1-1-1977

Two-temperature membrane filter method for enumeration of fecal coliform bacteria from chlorinated effluents.

Barbara L. Green
University of Massachusetts Amherst

TWO-TEMPERATURE MEMBRANE FILTER METHOD FOR ENUMERATION OF FECAL COLIFORM BACTERIA FROM CHLORINATED EFFLUENTS

A Dissertation Presented

By

BARBARA L. GREEN

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

November 1977

Plant and Soil Sciences

TWO-TEMPERATURE MEMBRANE FILTER METHOD FOR ENUMERATION OF FECAL COLIFORM BACTERIA FROM CHLORINATED EFFLUENTS

A Dissertation Presented

By

BARBARA L. GREEN

Approved as to style and content by:

Warren Litsky
Chairman of Committee

Haim B. Gunner, Member

David A. Evans, Member

Allen V. Barker
Department Head
Plant and Soil Sciences

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Warren Litsky for the encouragement and constructive advice so generously offered throughout the years that this study was in progress. My thanks are conveyed to Drs. Haim Gunner and David Evans for serving as committee members.

I also would like to express my appreciation to Dr. Karl Sladek for his contributions to this research, which included aid in the analysis of data and many helpful discussions concerning membrane filters.

I would like to thank Mildred Ralicki for her willing assistance in the laboratory and Elizabeth Beaudoin for her participation and interest in this work. To my husband and family, I wish to express thanks for their encouragement and patience that made this endeavor possible.

Finally, I would like to thank Millipore Corporation, Bedford, Massachusetts, for providing funding for this study.

A B S T R A C T

Two-Temperature Membrane Filter Method for Enumeration of Fecal Coliform Bacteria from Chlorinated Effluents

(February 1978)

Barbara L. Green
B.A., University of New Hampshire
M.S., University of Massachusetts
Ph.D., University of Massachusetts

Directed by: Professor Warren Litsky

Reports indicate that the standard membrane filter (MF) technique for recovery of fecal coliform bacteria from chlorinated sewage effluents is less effective than the multiple-tube (MPN) procedure. Tests were designed to evaluate the effects of several parameters (i.e., culture media, incubation temperature, membrane extractables, stress due to chlorination) on MF recovery of fecal coliforms. It was determined that immediate exposure to the 44.5°C incubation required by the standard MF method was the critical factor influencing recovery of sublethally injured coliforms.

A modified membrane filter method was developed which requires a preincubation period of five hours at 35°C followed by 18±1 hour at 44.5°C. This procedure was evaluated using both laboratory- and plant-chlorinated primary and secondary effluents. Results obtained by the modified MF method

compared favorably with the MPN technique for the enumeration of fecal coliforms from chlorinated effluent. Agreement between these two methods was greatest with samples from secondary treatment plants. Average recovery of fecal coliforms by the standard MF procedure was only 14 per cent of the MPN, whereas, using the modified technique recovery was increased to 68 per cent of the MPN counts. Enhanced recovery resulting from a simple modification of the incubation schedule makes the MF method a valuable adjunct for the enumeration of fecal coliforms from chlorinated effluents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Membrane filtration (MF) techniques have gained wide acceptance in the analysis of water and wastewater for bacterial indicators of pollution. The unique ability of the filter to concentrate bacteria in low density samples, together with a substantial reduction in time, equipment, labor and culture media have caused many workers in this field to prefer membrane methods to the more cumbersome multiple-tube most probable number (MPN) procedure. However, there are indications in the literature, dating back to the 1950s, that problems exist in recovering chlorine-injured bacterial cells by membrane filtration. McKee (69) in 1953 indicated that the standard MF technique for the recovery of total coliforms from chlorinated effluent was less effective than the MPN method.

Lin (56) in 1973 evaluated methods for enumerating fecal coliform bacteria from chlorinated sewage effluents and likewise concluded that the MF technique using M-FC medium at 44.5°C was less efficient than the EC (elevated coliform) multiple-tube procedure for fecal coliform recovery.

From the literature, it is apparent that difficulties in the recovery of chlorine-injured coliforms can be related to the general problem of quantitation of injured organisms

with selective media. Maxcy (62) suggested that non-lethal injury of coliform bacteria by various treatments including chlorination reduced the ability of cells to grow on selective media.

It has been postulated (56) that the broth medium of the presumptive MPN test provides a more favorable environment than the membrane filter surface for the repair of sublethally injured cells. In addition, damaged cells may undergo repair more readily at 35°C than when subjected immediately to the 44.5°C incubation temperature required by the standard MF method for enumeration of fecal coliforms.

The 14th edition of Standard Methods for the Examination of Water and Wastewater (5) does not recommend employing membrane filtration analysis for certain types of samples which have demonstrated coliform recovery problems. These include wastewaters that have received only primary treatment followed by chlorination, or industrial effluents containing toxic metals or phenols. Chlorinated secondary and tertiary effluents may be analyzed by the two-step MF procedure for total coliforms. The choice of method for the fecal coliform group "should be governed by methodology used for total coliform enumeration," though no specific procedures are included.

Because chlorination is currently the most common form of sewage disinfection and is now required throughout the year in many states, the U.S. Environmental Protection

Agency has recently suggested that the multiple-tube test be the method of choice for fecal coliform determination on effluent containing chlorine (8,29,30,108).

At the present time the Agency permits the use of the MF for self-monitoring provided some parallel MPN tests have been performed. However, the following qualifying statement has been appended to the method in the Federal Register (108): "Since the membrane filter technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies."

Some time ago it was demonstrated that reactivation of chlorine-treated Escherichia coli cells was in fact possible by immediate plating on nutritive media (70). Several procedures have been proposed to increase membrane filter recovery of fecal coliforms utilizing enrichment and/or temperature acclimation steps. Some methods require the manual transfer of membranes from one medium to another. None of these proposed techniques has been generally accepted as a satisfactory alternative to the MPN test.

Preliminary laboratory tests and an extensive body of literature strongly suggest that poor recovery of fecal coliform organisms from chlorinated effluent is due to the inability of injured cells to grow on the selective medium at an elevated temperature. This study was designed to evaluate the effects of incubation temperature, the M-FC medium, and the membrane filter, in order to propose a modified pro-

cedure for the enhanced recovery of fecal coliforms from chlorinated wastewater. Attention was focused on providing a practical membrane filter procedure that would be comparable to the MPN technique.

LITERATURE REVIEW

Development and use of membrane for water analysis. Since the development of the MF technique by Goetz and his co-workers (31-33) after World War II, much research has been devoted to improving methodology for its use in the bacteriological analysis of water. A membrane procedure for the enumeration of total coliforms was first included as a tentative method in the 10th edition of Standard Methods for the Examination of Water, Sewage and Industrial Wastes (2) in 1955. The technique was adopted as a standard method and an alternate to the MPN test in 1960 with the publication of the 11th edition of Standard Methods (3). Since that time membrane filtration has gained widespread acceptance as a convenient, time-saving, and precise technique for water and wastewater analysis.

Numerous reports have appeared which compare the membrane filter procedure with the MPN method of coliform determination. Yee et al. (116) found good correlation between the two methods when examining surface, ground and finished waters. Shipe and Cameron (95) added cultures of E. coli to several types of water and compared recovery by the two procedures. Fewer coliform bacteria were detected by the MF method from one river water sample, while recovery was com-

parable from the other two waters tested. The difficulty was attributed either to the accumulation of toxic substances on the filters or the inability of "weakened cells" to recover on the filter surfaces. Presnell et al. (81) examined sea water for coliform organisms and reported 87.1 per cent agreement between the two methods, with greater agreement in samples containing higher numbers of coliforms. Other comparisons on sea water show results varying from equality of the two methods to generally lower counts by the membrane filter test (14,45,47).

Kabler (49), reporting on the simultaneous examination of 1,706 water samples by MF and MPN procedures, found agreement in 1,260 samples, with a trend to lower coliform recovery by the filter technique. Adams (1) concluded that with raw waters having high coliform populations the two methods gave approximately the same results. He also found 88.4 per cent agreement in tests performed on 315 samples of prechlorinated Allegheny River water. Mallmann and Peabody (61), in their assessment of the data of both Kabler and Adams, excluded all samples in which no coliforms were detected by either method since these tests yielded no information on the comparative value of the two procedures. The 74 per cent agreement reported by Kabler was then reduced to 60 per cent. When 129 negative tests were eliminated from Adams' 315 river water samples the per cent agreement dropped to 56. Mallmann concluded from his own work that the MF

technique for recovery of coliform bacteria using M Endo MF agar (sic) did not yield data comparable to those obtained by the multiple tube test.

Streeter and Robertson (102) evaluated a 20 month comparison of the MF and MPN methods for coliform recovery in which eight laboratories participated in analyzing Ohio River quality. The combined average agreement between the MF and MPN results for the six laboratories completing the study was 81 per cent. Favorable agreement between MF and MPN determinations led the authors to believe that membrane filtration was an acceptable alternative to the MPN method and even preferable because of its precision, speed, and economy of space. A media comparison indicated that the most promising selective culture medium for recovering coliform organisms was that of Fifield and Schaufus (22) designated MF-Endo. Mallmann and Peabody (61) also found this medium to give the best recovery.

In 1956, Eye et al. (21) studied the effects of four different media and varying temperatures and humidities on the recovery of coliform bacteria by the MF technique. Consistently higher counts resulted on all four media at 32°C than when plates were incubated at the recommended 35°C.

McKee and McLaughlin (68) in 1958, demonstrated that membrane filter techniques were capable of measuring the coliform density of raw settled sewage with results comparable to those obtained by conventional confirmed MPN proce-

dures. Another paper by McKee et al. (69) soon followed, which reported on the effects of physical and chemical disinfection on the use of molecular filters for the bacterial assay of sewage. When chlorine was used as the disinfecting agent, the pattern of agreement among data from MPN and MF tests changed drastically. Without exception the membrane filter results were lower than the MPN data, sometimes varying by a factor of ten or more. It was suggested that this discrepancy could be attributed to the partial reversibility of disinfection. Following chlorination some of the coliforms are permanently inactivated, while others when placed in a rich aqueous medium, such as lactose broth, are able to recover and grow. This resuscitation may not be possible when cells are deposited on a membrane filter surface and supplied with limited nutrients by capillary action.

Recognizing the lack of agreement between the MF and MPN techniques for coliform enumeration, McCarthy and his coworkers (63-66) analyzed the discrepancies between the tests and proposed an improved MF procedure for coliform recovery. It was believed that the failure of many coliforms to develop with the one step techniques was caused by the inhibiting and/or bactericidal influence of M-Endo broth (22) on weak organisms in the first hours of incubation. To compensate for this an enrichment step was included in the new method. After filtration of the sample, the membrane was placed on an absorbent pad saturated with lauryl tryptose broth and in-

cubated at 35°C for 2±0.5 hours, a period shown to be sufficient to provide adequate enrichment for the weakest coliforms from a variety of samples. The filter was then transferred to an M-Endo medium which had been reduced in strength by 25 per cent and contained 1.5 per cent agar (L.E.S. Endo agar MF, sic). Incubation continued for 22 hours at 35°C. Comparisons of the proposed method with the single-step MF and MPN tests for the analysis of river, pond, and lake samples indicated that the two-step MF procedure gave results practically equal to those obtained by the multiple tube method and higher than those derived by the conventional one-step test. It was found, however, that the enrichment phase did not increase significantly the coliforms recovered from sewages and polluted rivers. These results seem to confirm the theory that the nearer the source of pollution, the less attenuated are the coliform organisms. Therefore, higher numbers are likely to withstand the inhibitory effects of the culturing procedures.

Application of MF technique to fecal coliform enumeration. The coliform bacteria comprise a heterogenous group which includes biotypes of sanitary significance and those of limited significance that are widely distributed in nature. The fecal coliforms are part of the total coliform group and have a high positive correlation with fecal contamination from warm-blooded animals (27). It has been shown that fecal

coliforms represent over 96 per cent of the coliforms isolated from human feces, and from 93 to 98.7 per cent of the total coliform population in the feces of other warm-blooded animals, including livestock, poultry, cats, dogs, and rodents (25,28). They are defined as gram-negative nonspore-forming rods that ferment lactose in 24±2 hours at 44.5°± 0.2°C with the production of gas (2-5). The most numerous fecal coliform biotype is Escherichia coli although other genera, especially the Klebsiella, may conform to the above definition.

Eijkman (20), in 1904, reported that only fecal Bacillus coli was capable of fermenting glucose at 46°C, and recommended a test with an elevated incubation temperature to differentiate between fecal coliforms and those of non-fecal origin. This early work formed the basis for the EC multiple-tube test for fecal coliforms that is widely used at the present time. A presumptive lactose fermentation at 35°C is followed by a confirmatory test in the EC broth medium of Hajna and Perry (38,39) at 44.5°C.

In 1962 Delaney et al. (16) developed a membrane filter procedure for the enumeration of E. coli Type I. The membrane was first incubated on tryptone bile agar at 44.5°C for 20 to 24 hours and then transferred to an absorbent pad containing a solution of para-dimethylaminobenzaldehyde and oxone. Indol-forming colonies turned a deep red color within thirty seconds and were counted as E. coli Type I. Al-

though this method proved highly selective and more rapid than the MPN test it was not generally adopted because: (1) recovery was limited to one member of the fecal coliform group, (2) transfer of the filter to a second medium was required, and (3) toxicity of the indol reagent prevented further verification of colonies.

In 1965 Geldreich et al. (26) formulated M-FC medium for the direct enumeration of the fecal coliform group using the membrane filter technique. An indicator system of aniline blue and the sodium salt of rosolic acid, as proposed by Bronfenbrener et al. (11), was added to an enriched lactose broth base. M-FC medium, which was reported to contain no inhibitory substances for fecal coliform bacteria, depended on an elevated incubation temperature of 44.5°C for 24±2 hours for its selectivity (24). This method made possible the direct enumeration of organisms of the fecal coliform group in one third of the time required by the MPN procedure and was included in the 13th edition of Standard Methods (4) in 1971 as an alternate procedure for the detection of fecal coliform organisms in water. The method was accepted enthusiastically at a time when adoption of the fecal coliform group as a pollution indicator was gaining popularity.

The number of papers published during the 1970s indicates a renewed interest in comparisons of coliform recovery by the membrane filter and multiple-tube techniques. Lin (56,57) concurred with earlier reports of McKee (69) that

total coliform yields from unchlorinated wastewater were similar by both methods. He also concluded that the L.E.S. M-Endo (two-step) procedure of McCarthy et al. (66) was comparable to the multiple-tube test for assaying total coliform densities in chlorinated secondary effluents. In addition, the MF method was favored over the MPN technique from the standpoint of time, convenience, equipment needs, and degree of statistical bias. Fecal coliform recovery by the two methods was evaluated using 96 samples from four chlorinated effluents. Results indicated that the MF procedure with M-FC medium was less efficient than the confirmed MPN test. In several cases the discrepancy was by a factor of 10 or more.

In a similar comparison, Greene et al. (37) found that total coliform MF results were in agreement with the corresponding MPN data for unchlorinated but not for chlorinated effluents. The L.E.S. two-step procedure of McCarthy et al. (66) increased recovery of total coliforms from both chlorinated and unchlorinated wastewaters. Fecal coliform MF densities did not agree with the MPN values for either effluent type. They evaluated several enrichment media and together with preincubation at 25°C for 2 to 6 hours provided closer agreement between the MF and MPN techniques. However, details of this work have not been published (Greene, Masters Thesis, University of Cincinnati, Ohio, 1973).

Moran (72) also reported that membrane filter recovery of fecal coliform bacteria was very low compared to the standard MPN method when chlorinated sewage effluent as well as non-chlorinated stream and lake water samples were tested. Additional studies (82,101) indicate that similar problems exist for MF recovery of fecal coliforms from marine and estuarine environments.

British microbiologists have reached similar conclusions regarding the use of membrance filtration for analysis of chlorinated waters (85). Although culturing procedures, media, and indicator groups differ from those used in the United States, the failure of the membrane technique has been linked to incubation at an elevated temperature (44°C). The following two incubation schemes have been suggested to increase counts of E. coli using enriched teepol medium: (1) incubation at 30°C for 4 hours followed by 14 hours at 44°C, and (2) incubation at 25°C for 6 hours followed by 18 hours at 44°C.

It has become common practice to refer to dead cells as those unable to grow on a rich medium such as trypticase soy agar (TSA). Those bacteria able to produce colonies on TSA but not on a selective medium have been designated "injured cells" (41,46,80,93). Hoadley and Cheng (46) reported that recovery of an ATCC strain of E. coli on M-FC medium was never equal to that on TSA during the first few hours following inoculation of the aqueous suspending medium. Using

membrane filter chambers, Bissonette et al. (6) exposed pure cultures of E. coli to aquatic environments and found that a significant proportion of cells lost their ability to produce colonies on a selective medium, but retained that capability on a nutritionally rich, nonselective medium. In a later study (7), pure cultures of E. coli or raw sewage suspensions were immersed in a stream environment. Samples were withdrawn from the chambers at regular intervals for evaluation of several coliform recovery methods. Multiple-tube fermentation techniques were found to be more efficient than membrane filter procedures, especially after prolonged exposure to the aquatic environment.

Scheusner et al. (71) observed that E. coli, injured as the result of exposure to hypochlorite sanitizers, failed to form colonies on a selective medium. Another study (9) showed that E. coli injured during chlorination of secondary sewage failed to produce colonies on membrane filters incubated on M-FC medium or to grow and produce gas in lactose broth. However, recovery of unstressed cells occurred equally well by all methods employed.

Using preparations of presumably uninjured cells, Hufham (48) found that a large discrepancy existed between the number of E. coli cells present in a particular suspension and the number that could be detected using the M-FC procedure at 44.5°C. There was no apparent difference between counts of E. coli made on total count broth and M-FC

broth at 35°C, although a comparison of two brands of membrane filters indicated a significant difference in recovery at 44.5°C that was not evident at 35°C. It was concluded that the temperature was an important factor but it was not clear whether the effect of temperature was directed toward the cell, the membrane, or the medium. Hartman et al. (42, 43) observed that a reduced incubation temperature facilitated coliform recovery from surface waters, milk and food samples. Violet red bile agar was incubated at 30°C. Related work with Salmonella (50) demonstrated that a primary, non-selective, ambient-temperature enrichment procedure was superior to elevated-temperature selective enrichment for isolation of these organisms from estuarine water samples. In the process of developing a seven-hour membrane filter test for quantitation of fecal coliforms, Van Donsel et al. (109) performed thermal gradient studies to select the optimum temperature for incubation. Since slower growing strains exhibited maximum cell density between 40 and 42°C, and rapidly growing strains peaked between 42 and 44°C, a compromise temperature of 41.5°C was chosen for rapid fecal coliform enumeration.

Davenport et al. (15) included a comparison of recovery methods in their study of fecal indicator bacteria persistence in an ice-covered arctic river. Fecal coliform recovery by the MF procedure was consistently less efficient than by the MPN method at stations near the pollution source.

However, the results became more comparable with increasing flow time. It was suggested that a heat-shock effect on uninjured cells resulting from the 44.5°C incubation was a major factor in suppression of fecal coliform counts on the membrane filters. It appears likely that the elevated temperature may be inhibiting injured cells, thereby explaining the more comparable results downstream where fewer injured cells persist.

Mechanism of action of chlorine. In order to understand the problems associated with recovery of coliform bacteria from chlorinated sewage effluents it is necessary to consider the mechanism of action of chlorine on the bacterial cell. A great deal is known about the physical chemistry of chlorine in an aqueous solution, but little is understood about the interaction between chlorine or chlorine compounds and the microbial cell which results in injury or death to the organism (53,112). As recently as 1977, Dychdala (19) claims that in spite of much research done in the field the mechanism of chlorine disinfection has not been fully elucidated.

When chlorine gas or sodium hypochlorite (the most widely used of the hypochlorites for potable water and wastewater treatment) are added to an aqueous solution similar results are achieved as is shown by the following reactions (19,112):

$$Cl_2 + H_2O \longrightarrow HOCl + H^+ + Cl^-$$

$$NaOCl + H_2O \longrightarrow HOCl + Na^+(OH)^-$$

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

Hypochlorous acid is the most active fraction formed and is responsible for the death or injury to microorganisms. The dissociation of HOCl is pH dependent and so rapid that equilibrium between HOCl and OCl^- ion is maintained, even though HOCl is being continuously used in the disinfection process (19,112). The efficiency of chlorine as a germicide increases with a decrease in pH since the equilibrium shifts to the left. The effectiveness of hypochlorous acid, which is similar in structure to water, is due to the relative ease with which it can penetrate cell walls. This entry is facilitated by its low molecular weight and by its absence of electrical charge (55,112).

Green et al. (36) in 1946 suggested that chlorine must be an inhibitor of some key enzymatic process within the cell because of the trace level at which it exerts its bactericidal action. Correlation was found between the effect of chlorine on bacterial growth and its effect on the rate of glucose oxidation by the bacterial cell. They observed that the minimal amount of chlorine necessary to prevent glucose oxidation in a bacterial suspension was consistently that concentration which sterilized the suspension. To explain these results they hypothesized that the enzyme triose-phosphate dehydrogenase, which is involved in the first step of glucose oxidation, must have been inhibited. Further ex-

periments showed that the triosephosphate dehydrogenase extracted from rabbit muscle was inhibited by bactericidal concentrations of chlorine. It was shown by Knox et al. (52) that chlorine in bactericidal amounts or less inhibited various sulfhydryl enzymes or other enzymes sensitive to oxidation. Inhibition of these essential enzymes was responsible for death of the microbial cell. It is interesting to note that attempts to reverse the chlorine effect on enzymes or bacteria, after it had been established, by the addition of cysteine or glutathione were unsuccessful. Wyss (115) favors the theory that the phenomenon of "unbalanced growth" is probably the cause of death in microorganisms exposed to chlorine. The destruction of part of the enzyme system may throw the cell so "out of balance" that the cell dies by "progress of its own metabolism" before the necessary repairs are made.

Using sophisticated biochemical techniques, Venkobachar et al. (110) recently studied the effect of active chlorine on the intact cells and cell-free extracts of E. coli. In whole cells the total dehydrogenase activity was observed to decrease in proportion to the viable cell count during chlorination. Exposure to chlorine also reduced the activity of membrane-bound succinic dehydrogenase (SDH) in crude cell extracts. Data indicated that the inactivation of these enzymes may be due to the oxidation of sulfhydryl groups as earlier researchers have suggested. It was also

found that reduced glutathione could reverse the inhibition of SDH caused by lower doses of chlorine. Bactericidal concentrations of chlorine had no effect on ATP-ase (membrane-bound) or catalase (non-SH) activity.

Problems associated with recovery of stressed cells. It is becoming apparent that the recovery of chlorine-injured coliforms can be related to the general problem of recovery of microorganisms which have been sublethally injured by a variety of physical or chemical means. Selective media formulated to inhibit some groups of bacteria, while allowing growth of others, have been shown to inhibit weakened cells of those same groups of bacteria for which they were designed to recover. Food microbiologists also have realized recently that accepted procedures for enumeration of bacteria from foods which had been subjected to heat, refrigeration, freezing, drying, irradiation, or high osmotic pressure are inadequate for recovering environmentally stressed cells (78, 79).

For some time a controversy existed as to whether chlorine-inactivated bacteria could be resuscitated and made to reproduce (23,44,52,69). Milbauer and Grossowicz (71) demonstrated unequivocally that plating on a rich medium could restore E. coli cells presumed killed by chlorination. When cultures were plated simultaneously, up to 1,000 times more colonies developed on nutrient agar than on minimal

agar. In an attempt to determine which of the nutrient agar ingredients were responsible for cellular repair, minimal agar was supplemented with individual nutrient agar ingredients. Yeast extract was responsible for the largest increase in counts, although the highest counts were obtained when the treated culture was plated on nutrient agar.

Moss and Speck (73) found that freezing and storing E. coli at -20°C also resulted in nonlethal or "metabolic" injury to a proportion of the surviving population. Injured cells could not be recovered on a minimal agar medium but could develop on trypticase soy agar. The component found responsible for the recovery of impaired cells was trypticase which contained five closely related peptides that possessed most of the biological activity. Surviving coliforms which are injured by freezing are inhibited from forming colonies on the selective medium violet red bile agar (VRBA). A later report (111) showed that the numbers of coliforms recovered from commercially frozen foods could be increased by mixing the thawed samples with trypticase soy broth (TSB) and allowing repair to occur for one hour at 25°C before exposure to the selective medium. A number of other publications discuss the enumeration and recovery of coliform bacteria which have been injured by freezing (86-89,97,98).

It was observed by Bissonnette et al. (6,7) that, upon exposure to stresses of the aquatic environment, a significant portion of E. coli cells lost their ability to pro-

duce colonies on selective medium, yet retained this capability on nutritionally rich non-selective media. In addition Klein and Wu (51) reported that heterotrophic microorganisms from mountain streams were susceptible to the transient stress of warmed agar used in the standard methods pour plate procedure.

A membrane filter technique was developed by Claydon (13) and Goff et al. (34) for the revival and isolation of heat-injured bacteria from milk. Counts from membranes incubated on TSB represented the total population which survived heating, while counts on TSB plus NaCl (TSBS) represented the uninjured organisms. When membranes were transferred from TSBS to fresh TSB and reincubated, injured cells recovered and formed colonies. Mossel and Ratto (74) acknowledged that a pre-enrichment also was necessary for recovery of impaired Enterobacteriaceae from samples of dried foods, drugs and feeds. It was determined that incubation of the samples at room temperature for 1 to 2 hours in buffered tryptone soya peptone broth (TSB) was sufficient for resuscitation before exposure to a selective medium.

