August 1	1.20×10^3	3.50×10^3	2.40×10^4
11	August 3	6.00×10^1	1.70×10^2	8.00×10^1
12	August 5	1.60×10^3	3.20×10^3	4.90×10^3
13	August 8	1.12×10^3	3.90×10^3	7.90×10^3
14	August 10	9.00×10^1	3.10×10^2	4.90×10^2
15	August 18	4.00×10^2	1.35×10^3	2.20×10^3

a. Sewage from chlorine contact basin of Blacksburg-VPI Sewage Authority Treatment Plant, Blacksburg, Va.

b. Standard fecal coliform membrane filter technique (4).

c. Improved fecal coliform membrane filter technique (10).

d. Most probable number technique (4).

e. MPN results were all negative tubes.

* All MF counts based on the mean of four replicate plates. MPN based on 5-tube serial dilution.

tank and Appendix Table A-III and A-IV for the chlorine contact tank. Included with the data are the standard deviations. Data for the presumptive and confirmed tests of the MPN procedure for analyses on the secondary settling tank and the chlorine contact tank are presented in Appendix Tables A-V and A-VI. There was a high percentage of false positives in the presumptive test from both sampling points.

The fecal coliform concentrations, as determined by the MF procedures for trial numbers 1 through 9, included minute colonies that were less than 0.3 mm in diameter. These colonies were later found to be organisms other than fecal coliforms and were excluded when enumerations were made during the final six sampling periods.

Data comparisons. The fecal coliform concentrations recovered by the three testing procedures are compared in Tables IV and V. In Table IV the ratios of recoveries of the three techniques and the log differences are presented for samples analyzed from the secondary settling tank. The same data are presented in Table V for samples analyzed from the chlorine contact tank. The data show that usually there was increased recovery when the IF-MF technique was used when compared to the SF-MF procedure. However, no general statement can be made concerning a comparison of recoveries by the MPN and MF techniques. Therefore, recoveries by each procedure have been compared to recoveries by each of the other methods.

The recoveries by all three techniques are shown in Figure 3 for samples collected from the secondary settling tank and in Figure 4

TABLE IV

Ratio and Log Differences of Fecal
Coliform Recoveries from Secondary Settling Tank

Trial Number	IF-MF[a] / SF-MF[b] Ratio	IF-MF[a] / SF-MF[b] Log Difference[d]	IF-MF / MPN[c] Ratio	IF-MF / MPN[c] Log Difference[d]	SF-MF / MPN Ratio	SF-MF / MPN Log Difference[d]
1	0.9	-0.06	24.2	1.38	27.5	1.44
2	1.4	0.13	50.0	1.70	40.0	1.60
3	3.5	0.54	3.6	0.56	1.0	0.00
4	1.6	0.20	2.4	0.38	1.5	0.18
5	4.5	0.65	3.4	0.53	0.8	-0.10
6	3.6	0.56	5.3	0.72	1.5	0.18
7	5.1	0.71	8.2	0.91	1.6	0.20
8	5.2	0.72	3.3	0.52	0.6	-0.22
9	5.4	0.73	7.6	0.88	1.4	0.15
10	1.5	0.18	0.8	-0.10	0.5	-0.30
11	2.0	0.30	1.7	0.23	0.9	-0.05
12	1.6	0.20	1.6	0.20	1.0	0.00
13	1.4	0.15	0.5	-0.30	0.3	-0.52
14	1.2	0.08	1.5	0.18	1.2	0.08
15	1.3	0.11	1.2	0.08	0.9	-0.05

a. Improved fecal coliform membrane filter technique (10).

b. Standard fecal coliform membrane filter technique (4).

c. Most probable number technique (4).

d. Calculated as follows: $\log \left(\frac{\text{mean of test}}{\text{mean of test}}\right)$
Positive numbers indicate ratio is greater than one.

TABLE V

Ratio and Log Differences of Fecal
Coliform Recoveries from Chlorine Contact Tank

Trial Number	IF-MF[a] / SF-MF[b]		IF-MF / MPN[c]		SF-MF / MPN	
	Ratio	Log Difference[d]	Ratio	Log Difference[d]	Ratio	Log Difference[d]
1	39.5	1.60	3570.0	3.56	91.0	1.96
2	1.0	0.00	>2.0	>0.30[e]	>2.0	>0.30[e]
3	3.3	0.52	4.2	0.62	1.3	0.11
4	1.6	0.20	>15.5	>1.19[e]	>10.0	>1.00[e]
5	2.7	0.43	2.4	0.38	0.7	-0.15
6	2.5	0.40	>30.0	>1.48[e]	>12.0	>1.08
7	3.3	0.52	>29.5	>1.47[e]	>9.0	>0.95
8	0.8	-0.10	2.3	0.36	3.0	0.48
9	2.8	0.45	1.6	0.20	0.6	-0.22
10	2.9	0.46	0.2	-0.70	0.1	-1.30
11	2.8	0.45	2.1	0.32	0.8	-0.10
12	2.0	0.30	0.7	-0.15	0.3	-0.52
13	3.5	0.54	0.5	-0.30	0.1	-1.00
14	3.4	0.53	0.6	-0.22	0.2	-0.70
15	3.4	0.53	0.6	-0.22	0.2	-0.70

a. Improved fecal coliform membrane filter technique (10).

b. Standard fecal coliform membrane filter technique (4).

c. Most probable number technique (4).

d. Calculated as follows: $\log \left(\frac{\text{mean of test}}{\text{mean of test}}\right)$
Postive numbers indicate ratio is greater than one.

e. MPN results were all negative tubes.

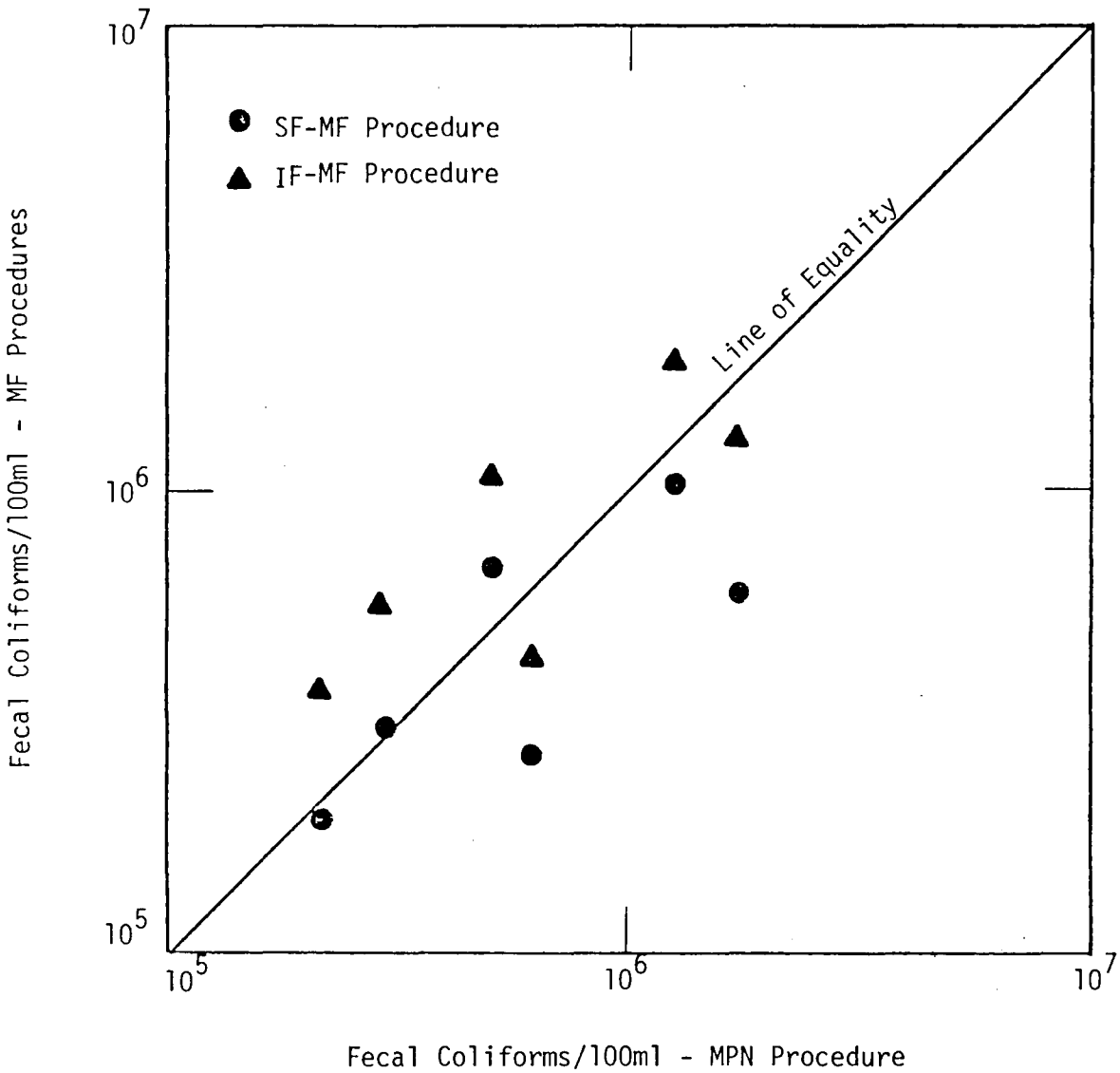

FIGURE 3. Comparisons of Fecal Coliform Recoveries From the Secondary Settling Tank Using Two MF Procedures and the MPN Procedure.

