A STUDY OF A MODIFIED MEMBRANE FILTER TECHNIQUE FOR THE ENUMERATION OF STRESSED FECAL COLIFORMS IN URBAN RUNOFF

by

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I. INTRODUCTION

Communicable diseases have always been a threat to man's health. Exposure of man to his fellow humans and other components of his environment increases the risk of illness. Communicable diseases are transmitted from one organism to another by vectors such as particulate matter, food, water, or by direct contact with the disease source. Because of increased city populations, it has become necessary to establish large public water supplies. Contamination of such water supplies with pathogenic organisms has been the cause of many epidemics throughout the world and may have been partially responsible for the great plagues.

Pathogens may enter a receiving stream from sewage treatment plant effluents, contaminated groundwater, or stormwater runoff and can contaminate other streams or impounded water supplies downstream of the original contamination site, causing, in many instances, serious and widespread public health problems.

Water-borne pathogens include: the bacteria responsible for typhoid (Salmonella typhosa), paratyphoid A (S. paratyphi), paratyphoid B (S. schottmuelleri); bacterial dysentery (Shigella dysenteriae); and the rather rare pathogenic strains of Escherichia coli, Paracolobacterium, or other enteric intermediates (1). Water-borne protozoan pathogens include those causing amoebic dysentery (Endamoeba histolytica) (1) and giardiosis (Giardia lamblia). Other pathogens which occasionally may be water-borne include viruses that cause infectious hepatitis, poliomyelitis, and histoplasmosis; parasitic worms such as tapeworms

(Taenia spp.), disease-producing blood flukes (Schistosoma spp.), and hookworms (Ascaris). Spore-forming bacteria, such as Bacillus arthracis that causes anthrax, may possibly occur in water (2).

The apparent absence of pathogenic organisms cannot be used as a basis for judging the safety of water for human consumption. A relatively elaborate series of procedures must be carried out for their isolation from mixed cultures. These procedures are not suitable for routine use. Instead, indicator organisms, i.e. bacteria native to the intestines of healthy humans and other warm-blooded animals, are used to indicate the existence of fecal pollution. Their presence evinces fecal pollution of some form and denotes the potential presence of pathogens. For control and enforcement purposes, demonstration of pathogenic organisms themselves is not necessary. The margin of safety afforded by the use of the indicator organisms depends on the ratio between them and human pathogens. This margin is quite wide under almost all circumstances (3), because the bacterial indicator organisms are usually many times more resistant than specific bacterial pathogens such as salmonellae.

In recent years, much concern has been expressed that indicator organisms, stressed by a host of environmental conditions in certain terrestrial and aquatic environments, may not be serving their intended purpose because the "stress" makes them susceptible to rigorous procedures used for their isolation that they normally could withstand. Bacteria entering the environment outside the intestines are subjected to a host of adverse environmental influences such as heat, cold,

exposure to disinfectants, irradiation, pH variation, the presence of minimal nutrients, antagonistic response due to other microflora, and toxic materials, such as heavy metals. These adverse conditions may generate excessive changes in physiological and morphological properties, creating a population of stressed organisms (4). Such stressed organisms may go undetected by routine tests when the relatively harsh differentation media for enumeration are employed. Failure to detect stressed indicators may seriously affect water quality classification based on designated standards. This is especially important since stressed pathogenic strains have been shown to maintain their virulence even though subjected to stresses.

The specific objectives of this study were, first, to compare a proposed assay procedure (to be described) for fecal coliforms in stormwater runoff from an urban area to the existing membrane filter (MF) and most probable number (MPN) techniques as prescribed by the American Public Health Association, et al. (5), and second, to relate, if possible, any observed differences in the relative recovery efficiencies to the heavy-metal concentrations in the urban runoff.

II. LITERATURE REVIEW

Much has been written on the use of coliform organisms as indicators of potentially dangerous polluted water. Extensive studies have been carried out on water samples from various sources, and the value of indicator organisms in pollution studies has been well established. Subpopulations of stressed coliforms, however, cause difficulties in accurately assessing the extent of fecal contamination loads. It has been suggested (4) that a period of recovery for these stressed organisms is needed before they will show up in standard analytical tests. The research area of this thesis is that of recovering this stressed subpopulation of coliform organisms. Topics that have been reviewed in the literature, which will be discussed in the following sections, are: historical aspects, the importance of the coliforms, coliform physiology, coliforms in runoff waters, tests used for detection, the concept of stressed organisms, heavy metal stress in urban runoff waters, and modified tests for stressed coliform recovery.

Historical Aspects of Pathogens Associated with Polluted Water

Disease epidemics have occurred at numerous occasions throughout history, one of the most extensive taking place in the fourteenth century A.D. when the "Black Death" claimed the lives of nearly 25 percent of the European population. An epidemic in the winter of 1664-1665 killed 14 percent of London's population. Polluted water may have partially been the cause of these epidemics (6).

Although water had long been suspected of carrying disease organisms, it was not until 1854 that this was proven epidemiologically. In that year, London had one of its recurrent cholera outbreaks, and 700 deaths occurred in the St. James parish alone. Dr. John Snow and his commission studied the nature of the outbreak and reported that the source of infection was the Broad Street Pump water supply. The well had been contaminated by sewage seeping in through a break in an adjacent masonry sewer. This incident established the fact that water was important in the transmission of Asiatic Cholera (6).

Water was proven to be a major medium for disease transmittance in 1875 by Robert Koch, who successfully isolated a pure culture of the anthrax-causing bacterium and, by doing so, initiated the science of bacteriology. He later isolated pure cultures of the pathogens causing Asiatic Cholera (in 1883) and typhoid (in 1884). Also in 1883, he described a method for estimating bacterial numbers in water. Modifications of this early method now comprise the standard plate count (SPC) technique (5).

Despite the effectiveness of modern filtration and disinfection practices in preventing water borne disease outbreaks, these defenses still occasionally break down and epidemics occur. A classic example of this took place in July, 1940, in Seymour, Indiana. The White River flooded following heavy rains. This, coupled with improper treatment plant operation, led to the occurrence of an epidemic. Before it was over, 2,250 cases of gastroenteritis and severe diarrhea had developed. Some 884 of these cases were diagnosed as typhoid and 27 deaths resulted (6).

Importance of the Coliform Bacteria

In 1885, Escherich isolated B. coli from the feces of a cholera patient (6). He insisted that the existence of these microorganisms in water supplies represented pollution because of their possible association with the enteric, disease-causing bacterial group present in the intestines of ill persons. The bacterial group described by Escherich were later classified in the Escherichia-Aerobacter genera. It was subsequently found that the group occurred regularly and in immense numbers in the feces of man and numerous other animals. Many species of the coliform group were isolated, and several species biochemically resembling the group were discovered in plants, soils, and similar sources. Due to the variation in environments in which coliform bacteria were found, confusion resulted as to their correlation with fecal pollution.

Smith (7) stated in 1895 that the presence of coliform bacteria in the physical environment was indicative of fecal pollution and hazardous to public health regardless of where they were detected. He maintained that all coliform organisms originated in the intestines of warm-blooded animals. In 1904, Eijkman presented his opposition to the accepted belief that only those coliforms, proven to be of fecal origin, typified dangerous pollution (6). He insisted that ignoring the source and native habitat of the coliform groups risked public health. He recommended an elevated-temperature culture incubation procedure. Fecal coliform organisms would grow at this elevated temperature, but non-fecal coliforms would not. Modern techniques

for coliform enumeration incorporate this "Eijkman reaction," as it came to be called.

Geldreich (8) assembled fecal samples from ten adults living in a controlled institutional environment. These residents ate the same food and worked at the same job. When he compared the coliforms in fecal samples from these adults to those in samples from 33 people from a variety of environmental backgrounds, he found the same coliform distributions. Human fecal samples had 11 of the possible 16 indole, methyl red, Voges-Proskauer, citrate (IMViC) contaminations of biochemical tests. He also studied the feces of livestock and poultry and found the coliform component to be almost exclusively Escherichia coliform (E. coli). Five other types appeared, but only occasionally. Later research along these lines demonstrated E. coli to be the dominant coliform in the feces of dogs, cats, and various rodents.

Large numbers of bacteria are contributed to drainage water from soil. The correlation between coliform bacteria from unpolluted soils and the fecal coliform test is therefore important. Geldreich (9) collected 251 soil samples from 26 states and 3 foreign countries and found that fecal coliforms were absent or present in relatively small quantities in unpolluted soils. Less than two fecal coliforms per gram were found in most of the samples. On the other hand, fecal coliform densities in soils from polluted areas such as animal feedlots, lands recently flooded with domestic wastewater, and the banks of heavily polluted streams, varied from 3,300 to 49,000 per gram.

Although rain falling to the earth contains insignificant bacterial contamination (10), it can cause any coliforms associated with vegetation to enter surface water via storm water drainage. Such organisms occurring on plants may, in turn, result from contact by insects. Geldreich (11) examined 152 species of plants and 40 insect specimens from various ecological environments. Although 13 different IMViC types on the vegetation specimens and 14 different types on the insects were found, no type predominated. Geldreich concluded that coliforms from the intestines of warm-blooded animals contributed a relatively minor percentage of the microorganisms associated with vegetation and insects.

Fresh water fish have no permanent coliform populations in the intestines. The occurrence of fecal coliform bacteria in fish is apparently related to the pollutional level of their food and the aquatic environment itself (11). Fish may act as carriers of warm-blooded animal pollution for up to approximately seven days, however, and can transport pathogens to areas of clean water (12).

Survival of coliform organisms outside the human body has been the subject of several studies. Geldreich (12) reported that many factors affect the persistence of the coliform organisms in the soil. Such factors as temperature, pH, and soil moisture may indirectly influence enteric bacterial survival by governing the growth of antagonistic organisms such as protozoa, fungi, and actinomycetes. Organic nutrients in the feces may also be conducive to the proliferation of these antagonistic organisms. Other factors of importance

include sunlight, soil exposure, and rainfall frequency (10).

Van Donsel et al. (13) studied E. Coli survival in both exposed and shaded outdoor soil plots over several years. The researchers reported a seasonal variation in the death rates at both sites. The 90 percent reduction times ranged from 3.3 days in summer to 13.4 days in the autumn. Their results indicated that fecal coliform bacteria could persist in the soil environment for several days after contamination and could result in subsequent water contamination. Work by Geldreich et al. (10) supported these observations.

McFeters et al. (14) studied the survival of indicator bacteria in the water environment. Using a membrane chamber technique they had developed, coliform bacteria, mixed natural populations of indicator bacteria, or enteric pathogens were immersed in a flowing supply of well water. The viability of each organism was determined as a function of time. A total of 29 fecal and non-fecal cultures, isolated from water and from fecal specimens from man and animals, were used. The studies showed a lack of close agreement in survival patterns among coliforms grouped together according to species, source type, or characterization as fecal or non-fecal. This observed lack of uniform die-off rates was also reported by Gordon (15). McFeters and his group also found no sigificant difference in persistence when fecal cultures were compared to non-fecal organisms. Previous studies by Geldreich et al. (10) and the ORSANCO Water Users Committee (16), however, reported a more rapid die-off of a fecal coliform culture as compared with a non-fecal variety at $20^{o}C$. The discrepancy in these reports

is probably caused by the diversity of the coliform group.

A few studies also have been carried out to compare the persistence of some pathogenic organisms with the indicator organisms (10,14,15,17). The general conclusion from these studies was that a few indicator bacteria survive somewhat longer than some enteric pathogens. Few concrete relationships could be determined. Work by McFeters et al. (14) indicated that the survival of the coliforms was on the same order as some of the salmonellae they tested. Some of the shigellae persisted in water slightly longer than some of the more stable coliforms. A much higher die-off rate was found for Salmonella typhi and Vibrio cholerae as compared to the coliforms, however. Work by Andre et al. (17) resulted in similar observations for S. typhi and V. cholerae.

Under some conditions, indicator bacteria entering the soil or water environment may proliferate (aftergrow). A good deal of disagreement has been expressed among investigators concerning the causes of aftergrowth. At least two conditions seem to promote it: the dilution of polluted water with clean water and the disinfection of sewage or effluent followed by discharge into the receiving stream. In both of these conditions, the numbers of indicator bacteria have been shown to increase, contrary to expectations (3). Sudden ecological upset between the indicators and their environment may be the cause of this phenomenon. The more complete destruction by disinfection of organisms preying on the bacteria or their dilution may be one mechanism for this upset.

Some investigators have estimated the aftergrowth period to be from 9 to 15 hours after initial discharge (18). Evans et al. (19) found maximum bacterial regrowth occurring in a period from 24 to 72 hours after discharge. Underhill (6) observed instream aftergrowth of total and fecal coliforms in less than three hours, but it could not be determined if these coliforms originated in the chlorinated sewage effluent or in runoff from pastureland located downstream of the sewage treatment plant. Kittrell (20) reported increases of from 10 to 100 times the initial population within one or two days downstream travel for some coliforms. These reported differential aftergrowth periods probably are largely a factor of the coliform group diversity and differences in the aquatic environments studied.

Aftergrowth has generally been associated with the non-fecal portion of the total coliform group (21). Geldreich (12) stated that aftergrowth is mainly a function of Enterobacter coliform (formerly Aerobacter), which can grow with very minimal nutrients. Geldreich concluded that non-fecal coliforms can survive and multiply in waters containing minimal quantities of nutrients, whereas the fecal coliform component requires more favorable environmental conditions. In one study of aftergrowth (6), coliforms attained a peak density, and then rapidly disappeared from the stream.

Coliform Physiology

Coliform bacteria have been defined as all of the aerobic and facultative anaerobic, Gram-negative, oxidase-negative, non-spore-forming, rod-shaped bacteria having the abilities to grow in a medium

containing bile salts and to ferment lactose with gas production within 48 hours at 35°C (22). The group includes all genera having the same structure and the ability to use either aerobic respiration or sugar fermentation as a metabolic pathway to derive energy. Included are the genera Erwinia, Enterobacter (Aerobacter), Escherichia, Pasteurella, Serratia, and Klebsiella. All of these genera can utilize sugars, amino acids, organic acids, or other simple substrates for aerobic metabolism.

The two major fermentative patterns characteristic of coliform bacteria are mixed acid fermentation and butylene glycol fermentation. The former pattern is characteristic of E. coli. This organism ferments glucose and forms formic acid, lactic acid, acetic acid, sucinic acid, ethyl alcohol, CO_2, and hydrogen gas. The CO_2 to H_2 ratio is 1:1 because they are derived solely from formic acid by the reaction: $HCOOH \rightleftarrows H_2 + CO_2$ (23). The enzyme formic hydrogenlyase catalyzes this reaction.

In butylene glycol fermentation, more ethyl alcohol is produced with 2,3-butylene glycol being the exclusive type. Less acid is formed in this fermentation pattern. Hydrogen and CO_2 are produced in a manner duplicate to that in mixed acid fermentation; however additional CO_2 is formed during the reactions that yield butylene glycol. The Voges-Proskauer reaction, the methyl red test, or a comparison of CO_2-H_2 gas ratios may be used to distinguish between the fermentation types.

E. coli is a common intestinal bacteria, and Erwinia is a plant pathogen. Of the other coliform groups, Enterobacter occurs in the

soil and on the plants, and Serratia is found in soil and water. Members of the genus Klebsiella may be found in human respiratory and intestinal tracts (23).

Coliforms Associated with Runoff

The traditional approach to water pollution control has been to eliminate discharges of raw wastewater and untreated industrial wastes as the main objective. In recent years, however, the importance of storm water runoff as a source of stream pollution has been given more and more consideration. According to Bradford (24), studies designated to quantify pollutant loadings in urban runoff first appeared in the 1950's. Some of these early studies presented substantial evidence that runoff waters can cause a shock load to the receiving stream that is 100 to 1000 times greater than that produced by sanitary wastewater (24).