Roth and Keenan (91) found that strains of E. coli exhibited an increased sensitivity to VRBA when sublethally treated in an acid environment. E. coli cells which had been treated with sanitizers were also inhibited by VRBA (93). Scheusner et al. (94) tested ingredients of this selective medium against cells damaged by a quaternary ammonium com-

pound. The bile salts mixture alone prevented as many injured cells from growing as did any combination of the selective agents and inhibited as many injured bacteria as were inhibited by the complete VRBA medium.

When coliforms were sampled from air they also were inhibited or restricted in growth on several selective media. In their studies of injured airborne coliforms, Stersky and Hedrick (100) found a direct relationship between the concentration of bile salts and the inhibitory effect of the medium. The effect of Bile Salts No. 3 (Difco) on airborne coliforms was determined by adding various concentrations, ranging from 0.1 to 2.0 grams per liter to standard plate count agar. As the concentration of bile salts increased coliform counts decreased, with inhibition discernible at levels much below the 1.5 grams per liter used in VRBA. Maxcy (62) also implicated bile salts as the primary constituent of VRBA responsible for its inability to detect injured cells. The inhibitory properties of several other salts were tested by Scheusner et al. (94). The addition of sodium lauryl sulfate (0.1 per cent) to a minimal agar medium resulted in no inhibition of untreated E. coli and limited inhibition of cells that had been treated with a quaternary ammonium compound. Concerned that the variability and toxicity of bile salt preparations often hampers the detection of unimpaired Enterobacteriaceae, Mossel et al. (75) suggested replacing the bile salts in EE-broth with the

chemically synthesized sodium lauryl sulfate. Test data indicated that in a concentration of 1 gram per liter the modified medium was selective without being inhibitory to pure cultures of Enterobacteriaceae or those occurring in foods subsequent to resuscitation. It was stressed, however, that this new medium should not be used directly when examining dried, frozen, heated, or acid foods. Direct exposure to even the best surfactant medium, the warning continues, will result in gross underestimation of the contamination level because of failure to recover sublethally injured cells.

Nelson (76) discussed the influence of several other factors on the recovery of injured cells. For several gram negative bacteria tested, pH 6.0 permitted maximum recovery of heat-stressed organisms, whereas non-stressed cells approximated the maximum viable count over a relatively broad pH range. Studies on the influence of incubation temperature on three representative species showed no demonstrable effect on unstressed organisms over the range of 21° to 42°C. However, maximum counts were obtained at 32°C for all the stressed organisms. It was found that omission of neutral red, lactose, or peptone from VRBA resulted in large increases in coliform counts, while omission of crystal violet or NaCl had a lesser effect. Although sugars are not usually considered inhibitory agents, the presence in medium of glucose and lactose may reduce under certain circumstances apparent survival of stressed organisms (76).

In view of these findings it is understandable that M-FC medium, which contains Bile Salts No. 3 (1.5 g/liter), lactose (12.5 g/liter), rosolic acid (0.1 g/liter), and aniline blue (0.1 g/liter), is inhibitory to injured fecal coliform organisms. Additional stress may be provided by the elevated incubation temperature (44.5°C) required by the membrane filter method. It has been postulated that the presumptive MPN test, which includes an incubation in broth for 24 to 48 hours at 35°C, provides a more favorable environment than the membrane filter surface for the repair of sublethally injured cells.

Resuscitation attempts. It has been shown that reactivation of environmentally-injured coliform cells was possible by exposure to a rich medium. Speck et al. (86,97,111) proposed a method for the repair and enumeration of freeze-injured coliforms from foods. Samples were surface plated on TSA, incubated for 1 to 2 hours at 25°C, overlaid with VRBA and reincubated at 35°C for 24 hours. This technique avoids the direct contact of impaired cells with the selective medium and the molten agar (45°C).

Several enrichment procedures have recently been proposed to increase membrane filter recover of fecal coliform bacteria. A two-day method was developed by Stevens et al. (101) which included an overnight acclimatizing period on a minimal (LES holding agar) medium at 25°C followed

by transfer to M-FC broth on absorbent pads for an additional 24 hour incubation at 44.5°C. Recovery of fecal coliforms from marine waters with this technique was equal to about 90 per cent of the MPN recovery when correction was made for the MPN bias. Selectivity was demonstrated by the 93 per cent confirmation rate. Preliminary attempts at pre-enrichment by these researchers included incubation of the membranes on lauryl tryptose broth at 35°C for varying time periods. This permitted excessive growth of non-fecal coliforms which often obscured the blue fecal coliform colonies.

Rose et al. (90) reported on an improved fecal coliform membrane filter method using M-FC agar with a lactose agar overlay. After incubating the plates at 35°C for two hours, the temperature was increased to 44.5°C for 22 to 24 hours. The two-layer agar method, when tested with a variety of water samples, yielded almost twice the number of fecal coliform colonies recovered by the standard MF procedure. However, the proposed method was not compared with the MPN test for recovery of fecal coliforms. The greatest improvement in fecal coliform detection with the two-layer agar technique was observed in the analysis of marine waters and chlorinated sewage effluents.

A two-step membrane filter method was described by Lin (59) that required pre-enrichment with phenol red lactose broth for 4 hours at 35°C. Membranes were then transferred to M-FC agar for an additional 18±2 hour incubation

at 44.5°C. Secondary and tertiary effluent samples from several treatment plants were chlorinated in the laboratory with calcium hypochlorite. Comparisons indicated that fecal coliform recovery using the two-step membrane filter method was comparable to recovery by the MPN procedure. In addition, the efficiency of M-FC broth was compared to M-FC agar for fecal coliform detection using the standard one-step MF method. For the 21 samples tested, fecal coliform counts were significantly higher (100 per cent) on M-FC agar than on the broth medium. Contrary to these findings, Davenport et al. (15) reported that the broth medium yielded 1.8 to 2.0 times more fecal coliforms than the agar medium when testing arctic river water. In an attempt to reduce the shock of a rapid temperature increase from the 0° river water to 44.5°C this group examined the possibility of including a preincubation step to the M-FC fecal coliform procedure. Filters were placed on the broth medium and left at either room temperature or 35°C for 2 hours prior to the 24 hour incubation at 44.5°C. Parallel samples were placed immediately in the water bath. Counts on membranes left at room temperature were 2.0 to 2.3 times greater and those preincubated at 35°C were 2.8 to 3.5 times greater than those from filters incubated immediately at 44.5°C. A 2 hour preincubation at 35°C was adopted and further comparisons of fecal coliform recoveries were made with the MPN procedure. Even with the preincubation step, the M-FC counts were below

the lower 95 per cent confidence limit of the MPN in all but 2 out of the 12 samples closer to the pollution source. The fecal coliform densities became more comparable after five days flow time downstream and were in agreement in the remaining 7 out of 9 samples. Presumably, as injured cells die off, resuscitation has a diminishing effect on the efficiency of the recovery technique.

Pressword and Strong (84) suggested eliminating rosolic acid from M-FC medium to increase fecal coliform recoveries. Analysis of 151 water samples from various sources including chlorinated and unchlorinated domestic sewage indicated that counts were slightly higher on the modified medium and selectivity was not effected.

Effect of the membrane on fecal coliform recovery. In the past several years it has been brought to the attention of workers in the field that the membrane filters themselves may influence coliform recovery and that significant differences may exist between filters produced by the various manufacturers for the same intended use. Brown (12) observed that the use of membrane filters for the enumeration of viable E. coli in suspensions resulted in lower and more variable colony counts than were obtained by standard pour or spread plating methods. Data indicated that Millipore membranes (HAWP, 0.45 µm pore size) had a variable inhibitory effect on certain strains of E. coli, although he was unable

to detect the presence of any diffusible toxic factors in the filters. Since inversion of the membranes on the agar surface produced colony counts equal to the spread plate controls, it was concluded that an uneven and insufficient supply of nutrients was inhibiting the growth of some of the E. coli strains on the filters.

Presswood and Brown (83) compared the ability of two leading brands of membrane filters to enumerate fecal coliform bacteria at both 35°C and 44.5°C. Colonial densities were consistently lower on Millipore (HAWG 04750) than on Gelman (GN-6) filters, but the disparity was greatest at the elevated incubation temperature. Using 25 strains of E. coli, counts on Gelman filters averaged 2.3 times higher than those on Millipore filters. Analysis of river water samples yielded similar data. Temperature studies performed with pure cultures and M-FC agar pour plates showed no statistical difference at the 5 per cent significance level between coliform counts on plates incubated at 35 and 44.5°C. These results led the authors to conclude that the higher temperature is not in itself detrimental to the growth of E. coli. However, their findings may have been influenced by preselecting temperature tolerant cultures. Test strains of E. coli were isolated from river water and sewage using membrane filters on M-FC broth at 44.5° C. Unlike Presswood and Brown (83), Hufham (48) was unable to find any differences in the recovery of E. coli with the two brands of fil-

ters when incubated on M-coliform broth (BBL) at 35°C. On the other hand Gelman membranes were significantly more efficient than Millipore when cultured on M-FC broth at 44.5°C. Schaeffer et al. (92) employed two statistical models for the analysis of coliform recovery on the same two brands of membrane filters. It was determined that fecal coliforms were recovered equally well by either membrane, but Gelman filters yielded higher numbers of total coliforms than did Millipore filters.

Dutka et al. (18) reported on still another comparison of the ability of several membrane filter brands to enumerate indicator organisms and heterotrophs. It was observed that Gelman membranes generally produced the highest counts of total coliforms, fecal coliforms, fecal streptococci and heterotrophs during the field studies, although fecal coliform data were inconclusive. Tests performed in March indicated that Gelman and Millipore filters did not differ significantly, while Sartorious membranes recovered fewer fecal coliforms. When the test was repeated in June, no significant filter-related differences in fecal coliform yield were apparent. Toxicity of the membranes was tested by comparing the MF recovery of an E. coli strain with corresponding pour plates using M-plate count medium at 35°C. Differences between membrane brands and among membranes of the same manufacturer were observed. When tests were performed at 44.5°C no membranes were able to recover more than

40 per cent of the test organisms. Brodsky and Schiemann (10) evaluated four brands of filters using as coliform sources positive EC broth cultures of routine water samples and Humber River (Ontario, Canada) water. It was concluded that the source of coliform bacteria has an important influence on the conclusions of membrane filter evaluation studies.

It was found by Sladek et al. (96) that membrane structure greatly affected the growth of fecal coliform bacteria. Maximum recovery was obtained on mixed cellulose ester filters with a surface opening diameter of 2.4 µm and a retention pore size of 0.7 µm. They suggested that the larger surface pore may enable the organisms deposited on the membrane surface to be more completely bathed in nutrient. Green et al. (35) compared the new membrane (Millipore HC) with five conventional membranes (including Millipore HA) for the enumeration of fecal coliforms from a variety of unchlorinated surface waters. Data indicated that fecal coliform densities were greatest on Millipore HC filters with Gelman GN-6 membranes ranking second in recovery. Electron micrographs of Standridge (99) and Tobin and Dutka (107) confirm that bacterial recoveries and flow rates correlate well with the surface pore structure of membrane filters. Surface pore openings of the Gelman GN-6 filter are slightly smaller than the HC but distinctly larger than those of the Millipore HA filters. Lin (58) also demonstrated that type

HC are superior to type HA membranes for fecal coliform recovery although the improvement was small when testing chlorinated sewage effluents with M-FC agar at 44.5°C. In a subsequent study Lin (60) compared ten membrane filter types for fecal coliform recovery from chlorinated effluents using his two-step procedure (4 hours preincubation on phenol red lactose broth at 35°C). Again Millipore HC filters were shown to provide most efficient recovery followed by the Gelman GN-6 membrane.

MATERIALS AND METHODS

Membrane filtration techniques. M-FC broth base (Difco and BBL) was used throughout these studies. This was prepared according to the manufacturer's directions with rosolic acid (Difco) and the addition of 15 g/liter agar. The medium was dispensed in 5 ml amounts in 50 mm plastic disposable petri dishes (Millipore Corp., Bedford, Mass.) and stored inverted in the dark at 5°C. Plates were usually prepared daily and always used within 48 hours of preparation. Care was taken not to use more than one production lot of medium for any one test run.

Type HC membrane (Millipore) filters with retention pore size of 0.7 µm and surface opening diameter of 2.4 µm were used for all tests unless otherwise noted. These membranes have been demonstrated (35,58,96,107) to provide the optimum pore structure for recovery of fecal coliform bacteria. HCWG 047 S1 filters are individually packaged and presterilized with ethylene oxide. For comparative purposes in several tests, GN-6 membranes (Gelman Instrument Co., Ann Arbor, Mich.) were employed. These were presterilized by the manufacturer by autoclaving. To assure that possible differences between membrane filter production lots would not influence test results, a single lot number was always

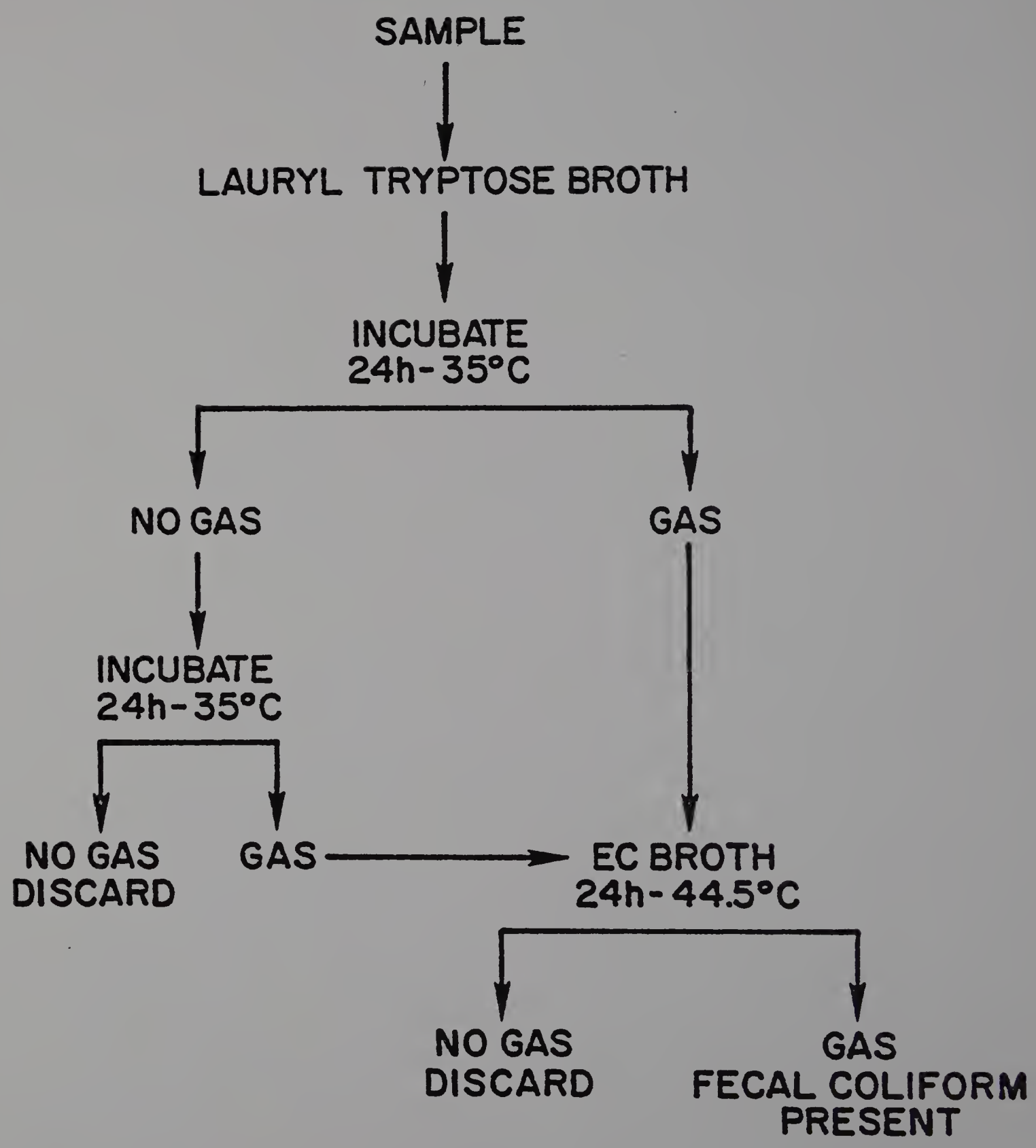
used for an entire experiment.

Membrane filtration tests were performed in accordance with the 14th edition of Standard Methods (5) using a six-place manifold, pyrex filter-holders, and bench-top vacuum pump. Peptone water (0.1 per cent) was used as a diluent and rinse. Samples were maintained in an ice bath to prevent multiplication while tests were in progress. Sample volumes were chosen to yield the recommended number of colonies (20-60) on the filters on M-FC agar plates.

Plates were incubated at 44.5±0.2°C in a Millipore solid-state heating block or submerged in a Blue M circulating water bath in zip-lock Bitran bags (Uly-Pak, Inc., Ulysses, Ks.). Plates requiring 35°C were placed in an air incubator. Colonies were counted with the aid of a stereomicroscope and fluorescent light source. Blue colonies recovered by the MF M-FC agar procedure were confirmed as fecal coliforms by gas production in lauryl tryptose broth at 35°C within 48 hours and in EC broth at 44.5°C within 24 hours. When possible all blue colonies on a plate were verified to avoid bias.

MPN test. Five-tube MPN tests were performed in accordance with Standard Methods (5) and as shown in Figure 1. Four serial dilutions of sample were inoculated into lauryl tryptose broth. Tubes showing gas production within 24 or 48 hours at 35°C were transferred with sterile wooden ap-

Figure 1. Protocol for the fecal coliform MPN test.


SAMPLE
LAURYL TRYPTOSE BROTH
INCUBATE
24h-35°C
NO GAS
INCUBATE
24h-35°C
NO GAS
DISCARD
GAS
GAS
EC BROTH
24h-44.5°C
NO GAS
DISCARD
GAS
FECAL COLIFORM
PRESENT

plicator sticks to EC broth. Tubes showing gas production within 24 hours at 44.5±0.2°C were considered positive for fecal coliforms. The fecal coliform most probable number was calculated using an MPN table.

Test organisms. E. coli ATCC 8739 was used for pure culture studies. In addition a fecal coliform strain (FC-10) was isolated from raw sewage by plating on eosin methylene blue (EMB) agar (Difco). Incubation was at 35°C so as not to select an elevated-temperature tolerant strain. The indole-methyl red-Voges-Proskauer-citrate utilization (IMViC) test was performed on a colony with the typical green metallic sheen. It was classified as E. coli type 1 (IMViC ++--) and fecal coliform by gas production in EC broth at 44.5°C.

Test cultures were standardized by transferring 0.1 ml of an overnight (35°C) trypticase soy broth (TSB) culture into a second 10 ml of TSB and incubating for 22-24 hours at 35°C.

Effect of membrane extraction on coliform recovery. The ideal membrane filter for microbiological applications should provide a non-toxic inert structure capable of retaining all organisms on the surface. Tests were performed to determine whether extractables are present on membrane filters which are inhibitory to injured fecal coliform bacteria. Membrane filters from two manufacturers (Millipore HC and Gelman GN-6) were extracted by placing 10-15 filters in a beaker contain-

ing approximately 800 ml of reagent grade water (Milli-Q System, Millipore Corp.) and bringing slowly to a boil. The water was then changed, brought slowly to a boil and simmered gently for 10 to 15 minutes. These filters were then compared to untreated membranes of the same lot number for fecal coliform recovery.

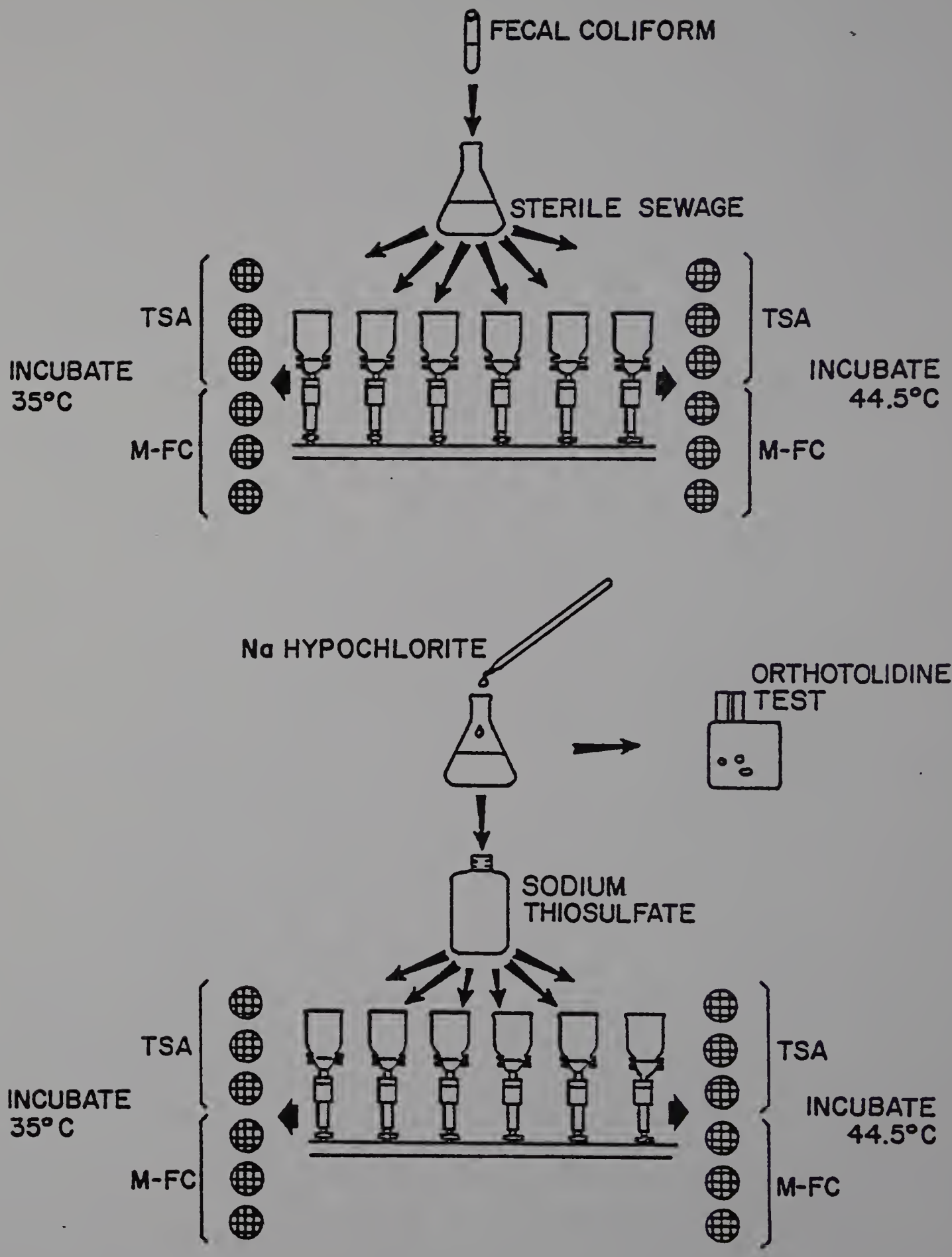
Raw primary sewage effluent from the Amherst treatment plant was chlorinated in the laboratory to provide a population of injured fecal coliforms. One-liter volumes of sewage were maintained at room temperature and dosed with chlorine in the form of sodium hypochlorite (0.5 per cent by weight). Sufficient hypochlorite was added to obtain a residual chlorine level (free and combined) of 0.1 mg/liter as measured by the orthotolidine test (4). Samples were mixed intermittently for 10 minutes before neutralization with an excess of sodium thiosulfate (0.2 per cent). One to 10 ml sample volumes were filtered using 3 to 5 replicates of extracted and untreated membranes.

Fecal coliform recovery on non-selective and selective media at two incubation temperatures. A series of tests were performed to determine the relative importance of culture medium and incubation temperature in the recovery of chlorine-injured fecal coliform organisms. The non-selective medium trypticase soy agar (TSA, BBL) was prepared according to the manufacturer's directions and dispensed in 5 ml amounts

in 50 mm disposable petri dishes. The selective medium, M-FC agar, was prepared as described previously. Employing a non-selective medium to enumerate both injured and uninjured organisms required the use of a pure culture as the inoculum. In order to simulate natural conditions, secondary sewage effluent (Amherst pilot treatment plant) was autoclaved in 500 ml amounts for 20 minutes at 15 pounds pressure (121°C) and seeded with appropriate dilutions of a 22 to 24 hour TSB culture of either E. coli ATCC 8739 or the wild strain FC-10 to yield approximately 100,000 cells per ml (Figure 2). The fecal coliform population before chlorination was determined by plating triplicate membranes on TSA and M-FC agar at both 35°C and 44.5°C. Sufficient hypochlorite (0.05 per cent by weight) was then added to the sewage to obtain a residual chlorine level of 0.3 mg/liter. After 6 to 7 minutes the sample was neutralized with sodium thiosulfate (0.2 per cent) and maintained in an ice bath during the filtration procedure. Triplicate membranes were plated on TSA and M-FC agar for incubation at 35°C and 44.5°C.

Effects of bile salts and rosolic acid at two incubation temperatures. The inhibition of injured fecal coliforms by M-FC agar is related to some degree to the chemical formulation of the medium. The effects of two suspect ingredients, Bile Salts No. 3 and rosolic acid were evaluated. Bacto-Bile Salts No. 3 (Difco) were added to TSA in the same concentra-

Figure 2. Protocol for the evaluation of fecal coliform recovery on TSA and M-FC agar at two incubation temperatures.