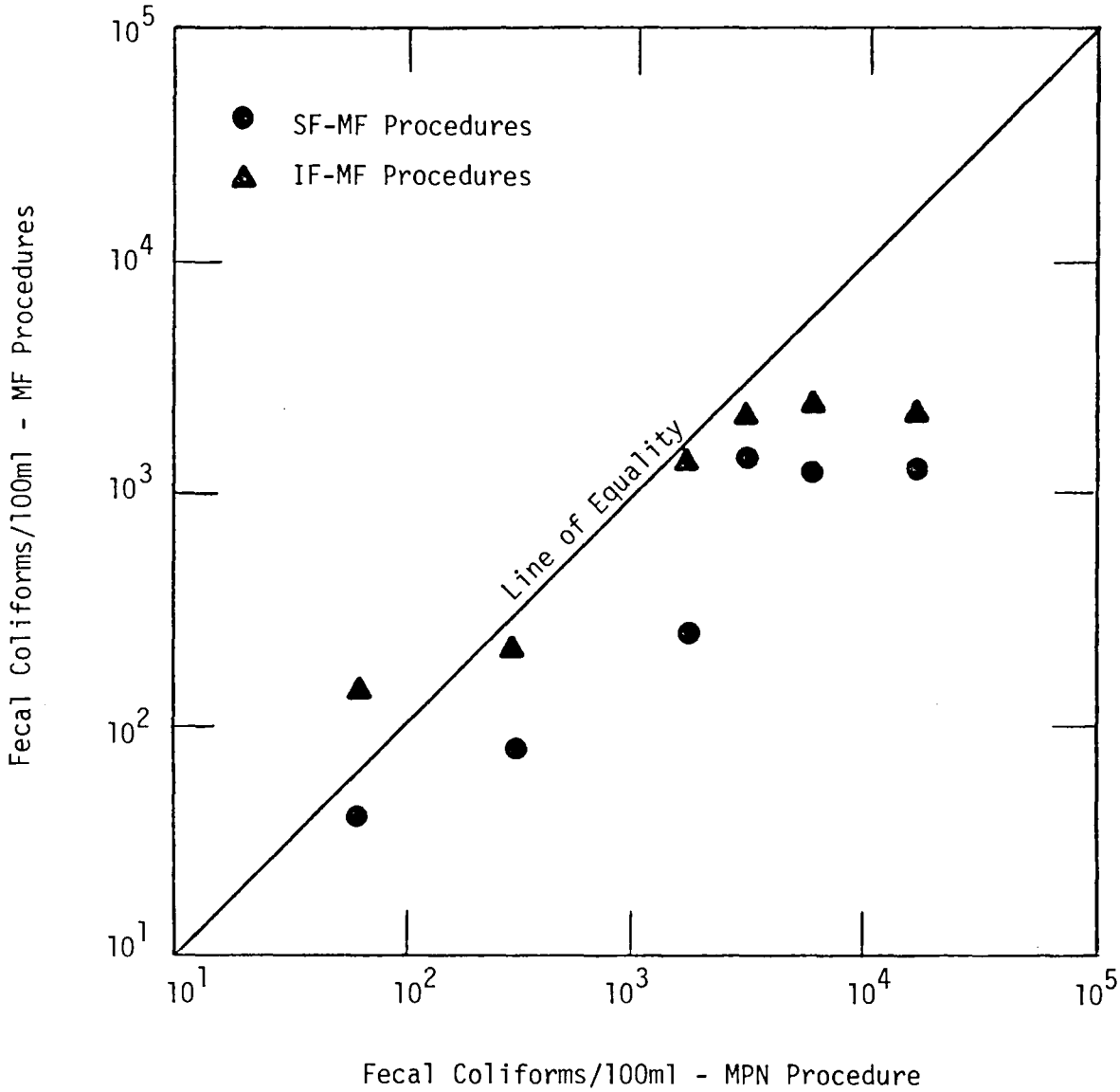

FIGURE 4. Comparisons of Fecal Coliform Recoveries From the Chlorine Contact Tank Using Two MF Procedures and the MPN Procedure.

for samples collected from the chlorine contact tank. This type of graphical comparison was adopted from Green et al. (55). In these figures the two MF procedures are compared with the MPN procedure. Because non-fecal coliforms (the small, blue colonies less than 0.3 mm in diameter) were included in the first nine trials, only the last six trials are indicated on the figures. It can be seen from these figures that recoveries by the IF-MF were greater than those by the SF-MF technique in all cases. Recoveries by the IF-MF approximately were equal to the recoveries by the MPN technique for samples analyzed from the secondary settling tank. In the samples analyzed from the chlorine contact tank the MPN gave higher results than the IF-MF except in one sample run.

Verification of fecal coliforms. During the study period fecal coliform colonies, which were isolated on the IF-MF plates, were selected randomly to determine if they were truly fecal coliforms. One hundred and one colonies were examined from the IF-MF plates used during analyses of samples from the secondary settling tank, while one hundred colonies were examined from the IF-MF plates used to analyze samples from the chlorine contact tank. The confirmation step showed 95 percent of the colonies selected from the secondary settling tank were fecal coliforms. In the confirmation step for the chlorine contact tank only 88 percent of all the colonies selected were found to be fecal coliforms. However, it should be noted that on July 8 most all of the colonies selected were very minute (0.3 mm or less in size). If this sample is ignored, the confirmation percentage increases

TABLE VI

Verification of Fecal Coliforms From the Secondary Settling Tank as Recovered by the IF-MF[a]

Date	Colonies Examined	Colonies[b] Verified	Percent Verification
July 5	10	10	100
July 8	10	10	100
July 12	13	13	100
July 23	8	8	100
July 25	10	9	90
August 1	10	9	90
August 3	10	10	100
August 5	10	9	90
August 8	10	8	80
August 10	10	10	100
August 18	10	10	100
		Mean	95
		Std. Dev.	6.9

a. Improved fecal coliform membrane filter technique (10).

b. Colonies were verified by transferring them to EC medium for 24 hours at 44.5°C. A test was considered positive when gas was formed.

TABLE VII

Verification of Fecal Coliforms from the Chlorine Contact Tank as Recovered by the IF-MF[a]

Date	Colonies Examined	Colonies[b] Verified		Percent Verification
July 5	10	10		100
July 8	10	2		20
July 12	12	11		92
July 23	8	8		100
July 25	10	10		100
August 1	10	9		90
August 3	10	10		100
August 5	10	10		100
August 8	10	9		90
August 10	10	9		90
August 18	10	10		100
			Mean	89
			Std. Dev.	23.5
		Excluding July 8 data:	Mean	96
			Std. Dev.	4.9

a. Improved fecal coliform membrane filter technique (10).

b. Colonies were verified by transferring them to EC medium for 24 hours at 44.5°C. A test was considered positive when gas was formed.

TABLE VIII

Statistical Comparison of Three Testing Procedures at Two Sampling Points

Location of Sample	Comparison	Statistical Comparison	Significance Level at or Greater than [c]	
			0.05	0.01
Sec. Settl. Tnk.	SF-MF with MPN[a]	MPN>SF-MF	Yes	No
Sec. Settl. Tnk	IF-MF with MPN[a]	IF-MF>MPN	No	No
Sec. Settl. Tnk.	SF-MF with IF-MF[b]	IF-MF>SF-MF	Yes	Yes
Chl. Con Tnk.	SF-MF with MPN[a]	MPN>SF-MF	Yes	Yes
Chl. Con. Tnk.	IF-MF with MPN[a]	MPN>IF-MF	Yes	No
Chl. Con. Tnk.	SF-MF with IF-MF[b]	IF-MF>SF-MF	Yes	Yes

a. Analysis by paired-t test.

b. Two-way analysis of variance.

c. See Appendix Tables B-I through B-VI.

to 96 percent. These data are presented in Tables VI and VII.

Statistical analysis of data. Data obtained by each of the three procedures during the final six sampling periods were analyzed statistically to determine which of the methods gave the best fecal coliform recoveries. The statistical analysis was conducted in two phases, one in which each MF procedure was compared with the MPN procedure and another wherein the two MF procedures were compared. A paired-t test (62) was used to compare the MF procedures and the MPN procedures. These data are presented in Appendix Tables B-I and B-II for the secondary settling tank and Appendix Tables B-III and B-IV for the chlorine contact tank. Data obtained by the SF-MF and IF-MF procedures were subjected to a two-way analysis of variance (ANOVA). The analysis was performed by a statistical Analysis System (SAS) computer program (SAS 76) at VPI&SU. These test results are presented in Appendix Table B-V for the secondary settling tank and Appendix Table B-VI for the chlorine contact tank. All plate counts were included in both analyses of the MF procedures. Only the means of the data were used in the analysis of the MF procedure and the MPN procedure. The results of these analyses are presented in Table VIII.