With the establishment of runoff, both rural and urban, as an important source of pollution, attention began to focus upon quantifying the pollutant sources in macro scale. Attempts were made by Weibel et al. (25) and Geldreich et al. (10), among others, to determine urban watershed area loads of various pollutants. Several studies appeared in the early 1970's stating that nonpoint pollution sources accounted for more than half of the observed total pollution (26). Since the late 1960's, efforts have mainly been focused on identifying and quantifying the individual sources of nonpoint water pollution and on the development of predictive models for determining the load that could be expected from a given area based upon several factors (24).

The 1972 Federal Water Pollution Act Ammendments included the requisite that area pollution control planning must include consideration of both point- and nonpoint sources of pollution.

Some workers have stated that storm water is the greatest intermittent source of bacterial pollution entering receiving streams (27). Numerous studies have been carried out to determine the quantities of coliform organisms in runoff waters (10,12,25,28,29,30). Whipple et al. (29) found coliform counts to be variable and greatly in excess of concentrations normally considered safe for water contact activities. Weibel et al. (25) studied storm water from a 27 acre residential and suburban area that was free of sanitary and industrial waste discharges, and found fecal coliform levels to average 11,000 per 100 ml. Faust (30) reported that in a study of the Rhode River watershed, the concentration of total and fecal coliforms changed seasonally and ranged from 17 to 24,000 per 100 ml, and from 4 to 11,000 per 100 ml, respectively. Various other studies have reported even greater ranges, with total coliform levels reported to be up to several million per 100 ml (10). The coliform levels entering water systems have been reported to depend on many factors, including: natural background coliform concentrations and soil types (10,13), the rates of water discharge (31), the hydraulic regime of the stream (32), seasonal differences (10,13,30), sediment load (33), and nutrient availability (34). It appears that concentrations of total coliforms and fecal coliforms are functions of the same variables (30). The main sources of fecal coliforms in urban storm water are animals associated with urban living: mainly cats,

dogs, birds, and various rodents. The soil contains naturally occurring coliform organisms and fecal coliforms from the excreta of the urban warm-blooded animals.

In recent years, several reports have been published stressing the importance of storm water runoff as a source of bacterial pollution in receiving waters. Burgess et al. (35) reported that rural and urban runoff generated significant inputs of fecal coliforms to Lake Burley Griffin in Australia, causing recreational use of the lake to often be interrupted because bacterial standards were violated. It had originally been thought that the high fecal coliform levels were solely the result of sewage discharge. Geldreich (36) studied a recreational lake in Texas having as its source a drainage basin receiving only 15 inches of rainfall a year. The basin supports around 15,000 people and 180,000 cattle. Here, the water quality was found to be excellent during dry weather, but storm water runoff introduced fecal coliform levels well in excess of the recommended levels. Work by Buckingham et al. (37) indicated that staggered contributing times caused by intermittent rainfall accounted for high fecal coliform loads entering receiving waters in a Tennessee Valley study. The problem of coliform concentrations in storm water has even generated interest in projects to disinfect runoff waters (38). The feasibility of such treatment has not been fully established.

Standard Tests for Coliform Detection

In 1904, Eijkman (6) found that coliform bacteria from the intestines of warm-blooded animals fermented glucose broth and produced

gas at 46°C, whereas non-fecal coliforms could not grow. Numerous improvements on the Eijkman reaction have since been made. Delaney et al. (39) summarized the early work on inconsistencies in the method. Strains of B. coli (now E. coli) failing to develop in glucose broth at 46°C were found. A number of methyl red-negative coliforms behaving in the Eijkman reaction were discovered, detracting from the specificity of the reaction. One report stated that only a small percentage of human fecal B. coli could ferment glucose at 46°C. The necessity of a more specific method for coliform detection prompted the development of a new medium with a reduced glucose concentration in 1933 and the replacement of glucose by lactose in the medium formulation in 1936. In 1943, an EC medium (buffered tryptose bile salt broth) was proposed. Subsequent investigations proved it to be unsatisfactory as a presumptive medium, however, and in 1948 brilliant green bile (BGB) broth was suggested for use. It too proved to be unsatisfactory for use as a presumptive medium. In 1957, there was renewed interest in the use of lactose broth. The EC medium and BGB broth were adopted for the confirmed test in the MPN for fecal and total coliforms, respectively. In recent years, lauryl tryptose broth has been suggested for use instead of lactose because it has been reported to yield fewer false positive results (4). The incubation temperature for fecal coliforms was changed from 46°C to 44.5°C. Geldreich (40) reported on the need for this temperature change. He stated that inhibition of the gas-forming mechanism of some fecal coliforms can be significant when the elevated incubation temperature exceeds 45.6°C.

He emphasized the need for incubation in a carefully-controlled water bath at 44.5°C (± 0.2°).

A statistically-based procedure was developed for determining coliform numbers in water samples using the fermentation of lactose as its basis. This most probable number (MPN) technique is described in Standard Methods (5). The technique consists of inoculating three, five, or more replicate samples of at least three dilutions of sample into a lactose or lauryl tryptose broth (Presumptive test). This presumptive test serves as a screening procedure in which a positive reaction, after incubation of samples at 35°C, indicates a probability of the presence of the coliform group and a negative reaction excludes its presence. This step also ensures an optimal cell density, generally in excess of 1,000 viable organisms, when culture transfers are made from positive tubes into the selective broths for coliform confirmation. Confirmation broths contain inhibitory substances such as ox bile, bile salts, surfactants, or dyes, allowing for growth of the coliforms, but excluding growth of other organisms. Brilliant green bile broth and an incubation temperature of 35.5 ± 0.5°C generally constitutes the confirmed test for total coliforms, while EC broth and an incubation temperature of 44.5 ± 0.5°C are used for confirmed fecal coliform determinations. A completed test may then be employed, followed by a Gram strain, if desired.

The IMViC series of biochemical tests may be used to differentiate between fecal and non-fecal coliforms (22). The series consists of various combinations of positive and negative reactions involving

indole production, the methyl red reaction, the Voges-Proskauer test, and citrate utilization. It has been found, however, that the elevated temperature (MPN) technique is superior for fecal coliform detection (41). The MPN technique does not require the isolation of a pure culture, multiple media inoculation, or the four biochemical reactions. Also, a five-day incubation period is necessary for the methyl red test in the IMViC series, whereas presumptive MPN results are obtained in 48 hours and confirmed fecal coliform results in 72 hours.

The membrane filter (MF) technique for bacterial water examinations was used in Europe for many years before it was formally introduced into the U. S. in 1947 (42). Since then, much work has been carried out on the technique to improve it and to evaluate its advantages and limitations. Several of the first investigators studied two-step membrane filter techniques for the elevated temperature reaction (39,43). Later, many media were proposed which were reported to give adequate results in a simpler one-step incubation (44,45). Fifield et al. (46) introduced the widely accepted M-Endo broth for the detection of the total coliform group in 1958. Geldreich et al. (47) developed the fecal coliform membrane filter procedure (M-FC) in 1965. This method allowed for direct quantitative enumeration of the fecal coliform organism group without the need for prior enrichment or subsequent chemical tests. The M-FC method was incorporated as an approved analytical procedure for water analysis into the 13th edition of Standard Methods (5). The MF

technique was shown to have several advantages over the MPN method , including quickness of analysis, less required labor, less expense, greater potential sample volumes, and lack of the statistical bias found in the MPN technique (39).

The initial M-Endo and M-FC techniques employed the placing of broths on sterile absorbent pads placed in Petri plates. McCarthy et al. (48), however, reported a suppressive effect when M-Endo broth was used in this manner. They found that the use of Endo agar (M-Endo medium + 1.5 percent agar-agar) gave consistently higher degrees of coliform recovery. They also reported that the use of M-Endo plus agar generated a more-pronounced sheen on the coliform colonies, making them easier to enumerate, and that this sheen persisted longer than when the liquid medium was used.

Although some researchers reported close agreement between data obtained by the MF and the MPN techniques when compared (42,48), the majority of the studies have demonstrated significantly poorer recoveries of the coliforms by membrane filter techniques, especially when the samples were toxic wastes or chlorinated effluents. McKee et al. (49) showed much lower coliform recoveries from chlorinated, settled sewage for the MF technique as compared with accepted MPN procedures. Lin (50) reported similar results when studying chlorinated, secondary sewage treatment plant effluents, and Shipe et al. (51) observed this same discrepancy in a study of river water containing toxic wastes. These observations prompted investigators to determine if an enrichment step prior to transfer of the sample to differential media would lead to a better correlation of the two

techniques. Lin (52) demonstrated that an enrichment two-step membrane filter technique, developed by McCarthy et al. (48) at the Lawrence Experiment Station, significantly improved total coliform recovery from chlorinated secondary effluent when compared to a one-step method. He found this LES procedure to be comparable to the completed MPN technique for total coliform enumeration for chlorinated, secondary sewage treatment plant effluents. Total coliform recovery in these effluents by the LES technique was around 1.5 times greater than results using the M-Endo, one-step technique. Lin concluded that the standard M-FC membrane filter assay for recovery of fecal coliforms from chlorinated effluents was less efficient than the confirmed MPN procedure. Dufour et al. (53) developed a modified membrane filter procedure (mC) for defining the quantities of coliforms in sea water. He showed this mC procedure to be comparable to the completed MPN method in their coliform recoveries, whereas the M-Endo procedure yielded substantially lower values.

Another improvement to the coliform enumeration tests was the development of a delayed-incubation membrane filter technique by Taylor et al. (54). This method employed a vitamin-free Casitone (VFC) holding medium, and provided an alternative procedure which could be used when raw samples could not be analyzed within the recommended six-hour period. Taylor and his group found this procedure to yield results comparable to those of the standard procedures.

A number of studies have been carried out to determine the most effective type of membrane filter to use with the MF procedure. Sladek et al. (55) found that the primary determinant of coliform growth on a membrane filter was that of surface pore morphology. Their data strongly suggested that neither the method of sterilization nor chemical composition of the filter had any sigificant effect on recovery. They suggested that an optimum membrane structure with surface pores slightly larger than the coliform organisms but also with internal retention pores smaller than the coliforms be used. This would allow for adequate cellular division, which could be affected by the extent and nature of the contact of the coliform with the membrane filter material. They further argued that nutrient supply by medium diffusion and subsequent metabolic waste products removal were also a function of pore morphology and membrane structure. Until this work, membranes for bacterial testing had been specified by a retention pore size of 0.45 μm. These membranes typically have surface opening diameters of one to two μm. Sladek and his coworkers suggested a change to an optimum membrane having a 2.4 μm surface opening and a 0.7 μm retention pore size. Subsequent work by Green et al. (56) and Lin (57) demonstrated the superiority of filters having these characteristics over the 0.45 μm filters.

The Concept of Stressed Coliform Organisms

As previously mentioned, a host of environmental factors can cause stresses on coliform organisms entering the terrestrial or aquatic environment. Numerous studies have been carried out to

determine the influence of these environmental factors on the coliform bacteria. Daubner (4) found that the chemical characteristics of the aquatic environment could be related to the lowering of E. coli metabolism. Maeda et al. (58) observed that sudden increases in temperature caused suppression of cell motility in an E. coli suspension. High temperatures can cause various cellular damages and can eventually bring about a collapse of the helical structure of DNA by a dissociation of the hydrogen bonds (59). The stressed condition of coliform bacteria not killed by chlorine disinfection has been documented by McKee et al. (49) and Lin (50,52). Recovery of organisms sublethally injured by freezing was studied by Ray et al. (60). Postgate et al. (61) studied the stress induced in bacteria in environments with low nutrient levels. Klein and Wu (62) also did work on the concept of starved bacterial populations and stated that this condition could lead to an increased susceptibility to secondary stresses such as heat. Mitra et al. (63) and Babich and Stotzky (64) have studied the stress generated by cadmium on bacteria. Mitra and his coworkers studied the ability of E. coli to alter its chemical physiology to compensate for cadmium toxicity. He found that E. coli appears to accommodate to the metal by exclusion of the ion from the cell and by cellular reversal of any damage generated by prior exposure to the ion. Bissonnette et al. (65) studied the influence of various environmental stresses on the indicator bacteria from natural waters. They observed that a substantial portion of the total coliform population may be physiologically injured by an assortment of stresses.

The nature of the damage the bacteria experience depends on the types of environmental stress the organism is exposed to. The exact characteristics of the damages are not well understood. The available literature along these lines is quite limited and is concerned mainly with disinfectants and related chemical agents. Some of the possible means of damage leading to populations of stressed organisms include (1):

a. Interference with the cell wall integrity.
b. Action against covalent bonds.
c. Disruption or loss in integrity of the cytoplasmic membrane.
d. Action upon cell protein.
e. Interferences with enzyme activity.
f. Interferences with polymerases.

All the environmental factors causing stress in organisms probably act in one or more of these manners. More work is warranted along these lines.

Laboratory procedures may also be responsible for stressed organisms. Such things as excessive sample transit time, extended holding times in dilution water, types of dilution water, rapid changes in environmental temperatures, and improper media formulations may cause stress on the organisms. Precautions must be taken to avoid such stress conditions.

Heavy Metal Stress in Parking Lot Runoff

The major constituent of street and parking lot surface contaminants has been consistently found to be inorganic mineral-like matter quite similar to common sand and silt (66). Most of this material is blown, washed, or carried in from nearby land areas. A small amount of organic matter is also usually found. Other constituents of the surface contaminants include heavy metals, various inorganic nutrients, oil and grease, small quantities of pesticides, and microorganisms, including both fecal and non-fecal coliforms (66,67). The heavy metals, plus some of the other constituents of runoff, can create subpopulations of stressed coliforms.

Significant amounts of heavy metals have been detected by numerous investigators in surface contaminants of streets and parking lots. Sator et al. (66) found zinc and lead to be the most prevalent metals, followed by copper. Other reports listed the highest concentrations of metals to be iron, lead, manganese, and zinc (68).

The main source of the lead found on street and parking lot surfaces is combustion of leaded gasoline by motor vehicles. Lead is added to gasoline as alkyl lead compounds to improve the fuel's antiknock quality. In 1965, it was estimated that the annual motor vehicle exhaust of lead in North America was around 240,000 tons (69). Assuming deposition of 50 percent of this on the roadbed, and assuming all of this is carried off in runoff waters, the average concentration of lead in street runoff would be a significant 0.23 mg/l (70). Actual

concentrations on heavily-traveled roadsides have been found to be much greater (70). Zinc and cadmium on street and parking lot surfaces are derived mainly from motor vehicle tires and oil (71). Zinc levels in street and parking lot runoff waters are often relatively high (66,71), whereas cadmium levels are generally low (71). The presence of chromium and copper levels are generally from wear on various parts of motor vehicles. Their concentrations are generally relatively low (66). Other heavy metals that may be present in low quantities include nickel from nickled gasoline and nickel-containing automotive parts (71), magnesium from parts (68), and iron in the form of rust from the vehicles.

The volume and type of traffic moving over the streets or parking lots has an effect on the kinds and amounts of contaminants present (68). Logically, more contaminants will occur on crowded parking lots than on rural roads. The nature of the road material is also important. Sator et al. (66) found that asphalt surfaces had up to 80 percent greater contaminant loadings than concrete surfaces. The surface physical conditions also exert an influence. Higher contaminant loadings are generally found on poor surfaces as compared to good surfaces (68). Other factors of importance concerning the quantities of surface contaminants in runoff waters are the quantities of air pollution fallout (68), the intensity and duration of the storm, the length of the antecedent dry period, and the management practices of the public works department (72).