FECAL COLIFORM
STERILE SEWAGE
TSA
INCUBATE
35°C
M-FC
TSA
INCUBATE
44.5°C
M-FC
Na HYPOCHLORITE
ORTHOTOLIDINE
TEST
SODIUM
THIOSULFATE
TSA
INCUBATE
35° C
M-FC
TSA
INCUBATE
44.5°C
M-FC

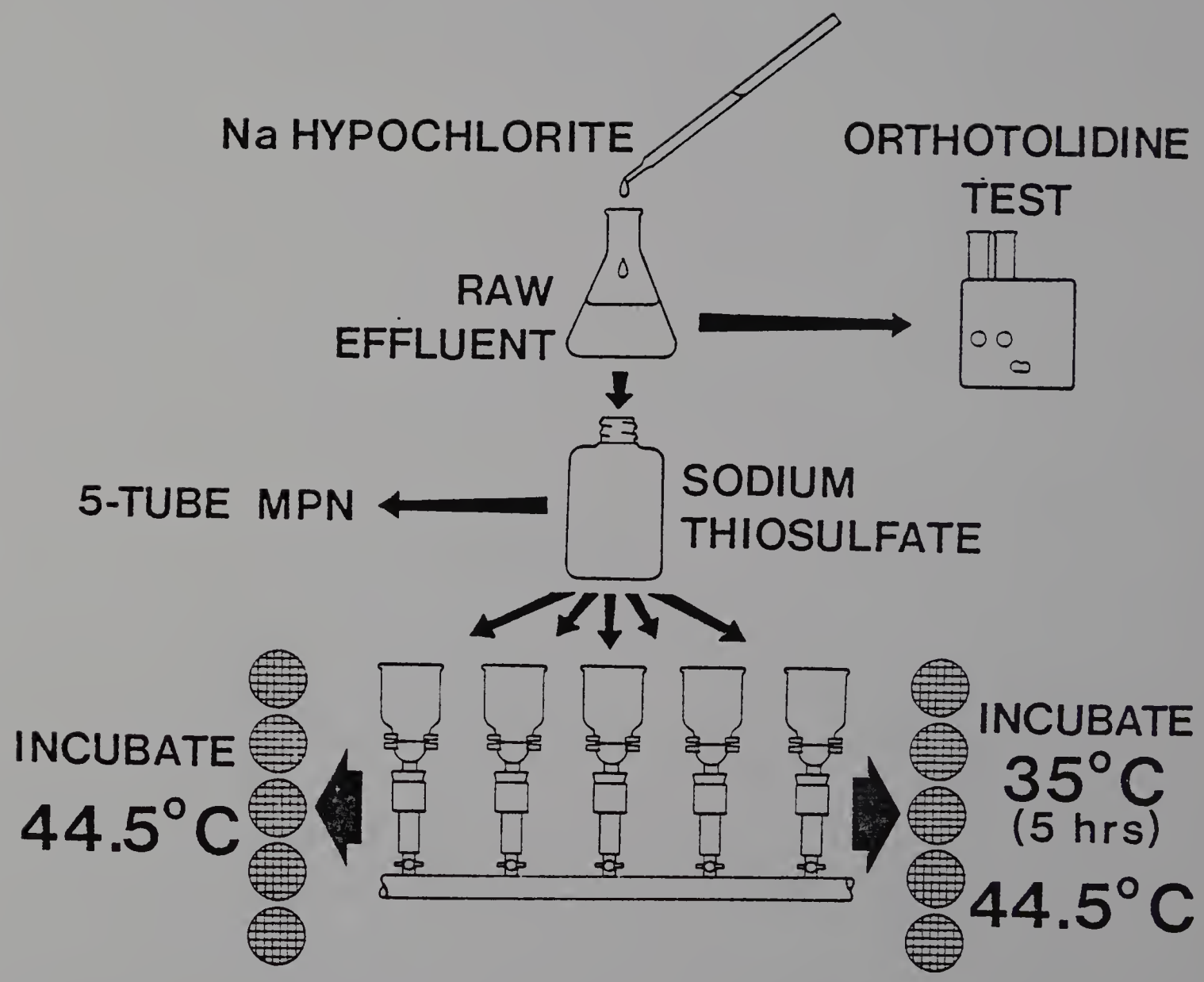
tion (1.5 g/liter) as found in M-FC medium. M-FC agar plates were prepared with and without rosolic acid. Autoclaved secondary sewage was seeded with an appropriate dilution of a pure culture of fecal coliform as previously described. Triplicate TSA spread plates (100 mm) were made to determine the initial count. Samples were chlorinated to obtain residual levels of 0.3 to 0.4 mg/liter for a contact period of 7 to 9 minutes. After neutralization sample volumes were filtered and triplicate membranes placed on duplicate sets of the four recovery media for incubation at both 35°C and 44.5°C. Triplicate MPNs also were inoculated with chlorinated samples for comparison with MF recoveries.

Sodium lauryl sulfate as substitute for bile salts. Sodium lauryl sulfate has been mentioned in the literature (75) as a non-toxic substitute for bile salts in selective media. The same experimental design as described above was used to evaluate the possibility of making this substitution in the formulation of M-FC agar. TSA plates (50 mm) were prepared in the conventional manner and with the addition of Bacto-Bile Salts No. 3 (1.5 g/liter) or sodium lauryl sulfate (SLS, laboratory grade, Fisher Scientific, Medford, Mass., 1 g/liter). In addition several tests were performed with SLS of higher purity (dodecyl sodium sulfate, Eastman Kodak Co., 1 g/liter). Duplicate or triplicate TSA spread plates were made from dilutions of the sewage seeded with a pure

culture of fecal coliform to determine the initial count. Samples were then dosed with hypochlorite to maintain residual chlorine levels of 0.3 to 0.35 mg/liter. After a 5 to 7 minute contact period, samples were neutralized and volumes filtered to provide four to six replicate membranes for each of three recovery media. Parallel sets of plates were incubated at 35°C and 44.5°C.

Effect of incubation temperature. The effect of temperature on the recovery of fecal coliform bacteria by the membrane filter (M-FC) procedure was investigated by evaluating various incubation time and temperature combinations. Primary sewage effluent was collected at the Amherst treatment plant and chlorinated in the laboratory. This facilitated standardization of samples and greater predictability in chlorine levels and exposure time. Sodium hypochlorite (0.5 per cent by weight) was added to one-liter samples to obtain residual chlorine levels up to 2 mg/liter (Figure 3). Samples were mixed intermittently for contact times varying from 5 to 20 minutes before being neutralized with an excess of sodium thiosulfate (0.2 per cent). Single or duplicate five-tube MPN tests were performed by inoculating four serial dilutions into lauryl tryptose broth. Simultaneously, 20 replicate volumes of sample were filtered and membranes were placed on M-FC agar plates. Five plates were incubated directly at 44.5°C in the solid-state heating block. The re-

Figure 3. Protocol for standard MF/modified MF/MPN comparisons.


Na HYPOCHLORITE
ORTHOTOLIDINE
TEST
RAW
EFFLUENT
5-TUBE MPN
SODIUM
THIOSULFATE
INCUBATE
44.5°C
INCUBATE
35°C
(5 hrs)
44.5°C

maining 15 plates were incubated in a 35°C air incubator. At 3, 4, and 5 hours, sets of five plates were transferred to the 44.5°C incubator for the remainder of the incubation period. Plates were counted and blue colonies recovered by the modified procedure were confirmed as fecal coliforms as described above.

Incubation at 35°C for 5 hours followed by 18±1 hour at 44.5°C was determined to be the most productive temperature-time combination yielding the highest recovery of fecal coliforms without excessive background growth. After establishing the optimum conditions, a study was carried out to compare the modified method with the standard MF and multiple-tube methods using laboratory-chlorinated primary sewage effluent. In addition several tests were performed to determine the effect of a five hour preincubation at 35°C on the recovery of fecal coliforms from unchlorinated primary effluent. The modified method was again compared with the standard MF and MPN procedures using dilutions of sewage from the Amherst treatment plant.

Field study--plant-chlorinated samples. The study was extended to include an evaluation of the modified method for fecal coliform recovery from plant-chlorinated effluents. Samples were collected from several primary and secondary treatment plants at various times after normal chlorination. Treatment plants cooperating in this project were in the

towns of Amherst, Sunderland, Northampton, Greenfield, Millers Falls and South Deerfield, Massachusetts. Additional samples from Bozeman and Trident, Montana plants were analyzed at Montana State University to achieve sample diversity. Samples were neutralized immediately or after holding from four to 25 minutes depending upon chlorine residual measurements and the distance they were collected from the chlorinator. Following dechlorination the samples were analyzed within two hours of collection as previously described.

The combined effect of the five hour preincubation and the omission of rosolic acid from the M-FC agar was determined using 13 of the same plant-chlorinated samples. Five additional membranes were prepared from each sample and placed on M-FC agar without rosolic acid. Plates were incubated at 35°C followed by 18±2 hours at 44.5°C.

Field study--marine samples. The suitability of membrane filtration for fecal coliform analysis of marine water is not clearly defined. However, reports indicate that the standard membrane filter procedure is less efficient than the MPN method for recovery of this group from sea water (82, 101). The marine environment may cause a similar stress on fecal coliform cells which reduces their ability to grow on a selective medium at the elevated temperature. The application of the modified MF technique to the analysis of marine

samples was evaluated. Forty samples were obtained from several locations along the northeastern coast including Rockport, Gloucester and Provincetown, Massachusetts, and Seawaren and Perth Amboy, New Jersey. Nine additional samples were withdrawn from a membrane-filter diffusion chamber (67) which was seeded with a 1:10 dilution of raw primary sewage, suspended in a flowing sea water tank (University of Massachusetts Marine Station), and sampled over a 48 hour period.

All samples were processed within four hours of collection. Five replicate M-FC plates were prepared for both the standard and modified MF tests. Simultaneously one to three five-tube MPNs were inoculated. The procedure, incubation, and colony verification was the same as previously described.

Comparison of modified MF and IM-MF methods. Concurrent to this research a study at Montana State University (Stuart et al., personal communication, June 1975) also was being conducted to develop a membrane filter technique for the enumeration of stressed fecal coliforms. A two-layer medium was proposed which contained metabolic intermediates (glycerol and acetate) in the glycolytic pathway plus reducing agents in both layers (see Appendix B for formulation). After filtration the membranes were rinsed with a rich resuscitation medium, placed on the agar and held at ambient temperature

for 1.5 hours, followed by 4.5 hours at 35°C, and 18 hours at 44.5±0.2°C. This medium has been designated IM-MF (injury-mitigating) in a recent publication (104).

The efficiency of this medium and technique for the recovery of fecal coliforms was evaluated by comparing it to the standard MF, the modified MF, and the MPN methods using laboratory-chlorinated primary effluent from the Amherst treatment plant. Hypochlorite was added to samples to maintain a residual chlorine level of 1.5 mg/liter for 13 to 19 minutes. After neutralization five replicate filters were prepared for each MF method and a five-tube MPN was inoculated with four serial dilutions of sample. Plates and tubes were incubated as required for each method.

RESULTS

Effect of membrane filter extraction. Seventeen pairs (extracted and untreated) of Millipore HC and eight pairs of Gelman GN-6 membrane filters were evaluated for efficiency of fecal coliform recovery from laboratory-chlorinated primary sewage effluent. Data in Table 1 indicate that the mean fecal coliform count per 10 ml on untreated Millipore filters was 46.4 or 51 per cent of the mean count (90.4) observed on Millipore extracted membranes. Untreated Gelman filters yielded a mean count of 23 or 62 per cent of the count (37) per 10 ml on extracted filters. The data suggest that (i) a chemical residue was removed from the membrane filters of both manufacturers by the boiling process, and that (ii) removal resulted in increased recovery of fecal coliform bacteria.

Fecal coliform recovery on non-selective and selective media at two incubation temperatures. Average fecal coliform counts for three test runs using two organisms are shown in Tables 2A, B, and C. The total viable population (i.e., uninjured plus injured cells) is represented by the number of colonies recovered on membranes incubated on TSA at 35°C for both the unchlorinated and chlorinated samples. Recovery on TSA at 44.5°C and on M-FC at 35° and 44.5°C are expressed

TABLE 1. Effect of filter extraction on fecal coliform recovery from laboratory-chlorinated primary effluent* by standard MF method. (Fecal coliforms per 10 ml)

DATE OF TEST	MILLIPORE TYPE HC			GELMAN GN-6		
	UNTREATED	LOT #	EXTRACTED	UNTREATED	LOT #	EXTRACTED
12/20/76	41	17104-9	102	21	81542	52
	53		113	40		75
	42		191	57		57
12/21/76	17		41	16		33
	14		47	5		20
	12		16	15		16
	28		73	16		20
	11		131	14		23
12/14/76	91	17104-21	127			
	104		146			
	75		98			
12/15/76	6		13			
	2		13			
	3		26			
12/14/76	110		160			
	70		90			
	110		150			
TOTAL	789		1,537	184		296
MEAN	46.4		90.4	23.0		37.0

*Chlorine residual 0.1 mg/liter; contact time 10 minutes.

TABLE 2. MF recovery of fecal coliforms before and after chlorination (residual chlorine 0.3 mg/liter; mean fecal coliform counts per 100 ml*).

A Test organism: FC-10; Contact time: 6 minutes.

INCUBATION TEMPERATURE	UNCHLORINATED		CHLORINATED	
	TSA	M-FC	TSA	M-FC
35°C	110,000	121,000	510	103
44.5°C	102,000	101,000	32	18

B Test organism: E. coli ATCC 8739; Contact time: 6 minutes.

INCUBATION TEMPERATURE	UNCHLORINATED		CHLORINATED	
	TSA	M-FC	TSA	M-FC
35°C	103,000	92,000	1,820	937
44.5°C	92,000	78,000	258	157

C Test organism: E. coli ATCC 8739; Contact time: 7 minutes.

INCUBATION TEMPERATURE	UNCHLORINATED		CHLORINATED	
	TSA	M-FC	TSA	M-FC
35°C	115,000	98,000	171	29
44.5°C	96,000	66,000	174	10

*Figures shown are averages of triplicate filters.

in Figures 4A, B, and C as percentages of the total population. Considering the inherent variability of bacteriological tests, the three bar graphs show only minor variations in coliform counts from the unchlorinated samples. When the majority of cells are undamaged, recovery by the membrane filters appears to be influenced to a small degree by temperature and culture media. After chlorination, however, the differences in counts are more dramatically affected by both temperature and media. When TSA was incubated at 44.5°C only 6 per cent, 14 per cent, and 17 per cent of the coliform cells were viable as compared to the 35°C incubation. Growth of chlorine-injured organisms on M-FC agar at 35°C was more erratic with average recovery rates of 20 per cent, 52 per cent, and 102 per cent. At the elevated temperature colony counts decreased significantly to 4 per cent, 9 per cent, and 6 per cent of the total population recovered from these samples on TSA at 35°C. These studies indicate that the 44.5°C incubation is more critical than the media to the growth of fecal coliforms that have been subjected to chlorination.

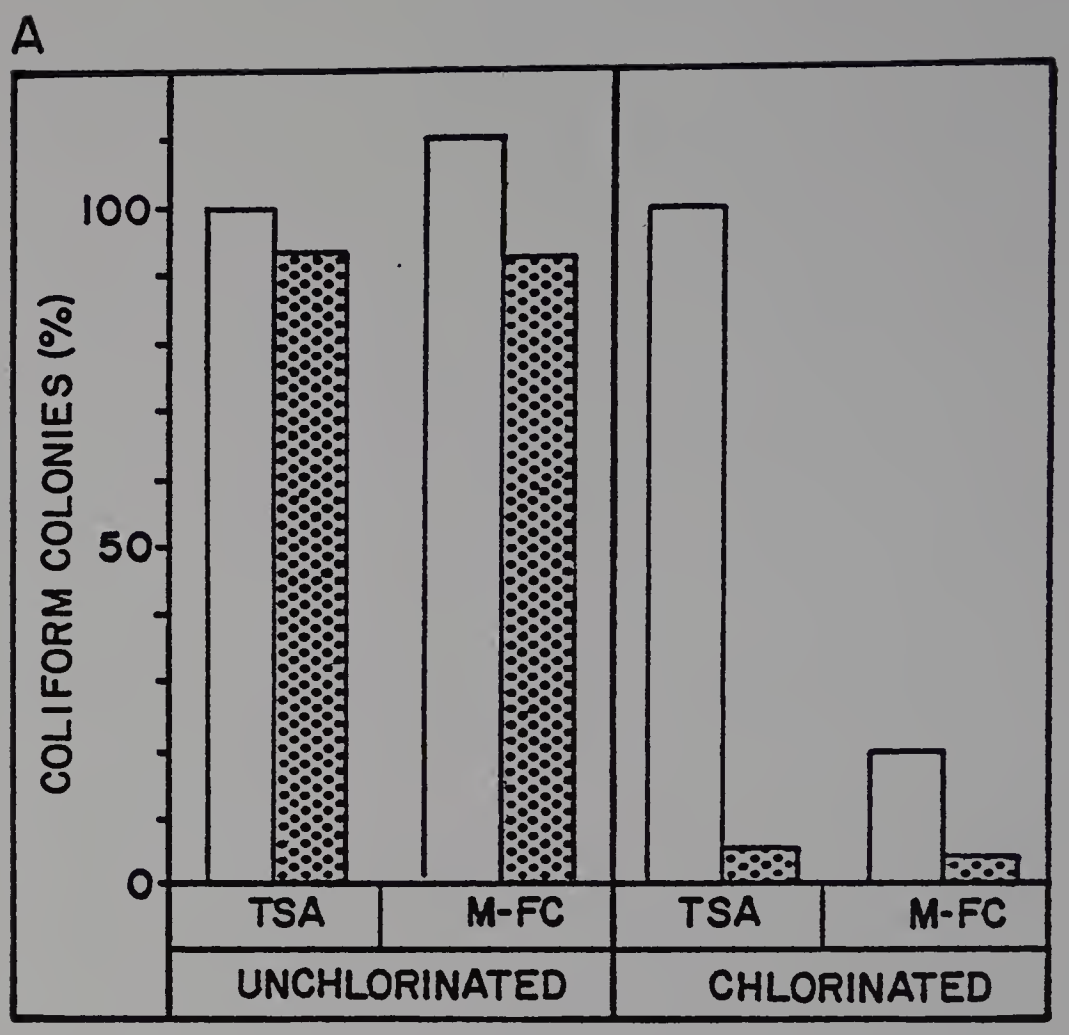
Effects of bile salts and rosolic acid at two incubation temperatures. Average fecal coliform counts per 100 ml for four test runs using E. coli ATCC 8739 are shown in Tables 3A, B, C, and D. Data for two tests using the wild strain FC-10 are in Tables 4A and B. The population before chlorination

Figure 4. Membrane filter recovery of fecal coliforms before and after chlorination (residual chlorine 0.3 mg/liter).

A. FC-10; chlorine contact time 6 minutes.

B. *E. coli* ATCC 8739; chlorine contact time 6 minutes.

C. *E. coli* ATCC 8739; chlorine contact time 7 minutes.


A
100
50
0
COLIFORM COLONIES (%)
TSA
M-FC
TSA
M-FC
UNCHLORINATED
CHLORINATED
B
100
50
0
COLIFORM COLONIES (%)
TSA
M-FC
TSA
M-FC
UNCHLORINATED
CHLORINATED
35 C
44.5 C

C

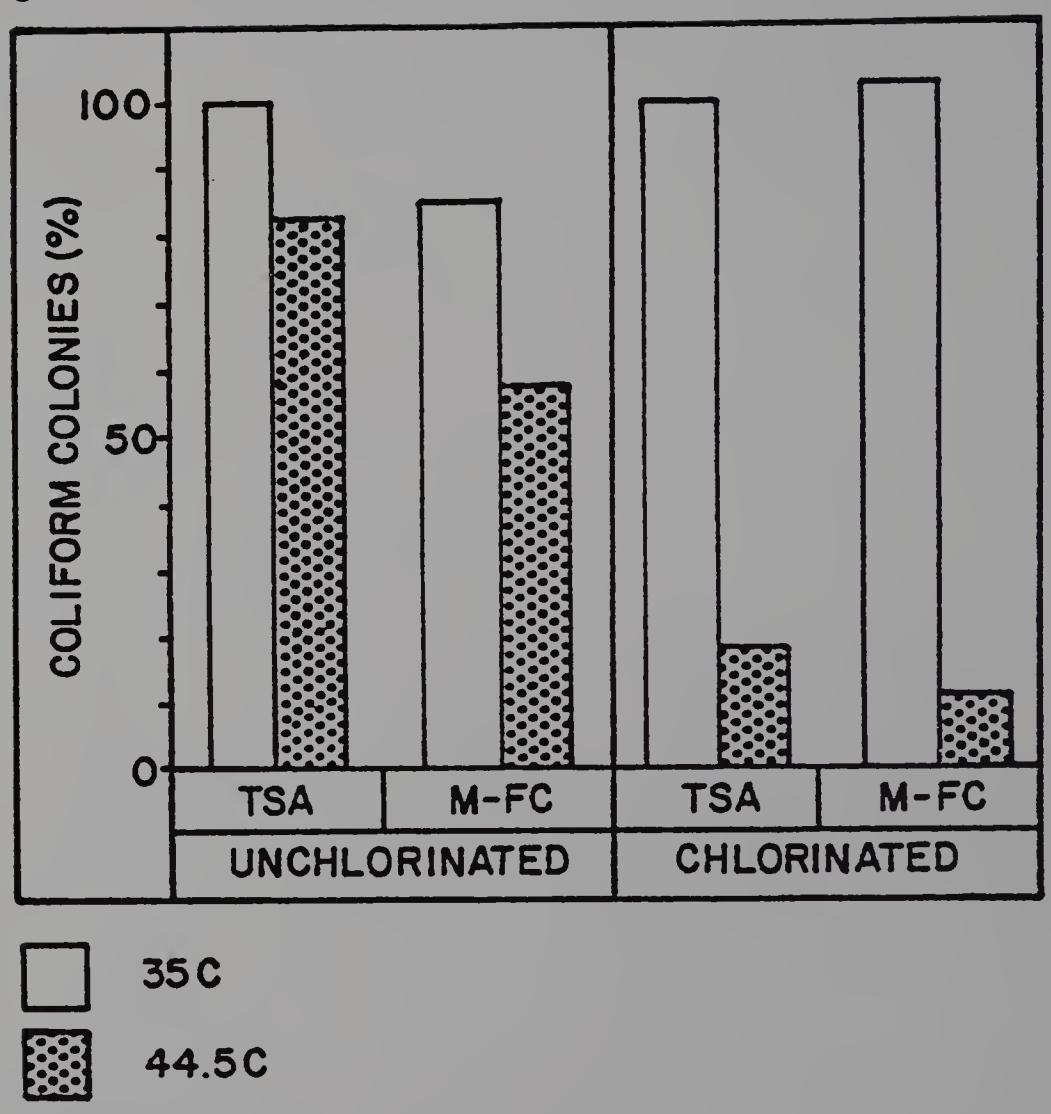

100
50
0
COLIFORM COLONIES (%)
TSA
M-FC
TSA
M-FC
UNCHLORINATED
CHLORINATED
35C
44.5C

TABLE 3. MF recovery of E. coli ATCC 8739 on four media after chlorination for 7-9 minutes (chlorine residual 0.3-0.4 mg/liter; mean fecal coliform counts per 100 ml[+]).

A Population before chlorination: 7.5×10^6/100 ml.
Mean MPN after chlorination: 340,000/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	8,967	8,533	3,400	7,900
44.5°C	760	780	327	940

B Population before chlorination: 7.3×10^6/100 ml.
Mean MPN after chlorination: 255,000/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	32,000	34,300	7,200	16,300
44.5°C	4,000	4,030	1,230	3,270

C Population before chlorination: 12×10^6/100 ml.
Mean MPN after chlorination: ≥187,000/100ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	6,430	8,900	2,030	5,230
44.5°C	330	240	67	243

D Population before chlorination: 7.5×10^6/100 ml.
Mean MPN after chlorination: ≥95,000/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	1,480	1,610	670	1,527
44.5°C	47	33	13	37

[+]Average of triplicate plates.

*Bacto-Bile Salts No. 3 (1.5 g/liter).

TABLE 4. MF recovery of FC-10 on four media after chlorination for 7 minutes (chlorine residual 0.35-0.4 mg/liter; mean fecal coliform counts per 100 ml[+]).

A Population before chlorination: 3.8×10^6/100 ml.
Mean MPN after chlorination: 8,300/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	3,230	2,830	2,130	2,030
44.5°C	367	343	67	150

B Population before chlorination: 3.9×10^6/100 ml.
Mean MPN after chlorination: 21,000/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	M-FC	M-FC w/o ROSOLIC ACID
35°C	20,000	20,700	8,740	11,920
44.5°C	3,900	4,100	533	1,494

[+]Average of triplicate plates.

*Bacto-Bile Salts No. 3 (1.5 g/liter).

ranged from 3.8×10^6 to 1.2×10^7 per 100 ml as determined by triplicate TSA spread plates. Fecal coliforms were enumerated after chlorination by plating six replicate membrane filters on each of four media. Parallel sets were incubated at 35°C and 44.5°C. The MPN tests varied greatly among triplicates and the average counts, which ranged from 8,300 to 340,000 per 100 ml, always exceeded corresponding counts on membrane filters incubated on TSA at 35°C.

In order to compare membrane filter recoveries on several media at two incubation temperatures the TSA counts at 35°C were chosen to represent 100 per cent of the viable population. The recovery on TSA at 44.5°C and on the other three media at both temperatures are expressed as percentages of the total populations in Figures 5A, B, C, and D and 6A and B. All six bar graphs indicate that the addition of Bacto-Bile Salts No. 3 to TSA does not inhibit growth of fecal coliforms when plates are incubated at 35°C. In four out of six tests growth appeared to be enhanced by the presence of bile. When membranes were incubated on M-FC agar at 35°C the recovery rate ranged from 23 to 66 per cent of the TSA. The omission of rosolic acid from the M-FC medium resulted in a slight improvement in recovery which ranged from 51 to 103 per cent of the TSA counts.