Physical Parameter Measurements

The physical and chemical parameters measured in samples from the secondary settling tank and chlorine contact tank are presented in Tables IX and X. Some problems were encountered with obtaining total suspended solids concentrations due to inaccuracies in drying and

TABLE IX

Physical Parameter Measurements of

Samples from Secondary Settling Tank

Trial Number	Total Suspended Solids,[a] mg/l	pH	DO, mg/l	Temperature, °C	Turbidity,[b] NTU [c]
1	17	6.8	2.8	19.0	8.5
2	8	6.8	2.2	22.0	7.0
3	29	7.0	2.8	21.5	12.0
4	8	6.6	2.3	23.0	7.5
5	7	6.6	3.4	23.0	8.6
6	-	6.7	3.4	23.0	9.4
7	-	6.8	3.6	23.0	7.1
8	-	6.8	4.5	22.5	9.2
9	-	6.8	3.7	23.0	12.0
10	5	6.9	4.0	23.0	11.0
11	28	6.8	3.6	23.0	12.0
12	10	7.1	4.5	23.5	10.0
13	13	6.9	4.2	23.5	11.0
14	2	6.8	3.4	23.5	10.0
15	19	6.8	3.8	23.5	13.0

a. Total Suspended Solids - mean of three measurements.

b. Turbidity - mean of three measurements.

c. NTU = nephelometric turbidity units.

TABLE X

Physical Parameter Measurements of
Samples from Chlorine Contact Tank

Trial Number	Total Suspended Solids,[a] mg/l	pH	DO, mg/l	Temperature, °C	Turbidity,[b] NTU[c]	Total Chlorine Residual,[d] mg/l	Chlorination Time, Min.
1	23	6.8	3.9	19.0	13.0	1.37	19
2	3	6.7	3.3	22.0	7.0	1.40	43
3	38	6.9	3.9	21.5	13.0	1.00	21
4	11	6.6	3.3	23.0	9.6	1.55	21
5	14	6.7	4.1	23.0	9.1	1.10	27
6	-	6.7	4.5	23.0	10.0	1.23	23
7	-	6.7	4.5	23.0	9.1	1.48	24
8	-	6.8	5.4	22.5	9.0	1.20	29
9	-	6.7	4.7	23.0	11.0	1.00	21
10	5	6.8	4.7	23.0	10.0	1.08	23
11	19	6.8	4.6	23.0	13.0	2.05	24
12	21	7.0	5.1	23.5	12.0	1.45	24
13	12	6.8	4.8	23.5	12.0	1.15	24
14	5	6.8	4.4	23.5	10.0	1.25	23
15	28	6.8	4.6	23.5	13.0	1.00	23

a. Total Suspended Solids - mean of three measurements
b. Turbidity - mean of three measurements
c. NTU - nephelometric turbidity units.
d. Total Chlorine Residual - mean of two measurements.
e. Chlorination Time - calculated as a plug flow reactor.

weighing of filters. This is the reason no data were obtained in trial numbers 6 through 9. Total chlorine in the chlorine contact chamber effluent varied considerably during the study period. Turbidity, DO, and suspended solids measurements also varied greatly. Measurements of pH and temperature were relatively constant during the study period.

Non-Fecal Coliform Testing Results

The minute colonies that were seen in all of the samples were isolated from several MF plates in an effort to determine the nature of the organisms. Several colonies less than 0.3 mm diameter were picked at random.

The organisms grew first on the enriched T-soy agar medium. Upon isolation of the colonies, it was found that the organisms were either Gram-positive cocci or Gram-negative rods. Both types of organisms grew when placed in lauryl tryptose broth and incubated at 35°C but only the Gram-positive organisms produced gas. When the Gram-positive organisms were placed in EC broth at 44.5°C the growth was seen again but with no gas production. The Gram-negative, rod, shaped bacteria appeared to be of the genus Pseudomonas (based on color, odor and colony characteristics), while the genus of the Gram-positive organisms was not determined.

Comparisons of Environmental Conditions to Fecal Coliform Recoveries

In the comparisons of environmental conditions to fecal coliform recoveries from the secondary settling and chlorine contact tanks,

temperature and pH were not considered because these parameters were relatively constant throughout the study. No comparisons were made of the parameters measured in the secondary settling tank with fecal coliform recoveries because of the inconsistent variations.

In Figure 5, the fecal coliform recoveries obtained with the IF-MF from the chlorine contact tank are compared with the total chlorine residual. Only the last six trials are shown. From this graph it can be seen that the variation is very large between chlorine residuals and it is not possible to discern a well-defined trend in the relationship between chlorine residual and the number of fecal coliforms recovered.

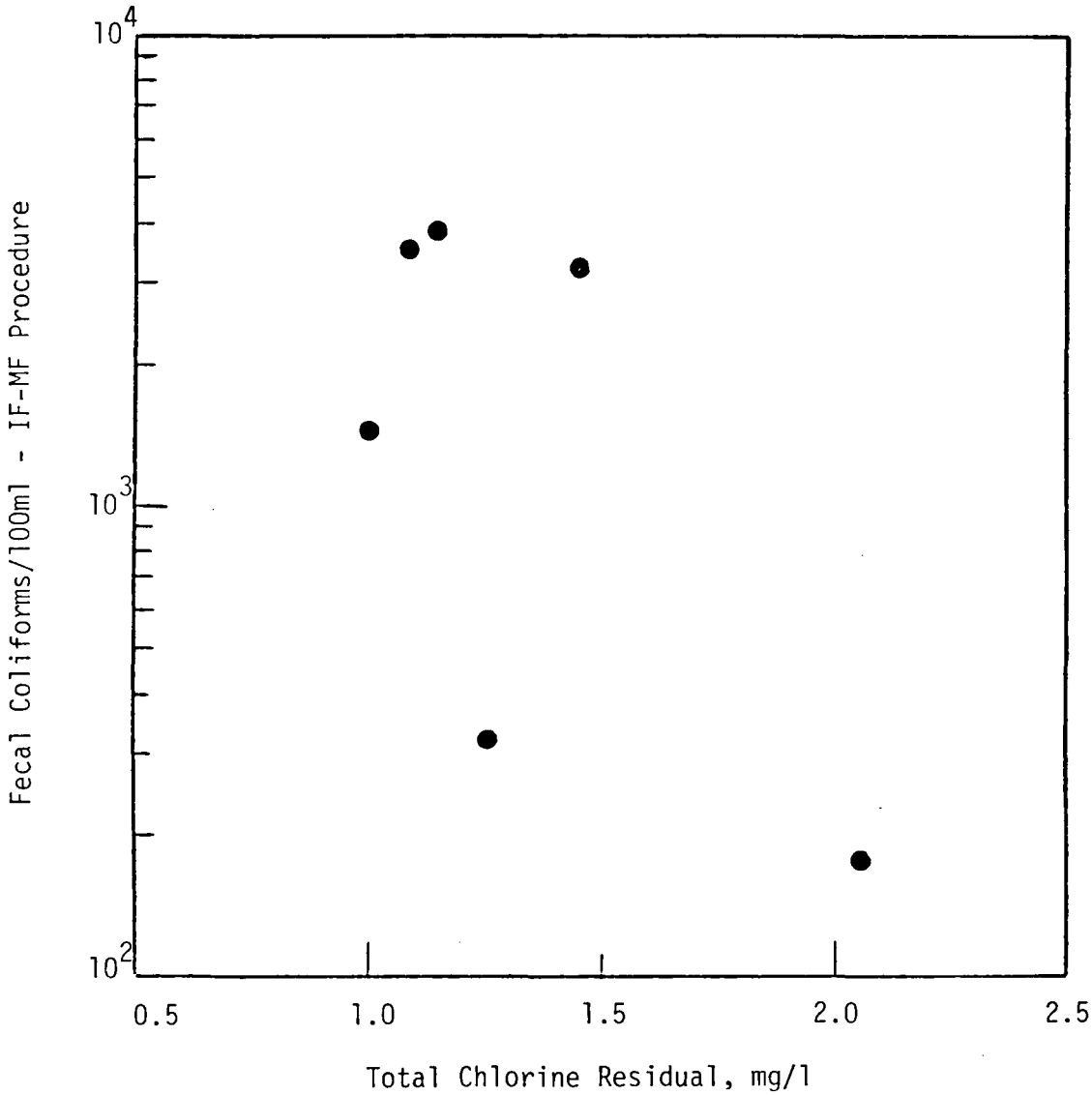

FIGURE 5. Comparisons of Fecal Coliform Recoveries by the IF-MF Procedure with Total Chlorine Residuals.

V. DISCUSSION OF RESULTS

Fecal Coliform Enumerations

Recoveries by the three testing procedures. The fecal coliform concentrations which were determined by the three testing procedures in the effluents from the secondary settling tank and the chlorine contact tank varied greatly during the study period. Samples were collected each day at approximately the same time in order to minimize coliform density variations caused by variations in flow and waste strength. However, due to the large fluctuations that do occur naturally in a sewage treatment plant from day to day, the variations could not be totally eliminated.

Samples from the secondary settling tank and the chlorine contact tank, when analyzed by both of the MF procedures, yielded very minute, blue colonies in addition to the larger blue colonies produced typically by the fecal coliforms. The percentage of the smaller colonies varied from sample to sample. However, actual counts of the minute colonies, distinct from the others, were never obtained in the first nine tests because they were not recognized as "atypical". In some instances when the MPN results in the chlorine contact tank effluent were extremely low (indicating that very good disinfection had occurred) the small colonies appeared in very high numbers relative to the number of typical coliform colonies. The apparent higher recoveries by the MF procedures, relative to the MPN technique indicates that some organism or group of organisms are more resistant to disinfection by chlorination than the

fecal coliform organisms and do produce small, but visible colonies on the selective MF media. In some instances, recoveries of these minute colonies by the IF-MF procedure were greater than those by the SF-MF procedure in samples from the secondary settling tank.

