Introduction to Environmental Microbiology

Manual to
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Coliform Bacteria

Bacteria are classified into four major groups according to their feeding habits.

1. *Saprophytic* — these feed on dead **organic** matter and are commonly called "decomposers." Saprophytic bacteria play a significant role in water polluted with biodegradable wastes because they use up great amounts of oxygen as they break down the wastes.

Organic wastes all contain carbon. Anything that is biodegradable (once part of a living thing is organic.

2. *Commensal* — Commensal bacteria take food from a living organism but in the process provide a valuable service to that organism. For example, bacteria present in the intestines of humans assist in the digestion of many foods. These bacteria couldn't live without us and, indeed, we would have difficulty digesting our food without them!

3. *Parasitic* — Parasites live off another organism at the expense of its health. All **pathogenic** bacteria fall into this category.

Pathogenic organisms are those which "cause disease."

4. *Autotrophic* — These bacteria make their own food from simpler **inorganic** substances. Some autotrophic bacteria contain chlorophyll and can carry on photosynthesis.

Inorganic compounds do not contain carbon.

Autotrophic bacteria live in water or soil containing iron and sulfate compounds. The end products produced by the bacteria lower the pH (make it more acid) of water and soil. This may harm some plants and animals. Because potatoes like an acid soil, potato growers often add sulfur to their soil so sulfur bacteria (autotrophs) will lower its pH.

Pathogenic bacteria are our greatest concern because of the health problems they pose. Unfortunately, pathogens are hard to detect in water because: 1. There usually aren't very many of them, and 2. They can't survive for very long outside the warm confines of the human or animal body. Nevertheless, if pathogens get into a water supply from animal wastes or through the release of unprocessed toilet wastes, they may live long enough to find and infect a human or animal.

Even if we could test easily for the presence of pathogens in water it would not be a good idea because we might get sick from exposure to the harmful bacteria. For this reason, we test for the presence of a relatively harmless (commensal) form of bacteria, called **coliform** bacteria which, like pathogens, can live in the human body.

Coliform: a relatively harmless bacteria found in the human intestine (colon).

Coliform bacteria are common in the intestines of both warm- and cold-blooded animals and aid in the digestion of foods. When animals "relieve themselves," some of these coliform bacteria (and pathogenic **microbes,** too, if present) pass out of their bodies with the waste. If these wastes find their way into a water supply, they will bring the bacteria and other microbes with them.

Microbes are too small to be seen without a microscope. They include bacteria, algae and tiny animals called *protozoa*.

Consequently, if we find coliform bacteria in a water sample we can presume there also is human or animal **excrement** — and in all probability, pathogens — there, too. Large numbers of coliform organisms therefore indicate the possible presence of pathogens.

Excrement is body waste. What goes down the toilet is "excrement."

As previously mentioned, coliform bacteria are found in the intestines of warm and cold-blooded animals. However, cold-blooded animals don't carry the same diseases as warm-blooded ones. For this reason, the **APHA** requires a special *fecal* test be used to separate out those bacteria found in the gut of warm-blooded animals.

APHA is the American Public Health Association.

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The *fecal* test is easy to do but requires precise incubation temperatures — 44.5 degrees Celsius and not varying more than 0.2 degrees Celsius either way. Consequently, we'll use the less-temperature dependent *total* test to determine the presence of coliform bacteria in our experiments. The specifics of the Total Coliform Test are explained in *Experiment 5*, which you may obtain from your teacher.

Coliform Standards

EPA is the U.S. Environmental Protection Agency.

Coliform standards for drinking water are given below. They are the same for all the states and are administered by the **EPA.** Standards for swimming waters and other recreational uses generally are set by the states and are close to the limits given below:

For drinking water: No more than one *total* coliform per 100 milliliters (mL) of water tested.

For swimming pools: Same as drinking water — no more than one total coliform per 100 mL of water tested. (This is the standard for swimming pools in Colorado and is similar to the standard for many other states.)

For "primary contact" waters (swimming beaches): An average of no more than 200 *fecal* coliforms per 100 mL. (Some states use 1000 *total* coliforms per 100 mL sample.)

For boating and general enjoyment (not swimming): The standard usually used is the one recommended by the Committee on Water Quality Criteria for the U.S. Secretary of the Interior — an average of no more than 2000 *fecal* coliforms per 100 mL of water tested. (*Total* coliform numbers will be *much* higher.)

The above standards are taken from Colorado's "Water Quality Standards" and *Water Pollution Microbiology.** Use them to interpret your coliform bacteria findings according to the procedures outlined in *Experiment 5.*

A weak positive relationship exists between the number of total (or fecal) coliform bacteria in a swimming area and your chances of getting sick. In fresh water, 2000 to 3000 total coliform bacteria per 100 mL water may increase your chances of becoming ill. However, most of the illnesses among swimmers are diseases of the eye, ear, nose and throat and are *not* the result of coliform bacteria or intestinal **pathogens.** This information suggests that we need better tests than the total coliform and fecal coliform tests when we check swimming pools and bathing beaches!