When all membranes, including those on TSA, were incubated at the elevated temperature, a sharp decline in colony counts was observed in all tests regardless of test

Figure 5. Membrane filter recovery of *E. coli* ATCC 8739 after chlorination.

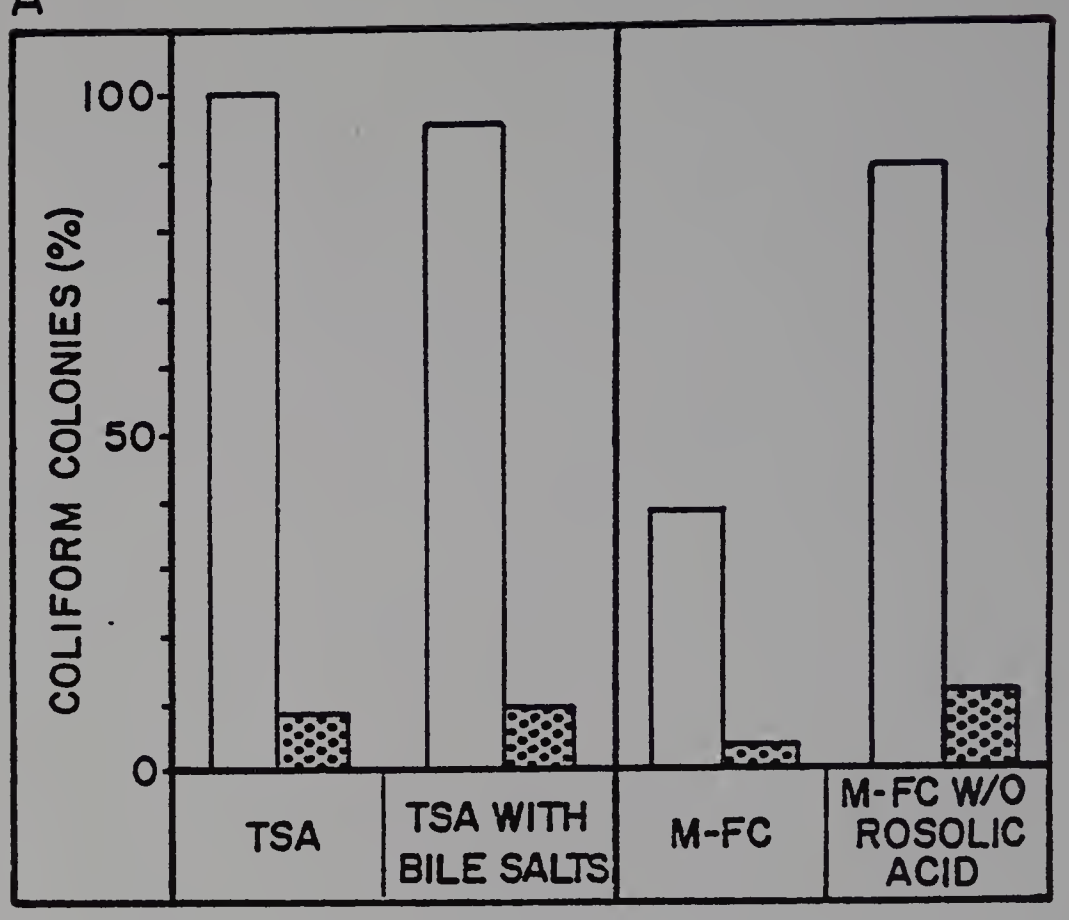
A. Residual chlorine 0.3 mg/liter; contact time 7 minutes.

B. Residual chlorine 0.4 mg/liter; contact time 7 minutes.

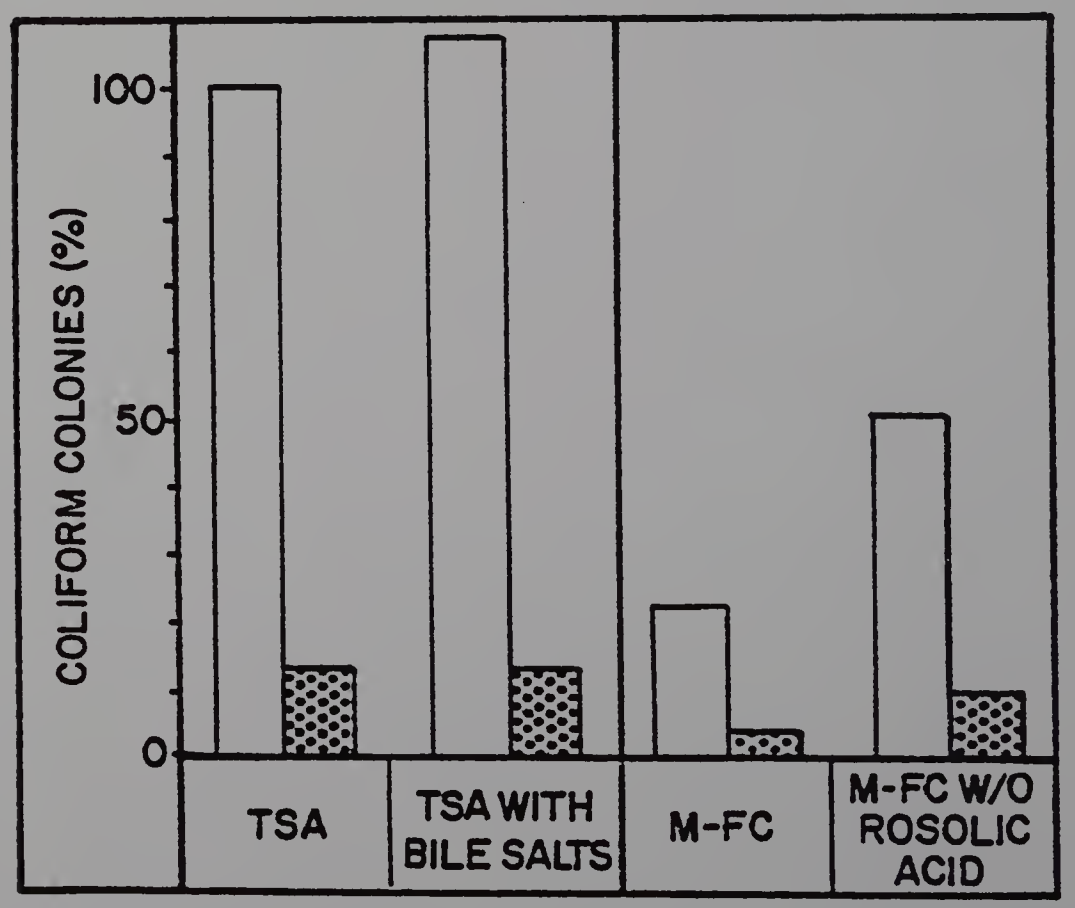
C. Residual chlorine 0.4 mg/liter; contact time 8 minutes.

D. Residual chlorine 0.4 mg/liter; contact time 9 minutes.

A


100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

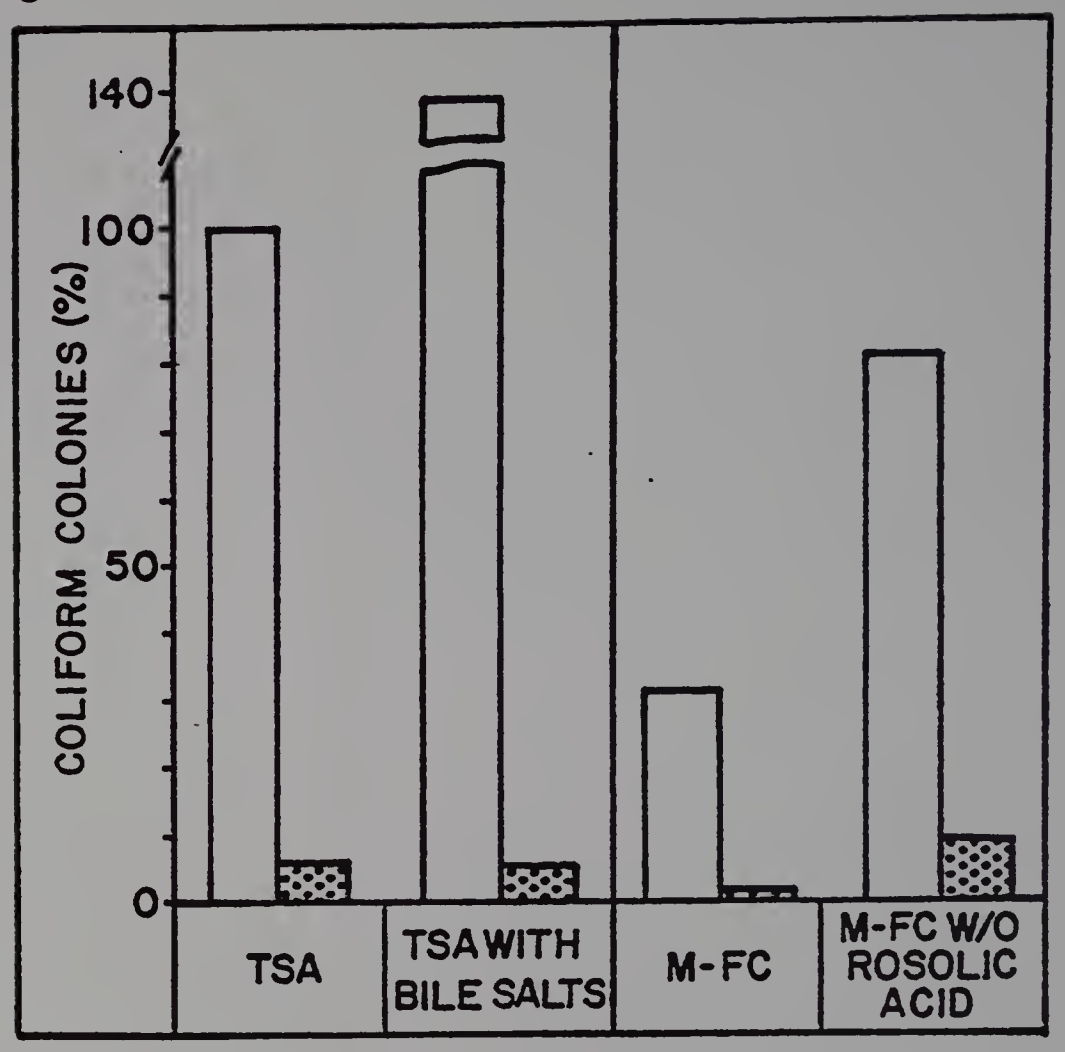
B


100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

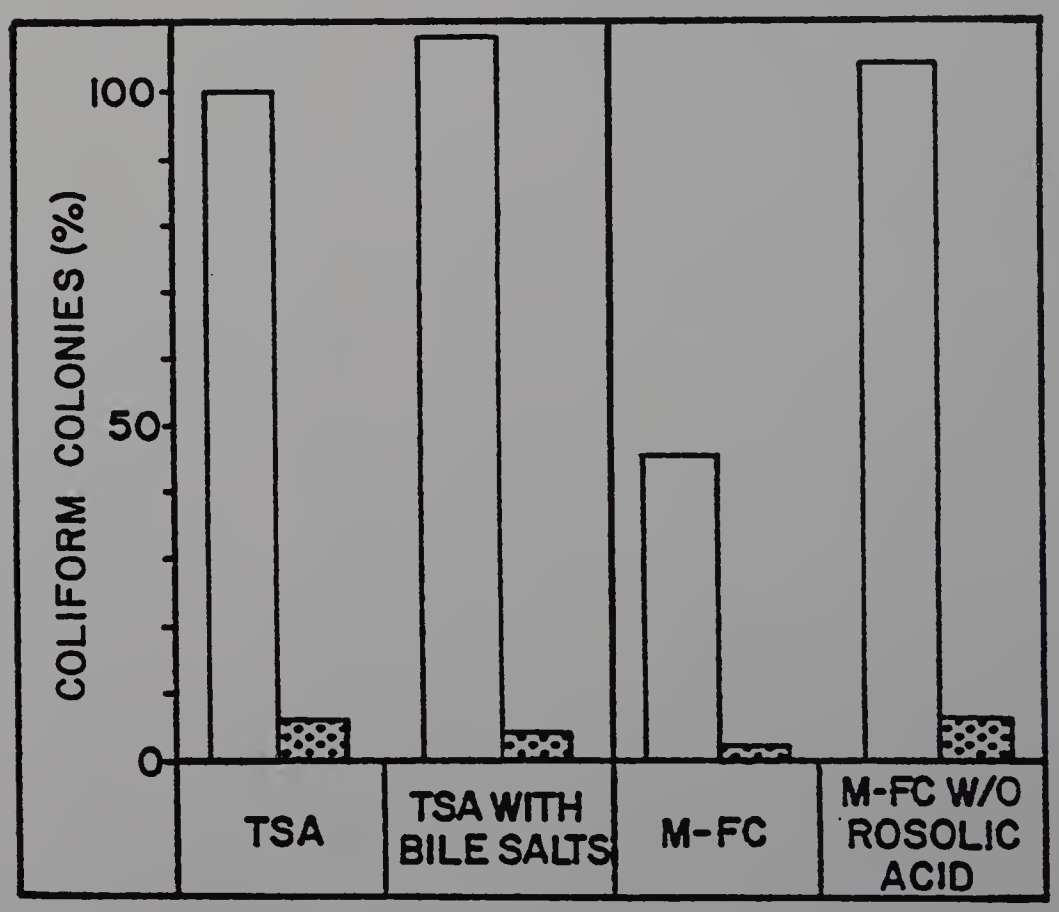
35C

44.5C

C


140
100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

D


100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

35 C

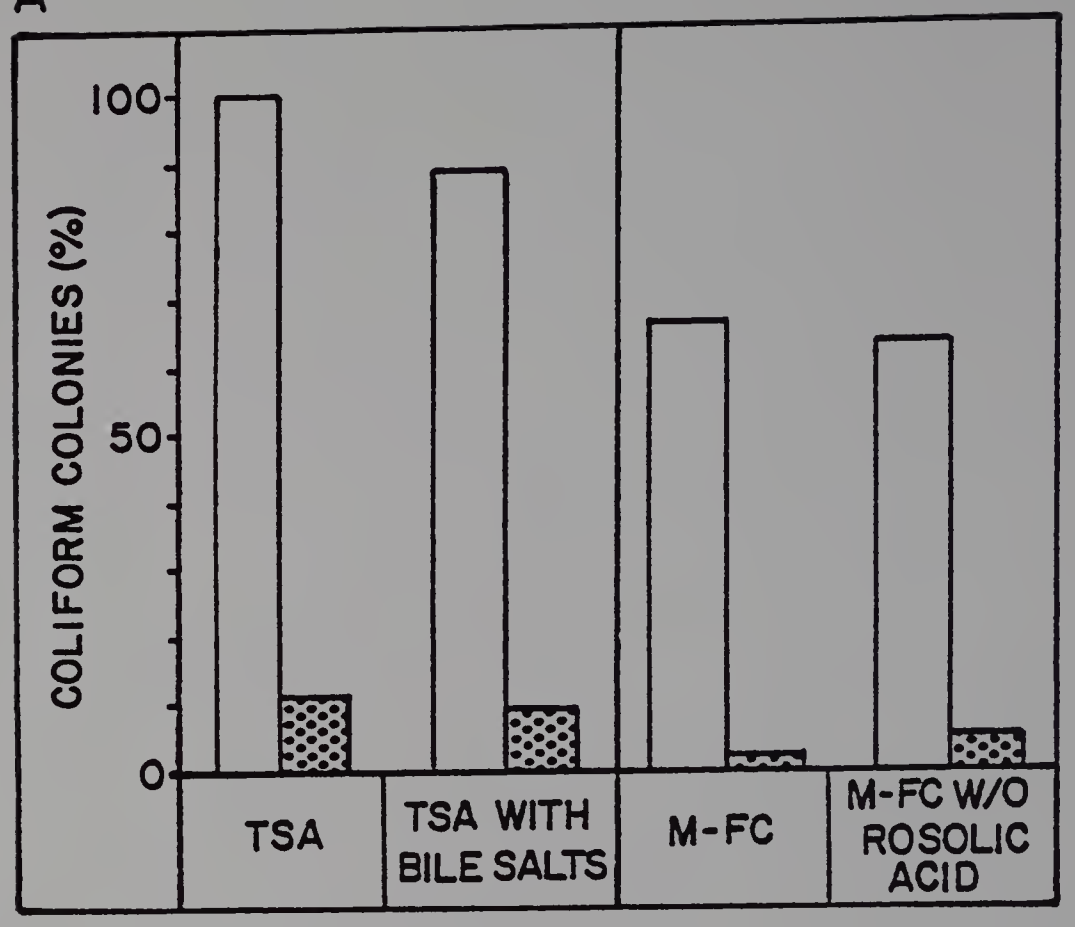
44.5 C

Figure 6. Membrane filter recovery of FC-10 after chlorination.

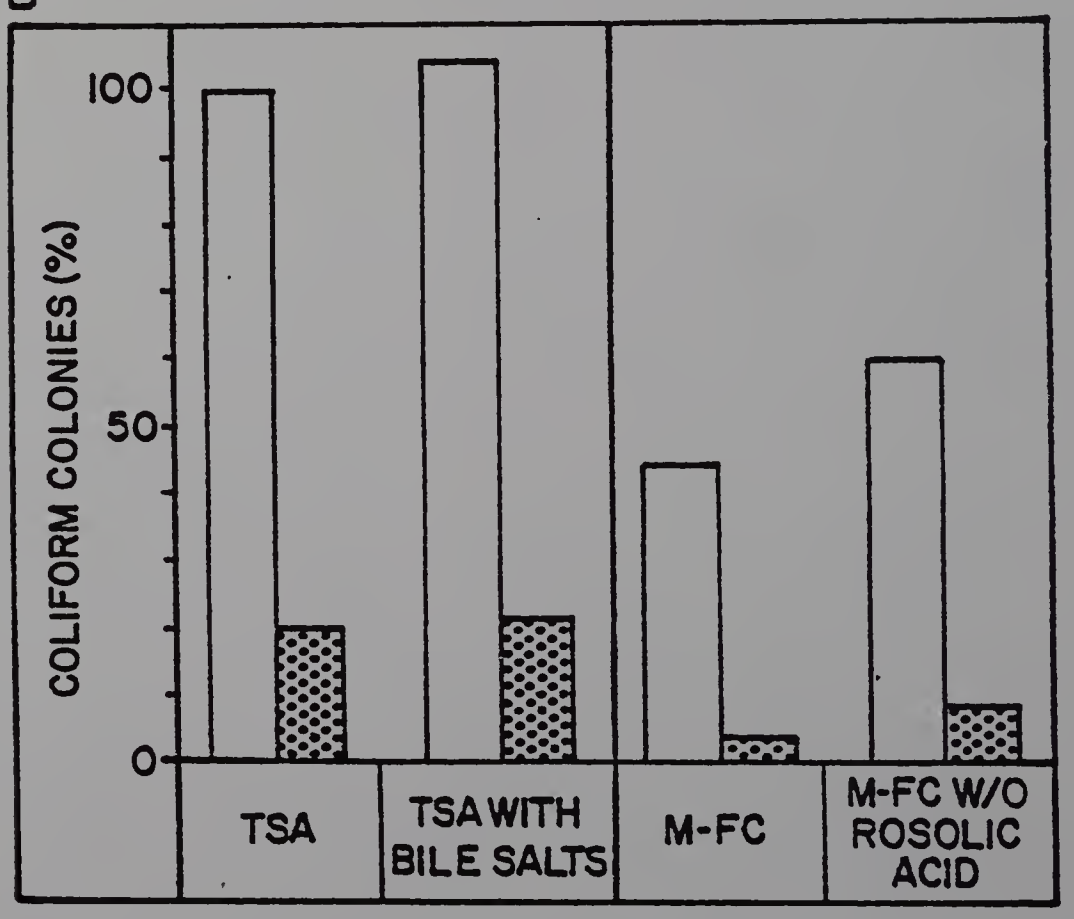
A. Residual chlorine 0.4 mg/liter; contact time 7 minutes.

B. Residual chlorine 0.35 mg/liter; contact time 7 minutes.

A


100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

B


100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
M-FC
M-FC W/O ROSOLIC ACID

35C

44.5C

organism or chlorination level. Average recovery did not exceed 21 per cent of the total population on any culture medium. In general the average number of colonies on TSA without and with bile salts was only slightly greater than on M-FC agar when incubation was at 44.5°C.

Sodium lauryl sulfate as substitute for bile salts. Two tests were performed using laboratory grade sodium lauryl sulfate (Fisher Scientific) and three using chemical of higher purity (Eastman Kodak Co.) in the TSA recovery medium. Data from the former are shown in Tables 5A and B, and from the latter in Tables 6A, B, and C. The fecal coliform populations before chlorination ranged from 4.3×10^6 to 1.3×10^7 per 100 ml. Colony counts on membrane filters incubated on TSA at 35°C represent the total viable population as in the previous studies. Fecal coliform recoveries on TSA at 44.5°C and on TSA with sodium lauryl sulfate (SLS) at both incubation temperatures are expressed as percentages of the total population in Figures 7A and B and 8A, B, and C. Bar graphs 7A and B show inconclusively the effect of SLS on coliform growth at 35°C. However slightly lower recoveries on TSA with SLS as compared with TSA with bile salts can be observed on graphs 8A, B, and C. In general, recovery is comparable on the three test media at the 35°C incubation temperature.

When plates were incubated at 44.5°C, the same sharp

TABLE 5. MF recovery of FC-10 on three media after chlorination for 5-6 minutes (chlorine residual 0.3 mg/liter; mean fecal coliform counts per 100 ml[+]).

A Population before chlorination: 4.3×10^6/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	TSA WITH SLS**
35°C	160	120	85
44.5°C	25	20	6

B Population before chlorination: 4.6×10^6/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	TSA WITH SLS**
35°C	190	160	215
44.5°C	22	20	10

[+]Average of duplicates.

*Bacto-Bile Salts No. 3 (1.5 g/liter).

**Sodium lauryl sulfate, laboratory grade (1 g/liter), Fisher Scientific.

TABLE 6. MF recovery of fecal coliforms on three media after chlorination for 7 minutes (chlorine residual 0.35 mg/liter; fecal coliforms per 100 ml[+]).

A FC-10; Population before chlorination: 4.5×10^6.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	TSA WITH SLS**
35°C	50,000	43,700	41,700
44.5°C	2,160	1,294	1,074

B *E. coli* ATCC 8739; Population before chlorination: 1.3×10^7/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	TSA WITH SLS**
35°C	28,700	29,300	26,300
44.5°C	2,040	514	73

C *E. coli* ATCC 8739; Population before chlorination: 9.7×10^6/100 ml.

INCUBATION TEMPERATURE	TSA	TSA WITH BILE SALTS*	TSA WITH SLS**
35°C	27,300	31,000	26,000
44.5°C	7,300	4,270	2,370

[+]Average of triplicate plates.

*Bacto-Bile Salts No. 3 (1.5 g/liter).

**Dodecyl sodium sulfate (reagent grade), Eastman Kodak Co. (1 g/liter).

Figure 7. Membrane filter recovery of FC-10 after chlorination. Bacto-Bile Salts No. 3 (Difco) was added to TSA in concentration of 1.5 g/liter. Laboratory grade sodium lauryl sulfate (Fisher Scientific) was added in concentration of 1 g/liter.