Modified Tests for Stressed Coliform Recovery

Since the realization that various environmental factors can lead to subpopulations of stressed coliform organisms, studies have been carried out to develop modified methods for their detection. Bissonnette et al. (65) reported that prolonged exposure of E. coli to certain aquatic environments often resulted in a substantially lower amount of recovery when differential media as opposed to rich, non-selective media were used for culturing. He reported that this nonlethally injured subpopulation was capable of recovering from injuries if exposed to a rich, non-selective broth before being placed on the harsh, selective media. Exposure to non-selective broth supposedly allows the organisms to repair cellular damage and subsequently multiply, whereas direct exposure to the secondary stress of the selective medium causes many of the injured cells to die.

The presumptive enrichment of the MPN procedure allows for such a period of repair and minimum stress. This fact, plus the built-in statistical bias of the MPN, probably account for the high numbers of coliform recovery at elevated temperatures with this method as compared with the standard membrane filter test (73). Application of enrichment techniques to improve the recovery of indicator organisms in the MF technique has been advocated by several researchers (48,52, 73,74). Rose and Litsky (74) employed a technique of incubating membrane filters on an absorbent pad containing an enrichment medium. This pad was placed over a base layer of selective agar. Cell recovery was reported to occur before the selective ingredients

diffused from the base agar to the membrane. Lin (52) found that the two-step enrichment LES membrane filtration method significantly improved recovery of coliforms from chlorinated secondary sewage treatment plant effluents when compared to the standard, one-step method. Hartman et al. (75) extended the studies of Rose and Litsky (73) by developing a method of using a base agar and a violet red bile (VRB) agar overlay, thus permitting direct-plating. Work by Speck et al. (76) showed that repair on trypticase soy agar (TSA), followed by a subsequent VRB agar, generally provided for increased coliform enumeration by allowing for repair of the injured subpopulation. Bissonnette et al. (77) recently compared several existing recovery methods for coliforms by applying their membrane filter chamber technique. They found that a two-hour enrichment on a rich, non-selective medium prior to exposure to selective media led to greater recovery of fecal coliforms with the MF technique. Substantially greater recoveries of fecal coliforms from raw sewage suspensions and of E. coli from pure-culture suspensions were observed when compared to values obtained by direct exposure to selective media.

Recently, Rose et al. (73) proposed a membrane filter technique involving two layers of agar. This technique minimized the inherent limitations of enrichment techniques, which include the need for more time, equipment, and manpower, and the greater possibility for contamination. The use of this two-layer procedure permits the culture to recover from injury as the selective agar constituents are diffusing into the enriched medium on which the organisms are growing.

The base medium in this technique is M-FC broth plus 1.5 percent agar. Five milliliters of this medium are added to a 50-mm-diameter, tight-fitting Petri dish and allowed to harden at room temperature. Then, two milliliters of lactose broth plus 1.5 percent agar are pipetted on top of this layer. The reason for the controlled thickness of the agar is that it has been shown that fecal coliform recovery may be a function of agar thickness (55). The ingredients of the two-agar medium will eventually diffuse into each other. This fact provides for the automatic shift of the culture contact from the enrichment medium to the selective medium. It also requires that the two-layer plates be prepared within one hour prior to use. The technique provides for repair and elevated temperature growth to be confined to a single Petri dish, thus avoiding unnecessary handling and possible contamination. Rose and his coworkers also incorporated the use of a two hour incubation at 35°C to allow for optimum growth and repair prior to a temperature increase to 44.5°C for 22 to 24 hours to achieve the necessary selectivity. Their preliminary results showed significant increases in coliform recovery through the use of this technique. Litsky (78) has suggested that the methodology of Rose et al. be broadened to include his research on a 5 hour preincubation period at 35°C, followed by 18 hours at 44.5°C.

III. MATERIALS AND METHODS

The objective of this study, as stated in the introduction, required the implementation of several different methods and procedures for data collection. Analyses performed included the standard MPN and MF fecal coliform enumeration procedures, a modified membrane filter technique for stressed fecal coliforms, and tests for the heavy metal concentration of cadmium, copper, chromium, iron, lead, and zinc.

Description of Study Area

Since the main objective of this study was to compare three methods for fecal coliform enumeration in urban storm water runoff, only one study site was needed. The nature of this research project did not necessitate determinations of all constituents of runoff, nor was there a need to collect samples from sites of varying characteristics. The site that was chosen for study was the parking lot of the Kroger grocery store in Blacksburg, Virginia. This site was chosen for several reasons, including its intensive use, well-defined drainage area, and good physical condition. The site is a high density parking lot with 239 spaces. It has a parking density of about 4.5 cars per space day (67). This density may vary significantly with the seasons of the year. The volume of traffic in the parking lot during the summer months is lower than during the fall and winter due to the reduced student population at Virginia Tech. The lot has an asphalt concrete pavement and is in good condition. The total drainage area of the site is 86,257 square feet. Of this, 79,306 square feet, or

92 percent is impervious (67). A map showing the location of the study site is exhibited in Figure 1, and a diagram of the site is shown in Figure 2.

Sampling Procedures

Twelve trials were made between May 25, 1977, and June 28, 1977. Samples were collected at the curb inlet in half-gallon plastic containers. Samples for heavy metal determination were decanted into 500-ml polyethylene bottles that had been washed sequentially with the following cleaning agents: Tap water plus detergent, 1:1 nitric acid, tap water, 1:1 hydrochloric acid, tap water, and deionized distilled water (79). These bottles contained 0.5 ml of concentrated nitric acid to reduce the sample pH to approximately 2. Samples for coliform enumeration were decanted into 500 ml polyethylene bottles previously washed sequentially with the following cleaning agents: Tap water plus detergent, tap water, and deionized distilled water.

Rainfall data was acquired from the Virginia Tech Department of Agricultural Engineering. This data was collected at their station 0.3 miles from the Kroger lot. An attempt was made to collect samples at more than one point on the storm hydrograph when practicable.

Heavy Metal Analysis

The quantities of extractable metals in the sample were determined. A 100-ml well-mixed sample was acidified with 5 ml of redistilled, concentrated nitric acid. The solution was heated for 15 minutes on a Corning Type PC-351 hot plate. The sample was then allowed to cool and

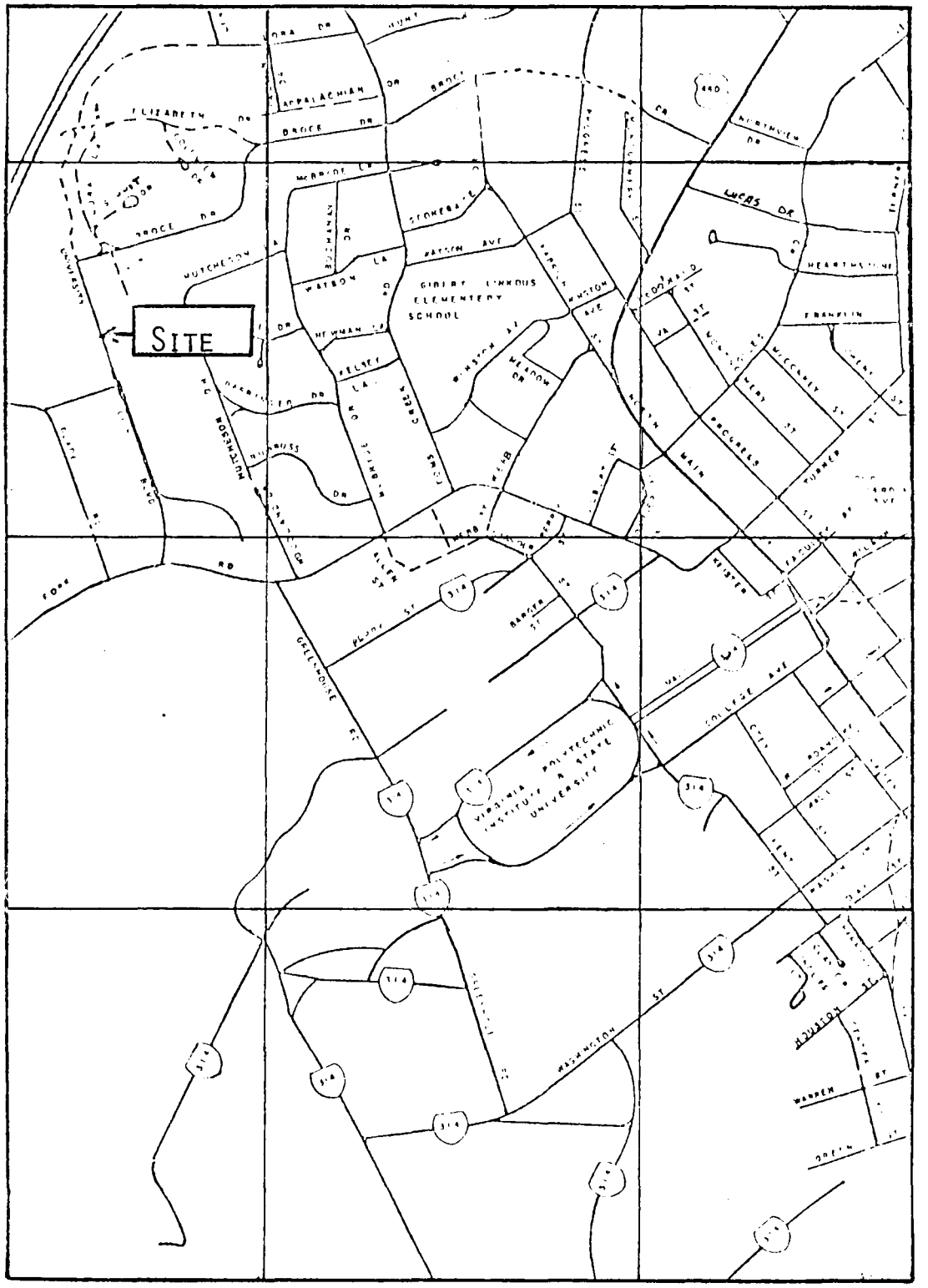

Figure 1. Location of Study Site, Blacksburg, Virginia.

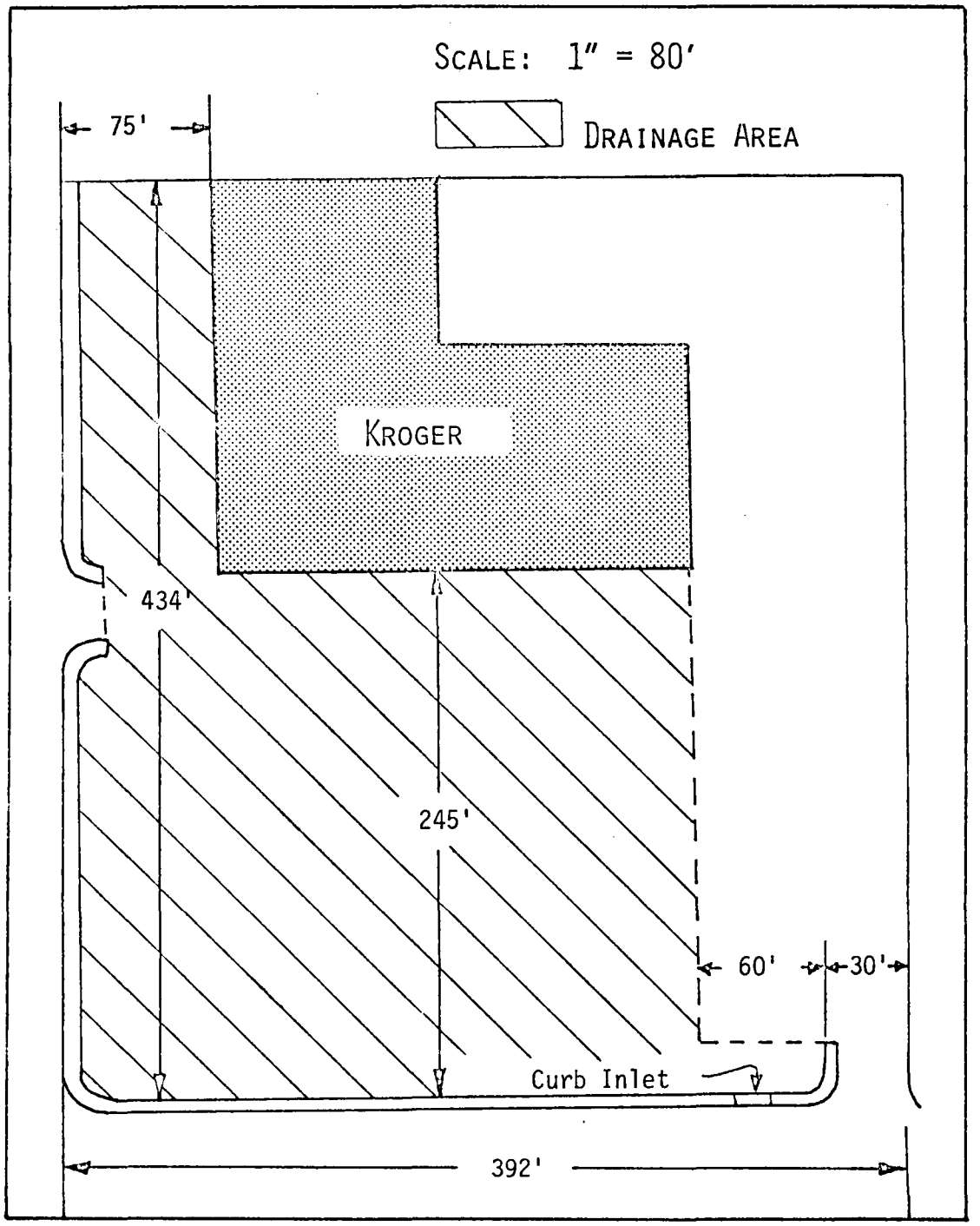

Figure 2. The Kroger's Parking Lot, Blacksburg, Va.

was filtered through a Millipore Type HA 0.45 μm membrane filter. The sample was then diluted to its original volume by the addition of deionized, distilled water. The sample was stored in a clean acid-washed polyethylene bottle until it was analyzed. The analysis was carried out using a Perkin-Elmer Atomic Absorption Spectrophotometer Model 403. Two samples consisting of distilled water plus nitric acid were run as controls to check the adequacy of the acid washing procedure in cleaning the sample containers.

Coliform Enumeration

A set of serial dilutions was made with the sample for fecal coliform enumeration. Due to its desirable characteristics (80,81,82), distilled water plus 0.1% peptone was used as the dilution water. Milk-dilution bottles containing the dilution water autoclaved at 121 psi for 15 minutes to provide a final volume of 99 ml were prepared. Dilutions made from the original sample ranged from 1:10 (10^{-1}) to 1:10,000 (10^{-4}).

A five-tube MPN procedure was employed. Inoculations were made from the serial dilutions of the original sample into tubes containing 10 ml of lauryl tryptose broth (BBL) and an inverted Durham tube. Dilutions made were 10^{0} to 10^{-4}. This constituted the presumptive test for the fecal coliforms. The tubes were incubated at 35°C in a Napco Model 330 incubator for 48 hours. At the end of this period, those tubes exhibiting growth and gas production were recorded as positive in reaction. Transfers were made from these positive tubes to tubes containing 10 ml of EC broth (BBL) and an inverted Durham tube. These

tubes were incubated in a GCA Precision Scientific circulating water bath at 44.5°C for 24 hours. Growth with gas production at the end of this incubation period constituted the confirmed test for fecal coliforms. Statistical tables found in Standard Methods (5) were then used to determine the fecal coliform concentrations.