Pathogens are organisms which cause disease.

Is it safer to swim in sewage-infested salt water than in sewage-infested fresh water? Evidently, yes. The **USPHS** discovered that bathing in sewage-polluted sea water carries only a negligible risk to health, even when measured coliform counts are over 10,000 per 100 mL water sample. Possibly the salt concentration of sea water kills many of the harmful bacteria. Also, oceans are big places — they quickly dilute the sewage to harmless levels. Your chances of encountering pathogens even in relatively crowded ocean waters are remote.

USPHS is the United States Public Health Service.

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Injured Coliforms in Drinking Water

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Coliforms were enumerated by using m-Endo agar LES and m-T7 agar in 102 routine samples of drinking water from three New England community water systems to investigate the occurrence and significance of injured coliforms. Samples included water collected immediately after conventional treatment, during the backwash cycle, at various points in the distribution system, and 1 week after the break and subsequent repair of a distribution main. Injured coliforms in these samples averaged >95%. m-T7 agar yielded 8- to 38-fold more coliforms than did m-Endo agar LES. The geometric mean of coliforms recovered by m-Endo agar LES was <1 confirmed coliform per 100 ml, although m-T7 agar yielded 5.7 to 67.5 confirmed coliforms per 100 ml. In addition, the majority of these samples giving positive results on m-T7 agar produced no detectable counts on m-Endo agar LES. These findings indicated that coliforms were injured and largely undetected by use of accepted analytical media in the systems examined.

The coliform group of bacteria has remained the cornerstone of the national drinking water regulations (25) and is used by many in the water supply industry as a criterion of operational parameters. However, some dissatisfaction has been expressed with the shortcomings of reliance upon coliform bacteria as indicators of water quality (7). Some of these concerns have been related to coliform occurrences in the absence of documented waterborne morbidity in the community (4), and others have cited outbreaks of waterborne disease where coliforms were not found (3, 24). The first situation represents a complex, unresolved problem of increasing dimensions that is frequently described as regrowth within the distribution system (4, 10, 18, 22). The latter situation relates to currently accepted methods that lead to underestimations in the detection of waterborne coliforms for a variety of reasons (9, 11, 12, 19, 23). However, the coliform is still regarded as a useful but imperfect criterion of drinking water quality (21, 25; E. E. Geldreich, ASM News **47**:23–27, 1981).

A number of chemical and physical factors common to drinking water systems are known to cause a form of sublethal and reversible injury that is responsible for the failure of waterborne coliforms to grow on accepted media used in the analysis of drinking water, such as m-Endo media (11, 14, 19). Factors found in drinking water that can cause injury include chlorine and other biocides, low concentrations of metals such as copper and zinc, extremes of temperature and pH, and interactions with other bacteria (14, 15). After exposure to these stressful factors, injured coliforms are uniquely susceptible to ingredients such as desoxycholate and bile salts that are found in most selective media used to isolate coliforms from water (19). This prompted the development of a selective medium that did not contain bile salts or desoxycholate for the enumeration of injured total coliform bacteria from drinking water (11). The medium was called m-T7. By using this medium and other approaches, surveys were conducted to determine the extent of injury in coliforms found in drinking water from different geographical locations. The results of an early comparative study of samples from community drinking water systems in Montana and Massachusetts by using m-Endo agar LES (Difco Laboratories, Detroit, Mich.) and m-T7 agar with a resuscitation step indicated that approximately half of the coliforms found were injured (14). A later study in Montana, comparing coliform recoveries from drinking water on m-Endo agar LES and m-T7 agar, revealed that 65% were injured (11). These results suggested that the majority of coliforms found in drinking water were injured. However, questions about the universality of that hypothesis remained, because injury results from the collective influence of many factors (6, 20, 21) that may be present in various levels in drinking water from different regions.

This study was initiated to learn more about the occurrence of injured coliforms and their significance in community water systems. Routine samples of drinking water from three New England water systems experiencing chronic or sporadic occurrences of coliform bacteria were analyzed for coliforms with m-Endo agar LES and m-T7 agar. The samples included water collected both during and immediately after conventional treatment, during the backwash cycle, and at various points in the distribution system, including 1 week after the break and subsequent repair of a distribution main. The results revealed that >90% of the coliforms isolated were injured. Recovery of confirmed coliforms on m-T7 agar was 8- to 38 times higher than that on m-Endo agar LES. In addition, the majority of samples analyzed on m-Endo agar LES yielded negative results, although confirmed coliforms were isolated by using m-T7 agar. These findings indicate that coliforms in routine distribution water samples, chlorinated water leaving treatment plants, and water associated with broken and repaired pipes are frequently undetected by accepted enumeration procedures. Further, these results have important consequences for drinking water systems experiencing coliform regrowth problems.