A. Residual chlorine 0.3 mg/liter; contact time 5 minutes.

B. Residual chlorine 0.3 mg/liter; contact time 6 minutes.

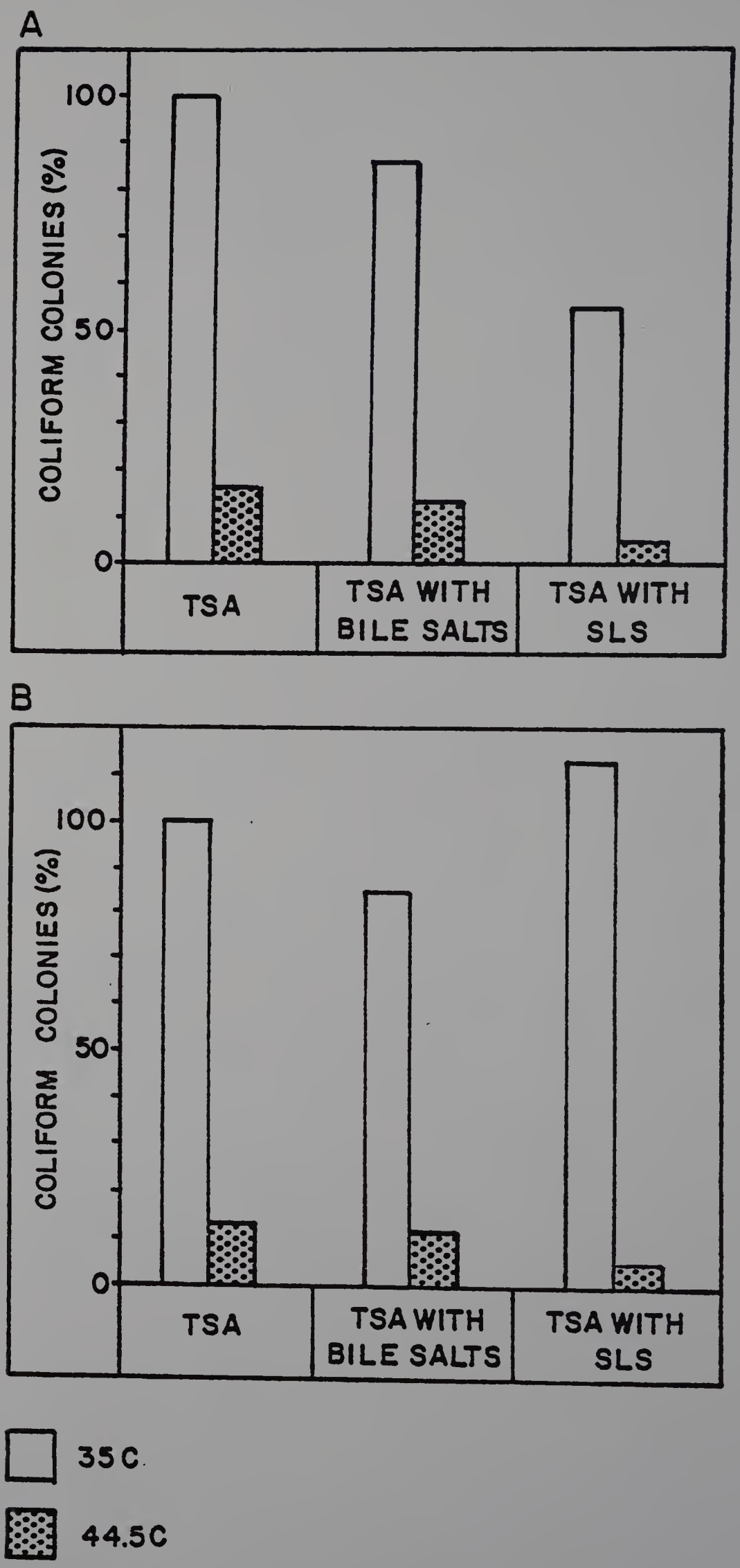

A
100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
TSA WITH SLS
B
100
50
0
COLIFORM COLONIES (%)
TSA
TSA WITH BILE SALTS
TSA WITH SLS
35 C
44.5 C

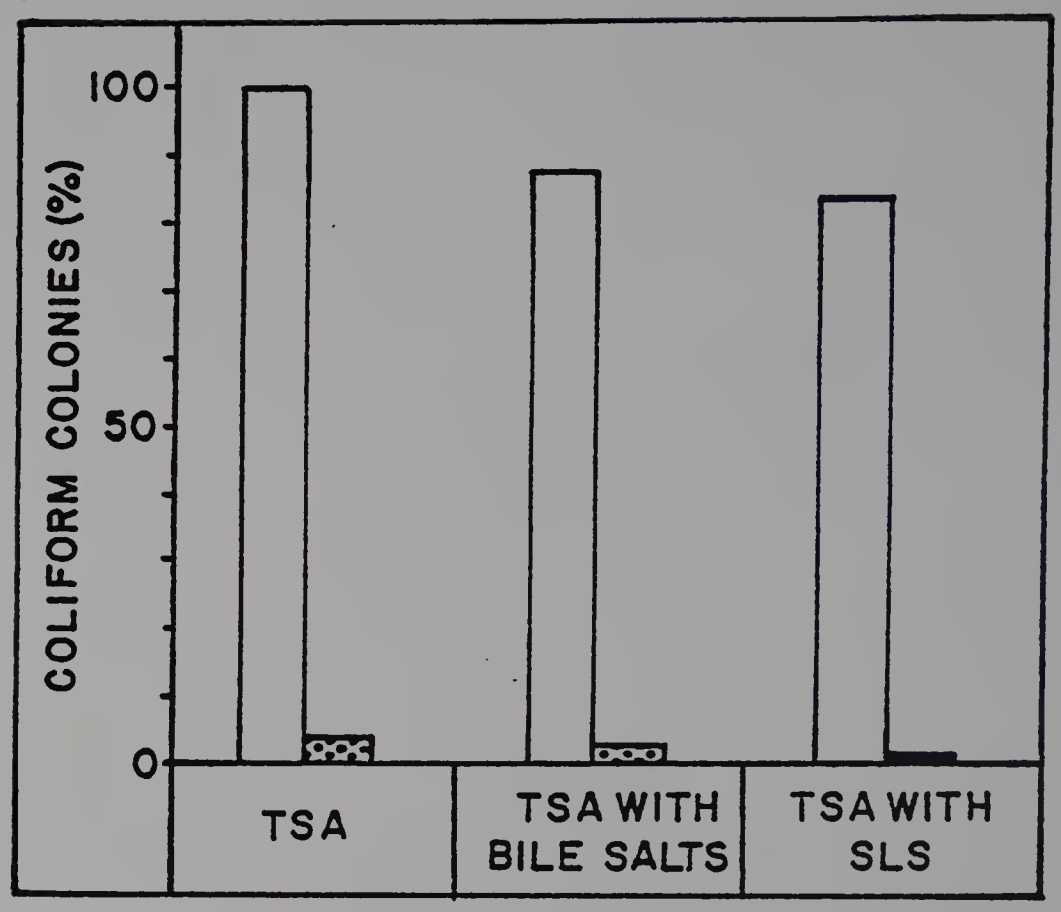
Figure 8. Membrane filter recovery of fecal coliforms after chlorination for 7 minutes (residual chlorine 0.35 mg/liter). Bile salts were the same as for Figure 7. Reagent grade dodecyl sodium sulfate (Eastman Kodak Co.) was added to TSA in concentration of 1 g/liter.

A. FC-10.

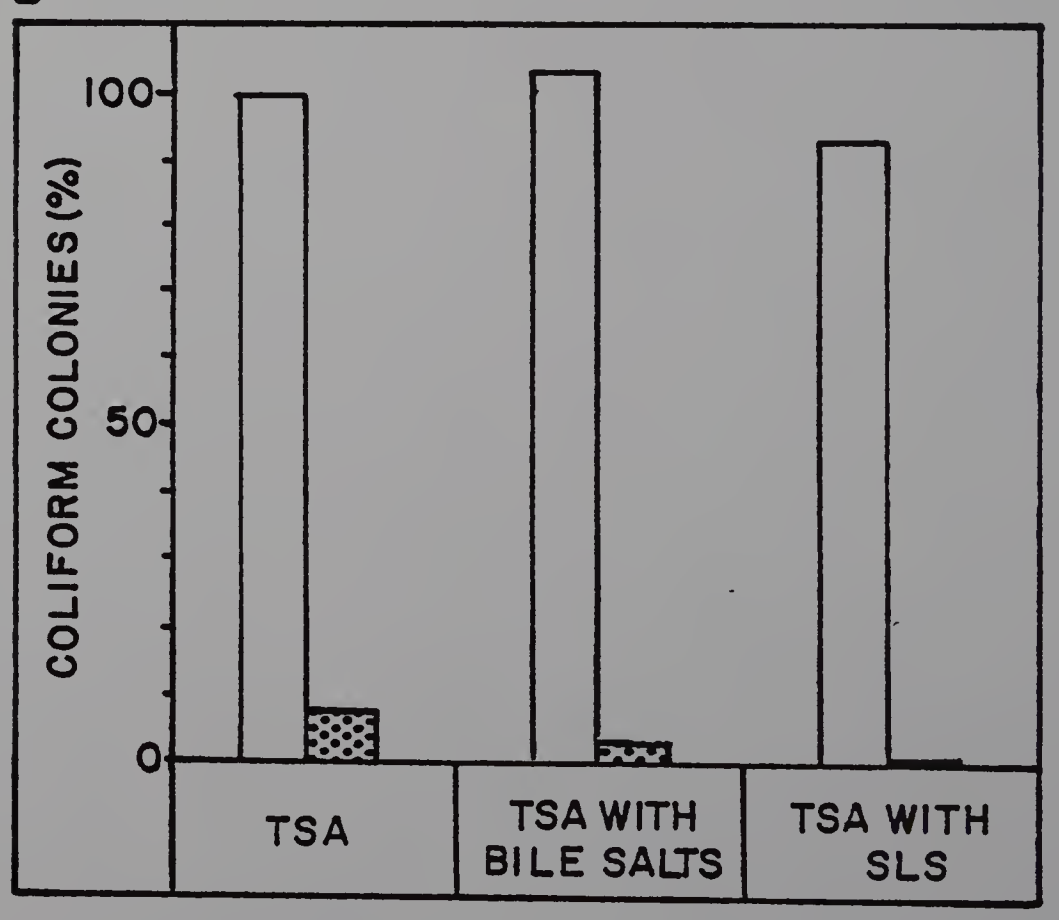
B. E. coli ATCC 8739.

C. E. coli ATCC 8739.

A

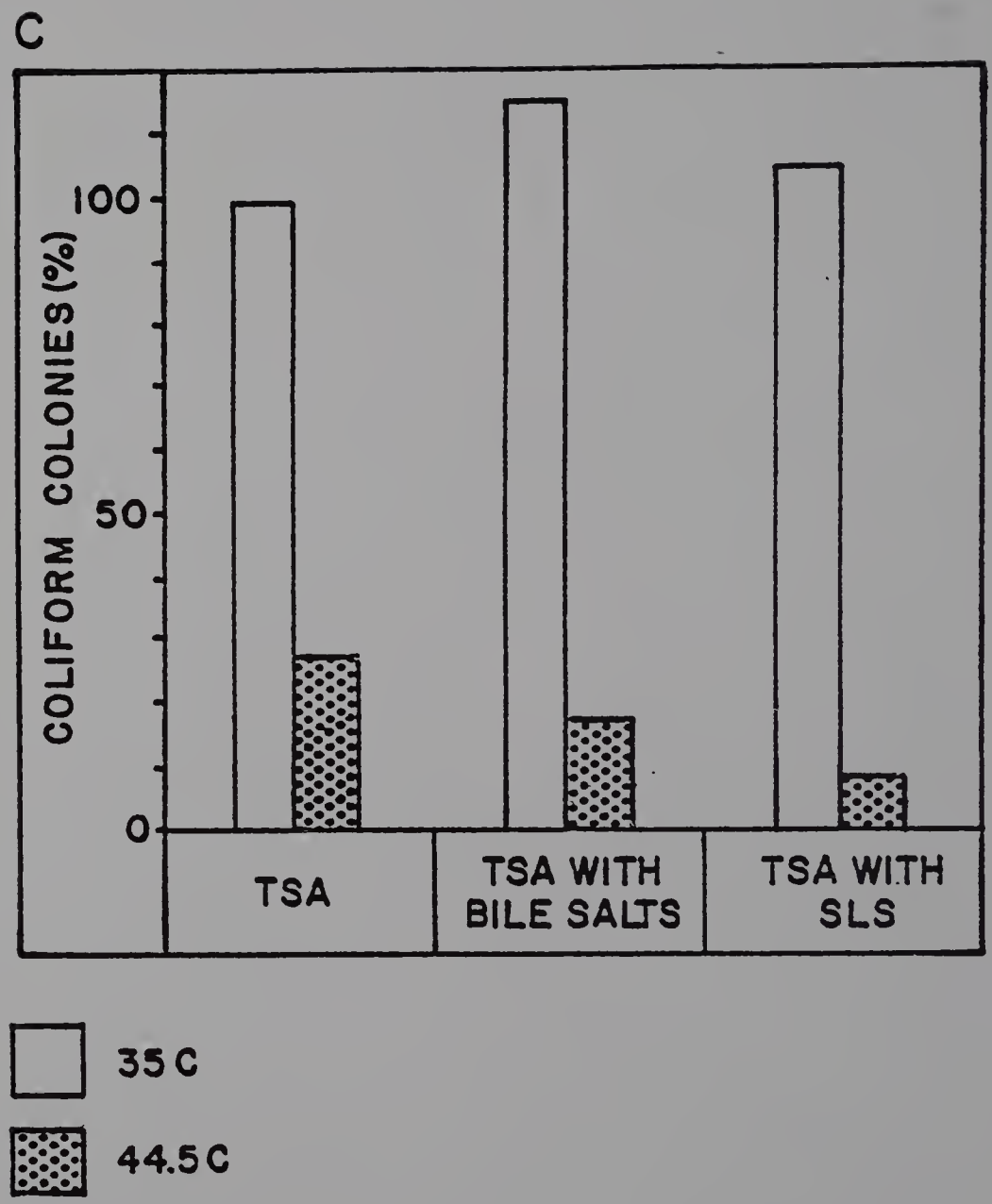

COLIFORM COLONIES (%)
100
50
0
TSA
TSA WITH BILE SALTS
TSA WITH SLS

B


COLIFORM COLONIES (%)
100
50
0
TSA
TSA WITH BILE SALTS
TSA WITH SLS

35C

44.5C


C
COLIFORM COLONIES (%)
100
50
0
TSA
TSA WITH BILE SALTS
TSA WITH SLS
35 C
44.5 C

decline in colony counts that was evident in the previous studies can be seen on all test media. Recovery ranged from 4 to 27 per cent on TSA, from 2 to 16 per cent on TSA with bile salts, and from 0.3 to 9 per cent on TSA with SLS. Neither the brand of SLS, the coliform strain, nor the chlorination level appeared to influence colony growth significantly. The general trend toward lower counts on plates containing SLS than on those with bile salts indicates that this substitution in M-FC medium would not be advantageous.

Effect of incubation temperature. The effect of various incubation periods at 35°C on the recovery of fecal coliforms from chlorinated effluents is shown in Table 7. Data are expressed as a per cent of the five-hour test with each figure representing the mean of 30 M-FC agar plates. It may be observed that recovery steadily increases with time while the confirmation rate remains stable. Preincubation for five hours at 35°C prior to the 44.5°C incubation appeared to provide the most favorable growth conditions. Preliminary tests indicated that preincubation exceeding five hours resulted in excessive background growth, spreading, and a significantly lower confirmation rate.

Having established the optimum conditions, 32 laboratory-chlorinated sewage samples were tested in order to compare the modified, the standard MF, and the multiple-tube procedures for the enumeration of fecal coliforms. These

TABLE 7. Effect of incubation temperature on recovery of fecal coliforms from chlorinated effluent.

HOURS AT 35°C	COUNT/100 ML AS % OF 5 HR*	NO. COLONIES PICKED	% CONFIRMED FECAL COLIFORM
0	24.7	132	93
3	70.8	267	87
4	76.6	278	87
5	100.0	279	89

*Based on 30 plates per incubation time.

results are shown in Table 8. Since fecal coliform densities covered many orders of magnitude, all data were expressed and analyzed as logarithms (base 10). For the purpose of comparing methods, it was found most illuminating to plot one method against the other as has been done by Lin (56). In this type of graph, the line of equality represents perfect agreement between the two methods. Figure 9 shows the fecal coliform counts by the standard MF procedure plotted against the MPN counts. Each point represents the average of five M-FC plates compared to one to three five-tube MPNs. All but one point fall below the line of equality indicating consistently greater recovery by the MPN procedure. Only 25 per cent of the 32 MF counts are within the 95 per cent confidence limits of the MPN tests. The correlation coefficient was calculated to be 0.52 by standard statistical procedures. When the modified MF method was compared with the MPN using the same samples, the points fell much closer to the line of equality (Figure 10). The correlation coefficient was calculated as 0.81 and 88 per cent of the 32 MF counts are now within the 95 per cent confidence limits of the MPN tests. The geometric mean fecal coliform count per 100 ml was 159 for the standard MF, 944 for the modified MF, and 1,250 for the MPN procedure. Based on these figures, the standard MF method yielded only 13 per cent while the modified MF method yielded 76 per cent of the coliforms recovered by the MPN technique. Ninety-one per cent of

TABLE 8. Fecal coliform counts by three methods; samples of raw sewage from Amherst Treatment Plant chlorinated in laboratory.

DATE OF RUN	CHLORINE RESIDUAL mg/l	CONTACT TIME min	LOG FECAL COLIFORM COUNT PER 100 ML		
			STANDARD MF METHOD	MODIFIED MF METHOD	5-TUBE MPN
11/3/75	1.5	6	3.083*	3.609*	4.041
"	1.5	8	2.681	3.114	3.519
11/5/76	1.5	6	3.593	3.873	4.380
"	1.5	8	3.029	3.477	3.690
11/19/75	1.5	13	2.146	2.571	2.342
"	1.5	15	1.602	2.365	2.041
11/20/75	1.5	15	2.215	2.617	2.690
"	1.5	17	2.265	2.692	2.380
12/3/75	1.5	17	2.193	2.617	2.519
"	1.5	19	2.146	2.683	2.663
12/8/75	1.5	16	1.903	2.694	3.041**
					2.845
"	1.5	18	1.924	2.516	2.544
					2.519
12/9/75	1.5	18	2.210	2.602	2.690
					2.898
12/10/75	1.5	6	2.255	2.763	3.041
					2.973
"	1.5	8	2.079	2.643	2.415
					2.690
12/15/75	1.4	6	2.982	3.606	3.690
					3.898
"	1.4	8	2.505	3.093	2.898
					2.898
"	1.5	6	2.763	3.305	3.362
					3.362
12/16/75	1.5	5	2.079	3.513	3.519
					4.041
12/29/75	1.75	4	2.000	3.283	3.491
"	2.0	7	1.301	2.623	3.230
12/30/75	1.5	4	2.079	3.808	4.146
"	1.5	5	1.602	3.210	3.898
"	1.5	7	1.602	2.441	2.845
"	1.5	7	1.204	2.537	2.690
1/6/76	1.25	7	1.447	2.681	1.301
2/17/76	1.5	5	2.422	3.459	3.898
"	1.5	5	2.465	3.459	4.041

TABLE 8--Continued

DATE OF RUN	CHLORINE RESIDUAL mg/l	CONTACT TIME min	LOG FECAL COLIFORM COUNT PER 100 ML		
			STANDARD MF METHOD	STANDARD MF METHOD	5-TUBE MPN
2/19/76	0.8	20	2.683	3.152	2.845
"	0.8	15	2.757	3.391	2.898
3/2/76	1.5	10	1.643	2.369	3.114
					3.230
					3.380
"	1.0	12	1.556	2.453	2.690
					2.519
					2.845

*Each figure represents the logarithm of the average of 5 replicate membranes.

**Additional numbers are replicate 5-tube MPNs.

Figure 9. Comparison of the standard membrane filter and MPN methods. Data are from laboratory-chlorinated primary effluent.

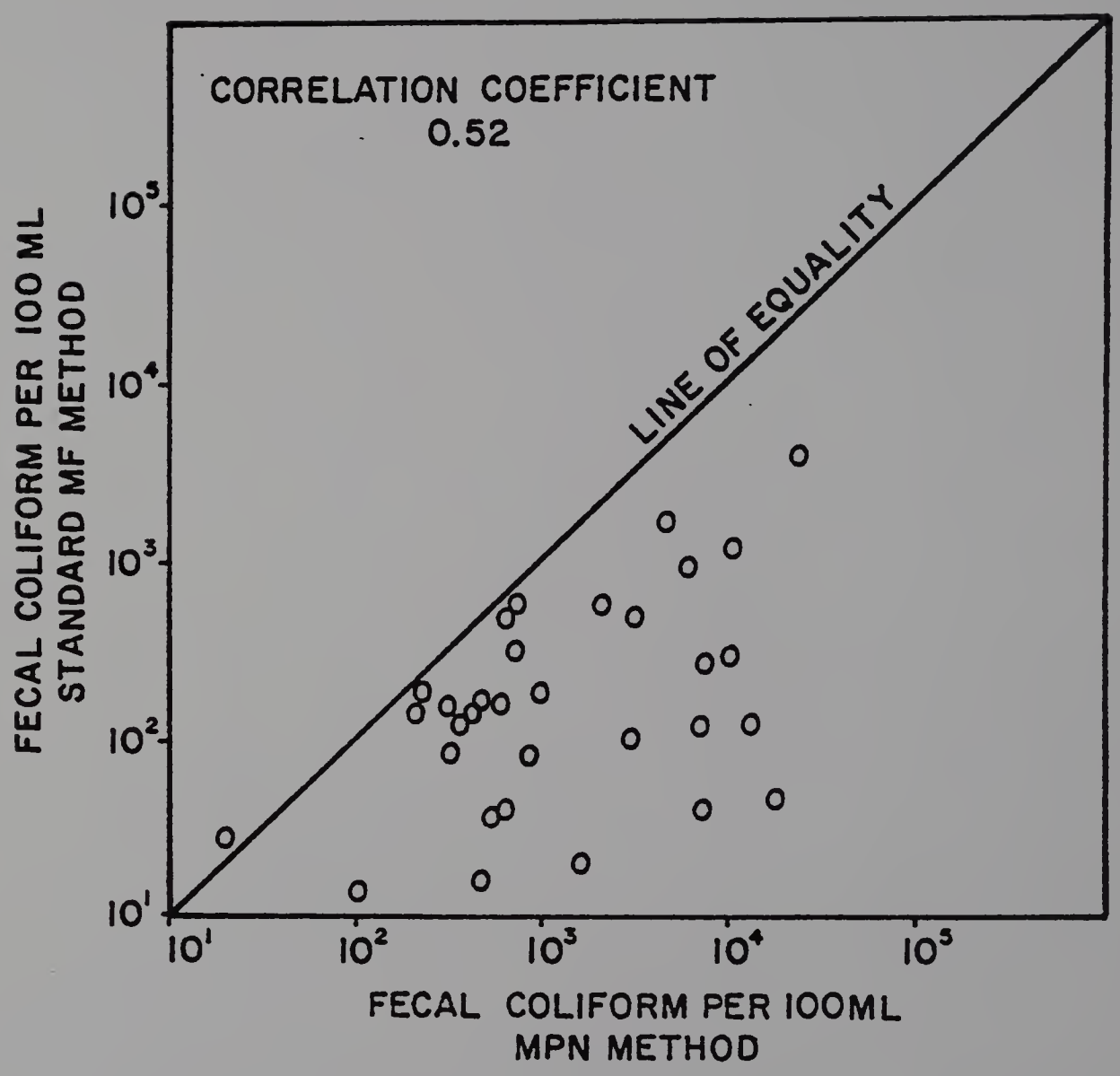
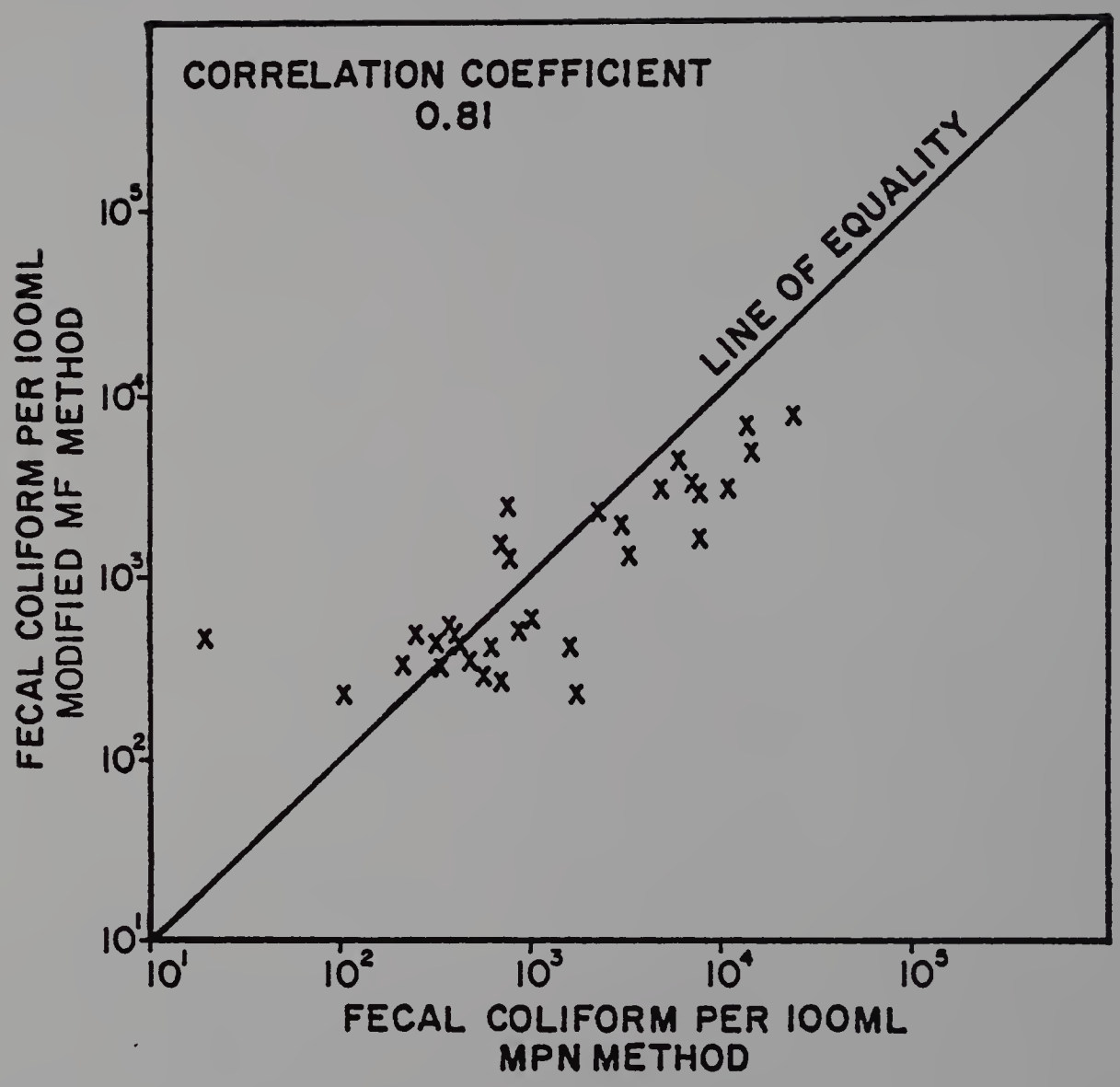

CORRELATION COEFFICIENT
0.52
LINE OF EQUALITY
FECAL COLIFORM PER 100 ML
STANDARD MF METHOD
10^5
10^4
10^3
10^2
10^1
10^1
10^2
10^3
10^4
10^5
FECAL COLIFORM PER 100ML
MPN METHOD

Figure 10. Comparison of the modified membrane filter and MPN methods. Data are from same samples as for Figure 9.