In addition, samples were analyzed by the standard membrane filter technique (MF) for fecal coliforms. A device was designed in the laboratory for preparing six samples simultaneously (Figure 3). Gelman magnetic filter funnels (47 mm) and 47 mm Millipore Type HC membrane filters (2.4 μm surface openings, 0.7 μm retention size) were used. The volume of the sample filtered was 10 ml in all cases. There were four replicates of each dilution in trials one through seven. Three 10-ml replicates were made in trials seven through twelve. Distilled water was used to thoroughly wash down the sides of the filter apparatus when the samples were filtered and between filtrations. At least two membrane filters per trial were prepared by filtering the distilled rinse water. These filtrations established controls to test the adequacy of this wash in preventing contamination of subsequent filters. The filter apparatus was exposed to UV light for 10 minutes between sample trials. After filtration, the filter membranes were transferred to tight-fitting 50 mm-diameter Petri dishes containing 5 ml of M-FC medium (BBL M-FC broth plus 1.5% Difco agar plus 1% MCB rosolic acid in 0.2 N NaOH). Aseptic techniques were employed at all times. The Petri plates were then placed in water-tight Nasco Whirl-Pak plastic bags, put in racks in the 44.5°C circulating water bath, and incubated 24

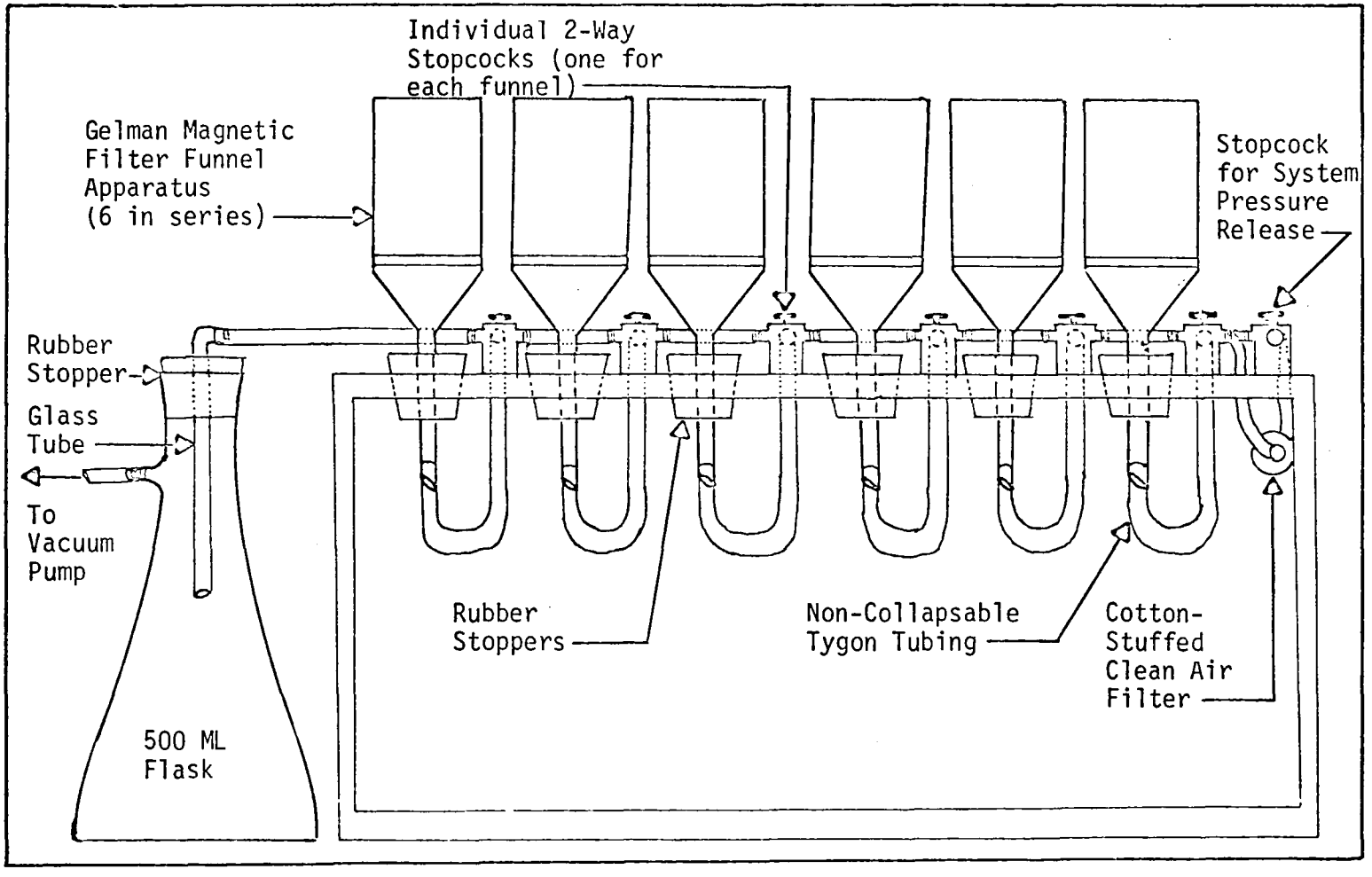

Figure 3. Membrane Filter Apparatus

hours. At the end of this period, plates from the dilution yielding counts closest to a range of 20 to 60 blue-colored colonies per plate were enumerated using a 15-power Bausch and Lomb dissecting microscope, a fluorescent light source, and a hand counter.

The stressed organism membrane filter technique described by Rose et al. (73) was also employed. The methodology was essentially that described for the standard MF technique. In this test, there four replicates of each dilution for the first seven trials and three replicates for the remaining trials. Five milliliters of M-FC medium were added to a 50 mm-diameter tight-fitting Petri dish and allowed to solidify at room temperature. Two milliliters of lactose broth (BBL), containing 1.5 gm of agar per 100 ml were then pipetted as a second layer onto the base agar. This overlay was added within one hour prior to use. After filtration, the membrane filters were placed on the media and were incubated for five hours at 35^{o}C. This incubation period has recently been proposed by Green, et al. (78), and was used in preference to the 2 hour incubation period used by Rose and his associates (73). Some tests were performed, however, to compare the 5-hour and 2-hour techniques. Both methods were used in trials seven through twelve. After this 5 hour period, the plates were placed in the water-tight plastic bags and incubated in the 44.5^{o}C circulating water bath for 19 hours. At the end of this period, the fecal coliform concentrations were determined in the same manner employed in the standard MF technique. The entire laboratory protocol is shown in Figure 4.

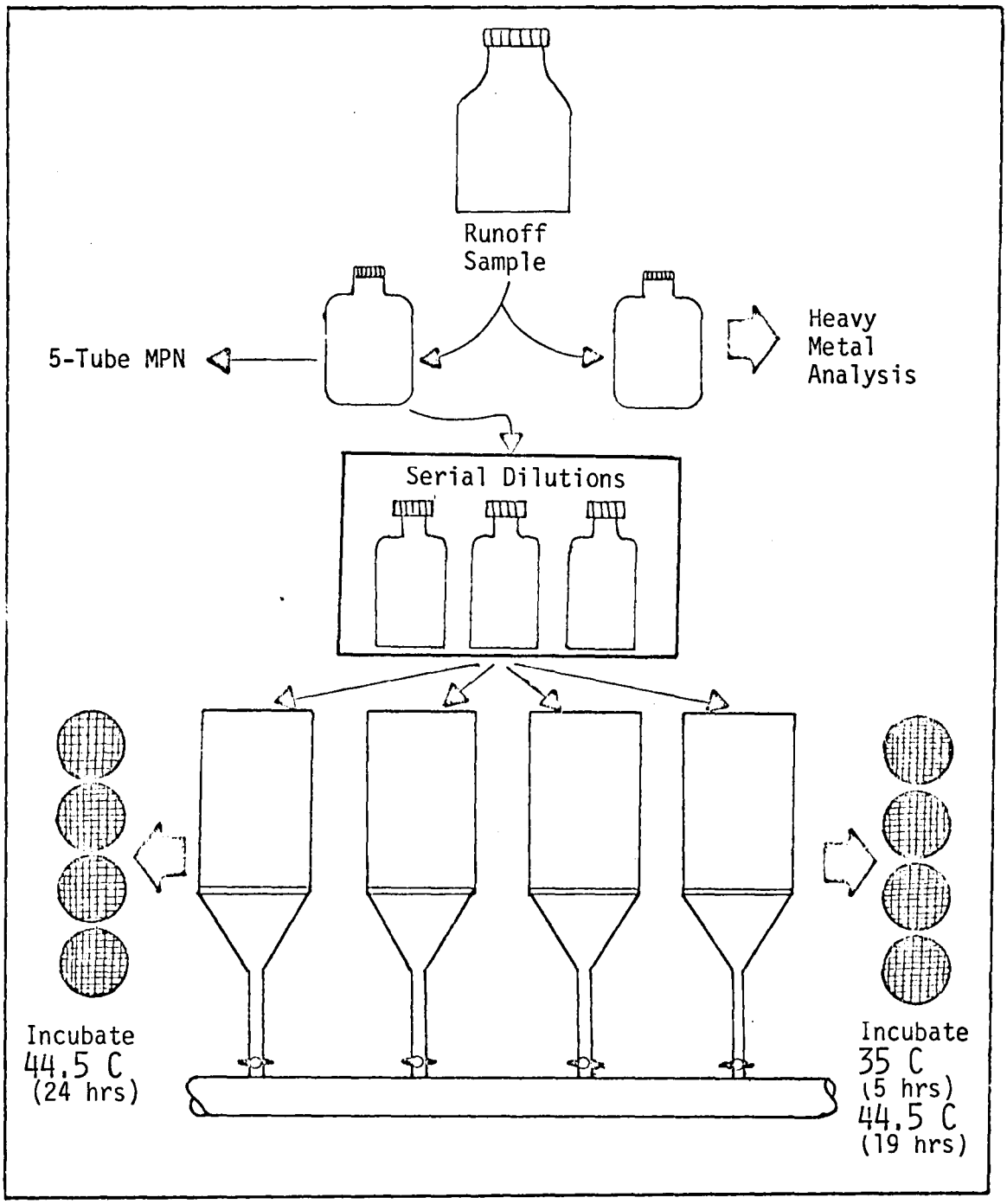

Figure 4. Protocol for Standard Membrane Filter-Modified MF-MPN Comparisons and Heavy Metal Analysis.

It has recently been suggested by Presswood et al. (83) that eliminating rosolic acid from the M-FC medium improves the M-FC procedure by allowing higher fecal coliform recoveries with greater ease in counting. To examine this idea, three replicate plates per dilution were prepared without rosolic acid in trials eleven and twelve. Verification of fecal coliform colonies isolated in the two MF techniques was performed by subculturing a percentage of the blue colonies into lauryl tryptose broth for 48 hours at 35°C. Tubes exhibiting growth and gas production at the end of this period were again subcultured to EC broth and incubated at 44.5°C for confirmation. In trials seven through twelve, the colonies were subcultured directly into EC broth and incubated at 44.5°C.

IV. RESULTS OF STUDY

Overview

During eight rainfall events, a total of twelve runoff samples was collected and analyzed for fecal coliforms by several techniques. The samples were also analyzed for selected heavy-metal concentrations.

In this section, the rainfall data and metals concentrations corresponding to the monitored rainfall events are presented. Also presented are the coliform data obtained by the various recovery procedures and a statistical analysis designed to determine if the recovery procedures gave significantly different results. Comparisons made include:

1) the standard MF method with the modified method involving a five-hour preincubation period at 35°C. The latter will be referred to as the "M-5hr" method.
2) the standard MF with the modified method involving a two-hour preincubation period at 35°C. The latter will be referred to as the "M-2hr" method.
3) the M-2hr method with the M-5hr method.
4) the M-5hr method with the MPN method.
5) the M-2hr method with the MPN method.
6) the standard MF with the MPN method.

An attempt was made to correlate the observed densities in recoveries by the various techniques with other environmental conditions, and those results are also presented.

Rainfall

The twelve runoff samples were collected during the period from May 25 through June 28, 1977. Rainfall data for this period (Table I) were obtained from the Department of Agricultural Engineering at VPI & SU. The rainfall events varied greatly in intensity and duration. The rainfall intensity calculations for each date were based on the total elapsed time from the beginning to the end of the rain on any given date. The times when samples were collected during the storm varied for each storm, and samples represented different stages on the hydrograph. On one date, samples were taken that represented early, middle, and late points on the hydrograph. The times of sample collection and their relationship to the hydrograph for each event are shown in Table II. As can be seen, the length of time between rainfalls varied from 4.4 hours to 462.4 hours. The elapsed time since the previous rain of at least 0.03 inches was used for these determinations because 0.03 inches has been mentioned as the amount of rain required to initiate runoff (67).

Fecal Coliform Determinations

Recovery data. Mean coliform concentrations, expressed as organisms per 100 ml, were determined by analysis of the samples by each MF technique. These data are presented in Tables III and IV. The MPN's for these samples, obtained by the multiple fermentation technique, are presented in Appendix Table A-1. Actual MF plate counts and standard deviations for all replicate tests are presented in Appendix Tables A-II through A-IV. The standard deviations, in most cases, were relatively large.

TABLE I

Characteristics of Rainfall During the Study Period

Date	Intensity (In/Hr)	Duration (Hours)*	Total Rain (Inches)
May 5	0.027	8.45	0.23
May 22	0.013	0.75	0.01
May 24	0.048	0.42	0.02
May 25	0.019	18.13	0.35
May 26	0.007	9.72	0.07
May 29	1.279	1.72	2.20
May 30	0.014	3.58	0.05
May 31	0.303	1.88	0.57
June 1	0.097	1.03	0.10
June 2	0.009	1.08	0.01
June 6	0.030	13.45	0.41
June 8	0.020	5.00	0.10
June 9	0.048	7.30	0.35
June 12	0.010	5.87	0.06
June 14	0.062	5.63	0.35
June 15	0.267	0.75	0.20
June 16	0.062	0.16	0.01
June 17	0.171	8.13	1.39
June 20	0.008	8.00	0.06
June 22	0.051	9.17	0.47
June 23	0.121	4.05	0.49
June 24	0.035	15.67	0.55
June 25	0.003	13.13	0.04
June 27	0.024	1.25	0.03
June 28	0.015	6.58	0.10

*Elapsed time from initial rain to end of rain on date, yielding an average overall rainfall intensity.

TABLE II

Sample Collection Times During Monitered Storms

Date	Begin Rain	End Rain	Sample Collection Times	Time Since Previous Rain[a] (Hours)
May 25	0:45[b]	19:23	8:00 9:30	462.4
May 31	21:00	22:53	9:00[c]	6.8
June 6	3:55	17:32	4:30 5:40 6:50 14:10	124.0
June 22	11:35	20:45	13:30	12.6
June 23	4:07	8:10	8:45	7.4
June 24	4:10	19:50	11:05	20.0
June 25	0:15	13:23	13:05	4.4
June 28	13:40	20:15	16:00	24.3

[a]Elapsed time since previous rain of at least 0.03 inches.

[b]All times based on a 24-hour clock.

[c]Sample collected day after storm from water remaining along storm curbing.

TABLE III

Fecal Coliform Concentrations in Runoff[a]
Determined During the Study Period

Trial Number	Date	Concentration (FC/100 ml)			Ratio
		Std. MF[b]	Mod. MF[c]	MPN[d]	Mod. MF:Std. MF
1	May 25 (#1)[e]	15,300	191,000	220,000	12.5:1
2	May 25 (#2)	8,800	83,000	110,000	9.4:1
3	May 31	3,900	30,000	35,000	7.7:1
4	June 6 (#1)	2,800	33,600	11,000	12.0:1
5	June 6 (#2)	7,200	400,000	17,000	55.6:1
6	June 6 (#3)	6,000	199,000	35,000	33.2:1
7	June 6 (#4)	1,040	11,900	5,400	11.4:1
8	June 22	1,000	6,900	7,900	6.9:1
9	June 23	2,600	9,400	9,200	3.6:1
10	June 24	3,000	7,400	13,000	2.5:1
11	June 25	5,900	17,400	24,000	3.0:1
12	June 28	1,600	8,300	9,400	5.2:1

[a]Runoff from the Kroger parking lot, Blacksburg, Va.