Isolates of these smaller colonies grown on the enriched T-soy medium did not appear to be fecal coliforms. Several were Gram-positive cocci (unidentified) and another was a Gram-negative rod whose colony growth characteristics and odor were similar to Pseudomonas. When these isolates were inoculated into lauryl tryptose broth and then EC broth; it was proven that at least some of the minute blue colonies observed on the MF plates indeed were not fecal coliforms.

As has been indicated previously, these minute colonies were included in the bacterial counts of the first nine samples tested. Because they were not coliforms, these data cannot be used when recoveries by the three procedures are compared. In the last six samples, colonies included in the counts were approximately 0.3 mm or greater because the non-fecal colonies which were isolated were smaller than 0.3 mm. In the first nine analyses, the inclusion of the minute colonies greatly increased the apparent recoveries by the MF procedures compared to the MPN procedure. This is demonstrated by the very high ratios of the MF procedures to the MPN procedures that occurred in five out of the first nine sample runs (Tables IV-V). In the last six sample analyses, the ratios were not as variable. Lin (54) observed what he termed "minute, atypical colonies" but no determinations were made as to their morphology.

Other non-fecal growth was also observed with both MF procedures.

The colony diameters ranged from approximately 0.5 mm to 3 mm or greater. The colonies were white, pink or grey rather than the typical blue color. Several of the pink colonies were tested by the Gram-stain test and were found to be Gram-positive cocci.

A high percentage of false positives were observed in the presumptive MPN test with samples from both the secondary settling tank and the chlorine contact tank (see Appendix Tables A-V and A-VI). No firm conclusions could be made as to the reasons for the large differences between the presumptive and confirmed tests, but it was noted that the Gram-positive cocci isolated on the enriched T-soy medium produced gas in lauryl tryptose broth but none in the E.C. broth.

In the samples from the secondary settling tank and the chlorine contact tank, where the dilutions were low and suspended solids concentrations had not been adequately removed, the particulate matter could be seen on the plates of both MF procedures as blue particles. These blue particles occasionally interferred with the enumeration of fecal coliforms at low dilutions. As a result the higher dilutions with less background interference had to be used for determining the fecal coliform concentrations.

In the last six sample runs, the coliform concentrations in samples from the secondary settling tank, determined by the three analytical procedures were highly variable. Recoveries were lowest by the SF-MF procedure. The IF-MF technique produced recoveries from 1.2 to 2.0 times greater than the SF-MF procedure (Table IV). The average ratio between the two procedures was 1.5:1 with a standard deviation of

0.3:1. Apparently, the current standard MF technique for enumerating coliforms in chlorinated sewage effluent gives lower concentrations than the improved method, and the consideration that is being given to adopting the modified technique as the standard is justified.

The recoveries by the MPN procedure were higher in four of the final analyses. Recoveries in the remaining two tests were not greatly different. The ratios of the means of the SF-MF to the MPN ranged from 0.3:1 to 1.2:1 (Table IV) with an average of 0.8:1 and a standard deviation of 0.3:1. No conclusion could be made as to the reason the ratios ranged from less than 1.0 to greater than 1.0.

Recoveries by the IF-MF procedure for samples from the secondary settling tank were higher four out of the six sample runs than those by the MPN. The ratios of the IF-MF to the MPN ranged from 0.5:1 to 1.7:1 with an average of 1.2:1 and a standard deviation of 0.5:1. No conclusion could be made as to the reason the ratios ranged from greater than 1.0 to less than 1.0. It would appear that the IF-MF would be a more desirable substitute for the MPN than the standard MF technique when analyzing effluents from secondary settled sewage. The use of the IF-MF would be particularly convenient when results are needed in 24 hours as compared to the MPN.

Variations in results obtained upon analyses of the last six samples taken from the chlorine contact tank were also high. As was true when effluents from the secondary settling tank were analyzed, the lowest number of fecal coliforms recovered was by the SF-MF technique. In none of the six samples analyzed did the SF-MF produce

fecal coliform recoveries greater than those obtained by the IF-MF or MPN procedures. The IF-MF procedure produced fecal coliform counts from 2.0 to 3.5 times greater than the SF-MF procedure (Table V). The average of the ratios was 3.0:1 and the standard deviation was 0.6:1.

Rose et al. (10) found the ratios between recoveries by their improved, two-layer agar method (with a two-hour incubation at 35° C) and the direct fecal MF coliform procedure (4) to range from 3.8:1 to 38:1 when chlorinated sewage effluents were analyzed. However, it should be noted that the direct MF procedure used by Rose and his associates as their "standard" technique did not include a pre-enrichment step before placing the membrane on the selective agar, a step that is suggested by Standard Methods (4) for analysis of chlorinated effluents. Green et al. (55) analyzed for coliforms in chlorinated sewage effluents by both the direct MF procedure (4) and the direct MF procedure that included a five-hour preincubation period at 35°C. They found the modified procedure yielded higher results. It should be emphasized that the procedure followed in the study for this thesis included both of these modifications (enrichment and preincubation) and resulted in much higher recoveries than were observed with the procedure outlined in Standard Methods (4) that was used. The higher recoveries obtained when the IF-MF procedure was used indicated that it should be instituted as the standard procedure instead of the currently recommended procedure by Standard Methods (4).

The ratios of the fecal coliform recoveries of the SF-MF and the MPN ranged from 0.05:1 to 0.8:1, while the ratios for the IF-MF and

MPN procedures ranged from 0.2:1 to 2.1:1 (Table V). In the latter instance, the data would appear to indicate that the MPN is the better analytical method for chlorinated effluents, but it should be recalled that each MPN has a 95 percent confidence interval associated with it, and in this study, the average recovery by the IF-MF procedure was within the range of the MPN confidence interval in five of the last six analyses. Therefore, it seems reasonable that the modified procedure for stressed organisms is a reliable substitute for the MPN procedure. Rose et al. (10) did not compare recoveries by their modified MF procedure (involving two-hour preincubation at 35°C and a two-layer agar medium) with the MPN procedure.

It should be noted that the IF-MF procedure is easier to perform than the SF-MF procedure. The former method does not require any transfer of the filter from one medium to another, thus eliminating the possibility of contamination that could occur with the SF-MF procedure. The relative simplicity of the IF-MF technique could become an important factor when many samples are being tested at one time.

The ratios of recoveries by the two MF techniques (IF-MF/SF-MF) appear to increase as the amount of stress the bacteria are subjected to is increased. From this study, the data indicate that there is some "stress" to the fecal coliforms that results from the passage of sewage through the sewage system and the treatment processes (excluding chlorination), as is evidenced by the improvement in coliform recovery by the IF-MF procedure over that observed by the SF-MF procedure (ratio approx. 1.5:1). However, the improvement was even greater when the

coliforms were subjected to additional stress by chlorination, the ratio (IF-MF/SF-MF) increased to approximately 3.0:1.

Verification of fecal coliform colonies. As stated previously, blue colonies were selected at random from the IF-MF plates of both the secondary settling tank and the chlorine contact samples, transplanted to E.C. broth, and incubated for 24 hours at 35°C to verify if they were fecal coliforms. The presumptive step was eliminated from the procedure as it was theorized the lactose agar overlay in the plates had previously provided the necessary enrichment to permit the stressed coliforms to recover sufficiently and to be able to withstand the higher incubation temperature in the E.C. broth. To check for cross contamination of the plates between sample filtrations, blank filters were washed with sterile water and incubated on the MFC medium as in the SF-MF procedure. No blank filters showed signs of any fecal coliforms, thus eliminating any possibility of data bias. The verification of colonies regarded as coliforms recovered from the secondary settling tank samples was good enough to indicate that identification of fecal coliforms by the IF-MF technique is quite accurate. One-hundred and eleven colonies were examined and the mean verification was 95 percent with a standard deviation of 6.9 percent. The verification percentage for coliforms isolated from the chlorine contact tank was much higher when all the data are considered (89 percent of 110 colonies examined); but, this low percentage is biased by the results of July 8 when nearly all the colonies examined were approximately 0.3 mm in diameter. If data

from this sample date are excluded from the analysis, the verification percentage increases to 96 percent (standard deviation 4.9 percent). Thus, it may be concluded that the IF-MF procedure may give very accurate fecal coliform counts if the minimum diameter of colonies regarded as fecal coliforms is limited to 0.3 mm.

Statistical analysis. There were two types of statistical analyses of the data. As was stated previously, the paired-t test was used to compare the means of the MF procedures with the means of the MPN procedures. This particular analysis was selected because there were large variations in the numbers throughout the study period and because there were unequal numbers of observations by the various procedures. Whereas the MPN procedure resulted in only one value per analysis, the analyses by each MF procedure consisted of four replicate plates per sample. In the paired-t test, the means of the four replicates of each MF procedure were compared to the means of the MPN.

The means of the MF procedures used to analyze samples from the secondary settling tank and the chlorine contact tank were compared statistically by a two-way analysis of variance using the computer program SAS 76 entitled ANOVA. In each analysis, all the replicate plates were used for the computations. With this type of analysis the variations between replicates on any given day and the variations in fecal coliform densities from day to day could be accounted for.

The comparisons between the means of the IF-MF procedure and the means of the SF-MF procedure revealed that the formed produced higher

recoveries at the 0.01 and 0.05 significance levels (Table VIII) when samples from both the secondary settling tank and the chlorine contact tank were analyzed. The results obtained at these high significant levels indicate that the IF-MF procedure is a much better recovery test for fecal coliforms as compared to the SF-MF procedure no matter what stress the bacteria have been subjected to throughout the treatment process.