CORRELATION COEFFICIENT
0.81
LINE OF EQUALITY
FECAL COLIFORM PER 100ML
MODIFIED MF METHOD
10^5
10^4
10^3
10^2
10^1
10^1
10^2
10^3
10^4
10^5
FECAL COLIFORM PER 100ML
MPN METHOD

1,356 colonies recovered by the modified procedure were confirmed as fecal coliforms.

The results from five unchlorinated primary effluent samples are shown in Table 9. Each figure is the logarithm of the average of five M-FC agar plates or a five-tube MPN test. The geometric mean fecal coliform count per 100 ml was 1.8×10^6 for the standard MF, 2.3×10^6 for the modified MF, and 2.4×10^6 for the MPN procedure. The three methods may be considered comparable for the enumeration of fecal coliforms from sewage effluents not containing chlorine. Although these data are limited, the comparative results for the standard MF and MPN procedures are in accordance with an earlier report of Geldreich et al. (26).

Field study: plant-chlorinated samples. Data from 66 comparisons performed on plant-chlorinated effluents are tabulated in Table 10. Figure 11 shows the fecal coliform counts by the standard MF method plotted against the MPN counts for a total of 98 combined laboratory- and plant-chlorinated primary and secondary sewage samples. Each point represents the average of five M-FC plates compared with one or more five-tube MPN tests. The majority of the points fall below the line of equality indicating consistently greater recovery by the MPN procedure. On the average, the standard MF method yielded only 8 per cent of the fecal coliforms recovered by the MPN technique, with a correlation

TABLE 9. Effect of preincubation on recovery of fecal coliforms from unchlorinated primary effluent.

	LOG FECAL COLIFORM COUNT PER 100 ML		
	STANDARD MF*	MODIFIED MF*	5-5-5 TUBE MPN
	6.474	6.193	6.380
	6.089	6.435	6.114
	6.223	6.418	6.362
	6.193	6.435	6.690
MEAN	6.245	6.370	6.387
GEOMETRIC MEAN	1.8×10^6	2.3×10^6	2.4×10^6

*Each figure represents logarithm of average of five replicates.

TABLE 10. Fecal coliform counts by three methods; sewage samples chlorinated by the treatment plant.§

DATE OF RUN AND LOCATION OF TREATMENT PLANT		CHLORINE RESIDUAL AND ADDITIONAL CONTACT TIME		LOG FECAL COLIFORM COUNT PER 100 ML		
		mg/l	min.	STANDARD M-FC METHOD	STANDARD MF METHOD	5-TUBE MPN
1/8/76	Amherst	1.3	7	1.079*	2.305*	3.041
1/21/76	Amherst	1.5	4	<2.000	2.602	3.380
1/21/76	Amherst	1.5	7	<1.301	1.602	2.447
1/21/76	Amherst	1.5	9	0.602	1.204	2.114
1/22/76	Amherst	0.8	5	0.903	2.538	3.380
1/26/76	Sunderland	1.0	0	2.274	2.703	2.898
1/27/76	Northampton	1.5	5	2.505	3.384	3.690
1/28/76	Amherst	0.9	10	2.158	3.759	4.732
2/3/76	Amherst	1.0	7	2.107	3.314	4.544
2/11/76	Northampton	3.0	5	0.602	2.633	3.898
2/12/76	Sunderland	0.9	0	2.176	3.086	3.898
2/12/76	Sunderland	0.9	0	2.086	3.025	3.041
2/18/76	Amherst	0.8	15	1.544	3.176	3.898
11/24/75	Bozeman	0.8	0	5.415	5.987	6.204
11/25/75	Bozeman	0.4	0	5.255	5.833	5.973
12/2/75	Bozeman	0.4	0	5.301	5.748	5.732
12/3/75	Bozeman	0.5	0	4.991	5.681	6.041
12/9/75	Bozeman	0.15	0	5.301	5.826	5.519
2/17/76	Bozeman	0.4	0	4.826	5.415	5.845
2/17/76	Bozeman	0.4	5	4.556	5.301	5.544
2/17/76	Bozeman	0.4	10	4.322	5.230	5.447
2/24/76	Bozeman	0.6	0	4.431	5.415	5.230
2/24/76	Bozeman	0.6	5	4.204	5.255	5.204
2/24/76	Bozeman	0.6	10	4.146	5.041	5.041

TABLE 10--Continued

DATE OF RUN AND LOCATION OF TREATMENT PLANT		CHLORINE RESIDUAL AND ADDITIONAL CONTACT TIME mg/l	min.	LOG FECAL COLIFORM COUNT PER 100 ML STANDARD M-FC METHOD	STANDARD MF METHOD	5-TUBE MPN
3/1/76	Trident	0.1	0	4.041	4.279	4.114
3/1/76	Trident	0.1	5	3.991	4.255	4.663
3/1/76	Trident	0.1	20	3.978	4.230	4.544
3/2/76	Amherst	1.5	10	1.342	2.580	3.898
3/2/76	Amherst	1.0	12	1.301	2.663	3.964**
						3.342
						4.204
3/10/76	Amherst	1.25	10	1.477	3.050	3.964
3/10/76	Amherst	1.25	15	<1.000	1.301	2.431
3/10/76	Amherst	1.25	20	0.602	1.380	1.301
3/11/76	Amherst	0.75	11	1.602	3.825	4.544
3/11/76	Amherst	0.75	15	<1.301	2.806	3.690
3/11/76	Amherst	0.75	20	0.778	1.477	3.380
3/16/76	Amherst	0.5	20	<1.000	2.362	2.519
3/18/76	Sunderland	1.5	0	0.301	1.892	1.519
3/22/76	Sunderland	1.5	0	2.845	3.792	3.544
3/22/76	Sunderland	1.5	0	1.415	2.706	3.380
3/23/76	Northampton	1.2	20	1.204	2.759	3.519
3/23/76	Northampton	1.2	25	2.021	2.593	3.732
3/23/76	Sunderland	0.5	0	1.681	2.556	3.041
3/30/76	Sunderland	1.6	0	0.778	1.556	2.146
3/30/76	Sunderland	1.3	0	<1.000	1.820	1.322
4/6/76	Greenfield	1.0	0	0.602	0.301	1.041
4/6/76	Sunderland	1.2	0	1.763	2.714	3.041

TABLE 10--Continued

DATE OF RUN AND LOCATION OF TREATMENT PLANT		CHLORINE RESIDUAL AND ADDITIONAL CONTACT TIME mg/l	min.	LOG FECAL COLIFORM COUNT PER 100 ML STANDARD M-FC METHOD	STANDARD MF METHOD	5-TUBE MPN
4/6/76	Sunderland	1.5	0	---	1.447	1.898
4/6/76	Millers Falls	1.5	0	<1.000	1.505	<-0.700
4/5/76	Bozeman	0.1	0	5.519	5.944	6.342
4/5/76	Bozeman	0.1	5	5.362	5.863	≥6.380
4/5/76	Bozeman	0.1	20	5.322	5.771	5.964
4/19/76	Bozeman	0.15	0	5.566	5.982	6.380
4/19/76	Bozeman	0.15	10	5.431	5.892	5.447
4/19/76	Bozeman	0.15	20	5.602	5.987	5.964
4/15/76	Sunderland	0.8	0	3.215	3.483	3.845
4/15/76	Sunderland	1.3	0	2.164	3.024	3.732
4/20/76	Sunderland	0.25	0	2.944	3.763	3.845
4/20/76	Sunderland	1.5	0	1.505	2.450	2.519
4/26/76	Sunderland	2.0	0	0.505	0.857	0.903
4/26/76	Sunderland	1.0	0	3.373	3.587	2.380
4/29/76	Sunderland	---	0	<1.000	1.663	2.362
4/29/76	Sunderland	---	0	1.301	2.380	2.663
4/29/76	So. Deerfield	1.6	0	1.447	1.806	2.230
5/3/76	Sunderland	1.5	0	0.602	1.342	1.342
5/4/76	Sunderland	0.8	0	1.602	2.000	2.690
5/4/76	Sunderland	1.3	0	1.973	2.310	2.519

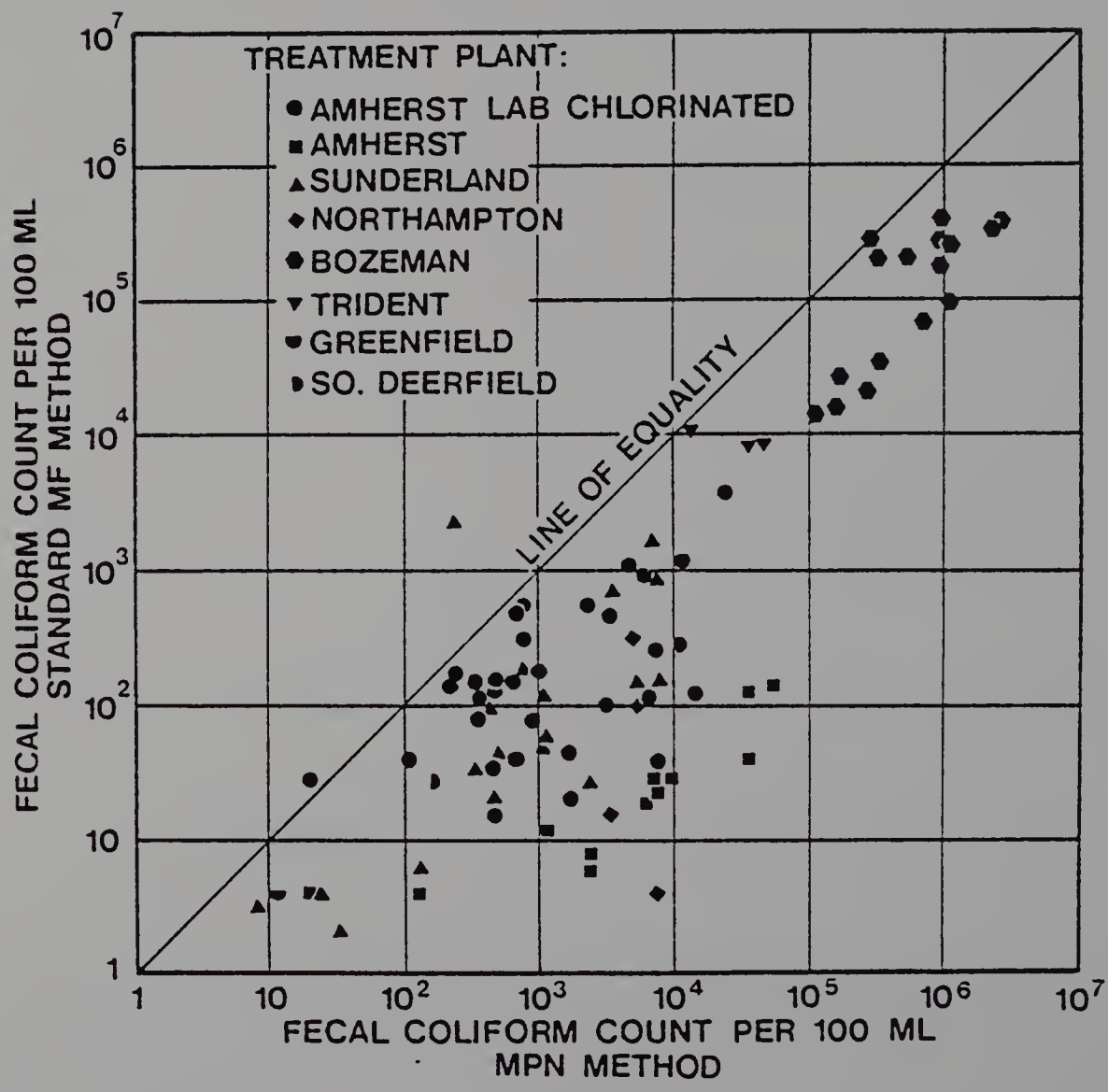
§All samples were chlorinated in the plant for a contact time which depends upon the volume and flow rate in the plant chlorinator; the additional contact time listed here is the time between taking the chlorinated sample and neutralizing it with thiosulfate.

TABLE 10--Continued

*Each figure represents the logarithm of the average of 5 replicate membranes.

**Additional numbers are replicate 5-tube MPNs.

Figure 11. Comparison of the standard MF with the MPN method. Data are from laboratory-chlorinated and plant-chlorinated primary and secondary effluents.


TREATMENT PLANT:
AMHERST LAB CHLORINATED
AMHERST
SUNDERLAND
NORTHAMPTON
BOZEMAN
TRIDENT
GREENFIELD
SO. DEERFIELD
LINE OF EQUALITY
FECAL COLIFORM COUNT PER 100 ML STANDARD MF METHOD
FECAL COLIFORM COUNT PER 100 ML MPN METHOD
1
10
10^2
10^3
10^4
10^5
10^6
10^7

coefficient of 0.84. When the modified MF method was compared with the MPN using the same samples, the points fell closer to the line of equality (Figure 12). The correlation coefficient was calculated as 0.92 and the mean count by the modified MF method increased to 49 per cent of the mean MPN count.

Upon closer examination of the data, it was found that agreement between the modified MF method and the MPN was greatest with samples from secondary treatment plants. Figure 13 shows the standard MF results of forty samples from plant-chlorinated secondary effluent. Here the correlation coefficient is high (0.96) but the recovery is only 14 per cent of the MPN. The same forty samples from secondary treatment plants, analyzed using the modified MF method, are plotted in Figure 14. The correlation coefficient is 0.97 and the recovery with the modified MF method is increased to 68 per cent of the MPN. The best fit straight line was computed by the method of least squares. The average distance separating the line of equality and the best fit line is a measure of productivity or recovery of one method with respect to another. The greater agreement between the modified MF method and the MPN from samples of secondary effluent could be related to the effectiveness of chlorination in effluent containing less organic material.

Referring again to Figure 14, it is possible that the scatter of points around the best fit line is due pri-

Figure 12. Comparison of the modified MF with the MPN method. Data are from same samples as Figure 11.

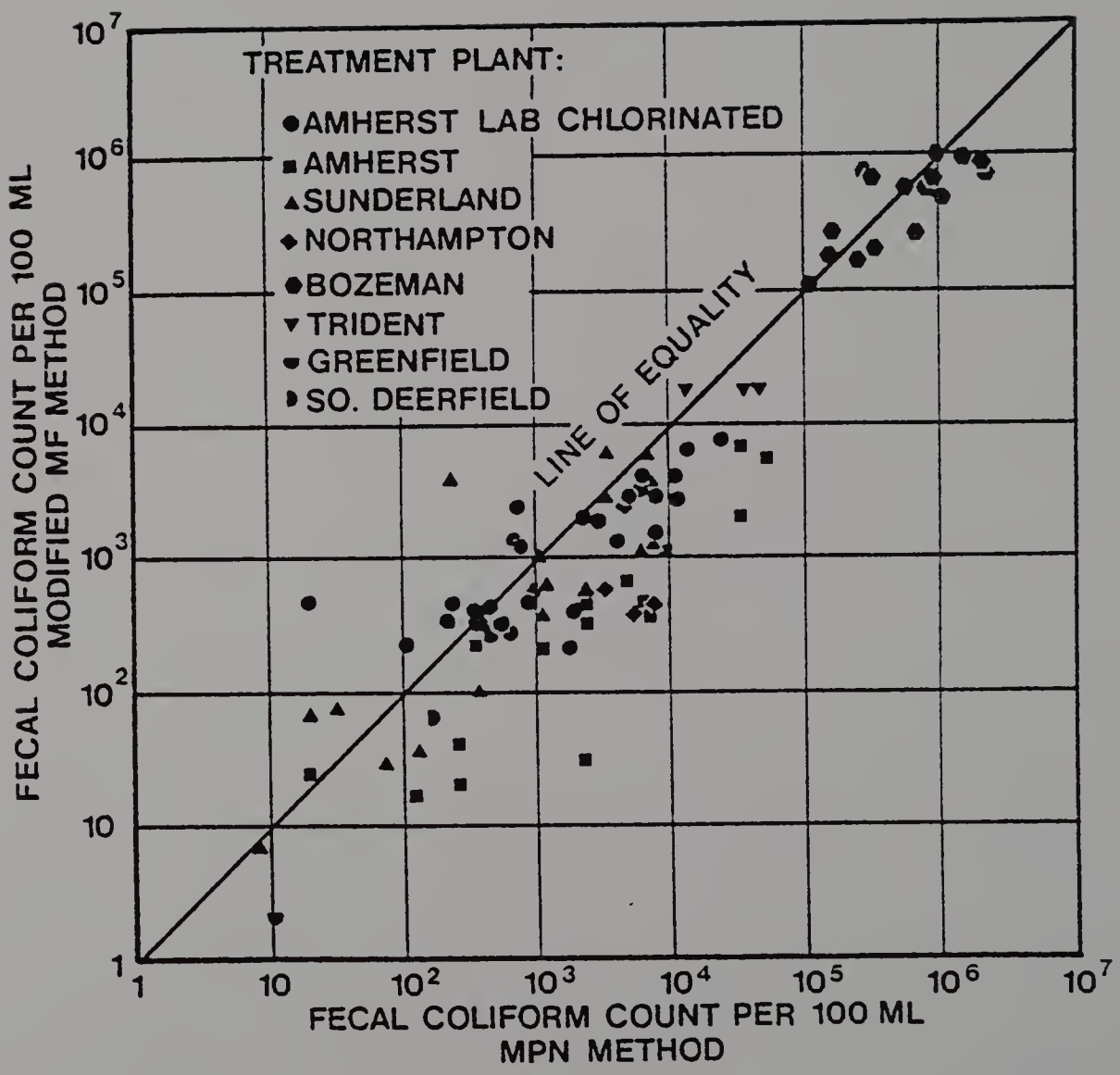

TREATMENT PLANT:
AMHERST LAB CHLORINATED
AMHERST
SUNDERLAND
NORTHAMPTON
BOZEMAN
TRIDENT
GREENFIELD
SO. DEERFIELD
LINE OF EQUALITY
FECAL COLIFORM COUNT PER 100 ML MODIFIED MF METHOD
FECAL COLIFORM COUNT PER 100 ML MPN METHOD
1
10
10²
10³
10⁴
10⁵
10⁶
10⁷

Figure 13. Comparison of the standard MF with the MPN method. Data are from chlorinated effluents from secondary treatment plants only.

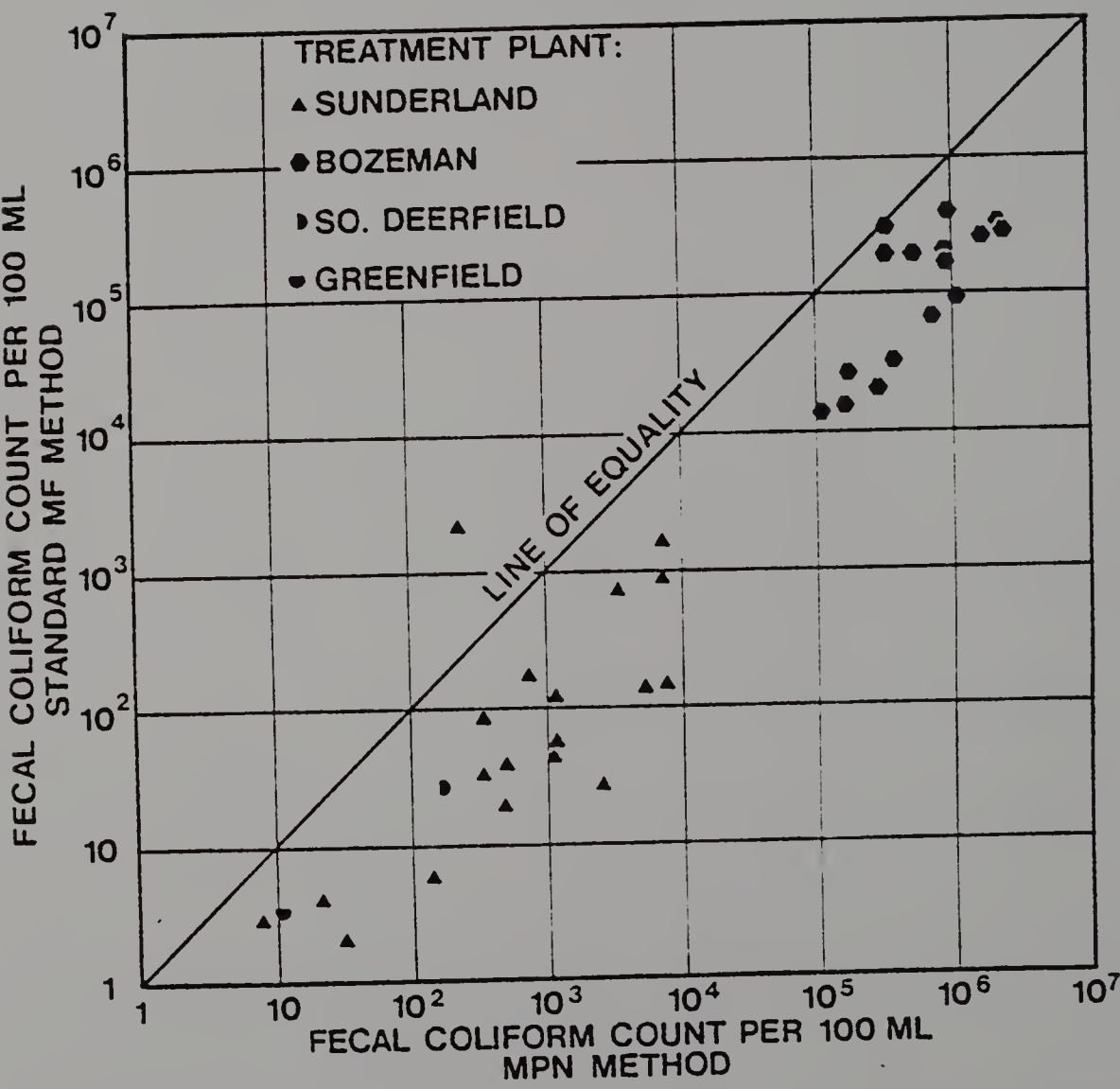
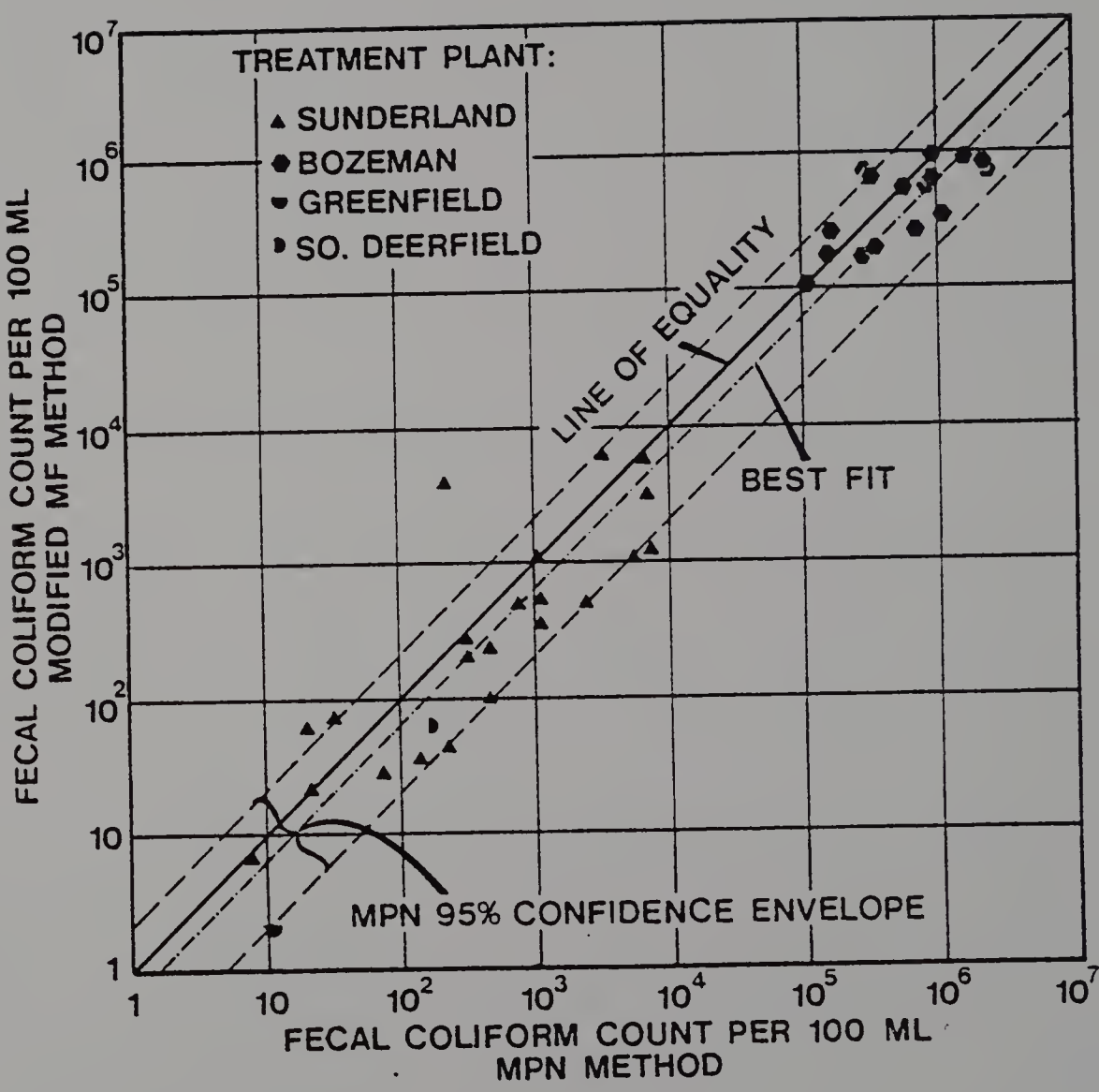

TREATMENT PLANT:
SUNDERLAND
BOZEMAN
SO. DEERFIELD
GREENFIELD
LINE OF EQUALITY
FECAL COLIFORM COUNT PER 100 ML
STANDARD MF METHOD
FECAL COLIFORM COUNT PER 100 ML
MPN METHOD
1
10
10²
10³
10⁴
10⁵
10⁶
10⁷

Figure 14. Comparison of the modified MF with the MPN method. Data are from same samples as Figure 13.