[b]Standard membrane filter technique, as described in Standard Methods (5).

[c]Modified membrane filter technique, with 2 ml lactose agar overlay and 5 hour preincubation at 35°C.

[d]Most probable number technique.

[e]Numbers in parenthesis represent trials at different times on a given date.

TABLE IV

Fecal Coliform Concentration in Runoff[a]
Determined Using Alternate Methods

Trial Number	Date	Concentration (FC/100 ml)			Ratio 2 Hr. Mod. MF :Std. MF
		Std. MF[b]	2 Hr. Mod. MF[c]	Std. MF-R.A.[d]	
8	June 22	1,000	1,900	Not Run	1.9:1
9	June 23	2,600	3,400	Not Run	1.3:1
10	June 24	3,000	5,000	Not Run	1.7:1
11	June 25	5,900	8,800	7,000	1.5:1
12	June 28	1,600	3,800	1,700	2.4:1

[a]Runoff from the Kroger parking lot, Blacksburg, Virginia.

[b]Standard membrane filter technique.

[c]Modified membrane filter technique, with 2ml lactose agar overlay and a 2-hour preincubation at 35°C.

[d]Standard membrane filter technique minus rosolic acid.

Graphical comparisons of the data. Recoveries by the various techniques for fecal coliform determinations are graphically compared in Figures 5-7. In each determination, the modified technique for stressed organisms resulted in greater recoveries than the standard MF procedure. Figure 8 shows the effects of increasing the preincubation period, which is part of the modified technique, from two hours to five hours. As can be seen, the recoveries when the five-hour period were used were higher in all instances.

Figure 9 shows a comparison of the recoveries of the three membrane filter techniques to the MPN data. This type of graphical comparison was adopted from Green et al. (78). It can be seen from this graph that the fecal coliform recoveries using the M-5hr technique closely approximated the MPN's recorded for most all trials. Recoveries with the standard MF and M-2hr techniques were consistently lower than the MPN values.

The differences in recovery by the various methods, expressed both as logarithms and as percentages, are shown in Table V. The technique that consistently produced the lowest recoveries, the standard MF technique, was used as the basis for comparison.

In two trials, M-FC medium without rosolic acid was prepared and used in parallel with the other techniques. The recoveries obtained using this medium did not appear to vary greatly from the standard MF recoveries (Table IV). The actual plate counts for this procedure are presented in Appendix Table A-V.

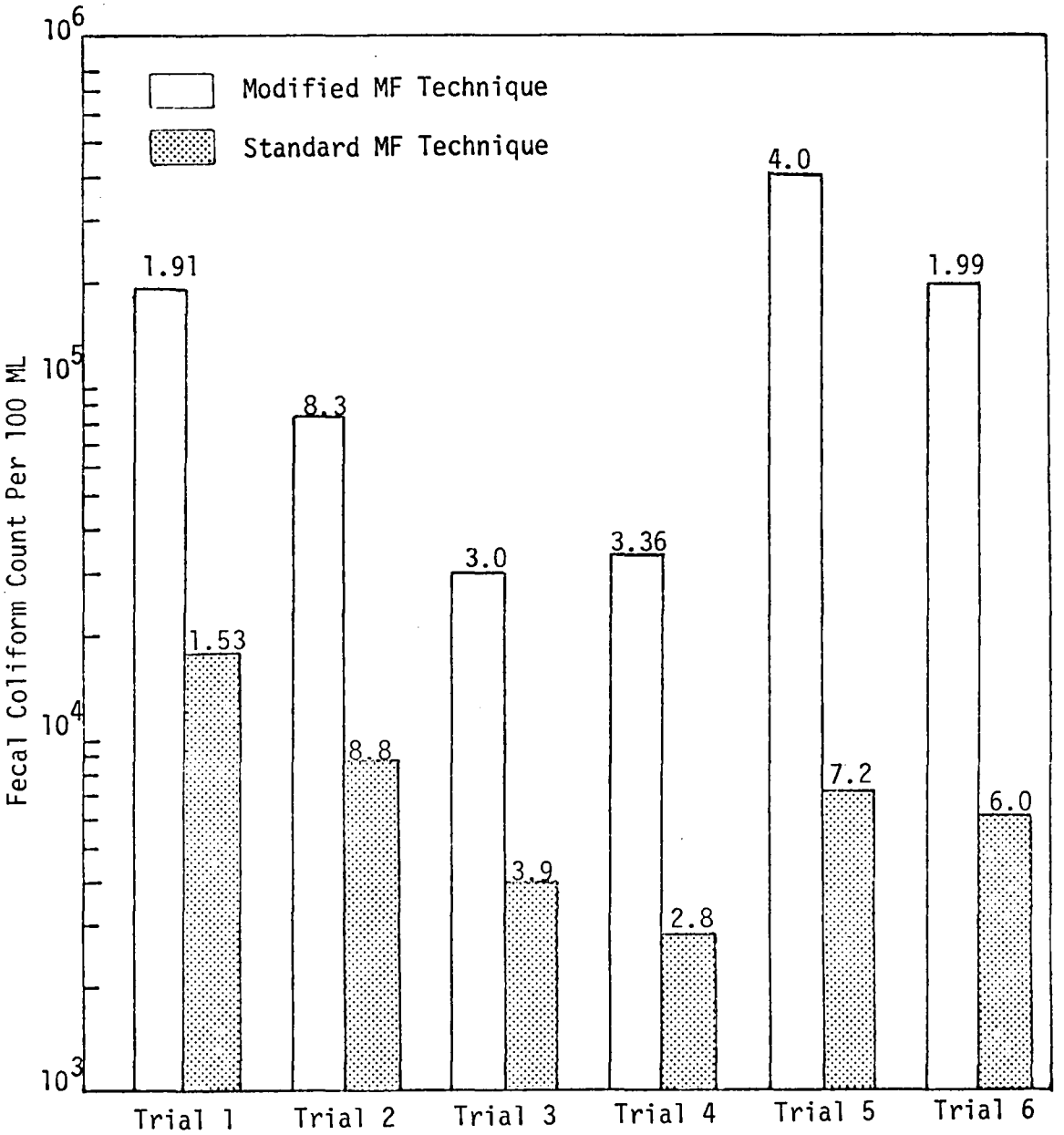

Figure 5. Comparisons of Fecal Coliform Recoveries from Kroger Parking Lot Runoff (Following Several Storm Events) Using the Standard Membrane Filter (MF) Technique and a Modified MF Technique Requiring a Five Hour Preincubation Step.

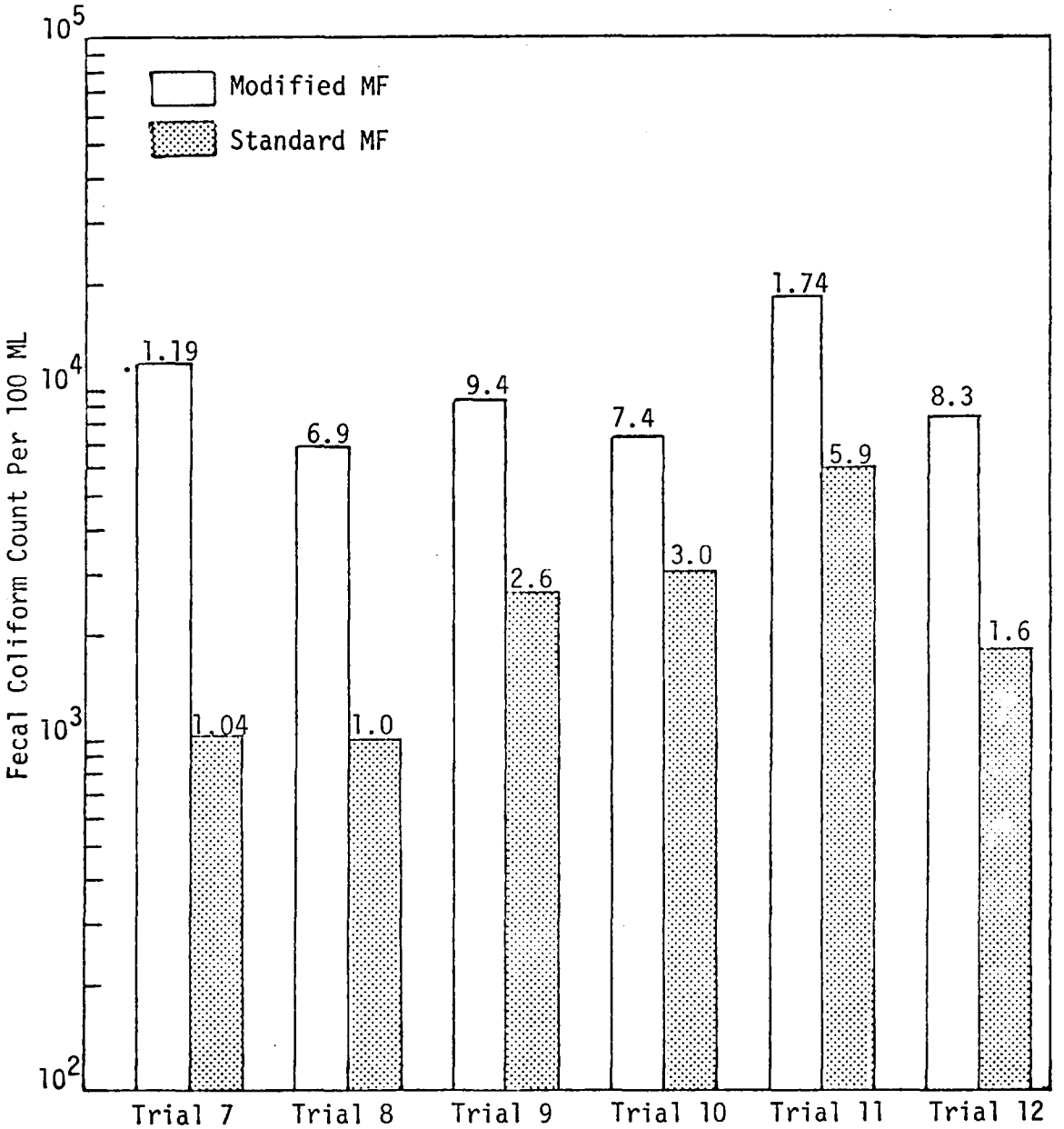

Figure 6. Comparisons of Fecal Coliform Recoveries from Kroger Parking Lot Runoff (Following Several Storm Events) Using the Standard Membrane Filter (MF) Technique and a Modified MF Technique Requiring a Five Hour Preincubation Step.

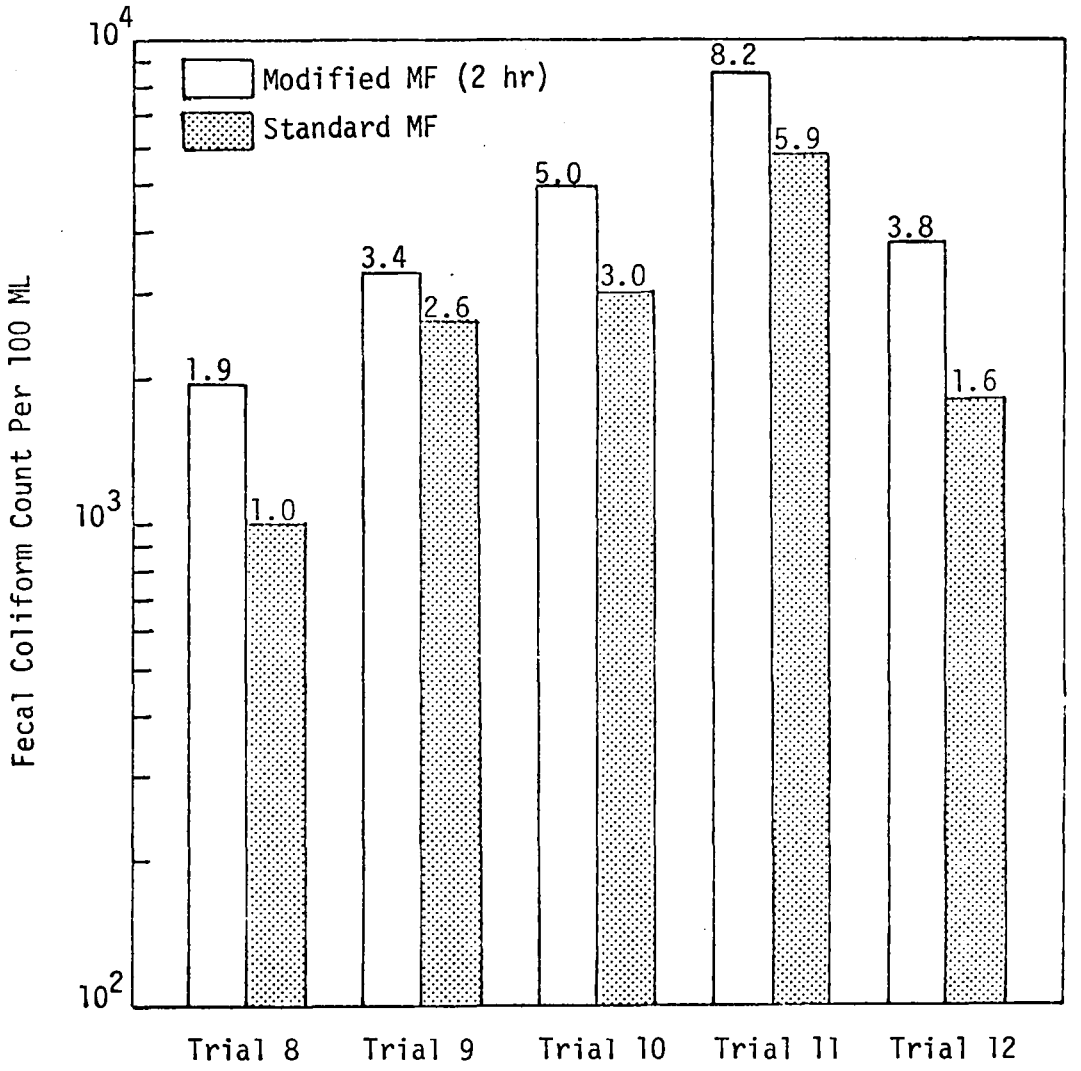

Figure 7. Comparisons of Fecal Coliform Recoveries from Kroger Parking Lot Runoff (Following Several Storm Events) Using the Standard Membrane Filter (MF) Technique and a Modified MF Technique Requiring a Two Hour Preincubation Step.