When the SF-MF technique was compared to the MPN procedure, the means of the MPN procedure for samples analyzed from the secondary settling tank were significantly higher at the 0.05 level only (Table VIII). However, when the results of the two procedures were compared for samples analyzed from the chlorine contact tank, the MPN gave higher recoveries at the 0.05 and 0.01 significance levels. These results would tend to indicate that the SF-MF procedure does not recover fecal coliforms adequately when the stress the bacteria are subjected to is increased as compared to the recoveries that are achievable with the MPN procedure. In any case the SF-MF procedure does not produce as high recoveries as the MPN procedure no matter what the degree of stress.

Although the IF-MF procedure gave higher recoveries than the MPN when samples from the secondary settling tank were analyzed, the means of the IF-MF were not greater than the MPN at the 0.01 or 0.05 significance levels. In the samples analyzed from the chlorine contact tank, the means of the MPN procedure were higher than the IF-MF procedure

means at the 0.05 significance level only. These analyses may indicate that the IF-MF procedure is an adequate substitute for the MPN procedure when samples from the secondary settling tank are to be analyzed but not for samples to be analyzed from the chlorine contact tank. However, it should be recalled that the IF-MF procedure results were within the 95 percent confidence interval of the MPN five out of the last six samples analyzed.

Physical Characterization of Sewage Samples

General. Each sample that was collected was analyzed for several physical and chemical parameters in an attempt to characterize the sewage at each sampling point as completely as possible. These data were desired because it was theorized that some relationship might exist between fecal coliform concentrations and one or more of the physical or chemical characteristics of the treated wastes.

Secondary settling tank. The samples from the secondary settling tank were analyzed for total suspended solids, pH, DO, temperature and turbidity (Table IX). The temperature of the sewage was between 23°C to 23.5°C throughout the last six sample runs while the pH was between 6.8 to 7.1 indicating a very good microbiological environment. The total suspended solids concentrations ranged from 2 to 28 mg/l and the turbidity varied from 10.0 to 13.0 N.T.U. The DO fluctuated from 3.4 to 4.5 mg/l, which is a very good aerobic environment. While these data did serve nicely to characterize the sample, there was no discernable relationship between the observed variations and the fecal coliform recoveries.

Chlorine contact tank. The physical parameters analyzed in samples from the secondary settling tank were also analyzed in the effluent from the chlorine contact tank. The total suspended solids varied from 5 to 28 mg/l and the turbidity varied from 10.0 to 13.0 N.T.U. The temperature of the wastewater ranged from 23.0°C to 23.5°C also, and the DO ranged from 4.4 to 5.1 mg/l. The pH variations were small, ranging from 6.8 to 7.0. All conditions were ideal for good microbial growth. The total chlorine residual, which was the major stress in this study, ranged from 1.00 to 2.05 mg/l. However, no relationships could be established between the variations in total chlorine residual and fecal coliform densities; and, as was the case with data collected from analyses of the effluents from the secondary settling basin, trends could not be established between variations in the fecal coliform densities with variations in physical and chemical characteristics of the wastes. The scatter of total chlorine residual data as a function of fecal coliform densities is shown in Figure 5.

VI. SUMMARY AND CONCLUSIONS

A comparative study of three coliform recovery techniques was conducted using samples obtained from the Blacksburg-VPI sewage treatment plant. The major objective was to determine if a modified, membrane filter analysis for stressed coliforms would improved recoveries over those observed by the standard procedures currently in use. The results indicate that higher estimates of the fecal coliform population can be obtained by this improved membrane filter technique. Some difficulties in enumerating fecal coliforms were encountered when the size of the colonies considered to be coliforms were not limited. Tiny (<0.3 mm) blue colonies, which proved to be other than fecal coliforms, interferred considerably. The following conclusions may be made based upon the results of this study for secondary treated sewage from a chlorine contact tank.

1. The improved membrane filter (IF-MF) technique, which consisted of a lactose agar layer over the MFC agar and a preincubation period at 35°C, permits higher fecal coliform recoveries than the standard fecal coliform membrane filter (SF-MF) technique when samples from the secondary settling tank and chlorine contact tank are analyzed. Four replicates for each technique were compared statistically and the mean recoveries with the IF-MF technique were significantly higher at the 0.05 and 0.01 levels.

2. Minute, non-fecal coliforms will grow on the membranes, regardless of whether the standard or modified procedure is used, and

will appear as blue colonies. To prevent these colonies from biasing the plate counts, the minimum diameter of colonies considered to be coliforms should be approximately 0.3 mm.

3. The MPN procedure gave higher recoveries than the SF-MF technique in samples from both the secondary settling and chlorine contact tanks. The IF-MF procedure gave as good results as the MPN technique when samples from the secondary settling tank were analyzed. The MPN was higher for samples from the chlorine contact tank, but in five out of six instances the mean IF-MF recovery was within the 95 percent confidence interval of the MPN.

4. Suspended solids can interfer with the enumeration of fecal coliforms when low dilutions are analyzed because the solids take up the stain in the selective medium and it is difficult to distinguish the particles from fecal coliforms. At higher dilutions this problem does not arise.

5. No correlations could be made between the physical and chemical characteristics of the sewage and the recoveries of fecal coliforms.

VII. RECOMMENDATIONS

1. Investigations should be made to determine if the minute colonies can be recovered from other treatment plants. A thorough investigation should establish whether the size of the colonies regarded by the investigator as typical coliforms is critical in obtaining accurate estimates of the coliform population.

2. The minute colonies that appear on the membranes should be studied to determine their exact identity and if they interject a bias in the coliform density.

3. A study should be conducted to determine the identity of colonies appearing to be coliforms on the membrane filters but do not produce gas in EC broth at 44.5°C.

VIII. LITERATURE CITED

1. Geldreich, E. E., Handbook for Evaluating Water Bacteriological Laboratories, U. S. Environmental Protection Agency, Cincinnati, Ohio (1975).

2. Sawyer, C. N. and McCarty, P. L., Chemistry For Sanitary Engineers, McGraw-Hill Book Company, New York, N.Y., 2nd Edition (1967).

3. Metcalf and Eddy, Inc., Wastewater Engineering Collection, Treatment, Disposal, McGraw-Hill Book Company, New York, N.Y. (1972).

4. Standard Methods for the Examination of Water and Wastewater, 14th Edition, American Public Health Association, Washington, D. C. (1975).

5. Geldreich, E. E., "Microbiology of Water," Jour. Water Poll. Control Fed., 49, 1222-1238 (1977).

6. McKee, J. E., McLaughlin, R. T., and Lesgourges, P., "Application of Molecular Filter Techniques to the Bacterial Assay of Sewage," Sewage and Industrial Wastes, 30, 245-252 (1958).

7. Hoffman, D. A., Kuhns, J. G., Stewart, R. C., and Crossley, E. I., "A Comparison of Membrane Filter Counts and Most Probable Numbers of Coliform in San Diego's Sewage and Receiving Waters," Jour. Water Poll. Control Fed., 36, 109 (1964).

8. Lin, S., "Evaluation of Coliform Tests for Chlorinated Secondary Effluents," Jour. Water Poll. Control Fed., 45, 498-505 (1973).

9. Mowat, A., "Most Probable Number Versus Membrane Filter on Chlorinated Effluents," Jour. Water Poll. Control Fed., 48, 724-728 (1976).

10. Rose, R. E., Geldreich, E. E., and Litsky, W., "Improved Membrane Filter Method for Fecal Coliform Analysis," Appl. Microbiol., 29 532-536 (1975).

11. Holden, W. S., Water Treatment and Examination, Williams and Wilkins Company, Baltimore, Maryland (1970).

12. Borchardt, J. A. and Walton, G., "Water Quality" in Water Quality and Treatment, 3rd Edition, American Water Works Association, McGraw-Hill Book Co., New York, N.Y. (1971).

13. Delaney, J. R., McCarthy, J. A., and Grasso, R. J., "Measurement of E. Coli Type 1 by the Membrane Filter," Water and Sewage Works, 109, 289-294 (1962).

14. Clark, H. F. and Kabler, P. W., "Re-evaluation of the Significance of the Coliform Bacteria," Jour. Am. Water Works Assn., 56, 931-935 (1964).

15. Geldreich, E. E., Bordner, R. H., Huff, C. B., Clark, H. F., and Kabler, P. W., "Type Distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals," Jour. Water Poll. Control Fed., 34, 295-300 (1962).

16. Geldreich, E. E., "Detection and Significance of Fecal Coliform Bacteria in Stream Pollution Studies," Jour. Water Poll. Control Fed., 37, 1722-1726 (1965).

17. Brock, T. D., Biology of Microorganisms, 2nd Edition, Prentice-Hall, Inc., Englewood Cliffs, N. J. (1974).

18. Geldreich, E. E., Huff, C. B., Bordner, R. H., Kabler, P. W., and Clark, H. F., "The Fecal Coli-Aerogenes Flora of Soils From Various Geographical Areas," Jour. Appl. Bacteriology, 25, 87-92 (1962).

19. Geldreich, E. E., "Fecal Coliform Concepts in Stream Pollution," Water and Sewage Works, 114, R-98 (1967).

20. Kehr, R. W., and Butterfield, C. T., "Notes on the Relation Between Coliforms and Enteric Pathogens," Pub. Health Rept., 58, 589 (1943).

21. Chambers, C. W., "Chlorination for Control of Bacteria and Viruses in Treatment Plant Effluent," Jour. Water Poll. Control Fed., 43, 228-240 (1971).