TREATMENT PLANT:
SUNDERLAND
BOZEMAN
GREENFIELD
SO. DEERFIELD
LINE OF EQUALITY
BEST FIT
MPN 95% CONFIDENCE ENVELOPE
FECAL COLIFORM COUNT PER 100 ML MODIFIED MF METHOD
FECAL COLIFORM COUNT PER 100 ML MPN METHOD
1
10
10^2
10^3
10^4
10^5
10^6
10^7

marily to the inherent variability of the MPN. MPN values are known to follow a log normal distribution with a standard deviation σ (log) of 0.25 for a 5-tube test (114). On Figure 14 this predicted variability has been represented as a 95 per cent confidence envelope drawn at $\pm 1.96\,\sigma$, or 0.48 log cycles from the best fit line. Of the 40 points on the graph, five fell outside this envelope, four of these being very close to its boundaries. Thus, the observed scatter does agree with that predicted from MPN theory.

The best fit line on Figure 14 lies below the line of equality by 0.17 log units, indicating an average modified MF recovery of 68 per cent of the MPN. However, it is known both theoretically (106) and experimentally (69) that MPN estimates are positively biased. Thomas (106) gives a factor of 0.851 to correct for the positive bias in 5-tube tests, and Lin (56) has used this factor in data analyses similar to Figure 13. Multiplying each MPN by 0.851 has the effect of moving the best fit line 0.07 log units closer to the line of equality. The discrepancy is then 0.10 log units, which corresponds to 79 per cent recovery.

A total of 3,133 colonies recovered by the modified MF method were verified as is shown in Table 11. Of these 2,903 (93 per cent) were confirmed as fecal coliforms indicating that the 35°C preincubation does not lower the confirmation rate below that expected when the standard membrane filter technique is used.

TABLE 11. Verification of fecal coliforms recovered by the modified MF method.

SOURCE	NO. COLONIES PICKED	NO. COLONIES CONFIRMED	VERIFICATION RATE
Amherst lab. chlor.	1,356	1,234	91%
plant chlor.	296	296	100%
Northampton	160	154	96%
Sunderland	234	219	94%
Trident	1,008	922	92%
Bozeman	79	78	99%
TOTAL	3,133	2,903	93%

Shown in Figure 15 are typical M-FC agar plates which demonstrate the dramatic difference in recovery by the two membrane filter methods.

Fecal coliform recoveries by the modified MF method using M-FC agar and M-FC agar without rosolic acid are compared to the MPN procedure in Table 12. The two membrane filter methods are plotted against the MPN counts in Figure 16. The locations of the two best fit lines indicate only slight improvement in the modified MF method when rosolic acid is omitted from the M-FC agar medium. These results are in agreement with those of Presswood and Strong (84) although the standard incubation schedule was used in their study.

Field study: marine samples. Data from 49 comparisons of the standard MF, the modified MF, and MPN methods using marine samples are listed in Table 13. Figure 17 shows the fecal coliform counts by the standard and modified MF methods plotted against results of the MPN tests. Each point represents the average of five replicate M-FC agar plates compared with single or average MPN counts. The modified membrane filter method yielded consistently higher fecal coliform counts than did the standard MF procedure, although the majority of the MF points fall below the line of equality. The correlation coefficients for both membrane methods were 0.97. It should be noted that the standard MF method was in

Figure 15. M-FC agar plates showing the difference in recovery by the two membrane filter methods. Plant-chlorinated secondary effluent from Sunderland treatment plant (4/6/76).


Standard Method 44.5° C
35° C (5 hrs) ⟶ 44.5° C

TABLE 12. Effect of rosolic acid on fecal coliform recovery by the modified MF method. Data are from plant-chlorinated effluents.

LOG FECAL COLIFORM COUNT PER 100 ML		
M-FC WITH ROSOLIC ACID	M-FC WITHOUT ROSOLIC ACID	5-TUBE MPN
1.362*	2.674*	2.519
1.892	2.093	1.519
3.792	3.844	3.544
2.706	2.771	3.380
2.846	2.845	4.204
2.759	3.128	3.519
2.593	2.724	3.732
2.556	2.651	3.041
1.556	1.176	2.146
2.714	2.727	3.041
1.447	1.447	1.898
3.587	3.513	2.380
0.857	1.017	0.903
LOG MEAN	2.359	2.509	2.756
GEOMETRIC MEAN	229	323	570

*Each figure represents the logarithm of the average of 5 replicate membranes.

Figure 16. Fecal coliform recoveries by the modified MF method using M-FC agar and M-FC agar without rosolic acid compared to the MPN method. Data are from plant-chlorinated effluents.

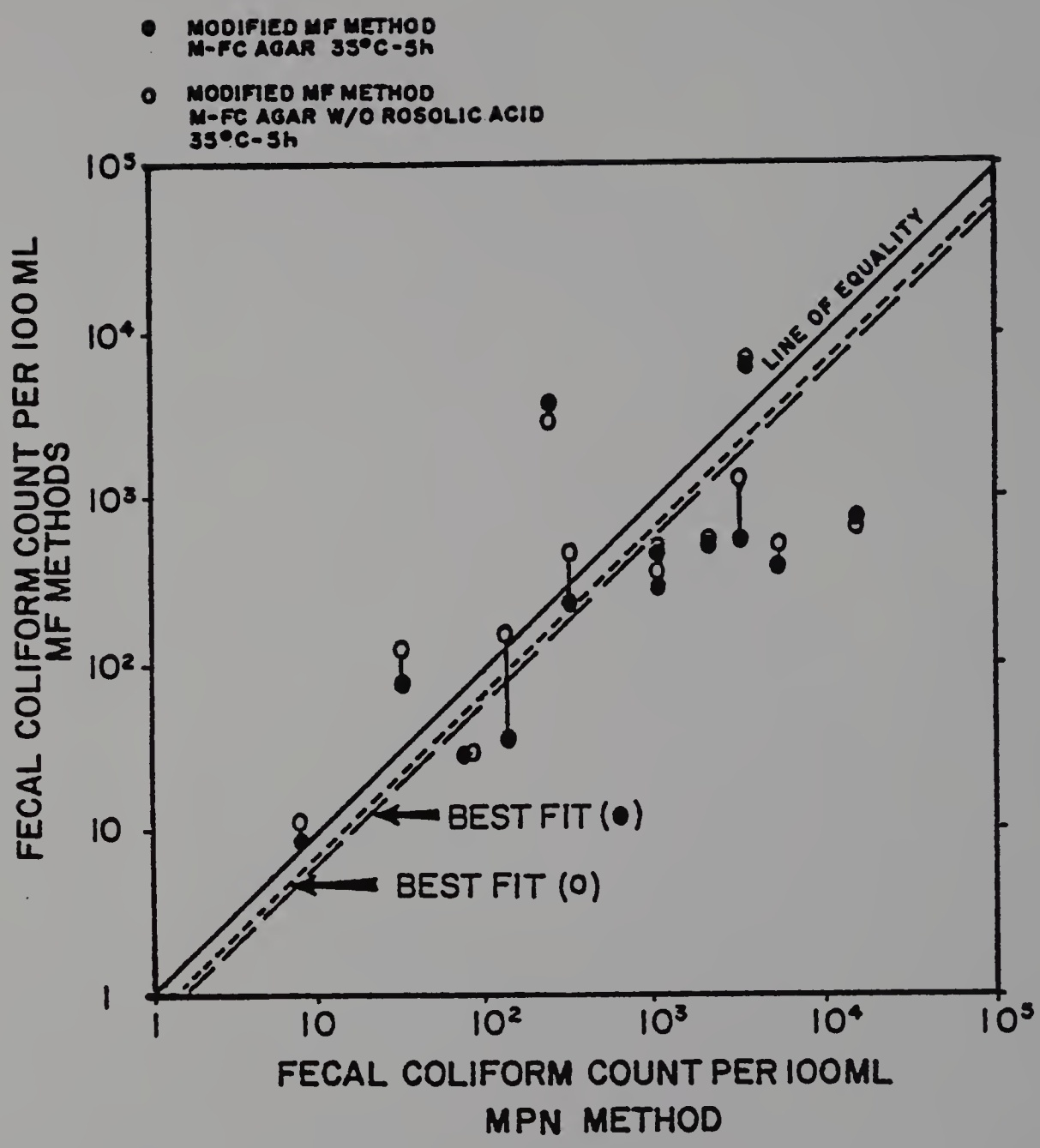

MODIFIED MF METHOD
M-FC AGAR 35°C-5h
MODIFIED MF METHOD
M-FC AGAR W/O ROSOLIC ACID
35°C-5h
FECAL COLIFORM COUNT PER 100 ML
MF METHODS
LINE OF EQUALITY
BEST FIT (●)
BEST FIT (o)
1
10
10²
10³
10⁴
10⁵
FECAL COLIFORM COUNT PER 100ML
MPN METHOD

TABLE 13. Fecal coliform counts by three methods from marine samples.

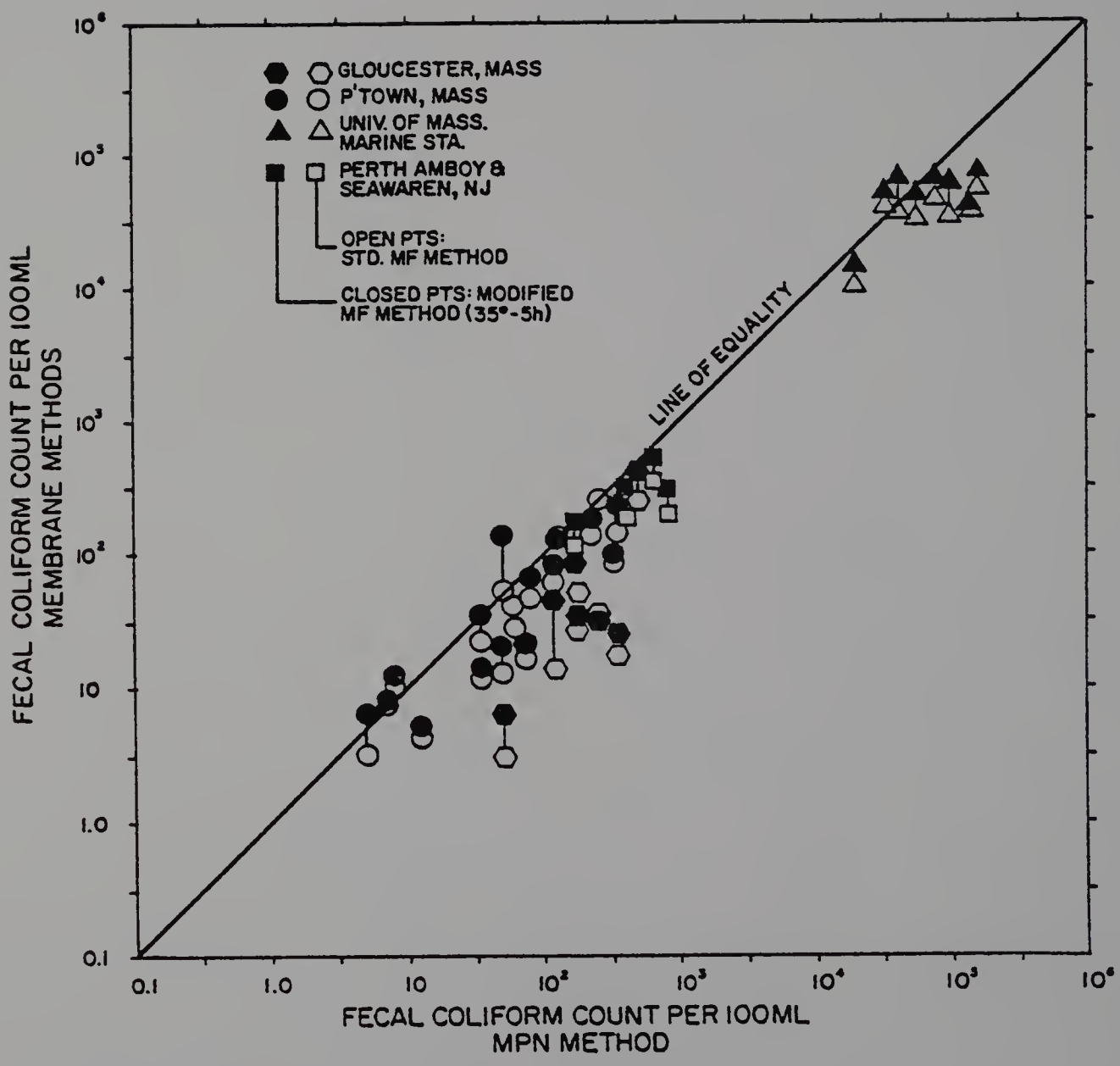
DATE	SAMPLE SITE	LOG FECAL COLIFORM COUNT PER 100 ML		
		STANDARD MF METHOD	MODIFIED MF METHOD	5-TUBE MPN
6/3/76	Rockport	2.441*	2.596*	2.690
	Gloucester	1.724	1.851	2.231
		1.255	1.380	2.519
		1.532	1.505	2.380
		1.146	1.644	2.041
		1.415	1.556	2.231
		0.477	0.778	1.690
7/5/76	Provincetown	1.690	2.143	1.690
		1.556	1.672	2.231
		1.663	1.806	1.898
		0.602	0.699	1.079
		1.041	1.079	0.903
		1.602	1.716	2.231
		2.134	2.104	2.114
		1.079	1.146	1.519
		0.903	0.903	0.845
		1.322	1.505	1.519
		1.653	1.756	2.041
		2.346	---	2.380
		1.231	1.279	1.845
		0.477	0.778	0.699
7/14/76	Marine Station	4.568	4.681	4.544
		4.634	4.672	5.137
		4.568	4.763	4.863
		4.591	4.845	5.167
		4.613	4.748	4.863
		4.544	4.810	4.684
		4.477	4.712	5.029
		4.362	4.568	4.740
		4.000	4.146	4.312
9/21/76	Provincetown	2.121	2.255	2.344
		1.969	2.188	2.348
		1.532	1.748	1.881
		1.919	2.053	2.509
		1.732	1.845	1.935
		1.114	1.301	1.602
		1.279	1.556	1.732
		2.100	2.292	2.496

TABLE 13--Continued

DATE	SAMPLE SITE	LOG FECAL COLIFORM COUNT PER 100 ML		
		STANDARD MF METHOD	MODIFIED MF METHOD	5-TUBE MPN
1/25/77	New Jersey	---	2.161	2.519
		1.544	1.987	2.344
		1.462	1.826	2.344
		2.314	2.596	2.663
		2.312	---	2.663
		2.301	2.556	2.898
		2.250	---	2.898
		2.250	2.459	2.690
		2.196	2.356	2.690
		2.041	2.366	2.362
		2.017	2.210	2.362

*Each figure represents the logarithm of the average of 5 replicate membranes.

Figure 17. Comparison of standard MF, modified MF, and MPN methods for enumeration of fecal coliforms from marine waters.


GLOUCESTER, MASS
P'TOWN, MASS
UNIV. OF MASS. MARINE STA.
PERTH AMBOY & SEAWAREN, NJ
OPEN PTS: STD. MF METHOD
CLOSED PTS: MODIFIED MF METHOD (35°-5h)
LINE OF EQUALITY
FECAL COLIFORM COUNT PER 100ML MEMBRANE METHODS
FECAL COLIFORM COUNT PER 100ML MPN METHOD
0.1
1.0
10
10²
10³
10⁴
10⁵
10⁶

closer agreement with the MPN when marine waters were tested than was the case with chlorinated effluents. When the standard MF procedure was compared to the MPN, 67 per cent of the samples tested fell within the 95 per cent confidence limits of the MPN. However, when the modified MF method was used for the same samples, 89 per cent fell within the confidence envelope. Ninety-six per cent of 1,040 colonies recovered by the modified procedure were confirmed as fecal coliforms.

Comparison of modified MF and IM-MF methods. Results from a four-way comparison of the standard MF, modified MF, IM-MF, and MPN methods for fecal coliform recovery are shown in Table 14. Each figure for the MF methods represents the average of five replicate membranes. Mean counts for the seven tests are 133, 380, 265, and 314, respectively. It was observed throughout these studies that agreement between MF and MPN methods generally was greatest with higher degrees of chlorination (as measured by residual chlorine or contact period). This explains the higher recovery rates by the membrane methods in this data set. Recovery by the standard MF method was 42 per cent of the average MPN count. The IM-MF procedure yielded 84 per cent, while the modified MF method yielded 121 per cent of the fecal coliforms recovered by the MPN procedure. These data indicate that although the IM-MF method is superior to the standard method, it is

TABLE 14. Comparison of four methods for recovery of fecal coliforms from laboratory-chlorinated primary effluent.* (Mean fecal coliform count per 100 ml**)

TEST DAY	CHLORINATION TIME (min)	STANDARD MF	MODIFIED MF 35°C-5 h	IM-MF+	5-TUBE MPN
1	13	140	372	312	220
	15	40	232	146	110
	17	104	252	164	350
2	15	164	414	217	490
	17	184	492	253	240
3	17	156	414	362	330
	19	140	482	400	460
	TOTAL	928	2,658	1,854	2,200
	MEAN	132.6	379.7	264.9	314.3
	NO. COLONIES CONFIRMED		188/200	191/200	
	VERIFICATION RATE		94%	95.5%	

*Chlorine residual 1.5 mg/liter.

**Each figure is average of 5 replicate membranes.

+Stuart et al. (103,104).

not as efficient as M-FC agar when this medium is preincubated for five hours at 35°C prior to the 44.5°C incubation.

DISCUSSION

The extraction procedure used in this study on two brands of membrane filters appears to have removed inhibitory residues remaining from the manufacturing process. Removal of these residues consistently resulted in improved MF recovery of fecal coliform bacteria from laboratory-chlorinated primary sewage effluent. The ideal membrane filter should remain chemically inert so as not to interfere with bacterial growth (29). The boiling process may have caused changes in the structure of the membranes which enhanced growth of fecal coliforms. To eliminate this possibility, further studies, including electron micrographs, should be performed.

Tests were designed to evaluate the effects of several parameters (i.e., incubation temperature, media, stress due to chlorination) on MF recovery of fecal coliforms. These experiments have demonstrated that when unchlorinated sewage samples are seeded and analyzed using membrane filtration, fecal coliform recovery is not greatly affected by either elevated incubation temperature (44.5°C) or selective culture medium (M-FC agar). However, after chlorination, fecal coliform organisms became much more sensitive to both temperature and medium, with temperature being the more inhibitory factor.

Other researchers have studied some but not all of these factors. Presswood and Brown (83), using unstressed pure cultures of E. coli, found no statistical difference between recovery on M-FC and plate count agar pour plates incubated at 35 and 44.5°C. As mentioned previously, it is possible that these test cultures were preselected for temperature tolerance. Hufham (48), also utilizing pure cultures of unstressed E. coli, found no apparent difference between MF counts made on plate count broth at 35°C and M-FC broth at 35°C. In another test, membranes incubated on plate count broth at 44.5°C yielded approximately 25 per cent of the number of E. coli recovered on plate count broth at 35°C. An even greater reduction in viable count was seen when M-FC broth was employed at 44.5°C. Although test organisms in this study were not exposed to chlorination, samples were prepared for filtration by dilution in sterile phosphate buffer. Exposure to this buffer has been shown to cause a reduction in bacterial populations (29). Maxcy (62) found that comparative counts on plate count agar and selective media were in very close agreement only when the cells were near the maximum growth phase and with only limited storage in the arrested state.

Bacto-Bile Salts No. 3, an ingredient in M-FC medium (Difco), was added to TSA to determine its effect on injured coliform organisms. Utilizing two strains of fecal coliforms, no significant inhibition of growth, as compared to

TSA, was observed at 35 or 44.5°C. Recovery on M-FC agar was consistently less efficient than on TSA with bile salts. The omission of rosolic acid from the M-FC medium resulted in an increase in coliform counts in most tests. These data are in agreement with that of Presswood and Strong (84). It was determined, however, that on all culture media used, including TSA, there was a drastic reduction in counts when plates were incubated at 44.5°C. These results indicate that although the effectiveness of M-FC medium may be improved slightly by eliminating rosolic acid, the elevated temperature required for its selectivity inhibits large numbers of stressed fecal coliform organisms from forming colonies.

Although the bile salt preparation used in these experiments was not shown to be toxic to sublethally injured coliforms, this natural substance may vary and has been found by other researchers to be an inhibitory constituent (61,62,75,86,87). Mossel et al. (75) proposed replacing the variable bile preparations in a selective medium (EE-broth) with the chemically synthesized surface active compound, sodium lauryl sulfate. In a concentration of 1.0 g/liter it was found to be selective and completely non-inhibitory for the Enterobacteriaceae. Test strains were cultured in tryptone soya peptone broth for up to 16 hours before being diluted for inoculation. Comparative tests were performed in selective broth media and incubated at 30°C. These results are contrary to those reported in this

study. Two brands of sodium lauryl sulfate were found to be consistently more inhibitory to stressed coliforms than the bile salt preparation. Differences in experimental conditions may explain the opposing conclusions. The severely stressed population resulting from chlorination was likely to be more sensitive to inhibitory factors than the broth cultures used by Mossel et al.

At this stage of the research it was concluded that modifications in the M-FC medium would not result in significant increases in fecal coliform recovery from chlorinated effluents. The sharp reduction in counts on non-selective TSA when incubated at 44.5°C seemed to indicate that temperature is the most critical factor influencing coliform growth. Using chlorinated effluents, several experiments were performed in which membrane filters were incubated on M-FC agar at various temperatures between 40 and 44.5°C for 24 hours. Improved recovery always resulted from reductions in incubation temperature. However, difficulty could be foreseen in acceptance of this modification of the standard method, since the definition of a fecal coliform includes its ability to grow at 44.5°C.

Several investigators had previously reported that pre-enrichment or preincubation of plates at temperatures from 25 to 35°C prior to the 44.5°C incubation resulted in improved fecal coliform recovery (37,85,90). A series of tests were performed to evaluate the effect of various pre-

incubation times at 35°C using conventional M-FC medium with the membrane. Recovery steadily increased with time at 35°C until non-coliform organisms appeared in significant numbers at six hours. Optimum recovery and selectivity were achieved with a five hour preincubation as shown in Table 7.

This modified MF method was compared to the standard MF and MPN techniques using laboratory-chlorinated primary sewage effluent. Recovery of fecal coliforms was increased by an average of six-fold compared to the standard MF method. A similar comparison using unchlorinated primary sewage yielded comparable results with the standard MF, the modified MF, and the MPN methods.

An extensive field test of the modified MF method using plant-chlorinated primary and secondary effluents demonstrated that this procedure consistently results in a significant increase in fecal coliform counts without a decrease in the confirmation rate. It was found that agreement between the modified MF method and the MPN was greatest with samples from secondary treatment plants. Data from both laboratory- and plant-chlorinated effluents indicate that the more effective the chlorination process, the better the correlation between the MF and MPN methods. Lower levels of chlorination resulting from dosage, contact time, or organic load, may result in a larger population of sublethally injured coliform organisms, many of which fail to grow on the membrane but recover in the broth of the MPN test.

Thirteen of the plant-chlorinated samples were utilized to determine the combined effect of the five-hour preincubation and the omission of rosolic acid from the M-FC medium. Again there was a slight increase in fecal coliform counts on M-FC agar without rosolic acid.

The modified MF method was found to be applicable to the analysis of marine waters for fecal pollutants. Preincubation for five hours at 35°C consistently resulted in improved fecal coliform recovery. Although average MF counts were usually below the corresponding MPN index, 89 per cent of the samples fell within the 95 per cent confidence envelope when the modified MF method was employed.

In these comparisons the multiple-tube procedure was used as the standard, but it must be noted that there are inherent shortcomings in this technique. The MPN method is based on probability statistics and estimates of bacterial density are known to vary over a ten-fold range in identical samples (77,106,114). Although the MPN index is a useful tool for expressing bacterial density in terms of a discrete number, all MPNs are not equally reliable or representative of the actual density of organisms in the sample. According to Woodward (114) many MPN values are actually improbable. To assist in interpretation of data, he computed precise confidence limits for three- and five-tube MPN tests. For a five-tube multiple dilution test, the 95 per cent confidence limits cover a thirteen-fold range from approximately

24 to 324 per cent of the MPN. After analyzing these tables, De Man (17) concluded that many of the results are so improbable that they seldom or never will be obtained and, therefore, it is better to reject a certain low percentage of correct determinations than to accept improbable results without question. He recently described a method for drawing up MPN tables restricted to results with a defined minimum probability.

At best the MPN is only approximate with the reliability increasing in direct proportion to the number of tubes inoculated per dilution (64,106). Application of the MPN technique is based on the assumptions that (i) organisms in the sample are randomly distributed, (ii) organisms exist independently of each other, and (iii) a single organism is sufficient to give a positive test. All these conditions may apply in some cases, but in others they may be completely lacking (54).

With laboratory experiments, McCarthy et al. (64) demonstrated that the degree of reproducibility associated with the direct colony count procedure greatly exceeds that of the MPN test. The precision of 15 replicate plate counts was at least three times that of replicate MPN values. In addition, the MPN was found consistently to overestimate the true coliform density (as determined by the plate count) to a degree almost equal to the positive bias predicted by mathematical analysis. Thomas (106) proposed a factor of

0.851 to correct for the bias of a five-tube MPN test. This figure has been cited often and was utilized in the discussion of data shown in Figure 14 of this study.