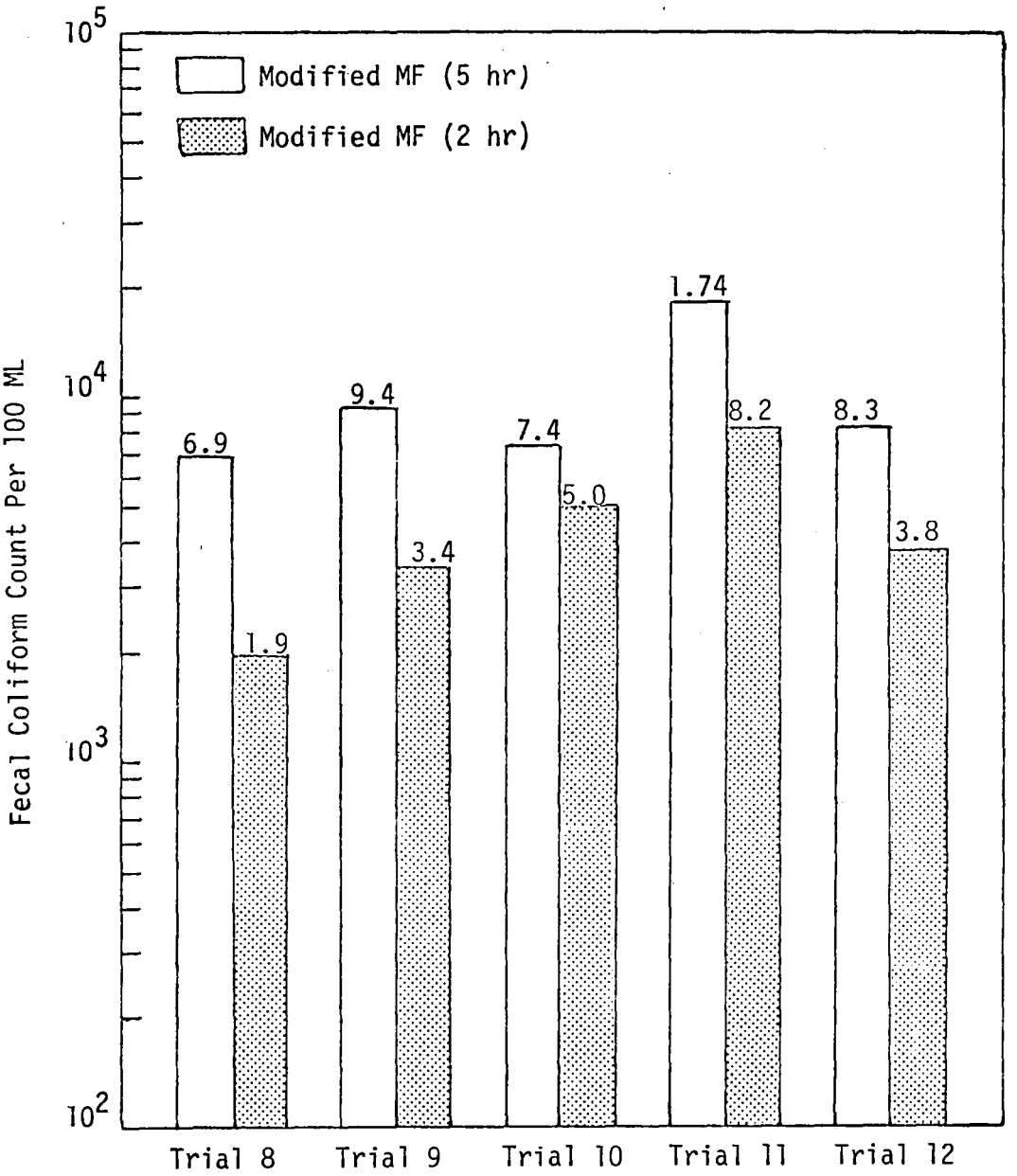

Figure 8. Comparisons of Fecal Coliform Recoveries from Kroger Parking Lot Runoff (Following Several Storm Events) Using the Modified MF Technique Requiring a Two Hour Preincubation Step and a Modified MF Technique Requiring a Five Hour Preincubation Step.

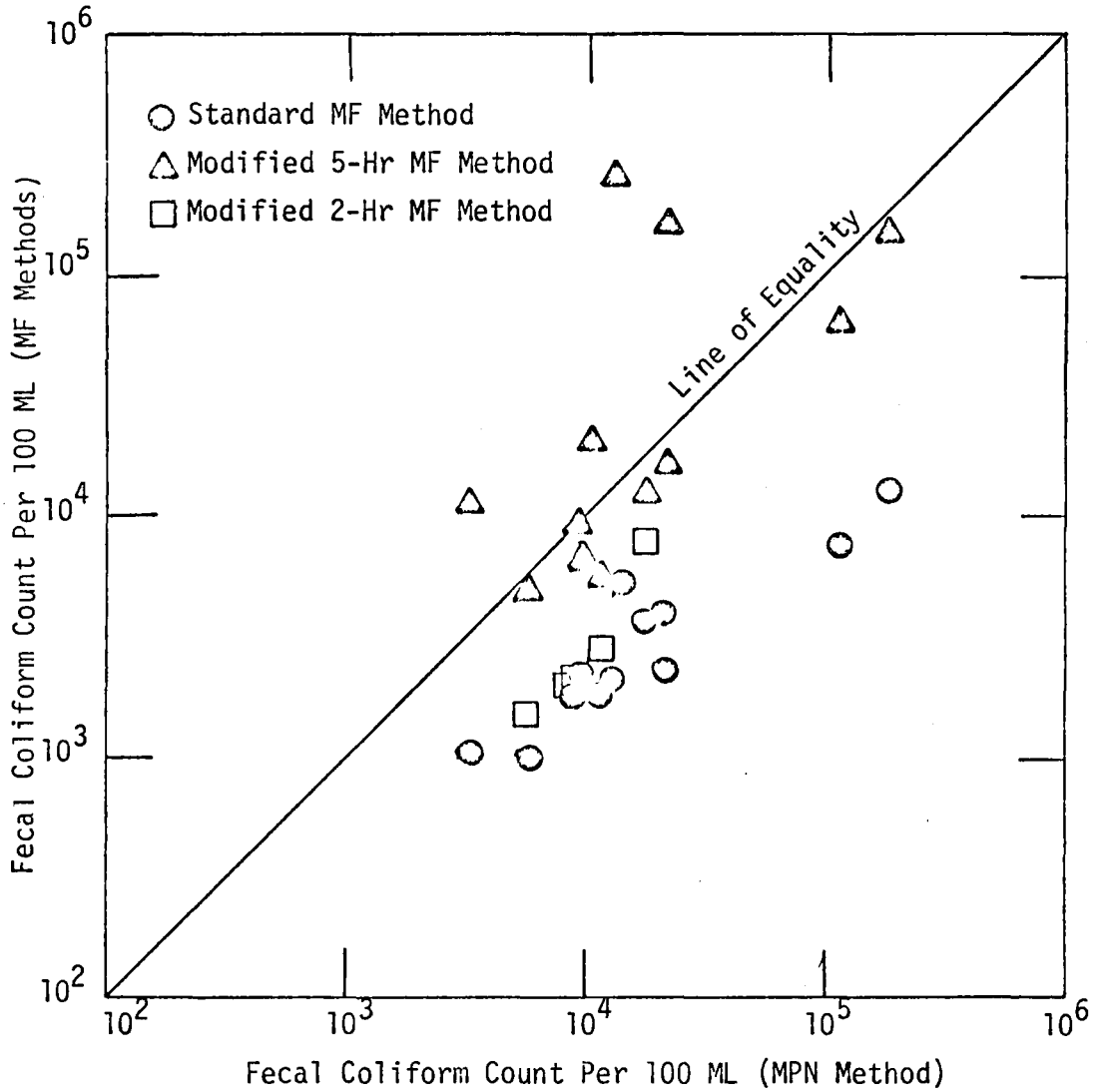

Figure 9. Comparison of Fecal Coliform Recoveries from Kroger Parking Lot Runoff Using Three Membrane Filter Techniques to the MPN Technique.

TABLE V

Log and Percent Differences in Fecal Coliform Recoveries by Various Techniques[a]

Trial Number	MPN[b]		Modified MF(5hr)[c]		Modified MF(2hr)[d]	
	Δlog	Δpercent	Δlog	Δpercent	Δlog	Δpercent
1	1.278[e]	1338[e]	1.263	1148	Not Run	
2	1.279	1150	1.249	843	Not Run	
3	1.265	797	1.248	669	Not Run	
4	1.171	293	1.313	1100	Not Run	
5	1.096	136	1.451	5456	Not Run	
6	1.201	483	1.402	3217	Not Run	
7	1.235	419	1.351	1044	Not Run	
8	1.300	690	1.280	590	1.093	90
9	1.161	254	1.164	262	1.035	31
10	1.181	333	1.112	147	1.063	67
11	1.162	307	1.125	195	1.045	49
12	1.241	488	1.225	419	1.119	138

[a]Using the standard membrane filter concentrations as basis for comparison.

[b]Most probable number technique.

[c]Modified membrane filter technique with 2 ml lactose agar overlay and 5 hour preincubation at 35°C.

[d]Modified membrane filter technique with 2 ml lactose agar overlay and 2 hour preincubation at 35°C.

[e]Positive numbers indicate recoveries greater than those by the standard membrane filter technique.

Statistical analysis of the data. The various analytical techniques for coliform recovery were compared statistically using the paired-t test (84). Appendix Tables B-I through B-VI show the actual comparisons. The results of these comparisons are summarized in Table VI.

Colony verification. One hundred and eighty nine colonies observed on the membranes used in the modified MF technique were verified (Table VII). Of these, 178 (93.7 percent) were confirmed as fecal coliforms. The standard deviation was 7.2 percent.

Heavy Metal Determinations

The concentrations of heavy metals in runoff varied greatly throughout the study, but, in most cases, they were low, rarely greater than 0.5 mg/l. The metal concentrations are presented in Table VIII.

Comparison of Recoveries to the Environmental Conditions

An attempt was made to determine if any correlation existed between the stresses suffered by coliforms present on the parking lot and 1) heavy metal concentrations in the runoff or 2) time between rainfall events. In this analysis, the ratio between the numbers of coliforms observed by the modified and standard MF techniques was used as the indicator of stress.

A plot of the ratio between the two coliform-recovery methods and four of the six metals monitored is shown in Figure 10. Cadmium and chromium were present in essentially insignificant concentrations and,

TABLE VI

Statistical Comparison of the Various Techniques for Fecal Coliform Recovery

Comparison	Number of Samples Compared	Statistical Comparison	Level of Significance
Standard MF with M-5hr	12	M-5hr > Std MF	0.025
Standard MF with M-2hr	5	M-2hr > Std MF	0.01
M-2hr with M-5hr	5	M-5hr > M-2hr	0.005
M-5hr with MPN	12	M-5hr > MPN	0.20
M-2h with MPN	5	MPN > M-2hr	0.01
Standard MF with MPN	12	MPN > Std MF	0.05

TABLE VII

Verification of Fecal Coliform Colonies
From the Modified MF Technique

Trial Number	Colonies Examined	Colonies Verified*	Percent Verification
1	20	20	100
2	20	19	95
3	25	24	96
4	15	15	100
5	15	14	93
6	15	15	100
7	15	13	87
8	12	9	75
9	12	11	92
10	12	11	92
11	12	12	100
12	16	15	94
Total	189	178	Mean 94
			Stnd. Dev. 7.2

*Colonies were subcultured to lauryl tryptose broth (35°C; 48hrs) and then to EC medium (44.5°C; 24hrs) in trials 1-7. Colonies were subcultured directly to EC medium in trials 8-12.

TABLE VIII

Metal Concentrations in Runoff[a] During the Study Period

Trial Number	Concentration (mg/l)					
	Cd	Cr	Cu	Fe	Pb	Zn
1	0.01	0.03	0.28	0.78	0.22	0.37
2	0.01	0.03	0.27	0.28	0.23	0.51
3	0.01	0.01	1.09	0.69	0.63	4.00
4	0.01	0.01	0.02	0.25	0.21	0.26
5	0.01	0	0.03	0.12	0.12	0.25
6	0	0.01	0.02	0.18	0.18	0.19
7	0	0.01	0	0.33	0.15	0.18
8	0.01	0	0.03	1.06	0.50	0.51
9	0	0	0.01	0.14	0.07	0.07
10	0.01	0.01	0	0.16	0.07	0.09
11	0.01	0.01	0.01	0.40	0.15	0.14
12	0.01	0	0.12	1.55	0.60	0.40
Blank #1[b]	0	0	0	0.01	0	0
Blank #2[b]	0	0	0	0.04	0	0.04

[a]Runoff from the Kroger parking lot, Blacksburg, Virginia.

[b]Distilled water plus 5ml concentrated nitric acid carried through the extraction process.

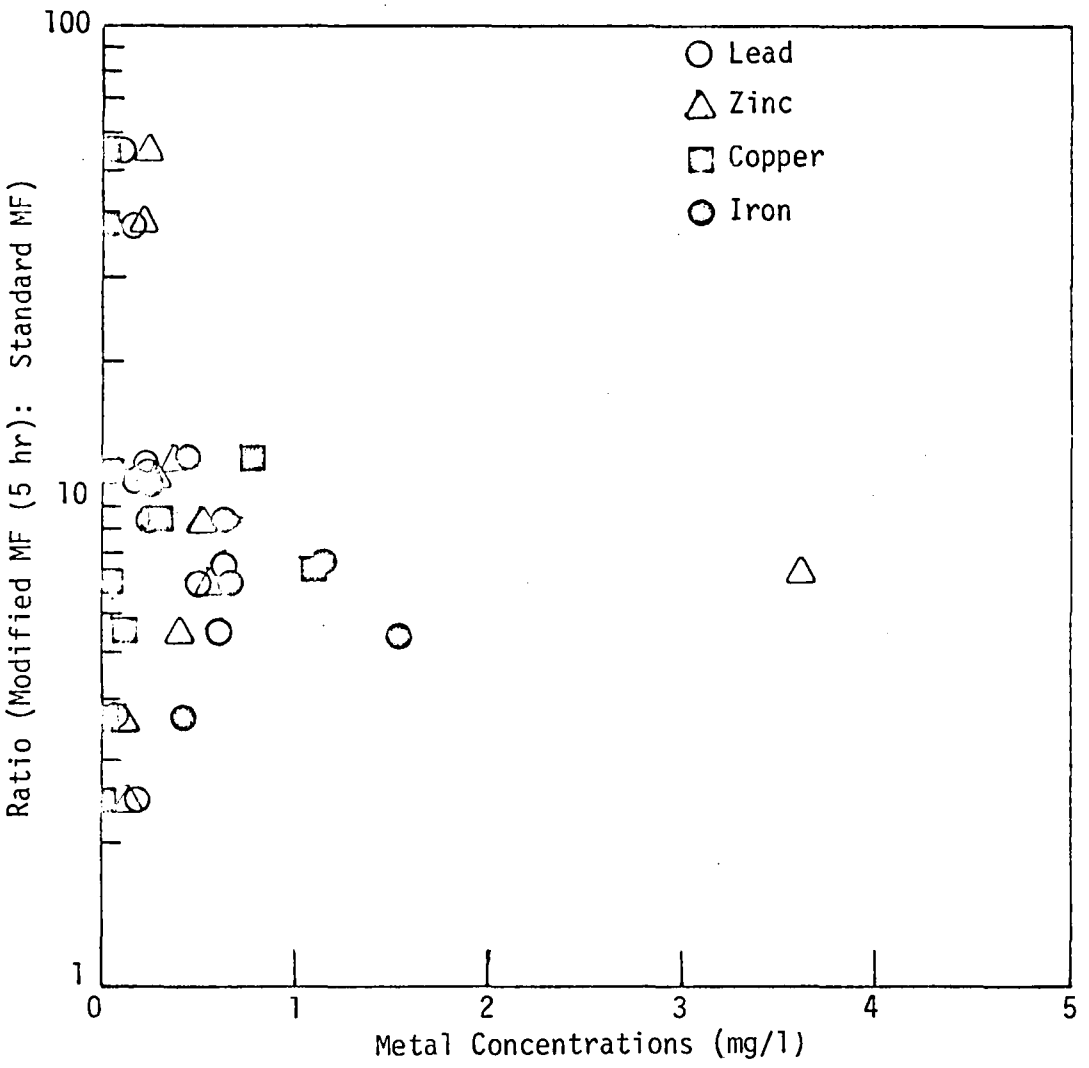

Figure 10. Comparison of the Modified MF (5 hr): Standard MF Fecal Coliform Recoveries to Metal Concentrations in Runoff Following Several Storm Events.

therefore, were not considered. As can be seen, there is no consistent relationship apparent. A comparison of the ratio to the time since the previous rain for the eight storm events is shown in Figure 11. Again, there was no obvious relationship that might be used to explain the variations in the ratio, which has been assumed to be a valid way of expressing the extent of stress the coliforms received prior to their collection and analysis.