22. Underhill, R. E., "The Regrowth of Coliform and Fecal Streptococcus Bacteria in a Stream Receiving a Municipal Sewage Treatment Plant Effluent," Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va. (1973).

23. Saunders, Kathleen G., "Regrowth of E. Coli and S. Faecalis in Treated Sewage After Chlorination in a Continuous-Flow Reactor," Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va. (1974).

24. Deaner, D. G. and Kern, K. D., "Regrowth of Fecal Coliforms," Jour. Am. Water Works Assn., 61 465-468 (1969).

25. Schiemann, D. A., Manley, J. L., and Arnold, M. O., "Coliphage Interference in the Recovery of Coliform Bacteria," Jour. Water Poll. Control Fed., 48, 533-539 (1976).

26. Water Quality Standards, Virginia State Water Control Board, Richmond, Va. (1974).

27. Sewerage Regulations, Commonwealth of Virginia, Richmond, Va. (1977).

28. White, G. C., Handbook of Chlorination, VanNostrant Rienhold Co., New York, N.Y. (1972).

29. Heller, Buford B., "Disinfection of Secondary Treated Sewage by Chlorine in a Continuous Flow Reactor," Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va. (1975).

30. Woodfin, Williams L. Jr., "A Comparison of the Bactericidal Efficiency of Chlorine and Bromine Chloride in Municipal Wastewater," Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Va. (1975).

31. Virginia State Water Control Board, Minute 6 of the proceedings of the Feb. 18-19, Richmond, Va., 1974.

32. Moore, E., "Fundamentals of Chlorination of Sewage and Wastes," Water and Sewage Works, 98, 130-136 (1951).

33. Butterfield, C. T. et al., "Influence of pH and Temperature on the Survival of Coliforms and Enteric Pathogens when Exposed to Free Chlorine," Pub. Health Rept., 58, 1837-1866 (1943).

34. Butterfield, C. T., and Wattie, F., "Influence of pH and Temperature on the Survival of Coliforms and Enteric Pathogens when Exposed to Chloramine," Pub. Health Rept., 61, 157-192 (1946).

35. Heukelekian, H. and Smith, M. B., "Disinfection of Sewage with Chlorine," Sewage and Ind. Wastes, 22, 1509-1517 (1950).

36. Heukelekian, H. and Day, R. V., "Disinfection of Sewage with Chlorine, III: Factors Affecting Coliforms Remaining and Correlations of Orthotolidine and Aperometric Titration Chlorine Residuals," Sewage and Ind. Wastes, 23, 155-163 (1951).

37. Anderson, D. A., Introduction to Microbiology, C. V. Mosby Co., St. Louis, Mo. (1973).

38. McCarthy, J. A., Thomas, H. A., and Delaney, J. E., "Evaluation of the Reliability of Coliform Density Tests," Am. Jour. of Public Health, 48, 1628-1635 (1958).

39. Braswell, J. R. and Hoadley, A. W., "Recovery of Escherichia Coli from Chlorinated Secondary Sewage," Appl. Microbiol., 28, 328-329 (1974).

40. Geldreich, E. E., "Applying Bacteriological Parameters to Recreational Water Quality," Jour. Am. Water Works Assn., 62, 113-120 (1970).

41. Goetz, A., "Application of Molecular Filter Techniques to the Bacterial Assay of Sewage, I. Proposed Technique," Sewage and Ind. Wastes, 25, 1136-1143 (1953).

42. Geldreich, E. E., Clark, H. F., Huff, C. B., and Best, L. C., "Fecal-Coliform-Organism Medium for the Membrane Filter Techniques," Jour. Am. Water Works Assn., 57, 208-213 (1965).

43. Standard Methods for the Examination of Water and Wastewater, 13th Edition, American Public Health Association, New York, N.Y. (1971).

44. Thomas, H. A., and Woodward, R. L., "Estimation of Coliform Density by the Membrane Filter and the Fermentation Tube Methods," Amer. Jour. of Pub. Health, 45, 1431-1437 (1955).

45. McCarthy, J. A., Delaney, J. E., and Grasso, R. J., "Measuring Coliforms in Water," Water and Sewage Works, 108, 238-243 (1961).

46. Shipe, E. L. and Cameron, G. M., "A Comparison of the Membrane Filter with the Most Probable Number Method for Coliform Determinations From Several Waters," Appl. Microbiol., 2, 85-88 (1954).

47. Maxcy, R. B., "Non-Lethal Injury and Limitations of Recovery of Coliform Organisms on Selective Media," Milk Food Technology, 33, 445-448 (1970).

48. Taylor, R. H., Bordner, R. H., and Scarpino, P. V., "Delayed-Incubation Membrane-Filter Test for Fecal Coliforms," Appl. Microbiol, 29, 363-368 (1973).

49. Bissonnette, G. K., Jezeski, J. J., McFeters, G. A., and Stuart, D. G., "Evaluation of Recover Methods to Detect Coliforms in Water," Appl. Microbiol., 33 590-595 (1977).

50. Presswood, W. G. and Brown, L. R., "Comparison of Gelman and Millipore Membrane Filters for Enumerating Fecal Coliform Bacteria," Appl. Microbial., 26, 332-336 (1973).

51. Sladek, K. J., Suslavich, R. V., Sohn, B. L., and Dawson, F. W., "Optimum Membrane Structures for Growth of Coliform and Fecal Coliform Organisms," Appl. Microbiol., 30, 685-691 (1975).

52. Green, B. L., Clausen, E., and Litsky, W., "Comparison of the New Millipore HC with Conventional Membrane Filters for the Enumeration of Fecal Coliform Bacteria," Appl. Microbiol., 30, 697-699 (1975).

53. Lin, S. D., "Evaluation of Millipore HA and HC Membrane Filters for the Enumeration of Indicator Bacteria," Appl. Microbiol., 32, 300-302 (1976).

54. Lin, S., "Membrane Filter Method for Recovery of Fecal Coliforms in Chlorinated Sewage Effluents," Appl. Microbiol., 32, 544-552, (1976).

55. Green, B. L., Clausen, E. M., and Litsky, W., "Two-Temperature Membrane Filter Method for Enumerating Fecal Coliform Bacteria from Chlorinated Effluents," Appl. Microbiol., 33, 1259-1264 (1977).

56. Geldreich, E. E., Personal Communication on April 19, 1977 to R. C. Hoehn, Assoc. Professor at Virginia Polytechnic Institute and State University, Blacksburg, Va.

57. Geldreich, E. E., Personal communication on July 7, 1977 to R. C. Hoehn, Assoc. Professor at Virginia Polytechnic Institute and State Univesrity, Blacksburg, Va.

58. Presswood, W. G. and Strong, D., "Modification of M-FC Medium by Eliminating Rosolic Acid," Paper presented at the Annual Meeting of the Amer. Soc. Micro., New Orleans, La., June 1977.

59. King, W. L., and Hurst, A., "A Note on the Survival of Some Bacteria in Different Diluents," Jour. Appl. Bacteriol., 26, 504-506 (1963).

60. Straka, R. P., and Stokes, J. L., "Rapid Destruction of Bacteria in Commonly Used Diluents and its Elimination," Appl. Microbiol. 5, 21-25 (1957).

61. Brown, E. R., "A Study of a Modified Membrane Filter Technique for the Enumeration of Stressed Fecal Coliforms in Urban Runoff," Master's Thesis, Virginia Polytechnic Institute and State University (1977).

62. Wine, R. L., Statistics for Scientists and Engineers, Prentice-Hall, Inc., Englewood Cliffs, N.J. (1964).

APPENDIX A

FECAL COLIFORM ENUMERATIONS

APPENDIX TABLE A-I

Standard MF Plate Counts for Secondary Settling Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml	
			Mean	Std. Deviation
1	1:1,000	TNTC*	--	--
	1:10,000	TNTC	--	--
	1:100,000	72,77,66,48	66	13
2	1:1,000	TNTC	--	--
	1:10,000	TNTC	--	--
	1:100,000	18,16,15,20	17	2
3	1:1,000	38,25,44,28	34	9
	1:10,000	3,2,2,6	--	--
	1:100,000	0,0,0,0	--	--
4	1:1,000	114,90,100,105	102	10
	1:10,000	9,7,9,7	--	--
	1:100,000	0,1,0,1	--	--
5	1:1,000	68,114,104,103	97	20
	1:10,000	9,10,9,9	--	--
	1:100,000	0,0,1,0	--	--
6	1:1,000	72,58,55,101	72	21
	1:10,000	11,10,4,9	--	--
	1:100,000	0,0,1,1	--	--
7	1:1,000	68,61,49,33	53	15
	1:10,000	2,8,2,6	--	--
	1:100,000	0,0,0,0	--	--
8	1:1,000	39,41,34,35	37	3
	1:10,000	2,3,3,4	--	--
9	1:1,000	39,55,35,54	46	10
	1:10,000	3,4,4,4	--	--
10	1:1,000	37,35,56,42	43	9
	1:10,000	4,4,4,3	--	--
	1:100,000	0,0,0,0	--	--
11	1:1,000	30,28,30,27	29	2
	1:10,000	3,2,3,3	--	--
	1:100,000	0,0,0,0	--	--

* Too numerous to count.

APPENDIX TABLE A-I (con't.)