In comparing the relative merits of the MF and MPN techniques, Thomas and Woodward (as cited in Laubusch, 54) estimated that the precision obtained with a single membrane filter is from two to five times greater than that of a 5-5-5 tube MPN. Since replicate MPN determinations are not usually made in routine analysis of wastewater, extensive errors are possible when this method is employed.

While this research was in progress, several other investigators also were attempting to develop improved membrane techniques for the enumeration of fecal coliforms from chlorinated effluents. Lin (59) developed a two-step procedure that required pre-enrichment with phenol red lactose broth for four hours at 35°C. Membranes were then transferred to M-FC agar for the remainder of the incubation period at 44.5°C. Recovery was found to be statistically comparable to the MPN, although the geometric mean MF count was 610 per 100 ml compared to 650 per 100 ml for the MPN test. The disadvantage of this procedure is that it necessitates preparation of two different culture media and the manual transfer of membranes from the pre-enrichment broth to the M-FC medium.

As mentioned previously, Davenport et al. (15) experimented with preincubation of M-FC plates at room tempera-

ture or 35°C for two hours to reduce the shock of a rapid temperature increase from 0° river water to 44.5°C. The 35°C preincubation was more effective, however, the majority of the MF counts were still below the 95 per cent confidence limit of the corresponding MPN. It is now evident that a two-hour preincubation period is insufficient for acclimatization of stressed coliforms on M-FC medium. It has been demonstrated that fecal coliform counts can be increased considerably by extending the incubation time at 35°C.

The IM-MF method developed by Stuart et al. (103, 104) was evaluated and compared with the modified MF method proposed in this study. Implementation of the IM-MF test required preparation of a two-layered enriched medium, the top layer of which was prepared within one hour of use. A recently prepared rinse medium was used in addition to the usual peptone rinse. It was theorized that if appropriate metabolic intermediates beyond aldolase in the glycolytic pathway were supplied to the injured organisms, enzymatic damage could be repaired enabling growth to continue. Glycerol and acetate were added to M-FC medium together with reducing agents to inactivate residual chlorine and reduce cellular components oxidized by exposure to chlorine. Plates were held at room temperature for 1.5 hours, followed by incubation at 35°C for 4.5 hours. Plates were then transferred to a 44.5°C incubator for the remaining 18 hours.

In the comparison reported here (Table 14), the IM-MF method was found to be more effective than the standard MF method for fecal coliform recovery. However in all cases coliform counts were still higher when the modified MF procedure was employed. These data appear to indicate that the additional ingredients included in the IM-MF medium were not responsible for repair of injured cells. Increased recovery with this method was most likely due to the preincubation period at ambient temperature and 35°C. These results are in agreement with earlier findings that incubation temperature is a more critical factor than culture medium in the MF recovery of stressed fecal coliforms.

The modified MF method provides the precision of a direct bacterial count and requires only 24 hours to complete, whereas the MPN test requires two to four days before final results are obtained. The modified MF method has the advantage of employing a culture medium which has already gained wide acceptance. This procedure has been proposed as a tentative method to be included in the 15th edition of Standard Methods.

In conclusion, these studies indicate that, while the standard MF technique for fecal coliforms from chlorinated effluents compares unfavorably with the MPN, a simple modification in the incubation procedure greatly enhances recovery. This modification, a five-hour incubation at 35°C, results in fecal coliform counts approximating the MPN in

laboratory-chlorinated sewage effluent, plant-chlorinated secondary effluent, and marine waters. Development of a temperature-programmed incubator to make the change from 35 to 44.5°C after the five-hour preincubation period would eliminate any inconvenience to laboratory personnel and provide a practical method for the analysis of samples containing injured fecal coliform populations.

LITERATURE CITED

1. Adams, R.B. 1957. Comparison of standard dilution and membrane filter methods. J. Am. Water Works Assoc. 49:1452-1458.

2. American Public Health Association, American Water Works Association, Federation of Sewage and Industrial Wastes Associations. 1955. Standard methods for the examination of water, sewage, and industrial wastes, 10th ed. American Public Health Association, New York.

3. American Public Health Association, American Water Works Association, Water Pollution Control Federation. 1960. Standard methods for the examination of water, sewage, and industrial wastes, 11th ed. American Public Health Association, New York.

4. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.

5. American Public Health Association. 1975. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Inc., New York.

6. Bissonnette, G.K., J.J. Jezeski, G.A. McFeters, and D.G. Stuart. 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. 29:186-194.

7. Bissonnette, G.K., J.J. Jezeski, G.A. McFeters, and D.G. Stuart. 1977. Evaluation of recovery methods to detect coliforms in water. Appl. Environ. Microbiol. 33:590-595.

8. Bordner, R. and J. Winter, ed. 1977. Microbiological methods for monitoring the environment. I. Water and wastes. U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio.

9. Braswell, J.R. and A.W. Hoadley. 1974. Recovery of Escherichia coli from chlorinated secondary sewage. Appl. Microbiol. 28:328-329.

10. Brodsky, M.H. and D.A. Schiemann. 1975. Influence of coliform source on evaluation of membrane filters. Appl. Microbiol. 30:727-730.

11. Bronfenbrenner, J., M.J. Schlesinger, and D. Soletsky. 1920. On methods of isolation and identification of members of the colon-typhoid group of bacteria. J. Bacteriol. 5:79-87.

12. Brown, O.R. 1973. Inhibition of Escherichia coli on cellulose acetate membrane filters. Microbiol. 7: 235-240.

13. Claydon, T.J. 1975. A membrane-filter technique to test for the significance of sublethally injured bacteria in retail pasteurized milk. J. Milk Food Technol. 38:87-88.

14. Connor, R.W. 1957. Comparison of fermentation tube and membrane filter techniques for estimating coliform densities in sea water. Appl. Microbiol. 5: 141-144.

15. Davenport, C.V., E.B. Sparrow, and R.C. Gordon. 1976. Fecal indicator bacteria persistence under natural conditions in an ice-covered river. Appl. Environ. Microbiol. 32:527-536.

16. Delaney, J.E., J.A. McCarthy, and R.J. Grasso. 1962. Measurement of E. coli type I by the membrane filter. Water & Sewage Works 109:289-294.

17. De Man, J.C. 1975. The probability of most probable numbers. European J. Appl. Microbiol. 1:67-78.

18. Dutka, B.J., M.J. Jackson, and J.B. Bell. 1974. Comparison of autoclave and ethylene oxide-sterilized membrane filters used in water quality studies. Appl. Microbiol. 28:474-480.

19. Dychdala, G.R. 1977. Chlorine and chlorine compounds, pp. 167-195. In S.S. Block (ed.), Disinfection, sterilization and preservation. Lea & Febiger, Philadelphia.

20. Eijkman, C. 1904. The fermentation test at 46°C as a means of testing potable water. Zentr. Bakteriol. Parasitenk., I. 37:742.

21. Eye, J.D., J.G. Gardner, and J.F. Neel. 1956. Effects of media, temperature, and humidity on the development of coliform organisms on molecular filter membranes. J. Am. Water Works Assoc. 48:1182-1188.

22. Fifield, C.W. and C.P. Schaufus. 1958. Improved membrane filter medium for the detection of coliform organisms. J. Am. Water Works Assoc. 50:193-196.

23. Garvie, E. 1955. The growth of Escherichia coli in buffer substrate and distilled water. J. Bacteriol. 69:393-398.

24. Geldreich, E.E., H.F. Clark, P.W. Kabler, C.B. Huff, and R.H. Bordner. 1958. The coliform group. II. Reactions in EC medium at 45°C. Appl. Microbiol. 6:347-348.

25. Geldreich, E.E., R.H. Bordner, C.B. Huff, H.F. Clark, and P.W. Kabler. 1962. Type distribution of coliform bacteria in the beces of warm-blooded animals. J. Water Pollut. Control Fed. 34:295.

26. Geldreich, E.E., H.F. Clark, C.B. Huff, and L.C. Best. 1965. Fecal-coliform-organism medium for the membrane filter technique. J. Am. Water Works Assoc. 57:208-214.

27. Geldreich, E.E. 1966. Sanitary significance of fecal coliforms in the environment. U.S. Dept. of the Interior, Federal Water Pollution Control Administration Publication WP-20-3. U.S. Government Printing Office, Washington, D.C.

28. Geldreich, E.E. 1970. Applying bacteriological parameters to recreational water quality. J. Am. Water Works Assoc. 62:113-119.

29. Geldreich, E.E. 1975. Handbook for evaluating water bacteriology laboratories. EPA-670/9-75, U.S. Environmental Protection Agency, Cincinnati, Ohio.

30. Geldreich, E.E. 1976. Fecal coliform and fecal streptococcus relationships in waste discharges and receiving waters. CRC Critical Reviews in Environmental Control. Oct:349-369.

31. Goetz, A. and N. Tsuneishi. 1951. Application of molecular filter membranes to bacteriological analysis of water. J. Am. Water Works Assoc. 43:943-969.

32. Goetz, A., R.H. Gilman, and A.M. Rawn. 1952. Application of molecular filter membranes to specific problems in water analysis. J. Am. Water Works Assoc. 44:471-483.

33. Goetz, A. 1953. Application of molecular filter techniques to the bacterial assay of sewage. I. Proposed technique. Sewage Ind. Wastes 25:1136-1144.

34. Goff, J.H., T.J. Claydon, and J.J. Iandolo. 1972. Revival and subsequent isolation of heat-injured bacteria by a membrane filter technique. Appl. Microbiol. 23:857-862.

35. Green, B.L., E. Clausen, and W. Litsky. 1975. Comparison of the new Millipore HC with conventional membrane filters for the enumeration of fecal coliform bacteria. Appl. Microbiol. 30:697-699.

36. Green, D.E. and P.K. Stumpf. 1946. The mode of action of chlorine. J. Am. Water Works Assoc. 38:1301-1305.

37. Greene, R.A. 1974. Applicability of the membrane filter and most probable number coliform procedures to chlorinated waste waters. Abs. Ann. Meeting, Amer. Soc. Microbiol. 34.

38. Hajna, A.A. and C.A. Perry. 1939. Optimum temperature for differentiation of Escherichia coli from other coliform bacteria. J. Bacteriol. 38:275-283.

39. Hajna, A.A. and C.A. Perry. 1943. Comparative study of presumptive and confirmative media for bacteria of the coliform group and for the streptococci. Am. J. Pub. Health 33:550-556.

40. Hamilton, M.A. and G.K. Bissonnette. 1975. Statistical inferences about injury and persistence of environmentally stressed bacteria. J. Hygiene 74:149-155.

41. Harris, N.D. 1963. The influence of the recovery medium and the incubation temperature on the survival of damaged bacteria. J. Appl. Bacteriol. 26:387-397.

42. Hartman, P.A., P.S. Hartman, and W.W. Lanz. 1975. Violet red bile 2 agar for stressed coliforms. Appl. Microbiol. 29:537-539.

43. Hartman, P.A. and P.S. Hartman. 1976. Coliform analysis at 30°C. J. Milk Food Technol. 39:763-767.

44. Heinmets, F., W.W. Taylor, and J.J. Lehman. 1954. The use of metabolites in the restoration of the viability of heat and chemically inactivated Escherichia coli. J. Bacteriol. 67:5-12.

45. Henderson, W.L. 1959. Studies on the use of membrane filters for the estimation of coliform densities in sea water. Sewage Ind. Wastes 31:78-91.

46. Hoadley, A.W. and C.M. Cheng. 1974. The recovery of indicator bacteria on selective media. J. Appl. Bacteriol. 37:45-57.

47. Hoffman, D.A., J.H. Kuhns, R.C. Stewart, and E.I. Crossley. 1964. A comparison of membrane filter counts and most probable numbers of coliform in San Diego's sewage and receiving waters. J. Water Pollut. Control Fed. 36:109-117.

48. Hufham, J.B. 1974. Evaluating the membrane fecal coliform test by using Escherichia coli as the indicator organism. Appl. Microbiol. 27:771-776.

49. Kabler, P. 1954. Water examinations by membrane filter and most probable number procedures. Am. J. Pub. Health 44:379-386.

50. Kaper, J.B., G.S. Sayler, M.M. Baldini, and R.R. Colwell. 1977. Ambient-temperature primary non-selective enrichment for isolation of Salmonella spp. from an estuarine environment. Appl. Environ. Microbiol. 33:829-835.

51. Klein, D.A. and S. Wu. 1974. Stress: a factor to be considered in heterotrophic microorganism enumeration from aquatic environments. Appl. Microbiol. 27:429-431.

52. Knox, W.E., P.K. Stumpf, D.E. Green, and V.H. Auerbach. 1948. Inhibition of sulfhydryl enzymes as the basis of the bactericidal action of chlorine. J. Bacteriol. 55:451-458.

53. Krusé, C.W., Y. Hsu, A.C. Griffiths, and R. Stringer. 1970. Halogen action on bacteria, viruses, and protozoa. In Proc. National Specialty Conference on Disinfection. Amer. Soc. Civil Engineers, New York.

54. Laubusch, E.J. 1958. MPN coliform index. Water & Sewage Works 105:334-338.

55. Laubusch, E.J. 1961. Rationale and accomplishments of chlorine disinfection, pp. 543-610. In Proc. Rudolfs Res. Conference on Public Health Hazards of Microbial Pollution of Water.

56. Lin, S. 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollut. Control Fed. 45:498-506.

57. Lin, S. 1974. Evaluation of methods for detecting coliforms and fecal streptococci in chlorinated sewage effluents. Illinois State Water Survey, Urbana. Report of Investigation 78.

58. Lin, S.D. 1976. Evaluation of Millipore HA and HC membrane filters for the enumeration of indicator bacteria. Appl. Environ. Microbiol. 32:300-302.

59. Lin, S.D. 1976. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. Appl. Environ. Microbiol. 32:547-552.

60. Lin, S.D. 1976. Comparing ten membranes for a two step membrane filter method for fecal coliform recovery in chlorinated effluents. Presented at 49th Annual Conference of the Water Pollution Control Federation.

61. Mallmann, W.L. and F.R. Peabody. 1961. Multiple-tube dilution and membrane filter methods. Water & Sewage Works 108:384-389.

62. Maxcy, R.B. 1970. Non-lethal injury and limitations of recovery of coliform organisms on selective media. J. Milk Food Technol. 33:445-448.

63. McCarthy, J.A. 1955. Comparative coliform densities in water by the membrane filter and by multiple tube technic. Am. J. Public Health 45:1569.

64. McCarthy, J.A., H.A. Thomas, and J.E. Delaney. 1958. Evaluation of the reliability of coliform density tests. Am. J. Public Health 48:1628-1635.

65. McCarthy, J.A. and J.E. Delaney. 1958. Basic studies on media for the membrane filter. Water & Sewage Works 105:292-296.

66. McCarthy, J.A., J.E. Delaney, and R.J. Grasso. 1961. Measuring coliforms in water. Water & Sewage Works 108:238-243.

67. McFeters, G. and D.G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. 24:805-811.

68. McKee, J.E. and R.T. McLaughlin. 1958. Application of molecular filter techniques to the bacterial assay of sewage. II. Experimental results for settled sewage. Sewage Ind. Wastes 30:129-137.

69. McKee, J.E., R.T. McLaughlin, and P. Lesgourgues. 1958. Application of molecular filter techniques to the bacterial assay of sewage. III. Effects of physical and chemical disinfection. Sewage Ind. Wastes 30:245-252.

70. Milbauer, R. and N. Grossowicz. 1959. Reactivation of chlorine-inactivated Escherichia coli. Appl. Microbiol. 7:67-70.

71. Milbauer, R. and N. Grossowicz. 1959. Effect of growth conditions on chlorine sensitivity of Escherichia coli. Appl. Microbiol. 7:71-74.

72. Moran, J.W. and L.D. Witter. 1976. An automated rapid method for measuring fecal pollution. Water & Sewage Works 123:66-67.

73. Moss, C.W. and M.L. Speck. 1966. Identifcation of nutritional components in trypticase responsible for recovery of Escherichia coli injured by freezing. J. Bacteriol. 91:1098-1103.

74. Mossel, D.A.A. and A. Ratto. 1970. Rapid detection of sublethally impaired cells of Enterobacteriaceae in dried foods. Appl. Microbiol. 20:273-275.

75. Mossel, D.A.A., G.A. Harrewijn, and C.F.M. Nesselrooy-van Zadelhoff. 1974. Standardization of the selective inhibitory effect of surface active compounds used in media for the detection of Enterobacteriaceae in foods and water. Public Health Lab. 11:260-267.

76. Nelson, F.E. 1971. Symposium on the restoration of sublethally impaired bacterial cells in foods. IV. Restoration of the normal physiology in sublethally impaired cells of bacteria in foods. Influence of pH and inhibitors. J. Milk Food Technol. 34:550-551.

77. Oblinger, J.L. and J.A. Koburger. 1975. Understanding and teaching the most probable number technique. J. Milk Food Technol. 38:540-545.

78. Ordal, Z.J. 1970. Current developments in detection of microorganisms in foods: Influence of environmental factors on detection methods. J. Milk Food Technol. 33:1-5.

79. Ordal, Z.J., J.J. Iandola, B. Ray, and A.G. Sinskey. 1976. Detection and enumeration of injured microorganisms, pp. 163-169. In M.L. Speck (ed.), Compendium of methods for the microbiological examination of foods. American Public Health Association, New York.

80. Postgate, J.R. and J.R. Hunter. 1963. The survival of starved bacteria. J. Appl. Bacteriol. 26:295-306.

81. Presnell, M.W., W. Arciez, and C.B. Kelly. 1954. Comparison of the MF and MPN techniques in examining sea water. Public Health Reports 69:300-304.

82. Presnell, M.W. 1974. Evaluation of membrane filter methods for enumerating coliforms and fecal coliforms in estuarine waters, pp. 127-131. In Proc. 8th National Shellfish Sanitation Workshop.

83. Presswood, W.G. and L.R. Brown. 1973. Comparison of Gelman and Millipore membrane filters for enumerating fecal coliform bacteria. Appl. Microbiol. 26:332-336.

84. Preswood, W.G. and D. Strong. 1977. Modification of M-FC medium by eliminating rosolic acid. Abs. Ann. Meeting, Amer. Soc. Microbiol. 272.

85. Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies. 1972. Comparison of membrane filtration and multiple tube methods for the enumeration of coliform organisms in water. J. Hygiene 70:691-705.

86. Ray, B. and M.L. Speck. 1972. Repair of injury induced by freezing Escherichia coli as influenced by recovery medium. Appl. Microbiol. 24:258-263.

87. Ray, B. and M.L. Speck. 1972. Metabolic process during the repair of freeze-injury in Escherichia coli. Appl. Microbiol. 24:585-590.

88. Ray, B. and M.L. Speck. 1973. Discrepancies in the enumeration of Escherichia coli. Appl. Microbiol. 25:494-498.

89. Ray, B. and M.L. Speck. 1973. Enumeration of Escherichia coli in frozen samples after recovery from injury. Appl. Microbiol. 25:499-503.

90. Rose, R.E., E.E. Geldreich, and W. Litsky. 1975. Improved membrane filter method for fecal coliform analysis. Appl. Microbiol. 29:532-536.

91. Roth, L.A. and D. Keenan. 1971. Acid injury of Escherichia coli. Canadian J. Microbiol. 17:1005-1008.

92. Schaeffer, D.J., M.C. Long, and K.G. Janardan. 1974. Statistical analysis of the recovery of coliform organisms on Gelman and Millipore membrane filters. Appl. Microbiol. 28:605-607.

93. Scheusner, D.L., F.F. Busta, and M.L. Speck. 1971. Injury of bacteria by sanitizers. Appl. Microbiol. 21:41-45.

94. Scheusner, D.L., F.F. Busta, and M.L. Speck. 1971. Inhibition of injured Escherichia coli by several selective agents. Appl. Microbiol. 21:46-49.

95. Shipe, E.L. and G.M. Cameron. 1954. A comparison of the membrane filter with the most probable number method for coliform determinations from several waters. Appl. Microbiol. 2:85-88.

96. Sladek, K.J., R.V. Suslavich, B.I. Sohn, and F.W. Dawson. 1975. Optimum membrane structure for growth of fecal coliform organisms. Appl. Microbiol. 30: 685-691.

97. Speck, M.L., B. Ray, and R.B. Read. 1975. Repair and enumeration of injured coliforms by a plating procedure. Appl. Microbiol. 29:549-550.

98. Speck, M.L. and B. Ray. 1977. Effects of freezing and storage on microorganisms in frozen foods: A review. J. Food Protection 40:333-336.

99. Standridge, J.H. 1976. Comparison of surface pore morphology of two brands of membrane filters. Appl. Environ. Microbiol. 31:316-319.

100. Stersky, A.K. and T.I. Hedrick. 1972. Inhibition of growth of airborne coliforms and other bacteria on selective media. J. Milk Food Technol. 35:156-162.

101. Stevens, A.P., R.J. Grasso, and J.E. Delaney. 1974. Measurement of fecal coliform in estuarine water, pp. 132-136. In Proc. 8th National Shellfish Sanitation Workshop.

102. Streeter, H.W. and D.A. Robertson. 1960. Evaluation of membrane filter technique for appraising Ohio River quality. J. Am. Water Works Assoc. 52:229-241.

103. Stuart, D.G., J.E. Schillinger, and G.A. McFeters. 1976. An improved membrane filter technique for the recovery of injured fecal coliform bacteria. Abs. Ann. Meeting, Amer. Soc. Microbiol. 196.

104. Stuart, D.G., G.A. McFeters, and J.E. Schillinger. 1977. Membrane filter technique for the quantification of stressed fecal coliforms in the aquatic environment. Appl. Environ. Microbiol. 34:42-46.

105. Thomas, H.A. 1946. Mathematics as a tool in the waterworks laboratory. J. New England Water Works Assoc. 60:153-160.

106. Thomas, H.A. 1955. Stream pollution: Statistical analysis of coliform data. Sewage Ind. Wastes 27:212-222.

107. Tobin, R.S. and B.J. Dutka. 1977. Comparison of the surface structure, metal binding, and fecal coliform recoveries of nine membrane filters. Appl. Environ. Microbiol. 34:69-79.

108. U.S. Environmental Protection Agency. 1975. Proposed amendments to 40 CFR, part 136, guidelines establishing test procedures for analysis of pollutants. Federal Register 40:24535-24539.

109. Van Donsel, D.J., R.M. Twedt, and E.E. Geldreich. 1969. Optimum temperature for quantitation of fecal coliforms in seven hours on the membrane filter. Bacteriol. Proc., Am. Soc. Microbiol. 25.

110. Venkobachar, C., L. Iyengar, and A.V.S. Prabhakara Rao. 1975. Mechanism of disinfection. Water Research 9: 119-124.

111. Warseck, M., B. Ray, and M.L. Speck. 1973. Repair and enumeration of injured coliforms in frozen foods. Appl. Microbiol. 26:919-924.

112. White, G.C. 1972. Handbook of chlorination. Van Nostrand Reinhold Co., New York.

113. Wu, S. and D.A. Klein. 1976. Starvation effects on Escherichia coli and aquatic bacterial responses to nutrient addition and secondary warming stresses. Appl. Environ. Microbiol. 31:216-220.

114. Woodward, R.L. 1957. How probable is the most probable number? J. Am. Water Works Assoc. 49:1060-1068.

115. Wyss, O. 1961. Theoretical aspects of disinfection by chlorine, pp. 400-427. In Proc. Rudolphs Res. Conference on Public Health Hazards of Microbial Pollution of Water.

116. Yee, G.S., W.B. Krabek, and C.P. Schaufus. 1953. New medium for bacteriological analysis with molecular filter membranes. J. Am. Water Works Assoc. 45: 945-951.

117. Ziegler, N.R. and H.O. Halvorson. 1935. Application of statistics to problems in bacteriology. IV. Experimental comparison of the dilution method, the plate count, and the direct count for the determination of bacterial populations. J. Bacteriol. 29:609-634.

A P P E N D I X A

COMPOSITION OF M-FC AGAR MEDIUM

M-FC Broth Base

Tryptose	10.0 g
Proteose peptone No. 3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salts	1.5 g
Aniline blue (Water Blue)	0.1 g
Distilled water	1000 ml

1.5 g agar was added to 100 ml base medium. Medium was heated to dissolve agar. One ml of a one per cent solution of rosolic acid (Difco) in 0.2 N sodium hydroxide was added. Medium was heated to boiling and boiled for 1 minute, cooled and dispensed.

A P P E N D I X B

COMPOSITION OF IM-MF MEDIUM OF STUART ET AL. (104)

Enriched M-FC Agar (1.5 strength)*

Dehydrated M-FC broth base	55.5 g
Sodium acetate	1.0 g
Glycerol	1.0 g
Thioglycolate	0.25 g
Glutathione	0.25 g
Sodium thiosulfate	1.0 g
Agar	15.0 g

Enriched Lactose Agar (overlay)**

Dehydrated lactose broth base	13.0 g
Sodium acetate	1.0 g
Glycerol	1.0 g
Thioglycolate	0.25 g
Glutathione	0.25 g
Sodium thiosulfate	1.0 g
Yeast extract	1.0 g
Agar	15.0 g

*Agar was dissolved in 1 liter of purified water containing 15 ml of 1 per cent rosolic acid in 0.2 N NaOH, heated to boiling, and promptly removed from the heat. It should not be autoclaved.

**This agar was dissolved in 1 liter of purified water and autoclaved. Enriched lactose medium without agar was used as a rinse.

A P P E N D I X C

CLASSIFICATION OF TREATMENT PLANTS FROM WHICH SEWAGE EFFLUENTS WERE OBTAINED

A. Primary Treatment

Amherst
Northampton
Trident (Montana)

B. Secondary Treatment

Sunderland
Greenfield
So. Deerfield
Millers Falls
Amherst Pilot Plant
Bozeman (Montana)