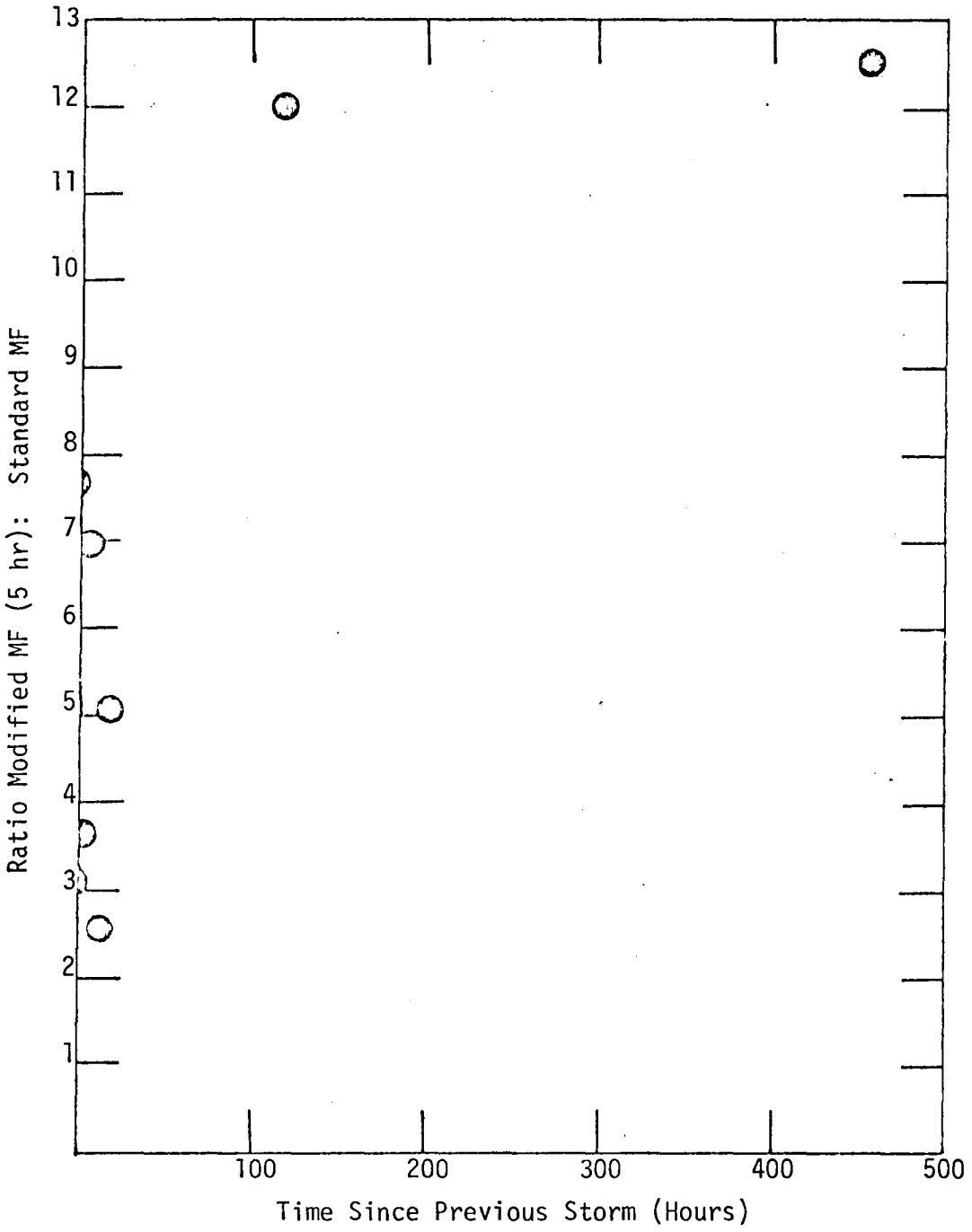

Figure 11. Comparison of the Modified MF (5 hr): Standard MF Fecal Coliform Recoveries to the Time Since the Previous Storm Event of at Least 0.3 Inches.

V. DISCUSSION OF RESULTS

Fecal Coliform Determinations

Recoveries by the various methods. Fecal coliform densities in runoff varied greatly with each rainfall event, as well as in runoff collected at different times during the same event. In each trial, however, the mean fecal coliform count per 100 ml determined with the modified MF (M-2hr) technique was greater than the recovery by the standard MF technique. The mean of all M-2hr trials was significantly greater at the 0.01 level. The ratios of M-2hr to standard MF counts ranged from 1.3:1 to 2.4:1. The average ratio for five samples (Table IV) was 1.8:1, with a standard deviation of 0.4:1. Rose et al. (73), who introduced this modified method in their paper, reported ratios of 1.1:1 to 1.3:1 in runoff. In various other waters, these workers found ratios of up to 38:1. The source and nature of their runoff samples were not specified, so it is difficult to compare their results to those of this study. It is clear, however, that subpopulations of stressed fecal coliforms existed in both their study and this one and that the M-2hr method was of some value in improving the recovery of these organisms. Both the use of an overlay of enriched agar and an acclimation period at 35°C prior to the incubation at 44.5°C appear to provide suitable conditions to permit recovery from stress and subsequent multiplication of injured fecal coliforms.

Lengthening the 35°C preincubation time in this double agar modified method to five hours (M-5hr) resulted in even higher recoveries. The mean population observed by the M-5hr method was statistically

greater at the 0.025 level than that observed when the standard MF technique was used. Ratios of recoveries by the M-5hr method to those by the standard MF method (Table III) ranged from 2.5:1 to 55.6:1. The average ratio for 12 samples was 13.6:1 but the standard deviation of 15.6:1 was quite high. No other reports have been located in which this M-5hr method has been studied. Green et al. (78) proposed and evaluated a method employing a five hour preincubation at 35°C. They found this step to be valuable in recovering stressed fecal coliforms. However, those investigators did not use the two-layered agar; standard M-FC agar was used instead. The present study has combined the favorable aspects of a nutrient-rich agar overlay and a five-hour preincubation period. The resulting M-5hr method yielded a mean fecal coliform count statistically greater than that of the M-2hr method at the 0.005 level, indicating the worth of the longer preincubation period.

It should be noted that there was a tremendous range of ratios when the M-5hr method was compared to the standard MF method (2.5:1 up to 55.1:1). This variation appears to indicate that the stress conditions were much greater at certain times, yielding the higher ratios.

One factor associated with the length of this preincubation that possibly can complicate enumeration of fecal coliforms is the growth of other types of organisms. Green and her associates (78) found that preincubation periods exceeding five hours resulted in excessive background growth and spreading. In the study reported in this thesis,

an increase in non-fecal-coliform organisms was noted when both the modified MF methods were evaluated. In a few instances, the background growth actually interferred with the enumeration of the fecal coliforms, and this problem may prove to be one of the factors that will limit the use of the M-5hr method in waters containing high densities of other bacteria. Green and her associates also noted that preincubation periods exceeding five hours caused a significant decrease in the number of colonies identified as coliforms that actually confirmed to be coliforms when transferred to lauryl tryptose broth.

In seven out of twelve instances the MPN technique yielded the greatest mean coliform concentration when compared to the other methods. In the other five trials, the M-5hr technique gave the greatest recoveries. When the 95 percent confidence range of the statistically-based MPN technique is considered in this comparison, however, (Appendix Table A-I), these two techniques compare relatively well. Only on two trials (numbered five and six), when the M-5hr data seemed to be unusually high, did the M-5hr data fall out of the 95 percent confidence interval of the MPN. The reason for the excessively high recoveries by the M-5hr technique during these two trials is not known. The M-2hr recoveries were within the MPN range in four out of five trials.

The recoveries by the standard MF technique were outside of the MPN 95 percent confidence interval in all trials. Because the MPN method is based on probability statistics, and because estimates of bacterial concentration by this method are known to vary as much as one order of magnitude on identical samples (78), a direct-count membrane filter

technique that would give more accurate estimates of coliform densities is highly desirable. The results of this study showed at no less than a 97.5 percent level of significance that the standard MF technique does not permit an accurate estimation of the coliform density when the nature of the environment has generated stressed subpopulations. Furthermore, this study has shown that the M-5hr method can be a valuable tool for obtaining a better indication of water quality under such conditions.

Statistical analysis. As previously mentioned, the paired-t test was used to compare the data obtained by the various methods. This approach, rather than the group-t test, was chosen because there were great variations among the coliform populations recovered by any one method during the twelve analyses. The paired-t test provides also for a reduction in variance over that obtained when the group-comparison method is used. Had the number of trials involving each method been the same, a randomized-block statistical design could have been employed.

The M-2hr technique was not introduced as a method for evaluation in this study until midway through the project. Consequently, there were only five instances where this method was evaluated. The possibility that the Duncan's Multiple Range Test could be used to analyze the data was also investigated, but this technique also would have been inappropriate because there was an unequal number of trials and the variation between the data pairs was large. It should be noted that the statistical analysis involved a comparison of means derived from a set of replicate plates (three or four replicates in each case). In all

cases, the means for each data pair were derived from the same number of replicates.

Because Presswood and Strong (83) suggested that the elimination of rosolic acid from the M-FC medium allows for higher fecal coliform recoveries, two trials during this study incorporated a comparison of fecal coliforms observed on this medium prepared with and without the inhibitor. As stated earlier, the recoveries obtained with this medium did not appear to be very different from the recoveries with the standard MF procedure. Recoveries in both instances were slightly higher when the medium without rosolic acid was used, and elimination of this substance, which was originally suggested as an addition to M-FC agar to aid in the inhibition of non-fecal-coliform organisms, may have merit. However, not enough experiments were performed during this study to confirm that eliminating rosolic acid offers any advantage. Presswood also stated that when rosolic acid is eliminated, intensely blue colonies develop that are easier to identify than those that develop on agar that includes it. Furthermore, deletion of rosolic acid would eliminate another possible source of stress to the fecal coliforms.

Plates prepared as blanks showed the use of UV light and distilled rinse water to be quite effective in removing any coliform organisms left on the filter apparatus between filtrations. Therefore, no errors in measured coliform densities were introduced by the filtration procedures. Twenty-eight blanks were prepared and all exhibited either one coliform colony or none at all after incubation at the prescribed temperature.

Colony verification. The 94 percent verification of the blue-colored colonies as fecal coliforms by the modified MF technique indicated that use of the 35°C preincubation period and the lactose overlay does not increase the likelihood that coliforms will be falsely identified more often than would be expected when the standard MF technique is employed. In trials 1-7, the colonies were confirmed by subculturing them into lauryl tryptose broth (35°C; 48 hrs) and then to EC medium (44.5°C; 24 hrs) as reported by Rose et al. (73).

In trials 8-12, colonies were confirmed by direct transfer to the EC medium. Omission of the lauryl tryptose step did not appear to lower the percent of verification. This seems logical, because the modified MF techniques have a preinrichment step built into them, precluding the need for the lauryl tryptose. It appears from this study that further evaluations of these modified MF methods could incorporate this verification by inoculating directly into EC-medium and thus save considerable time and expense.

Heavy Metal Determinations

As can be seen in Table VII, the heavy metal concentrations varied considerably from one sample to the next and, generally, were relatively low. Several factors could account for the variations. The length of the dry period during which the metals could accumulate, the rainfall intensity (its flushing ability), and differential traffic densities are all factors. Several observations can be made concerning the metals determined.

Cadmium and chromium were found to occur in the runoff waters in very low concentrations. In nine of the trials, the cadmium level was 0.01 mg/l, and in the other three none was detected. Chromium concentrations ranged from 0 to 0.03 mg/l.

Copper concentrations ranged from 0 to 1.09 mg/l. The high concentration was found in trial number three. Several other metal concentrations were uncharacteristically high for this sample also. The possible explanation for this is the fact that the sample was collected from a small stream of water along the storm curbing that persisted several hours after the rainfall had ended. This sample was highly turbid, and the high metal concentrations are probably the result of their adsorption to the particles in the water. Copper concentrations were below 0.15 mg/l in nine of the twelve trials.

Iron concentrations varied from 0.12 mg/l to 1.55 mg/l. Seven iron concentrations were 0.25 mg/l or greater. In general, iron was present in the greatest concentrations as compared with the other metals.

Lead concentrations varied from 0.07 mg/l in two trials to 0.63 mg/l. Nine of the twelve samples had lead concentrations below 0.25 mg/l.

Zinc was found in concentrations between 0.15 mg/l and 0.5 mg/l. Two samples taken during a storm that occurred after a short dry period contained zinc in concentrations less than 0.1 mg/l. An uncharacteristically high concentration of 4.0 mg/l zinc was found in the sample collected several hours after the rainfall had ended. The fact that

iron, lead, and zinc were the most prevalent metals agrees with previous reports (66,68). The lead concentrations found in this study were in relatively close agreement with the 0.23 mg/l theoretical value developed by Newton et al. (70) in their study of lead pollution in street runoff. In the future, lead concentrations could tend to decrease due to the increased use of unleaded gasoline in automobiles equipped with catolytic converters.

The metal concentrations determined in this study were representative of the amounts which can build up on the parking lot during the late spring and summer months in Blacksburg. The increased student population in the fall and winter could likely result in greater metal concentrations in the runoff waters.

Comparison of Recoveries to the Environmental Conditions

No correlations with coliform stress could be made with either metal concentrations in the runoff or the time between rainfall events. When the ratio between populations estimated by the M-5hr and standard MF techniques was plotted against the heavy metal concentrations (Figure 10), no correlation could be seen. Ratios varied greatly over a relatively narrow range of metal concentrations. Evidently, the heavy metals were not the main source of the stress in these samples, although there likely would be some correlation if metal concentrations were higher.

As can be seen in Figure 11, there also was no clearly defined relationship between the stressed condition and the time elapsed since the previous rain even though the two largest ratios did occur when the

interval between rainfall events was the greatest. It does seem logical, however, that the longer the organisms are exposed to the environmental conditions causing stress, the greater the stressed subpopulation would be.

It is probable that the stressed fecal coliform subpopulation in urban runoff results largely from other environmental conditions not monitored in this study. The effects of temperature and exposure to UV light, dehydration, and the lack of food probably should be considered. Stressed subpopulations likely result from a combination of numerous environmental conditions and their interactions and while further research is warranted, these conditions would be extremely difficult to monitor.

If enough environmental conditions causing stress affect a water source, a potentially-dangerous water supply could be regarded as safe if the standard MF technique is used to evaluate it. Because it has been demonstrated in this study and others (73,78) that a substantial portion of the fecal coliform population in certain types of waters may be injured, it is evident that a modified technique, such as the M-5hr method, is needed to recover these organisms and, thus, permit effective evaluation of the water quality. Further work should be carried out to determine which factors should be given major consideration in deciding if a modified procedure is needed to evaluate a particular water source. As suggested by Rose et al. (73), the decision to use the slightly more involved two-layered medium method in preference to the standard MF method should be based on a demonstration of increased verified

recoveries of fecal coliforms in the samples routinely examined. A modified technique, such as the M-5hr method, could prove to be valuable because a major interest in the fecal coliform test is related to the bacterial quality assessment of effluent waters under the National Pollution Discharge Elimination System. Should it be decided that a modified MF method is valuable in certain assessments, the use of a temperature-programmed incubator that automatically makes the temperature change from 35 to 44.5^{o}C after a prescribed preincubation period would be a helpful addition to the technique.