Standard MF Plate Counts for Secondary Settling Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml	
			Mean	Std. Deviation
12	1:1,000	39,55,54,42	48	8
	1:10,000	4,5,5,4	--	--
	1:100,000	0,0,0,0	--	--
13	1:1,000	86,81,72,71	78	7
	1:10,000	9,5,6,5	--	--
14	1:1,000	82,71,89,94	84	10
	1:10,000	9,7,10,10	--	--
15	1:1,000	101,109,99,98	102	5
	1:10,000	14,10,8,9	--	--

APPENDIX TABLE A-II

Improved MF Plate Counts for Secondary Settling Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml Mean	Std. Deviation
1	1:1,000	TNTC*	--	--
	1:10,000	TNTC	--	--
	1:100,000	59,60,54,59	58	3
2	1:1,000	TNTC	--	--
	1:10,000	TNTC	--	--
	1:100,000	40,10,30,10	23	15
3	1:1,000	TNTC	--	--
	1:10,000	11,11,11,14	12	2
	1:100,000	0,0,1,1	--	--
4	1:1,000	144,178,170,171	166	15
	1:10,000	7,10,19,13	--	--
	1:100,000	0,1,0,1	--	--
5	1:1,000	TNTC	--	--
	1:10,000	44,40,40,52	44	6
	1:100,000	3,2,4,4	--	--
6	1:1,000	TNTC	--	--
	1:10,000	20,28,25,29	26	4
	1:100,000	0,1,1,0	--	--
7	1:1,000	TNTC	--	--
	1:10,000	13,23,44,27	27	13
	1:100,000	1,2,2,1	--	--
8	1:1,000	TNTC	--	--
	1:10,000	12,17,14,19	16	3
9	1:1,000	TNTC	--	--
	1:10,000	15,41,18,25	25	12
10	1:1,000	77,60,50,67	64	11
	1:10,000	7,6,6,8	--	--
	1:100,000	0,0,0,0	--	--
11	1:1,000	50,69,61,47	57	10
	1:10,000	5,6,6,6	--	--
	1:100,000	0,0,0,0	--	--

* Too numerous to count.

APPENDIX TABLE A-II (con't.)

Improved MF Plate Counts for Secondary Settling Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml	
			Mean	Std. Deviation
12	1:1,000	86,73,69,70	75	8
	1:10,000	7,7,7,9	--	--
	1:100,000	0,0,0,0	--	--
13	1:1,000	105,136,97,107	111	17
	1:10,000	9,15,12,8	--	--
14	1:1,000	108,98,100,106	103	5
	1:10,000	14,12,10,15	--	--
15	1:1,000	120,133,129,130	128	6
	1:10,000	10,15,11,18	--	--

APPENDIX TABLE A-III

Standard MF Plate Counts for Chlorine Contact Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml	
			Mean	Std. Deviation
1	1:10	TNTC*	--	--
	1:100	TNTC	--	--
	1:1,000	16,20,14,29	20	7
2	1:10	5,4,3,6	4	1
	1:100	0,0,0,0	--	--
	1:1,000	0,0,0,0	--	--
3	1:10	TNTC	--	--
	1:100	6,9,12,14	10	4
	1:1,000	1,1,1,2	--	--
4	1:10	12,31,14,24	20	9
	1:100	2,2,3,2	--	--
	1:1,000	0,0,0,0	--	--
5	1:10	16,28,33,40	29	10
	1:100	3,3,4,3	--	--
	1:1,000	0,0,0,0	--	--
6	1:10	21,25,31,18	24	6
	1:100	3,4,2,2	--	--
	1:1,000	0,0,0,0	--	--
7	1:10	21,14,15,20	18	4
	1:100	2,5,3,2	--	--
	1:1,000	0,0,0,0	--	--
8	1:1	TNTC	--	--
	1:10	39,50,30,38	39	8
	1:100	5,6,7,3	--	--
9	1:1	TNTC	--	--
	1:10	TNTC	--	--
	1:100	91,95,122,70	95	21
10	1:10	12,7,11,18	12	5
	1:100	2,1,2,2	--	--
	1:1,000	0,0,0,0	--	--

* Too numerous to count.

APPENDIX TABLE A-III (con't.)

Standard MF Plate Counts for Chlorine Contact Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml	
			Mean	Std. Deviation
11	1:1	4,5,7,7	6	2
	1:10	0,1,1,0	--	--
	1:100	0,0,0,0	--	--
12	1:1	TNTC*	--	--
	1:10	19,13,16,15	16	3
	1:100	1,0,2,1	--	--
13	1:1	109,115,111,114	112	3
	1:10	6,11,7,4	--	--
	1:100	2,1,0,1	--	--
14	1:1	7,10,8,9	9	1
	1:10	0,0,0,0	--	--
15	1:1	41,39,45,35	40	4
	1:10	6,3,4,2	--	--

* Too numerous to count

APPENDIX TABLE A-IV

Improved MF Plate Counts for Chlorine Contact Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml Mean	Std. Deviation
1	1:10	TNTC*	--	--
	1:100	TNTC	--	--
	1:1,000	794,802,744,819	790	32
2	1:10	6,4,3,4	4	1
	1:100	0,0,0,0	--	--
	1:1,000	0,0,0,0	--	--
3	1:10	TNTC	--	--
	1:100	25,43,41,21	33	11
	1:1,000	11,5,2,7	--	--
4	1:10	21,24,36,41	31	10
	1:100	2,3,4,3	--	--
	1:1,000	0,0,0,0	--	--
5	1:10	80,66,106,64	79	19
	1:100	7,6,5,6	--	--
	1:1,000	0,0,0,0	--	--
6	1:10	50,66,65,58	60	7
	1:100	5,6,7,5	--	--
	1:1,000	0,0,0,0	--	--
7	1:10	61,54,58,63	59	4
	1:100	8,4,6,6	--	--
	1:1,000	0,0,0,0	--	--
8	1:1	TNTC	--	--
	1:10	33,30,22,36	30	6
	1:100	3,3,4,5	--	--
9	1:1	TNTC	--	--
	1:10	TNTC	--	--
	1:100	264, 269,251,261	261	8
10	1:10	39,37,38,27	35	6
	1:100	4,5,6,5	--	--
	1:1,000	0,0,0,0	--	--

* Too numerous to count

APPENDIX TABLE A-IV (con't.)

Improved MF Plate Counts for Chlorine Contact Tank

Trial Number	Dilution	Counts of Replicate Plates	Colonies per 10 ml Mean	Colonies per 10 ml Std. Deviation
11	1:1	20,9,23,15	17	6
	1:10	2,2,3,2	--	--
	1:100	0,0,0,0	--	--
12	1:1	TNTC*	--	--
	1:10	34,42,30,20	32	9
	1:100	3,2,3,3	--	--
13	1:1	TNTC	--	--
	1:10	39,27,59,30	39	14
	1:100	1,1,2,0	--	--
14	1:1	24,47,24,27	31	11
	1:10	1,2,6,3	--	--
15	1:1	147,137,122,135	135	10
	1:10	15,14,11,12	--	--

* Too numerous to count

APPENDIX TABLE A-V

MPN Determinations for Secondary Settling Tank

Trial Number	Positive Presumptive Tubes (of 5)				Positive Confirmed Tubes (of 5)				Fecal Coliforms/100ml by MPN	
	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	$1:10^3$	$1:10^4$	$1:10^5$	$1:10^6$	Presumptive	Confirmed
1	5	5	2	0	5	5	0	NR[a]	4.9×10^6	2.4×10^6
2	5	4	3	0	5	1	1	NR	2.8×10^6	4.6×10^5
3	5	5	0	0	5	1	0	0	3.1×10^6	3.5×10^5
4	5	5	3	1	5	2	1	NR	7.9×10^6	7.0×10^5
5	5	5	3	0	5	4	0	NR	7.9×10^6	1.3×10^6
6	5	5	3	0	5	2	0	NR	7.9×10^6	4.9×10^5
7	5	4	3	0	5	1	0	NR	2.8×10^6	3.3×10^5
8	5	5	1	0	5	2	1	NR	3.3×10^6	4.9×10^5
9	5	5	1	0	5	1	0	NR	3.3×10^6	3.3×10^5
10	5	5	4	0	5	3	0	NR	1.3×10^7	7.9×10^5
11	5	5	3	0	4	3	1	NR	7.9×10^6	3.3×10^5
12	5	5	4	0	5	1	1	NR	1.3×10^7	4.6×10^5
13	5	5	2	0	5	5	0	NR	4.9×10^6	2.4×10^6
14	5	5	3	0	5	2	1	NR	7.9×10^6	7.0×10^5
15	5	5	1	0	5	3	1	NR	3.3×10^6	1.1×10^6

a. Not run.

APPENDIX TABLE A-VI

MPN Determinations for Chlorine Contact Tank

Trial Number	Positive Presumptive Tubes (of 5)					Positive Confirmed Tubes (of 5)					Fecal Coliforms/100ml by MPN	
	$1:10^0$	$1:10^1$	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^0$	$1:10^1$	$1:10^2$	$1:10^3$	$1:10^4$	Presumptive	Confirmed
1	NR[a]	5	5	4	4	NR	4	2	0	0	$3.5x10^5$	$2.2x10^3$
2	NR	0	0	0	0	NR	NR	NR	NR	NR	$<2.0x10^3$	$<2.0x10^2$
3	NR	5	5	1	0	NR	5	3	0	NR	$3.3x10^4$	$7.9x10^3$
4	NR	2	0	0	0	NR	0	NR	NR	NR	$5.0x10^2$	$<2.0x10^2$
5	NR	5	5	1	0	NR	5	1	0	NR	$3.3x10^4$	$3.3x10^3$
6	NR	5	0	0	0	NR	0	NR	NR	NR	$2.3x10^3$	$<2.0x10^2$
7	NR	0	0	0	0	NR	NR	NR	NR	NR	$<2.0x10^2$	$<2.0x10^2$
8	5	5	4	1	NR	5	4	0	0	NR	$1.7x10^4$	$1.3x10^3$
9	5	5	5	5	NR	5	5	5	4	NR	$\geq 2.4x10^5$	$1.6x10^5$
10	NR	5	5	4	0	NR	5	5	0	NR	$1.3x10^5$	$2.4x10^4$
11	5	2	0	0	NR	3	0	NR	NR	NR	$7.9x10^2$	$8.0x10^1$
12	5	5	5	4	NR	5	5	2	0	NR	$1.6x10^5$	$4.9x10^3$
13	5	5	5	3	NR	5	5	3	0	NR	$9.2x10^4$	$7.9x10^3$
14	5	5	3	0	NR	5	2	0	NR	NR	$7.9x10^3$	$4.9x10^2$
15	5	5	3	2	NR	5	4	2	0	NR	$1.4x10^4$	$2.2x10^3$

a. Not run.

APPENDIX B

STATISTICAL ANALYSIS

APPENDIX TABLE B-I

Statistical Analysis of the SF-MF and the MPN for the Secondary Settling Tank

SF-MF*	SF-MF log	MPN*	MPN log	Difference
430,000	5.6335	790,000	5.8976	0.2461
290,000	5.4624	330,000	5.5185	0.0561
480,000	5.6812	460,000	5.6628	-0.0184
780,000	5.8921	2,400,000	6.3802	0.4881
840,000	5.9243	700,000	5.8451	-0.0792
1,020,000	6.0086	1,100,000	6.0414	0.0382
ΣY	34.6021		35.3456	0.7309

$\Sigma D^2 = 0.3100$

$\bar{D} = \Sigma D/6 = 0.7309/6 = 0.1218$

$$S_D = \sqrt{\frac{\Sigma D^2-(\Sigma D)^2/b}{b-1}} = \sqrt{\frac{.3100-0.0890}{5}} = .210$$

$$S_{\bar{D}} = \frac{S_D}{b} = \frac{.210}{6} = 0.0350$$

Assume differences of the means of the two groups equal zero.

$$t_s = \frac{\bar{D}-0}{S_{\bar{D}}} = \frac{0.1218}{0.0350} = 3.48$$

$t_{0.05}(5) = 2.571 < 3.48 < t_{0.01}(5) = 4.03$

Means significantly greater at 0.05 level but not at 0.01 level

*Mean fecal coliforms/100ml

APPENDIX TABLE B-II

Statistical Analysis of the IF-MF and the MPN for the Secondary Settling Tank

SF-MF*	SF-MF log	MPN*	MPN log	Difference
640,000	5.8062	790,000	5.8976	-0.0914
570,000	5.7559	330,000	5.5185	0.2374
750,000	5.8751	460,000	5.6628	0.2123
1,110,000	6.0453	2,400,000	6.3802	-0.3349
1,030,000	6.0128	700,000	5.8451	0.1677
1,280,000	6.1072	1,100,000	6.0414	0.0658
	35.6025		35.3456	0.2569

$\Sigma D^2 = 0.2544$

$\bar{D} = \Sigma D/6 = 0.2569/6 = 0.0428$

$$S_D = \sqrt{\frac{\Sigma D^2 - (\Sigma D)^2/b}{b-1}} = \sqrt{\frac{0.2544-0.0003}{5}} = 0.2254$$

$$S_{\bar{D}} = \frac{S_D}{b} = \frac{.2254}{6} = 0.0376$$

$$t_s = \frac{\bar{D}-0}{S_{\bar{D}}} = \frac{0.0428}{0.0376} = 1.139$$

$t_{0.05}(5) = 2.571 > 1.139 < t_{0.01}(5) = 4.03$

Means not significantly greater at 0.05 level or 0.01 level.

*Mean fecal coliforms/100ml

APPENDIX TABLE B-III

Statitical Analysis of the SF-MF and the MPN for the Chlorine Contact Tank

SF-MF*	SF-MF log	MPN*	MPN log	Difference
1,200	3.0792	24,000	4.3802	1.3010
60	1.7782	80	1.9031	0.1249
1,600	3.2041	4,900	3.6902	0.4861
1,120	3.0492	7,900	3.8976	0.8484
90	1.9542	490	2.6902	0.7360
400	2.6021	2,200	3.3424	0.7403
	15.6670		19.9037	4.2367

$\Sigma D^2 = 3.7540$

$\bar{D} = \Sigma D/6 = 4.2367/6 = 0.7061$

$$S_D = \frac{\Sigma D^2-(\Sigma D)^2/b}{b-1} = \frac{3.754-2.9916}{5} = 0.3905$$

$$S_{\bar{D}} = \frac{S_D}{b} = \frac{0.3905}{6} = 0.0651$$

$$t_s = \frac{\bar{D}-0}{S_{\bar{D}}} = \frac{0.7061}{0.0651} = 10.85$$

$t_{0.05}(5) = 2.571 < 10.85 > t_{0.01}(5) = 4.03$

Means significantly greater at 0.05 and 0.01 levels.

* Mean fecal coliforms/100ml

APPENDIX TABLE B-IV

Statistical Analysis of the IF-MF and the MPN for the Chlorine Contact Tank

SF-MF*	SF-MF log	MPN*	MPN log	Difference
3,500	3.5441	24,000	4.3802	0.8361
170	2.2304	80	1.9031	-0.3273
3,200	3.5051	4,900	3.6902	0.1851
3,900	3.5911	9,900	3.8976	0.3065
310	2.4914	490	2.6902	0.1988
1,350	3.1303	2,200	3.3424	0.2121
	18.4924		19.9037	1.4113

$\Sigma D^2 = 1.0189$

$\bar{D} = \Sigma D/6 = 1.4113/6 = 0.2352$

$$S_D = \sqrt{\frac{\Sigma D^2 - (\Sigma D)^2/b}{b-1}} = \sqrt{\frac{1.0189-0.3320}{5}} = 0.3707$$

$$S_{\bar{D}} = \frac{S_D}{b} = \frac{.3707}{6} = 0.0618$$

$$t_s = \frac{\bar{D}-0}{S_{\bar{D}}} = \frac{0.2352}{0.0618} = 3.81$$

$t_{0.05}(5) = 2.571 < 3.81 < t_{0.01}(5) = 4.03$

Means significantly greater at 0.05 but not at 0.01 level

* Mean fecal coliforms/100ml

APPENDIX TABLE B-V

Statistical Analysis of the SF-MF and the IF-MF for the Secondary Settling Tank

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Sum of squares of test procedures = 0.34067*
degrees of freedom = 1

Sum of squares of total error = 0.05373*
degrees of freedom = 5

Mean squares of test procedures = 0.34067

Mean squares of total error = 0.01075

$$F = \frac{\text{M.S. test procedure}}{\text{M.S. total error}} = 31.7$$

$$F_{1,5}^{(.05)} = 6.61 < 31.7 > F_{1,5}^{(.01)} = 16.3$$

Means significantly greater at 0.05 and 0.01 levels.

$\therefore$ Means of IF-MF > Means of SF-MF

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* Values determined by computer using SAS 76 program for two-way ANOVA test.

APPENDIX TABLE B-VI

Statistical Analysis of the SF-MF and the IF-MF for the Chlorine Contact Tank

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Sum of squares of test procedures = 2.64249*
degrees of freedom = 1

Sum of squares of total error = 0.54879*
degrees of freedom = 41

Mean squares of test procedures = 2.64249

Mean squares of total error = 0.01339

$$F = \frac{\text{M.S. test procedures}}{\text{M.S. total error}} = 197.42$$

$$F^{(.05)}_{1,47} = 4.03 < 197.42 > F^{(.01)}_{1,47} = 7.21$$

Means significantly greater at 0.05 and 0.01 levels.

$\therefore$ Means of IF-MF > Means of SF-MF

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* Values determined by computer using SAS 76 program for two-way ANOVA test.

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AN EVALUATION OF A MODIFIED MEMBRANE FILTER PROCEDURE FOR ENUMERATING STRESSED FECAL COLIFORMS IN CHLORINATED SEWAGE EFFLUENTS

by

Steven Paul Clark

(ABSTRACT)

Wastewater samples were collected from both the secondary settling and the chlorine contact tanks at a secondary sewage treatment plant (trickling filter) in Blacksburg, Virginia and analyzed for fecal coliforms using three procedures. Physical parameters including total suspended solids, DO, pH, turbidity, temperature and total chlorine residual were measured in effort to ascertain their effect on fecal coliform recoveries.

The three procedures employed included the multiple-tube fermentation technique that yields the most probable number (MPN), the standard MF technique (SF-MF), and a modified MF technique (IF-MF) which consisted of a lactose overlay and a 5-hour incubation period at 44.5°C. A statistical analysis of the data showed that the means of the recoveries by the IF-MF technique were significantly greater (0.01 level) than those by the SF-MF technique in both the secondary settling tank and the chlorine contact tank samples. Recoveries by the IF-MF technique were comparable to those by the MPN technique

when samples from the secondary settling basin were analyzed, but not in samples from the chlorine contact tank. However, the means of the IF-MF recovery procedure were within the 95 percent confidence interval associated with the MPN. No relationships could be established between the observed variations in the physical and chemical characteristics of the treated sewage samples and the fecal coliform densities.