VI. SUMMARY AND CONCLUSIONS

Urban runoff samples collected from a parking lot in Blacksburg, Virginia were analyzed by several techniques for fecal coliform recovery. The samples also were analyzed for heavy metal concentrations. The results of this study indicated that a substantial percentage of the fecal coliform population found in urban runoff may be injured by a host of environmental factors and are not recovered adequately by the standard membrane filter technique (MF). Based on the results of this study, the following conclusions are warranted regarding the analysis of fecal coliforms in stormwater runoff from a parking lot:

1. A modification of the standard MF method, employing a thin lactose agar layer over the M-FC agar and a preincubation period at 35°C permits recoveries of fecal coliforms that are consistently greater than those observed when the standard MF technique is used. The means of three replicates were significantly greater than those obtained by the standard MF technique in five experiments when a two-hour preincubation period was used (0.01 level of significance) and also in twelve experiments when a five-hour preincubation period was used (0.025 level of significance). The means for this comparison were obtained from either three or four replicates, depending on the trial number.
2. Increasing the incubation in the modified method from two to five hours (M-5hr) resulted in even greater recoveries of the stressed fecal coliforms (0.005 level of significance in five experiments of three replicates in each trial). It was also noted that the

range of differences between the modified and standard MF techniques was highly varied.

3. The standard MPN procedure permitted recoveries consistently greater than those observed when the standard MF and M-2hr techniques were used. The MPN means were significantly greater than those obtained by the standard MF in twelve experiments (0.05 level of significance) and significantly greater than those obtained by the M-2hr method in five experiments (0.01 level of significance). The M-5hr technique permitted recoveries significantly greater than those obtained by the MPN technique but the level of significance was only 0.2 in twelve experiments. Practically speaking, the 0.2 level is not a good enough justification to declare the M-5hr method superior to the MPN technique.
4. The use of a lactose agar overlay and a 35^{o}C preincubation period can result in substantial growth of non-fecal-coliform organisms that can make enumeration of these indicator bacteria more difficult. This conclusion agrees with statements by others (73,78).
5. No correlation could be made in this study when comparing heavy metal concentrations in the runoff waters and the elapsed time between rainfalls with the degree of stress.

VII. RECOMMENDATIONS

1. Investigations should be made to determine what environmental factors cause the stressed fecal coliform subpopulations in urban runoff. Such things as extreme temperatures, pH, the quantities of UV light received over a period of time, and the levels of pesticides could be examined. The significance of heavy metals and length of antecedent dry period should be further investigated.
2. The possibility of developing a predictive model for stressed fecal coliforms should be studied following the above investigations.
3. An investigation could be made to determine the identity of the non-fecal-coliform organisms that grow in the modified membrane filter methods.
4. Additional work should be done to determine the effect rosolic acid addition to the M-FC medium has on fecal coliform recoveries and whether or not it is actually needed.

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APPENDIX A

FECAL COLIFORM DETERMINATIONS BY VARIOUS TECHNIQUES

APPENDIX TABLE A-I

MPN Determinations

Trial Number	Positive Presumptive Tubes (of 5) Dilution 1:1	1:10	1:100	1:1000	1:10,000	Positive Confirmed Tubes (of 5) Dilution 1:1	1:10	1:100	1:1000	1:10,000	MPN (fc/100 ml) Range[a]	Average
1	NR[b]	NR	5	4	2	NR	NR	5	4	2	57,000-700,000	220,000
2	NR	NR	5	3	2	NR	NR	5	3	1	31,000-250,000	110,000
3	5	5	5	2	NR	NR	5	5	1	NR	12,000-100,000	35,000
4	NR	5	3	1	NR	NR	5	3	1	NR	3100- 25,000	11,000
5	NR	5	4	2	NR	NR	5	4	1	NR	4300- 49,000	17,000
6	NR	5	5	1	NR	NR	5	5	1	NR	12,000-100,000	35,000
7	5	5	2	NR	NR	5	5	2	NR	NR	1800- 14,000	54,000
8	NR	5	3	0	0	NR	5	3	NR	NR	2500- 19,000	7,900
9	5	5	3	0	NR	5	5	3	NR	NR	3000- 32,000	9,200
10	NR	5	5	0	NR	NR	5	4	NR	NR	3500- 30,000	13,000
11	NR	5	5	0	NR	NR	5	5	NR	NR	6800- 75,000	24,000
12	5	5	3	2	NR	NR	5	2	2	NR	2800- 22,000	9,400

[a]95% confidence interval.

[b]Not run.

APPENDIX TABLE A-II

Standard MF Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml Mean	Colonies Per 100 ml Std. Deviation
1	1:10	TNTC*	--	--
	1:100	156; 144; 151; 162	15,300	763
	1:1000	15; 13; 16; 15	--	--
2	1:10	TNTC	--	--
	1:100	94; 81; 95; 83	8800	727
	1:1000	5; 8; 6; 9	--	--
3	1:10	TNTC	--	--
	1:100	38; 35; 40; 42	3900	299
	1:1000	3; 5; 2; 2	--	--
4	1:100	27; 24; 29; 33	2800	377
	1:1000	4; 1; 3; 1	--	--
	1:10,000	0; 0; 0; 1	--	--
5	1:100	66; 89; 53; 78	7200	155
	1:1000	5; 5; 3; 3	--	--
	1:10,000	1; 0; 1; 0	--	--
6	1:100	74; 61; 44; 63	6000	1240
	1:1000	4; 6; 8; 3	--	--
	1:10,000	0; 0; 0; 0	--	--
7	1:10	115; 110; 95; 95	1040	103
	1:100	7; 14; 11; 17	--	--
	1:1000	1; 1; 0; 0	--	--
8	1:10	TNTC	--	--
	1:100	10; 11; 10	1000	58
	1:1000	1; 0; 0	--	--
	1:10,000	0; 0; 0	--	--

*Too numerous to count

APPENDIX TABLE A-II (con't)

Standard MF Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml Mean	Std. Deviation
9	1:10	TNTC	--	--
	1:100	25; 31; 23	2600	416
	1:1000	1; 2; 1	--	--
10	1:10	TNTC	--	--
	1:100	35; 26; 30	3000	451
	1:1000	4; 2; 4	--	--
11	1:10	TNTC	--	--
	1:100	58; 59; 59	5900	58
	1:1000	11; 10; 8	--	--
12	1:10	TNTC	--	--
	1:100	15; 19; 14	1600	265
	1:1000	0; 0; 1	--	--

*Too numerous to count.

APPENDIX TABLE A-III

Modified MF (5 Hr 35°C Preincubation) Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml Mean	Colonies Per 100 ml Std. Deviation
1	1:10	TNTC*	--	--
	1:100	TNTC	--	--
	1:1000	187; 192; 188; 195	191,000	3697
2	1:10	TNTC	--	--
	1:100	TNTC	--	--
	1:1000	82; 87; 79; 83	83,000	3304
3	1:10	TNTC	--	--
	1:100	TNTC	--	--
	1:1000	23; 30; 30; 35	30,000	4933
4	1:100	320; 331; 350; 344	33,600	1343
	1:1000	3; 7; 8; 10	--	--
	1:10,000	2; 1; 0; 0	--	--
5	1:100	TNTC	--	--
	1:1000	TNTC	--	--
	1:10,000	43; 46; 41; 29	298,000	74,554
6	1:100	TNTC	--	--
	1:1000	132; 166; 255; 243	199,000	59,581
	1:10,000	0; 1; 14; 6	--	--
7	1:10	TNTC	--	--
	1:100	112; 123; 125; 115	11,900	624
	1:1000	0; 4; 3; 0	--	--
8	1:10	TNTC	--	--
	1:100	71; 72; 65	6900	379
	1:1000	2; 1; 2	--	--
	1:10,000	0; 0; 0	--	--

*Too numerous to count.

APPENDIX TABLE A-III (con't)

Modified MF (5 Hr 35°C Preincubation) Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml Mean	Colonies Per 100 ml Std. Deviation
9	1:10	TNTC	--	--
	1:100	93; 100; 89	9400	557
	1:1000	5; 4; 3	--	--
10	1:10	TNTC	--	--
	1:100	63; 93; 65	7400	1677
	1:1000	5; 7; 5	--	--
11	1:10	TNTC	--	--
	1:100	188; 177; 156	17,400	1626
	1:1000	10; 9; 10	--	--
12	1:10	TNTC	--	--
	1:100	88; 62; 99	8300	1900
	1:1000	1; 3; 4	--	--

*Too numerous to count.

APPENDIX TABLE A-VI

Modified MF (2 Hr 35°C Preincubation) Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml Mean	Colonies Per 100 ml Std. Deviation
8	1:10	TNTC*	--	--
	1:100	19; 24; 15	1900	451
	1:1000	0; 2; 1	--	--
	1:10,000	0; 0; 0	--	--
9	1:10	TNTC	--	--
	1:100	29; 36; 36	3400	404
	1:1000	1; 1; 2	--	--
10	1:10	TNTC	--	--
	1:100	46; 49; 54	5000	404
	1:1000	7; 6; 5	--	--
11	1:10	TNTC	--	--
	1:100	82; 87; 94	8800	603
	1:1000	7; 6; 8	--	--
12	1:10	TNTC	--	--
	1:100	38; 32; 45	3800	651
	1:1000	2; 1; 1	--	--

*Too numerous to count.

APPENDIX TABLE A-V

Standard MF Without Rosolic Acid Plate Counts

Trial Number	Dilution	Counts in Replicate Plates	Colonies Per 100 ml	
			Mean	Std. Deviation
11	1:10	TNTC*	--	--
	1:100	84; 62; 65	7000	3208
	1:1000	12; 11; 11	--	--
12	1:10	TNTC	--	--
	1:100	22; 13; 15	1700	404
	1:1000	0; 0; 0	--	--

*Too numerous to count.

APPENDIX B

STATISTICAL ANALYSIS

APPENDIX TABLE B-I

Statistical Comparison of the Standard MF and the Modified M-5hr Techniques

Standard MF Count*	MPN Count*	Difference
153	1910	1757
88	830	742
39	300	261
28	336	308
72	4000	3928
60	1990	1930
10.4	119	108.6
10	69	59
26	94	68
30	74	44
59	174	115
16	83	67
ΣY 591.4	9979	9387.6

$\Sigma D^2 = 22,994,231$

$\overline{D} = \Sigma D/b = 9387.6 / 12 = 782.3$

$$S_D = \sqrt{\frac{\Sigma D^2 - (\Sigma D)^2/b}{b - 1}} = \sqrt{\frac{22,994,231 - 7,343,919}{11}} = 1193$$

$$S_{\overline{D}} = \frac{S_D}{b} = \frac{1193}{12} = 345$$

We assume the true differences between the means of the two groups, $\mu_1 - \mu_2$, equals zero.

$$t_s = \frac{\overline{D} - 0}{S_{\overline{D}}} = \frac{782.3}{345} = 2.27$$

$t_{.025}(11) = 2.201$

$t_{.01}(11) = 2.718$

Means significantly different at $0.025 > P > 0.01$

*Mean fecal coliform count per 100 ml reduced by a factor of 100 to simplify calculations (statistics not altered).

APPENDIX TABLE B-II

Statistical Comparison of the Standard MF and the Modified M-2hr Techniques

Standard MF Count*	M-2hr Count*	Difference
10	19	9
26	34	8
30	50	20
59	88	29
16	38	22
ΣY 141	229	88

$\Sigma D^2 = 1870$

$\overline{D} = 17.6$

$S_D = 8.96$

$S_{\overline{D}} = 4.0$

$t_s = 4.4$

$t_{.01}(4) = 3.747$

$t_{.005}(4) = 4.604$

Means significantly different at $0.01 > P > 0.005$

*Mean fecal coliform count per 100 ml reduced by a factor of 100.

APPENDIX TABLE B-III

Statistical Comparison of the M-2hr and M-5hr Techniques

M-2hr Count*	M-5hr Count*	Difference
19	69	50
34	94	60
50	74	24
88	174	86
38	83	45
ΣY 229	494	265

$\Sigma D^2 = 16{,}097$

$\overline{D} = 53$

$S_D = 22.7$

$S_{\overline{D}} = 10.1$

$t_s = 5.25$

$t_{.005}(4) = 4.604$

$t_{.0005}(4) = 8.610$

Means significantly different at $0.005 > P > 0.0005$

*Mean fecal coliform count per 100 ml reduced by a factor of 100.

APPENDIX TABLE B-IV

Statistical Comparison of the MPN and M-5hr Techniques

MPN Count*	M-5hr Count*	Difference
2200	1910	-290
1100	830	-270
350	300	-50
110	336	226
170	4000	3830
350	1990	1640
54	119	65
79	69	-10
92	94	2
130	74	-56
240	174	-66
94	83	-11
ΣY 4969	9979	5010

$\Sigma D^2 = 17,581,018$

$\overline{D} = 417.5$
$S_D = 1186.6$

$S_{\overline{D}} = 342.9$

$t_s = 1.22$

$t.2(11) = 0.876$
$t.1(11) = 1.363$

Means significantly different at $0.2 > P > 0.1$

*Mean fecal coliform counts per 100 ml reduced by a factor of 100.

APPENDIX TABLE B-V

Statistical Comparison of the M-2hr and MPN Techniques

M-2hr Count*	MPN Count*	Difference
19	79	60
34	92	58
50	130	80
88	240	152
38	94	56
ΣY 229	635	406

$\Sigma D^2 = 39,604$

$\overline{D} = 81.2$
$S_D = 40.7$

$S_{\overline{D}} = 18.2$

$t_s = 4.46$

$t.01(4) = 3.747$
$t.005(4) = 4.604$

Means significantly different at $0.01 > P > 0.005$.

*Mean fecal coliform counts per 100 ml reduced by a factor of 100.

APPENDIX TABLE B-VI

Statistical Comparison of the Standard MF and MPN Techniques

Standard MF Count*	MPN Count*	Difference
153	2200	2047
88	1100	1012
39	350	311
28	110	82
72	170	98
60	350	290
10.4	54	43.6
10	79	69
25	92	66
30	130	100
59	240	181
16	94	78
ΣY 591.4	4969	4377.6

$\Sigma D^2 = 5,471,365$

$\overline{D} = 364.8$
$S_D = 593.5$

$S_{\overline{D}} = 171.5$

$t_s = 2.13$

$t.05(11) = 1.796$
$t.025(11) = 2.201$

Means significantly different at $0.05 > P > 0.025$

*Mean fecal coliform counts per 100 ml reduced by a factor of 100.

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A STUDY OF A MODIFIED MEMBRANE FILTER TECHNIQUE FOR THE ENUMERATION OF STRESSED FECAL COLIFORMS IN URBAN RUNOFF

by

Edward Ryland Brown, Jr.

(ABSTRACT)

Urban runoff samples collected from a parking lot in Blacksburg, Virginia were analyzed by several techniques for fecal coliform recovery. The samples were also analyzed for heavy metal concentrations.

Statistical analyses of data by the paired-t test showed that the standard membrane filter technique (MF) yielded significantly lower recoveries (0.05 level) than the standard MPN procedure. A modified membrane filter technique employing a two-layer agar and a two hour 35^{o}C preincubation period (M-2hr) was found to yield recoveries consistently greater (0.01 level) than the standard MF technique. Increasing the preincubation period to five hours in this modified method (M-5hr) resulted in recoveries that were even greater (0.005 level). The recoveries of the M-5hr method were found to closely approximate the MPN recoveries in most cases.

It was concluded that a substantial percentage of the fecal coliform population found in urban runoff may be injured by a host of environmental factors, though no correlations could be made with metal concentrations or elapsed time between rainfalls. The M-5hr method was

concluded to be quite useful in the recovery of these stressed organisms, though growth of non-fecal-coliforms was increased and made enumeration of the fecal coliforms more difficult.