MATERIALS AND METHODS

Study sites and sample collection. Water samples were collected from various points within the drinking water treatment facilities and distribution systems of three New England communities. The systems studied were located at Salem and Beverly, Mass.; Bennington, Vt.; and Kennebunk,

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TABLE 1. Detection of injured coliforms in three New England drinking water treatment and distribution systems

Sample no.	Sample source	No. of samples	No. of confirmed colonies/100 ml detected on:		% injury	% false-negative
			m-Endo agar LES	m-T7 agar		
1	Throughout systems	71	0.3	9.5	96.8	79
2	Water leaving treatment plants	46	0.2	5.7	96.5	69
3	Filter backwash	1	18	136	86.7	
4	After backwash	1	5	42	97.4	82
5	Pipe break	28	0.9	35.3	97.4	82
6	1 wk after pipe break	11	0	67.5	100	100
7	After disinfection of new main	1	0	11	100	100

Maine. All three of these systems have experienced chronic or intermittent occurrences of excessive coliform populations in the past, including the time this study was conducted.

The system at Salem and Beverly used surface water from a lake and reservoirs that was conventionally treated by using aluminum sulfate, lime, and a phosphate-based corrosion inhibitor. The water was chlorinated before and after rapid sand filtration to maintain a free-chlorine residual concentration of approximately 1.0 mg/liter, although no chlorine was detected in some dead-end samples. This system served a population of approximately 75,000. Bennington received water from a brook in an agricultural watershed. Conventional treatment without prechlorination was followed by chlorination to a free-chlorine residual concentration of 0.5 mg/liter. This system served a population of approximately 16,000. The Kennebunk water district also used conventional treatment, with alum coagulation, soda ash, and a phosphate-based corrosion inhibitor. Pretreatment and postreatment chlorination to a level of 1.0 mg of free-chlorine residual per liter was practiced, but that concentration was not always found in some outlying areas of the distribution system.

Water samples were collected in 250-ml glass or polypropylene bottles with added sodium thiosulfate (0.008%) plus EDTA (1). Free and total chlorine levels were measured at the time of sampling by using a chlorine kit (DPD; Hach Chemical Co., Loveland, Colo.). Samples were placed on ice or in a cooler and transported to the laboratory, where most were analyzed within 4 h after collection. Samples from Bennington were analyzed within 12 h because of shipping requirements.

Microbiological analyses. Comparative analyses for total coliform bacteria were performed on each water sample by using m-Endo agar LES and m-T7 agar. m-Endo agar LES was prepared according to the specifications of the manufacturer. m-T7 agar was prepared as described, including penicillin, by LeChevallier et al. (11). Sample volumes of 100 ml each were filtered through membrane filters (HA WG 04721; Millipore Corp., Bedford, Mass.) and incubated at 35 ± 0.5°C. Sheen colonies on m-Endo agar LES and yellow colonies on m-T7 agar were counted by using a magnification of ×15 according to established guidelines (1, 11). Positive colonies were confirmed by Gram stain and the β-galactosidase-cytochrome oxidase method (1, 12). Additionally, approximately one-third of the confirmed colonies from both media were identified with the API 20E system (Analytab Products, Plainview, N.Y.).

Quality control and statistical comparisons. Accepted quality assurance practices (1, 2) were observed throughout this study. Statistical comparisons were made by using the paired t test on logarithmically transformed data.

RESULTS

Water samples collected from various locations within three drinking water treatment and distribution facilities in New England were analyzed for total coliform bacteria by using m-Endo agar LES and m-T7 agar. m-Endo agar LES was used because m-Endo media are most frequently applied in the enumeration of coliforms in drinking water in the United States (1). m-T7 agar was selected because it allows the resuscitation and recovery of damaged cells (11). Therefore, a comparison of the resulting data provided an opportunity to examine the occurrence of injured coliforms in operating drinking water systems, the utility of m-T7 agar, and the significance of injured coliforms in drinking water systems having chronic occurrences of indicator bacteria in three drinking water systems. The results show the comparative recovery of coliforms in 102 water samples from the three systems studied (Table 1). Results from a subset of 71 routine samples obtained from throughout the distribution systems revealed that a major portion (96.8%) of the confirmed coliforms recovered from finished drinking water were injured and not enumerated as either typical or atypical colonies on m-Endo agar LES (Table 1). The remainder of the sample categories, likewise, showed injury ranging from 86.7 to 97.4% (Table 1). It should also be noted that m-Endo agar LES detected no coliforms in 78% of samples showing positive results on m-T7 agar. Also, the mean coliform level determined with m-Endo agar LES was less than 1.0 confirmed coliform per 100 ml for most of the samples, although it ranged from 5.7 to 67.5 confirmed coliforms per 100 ml for m-T7 agar. The differences observed in the coliform enumerations with m-Endo agar LES and m-T7 agar were highly significant for all data sets ($P < 0.001$). Only 9 of the 102 samples analyzed yielded no detectable coliforms on both m-Endo agar LES and m-T7 agar.

Finished drinking water leaving the treatment plants was also examined to determine if injured coliforms were passing undetected into the distribution system. Results of the 46 sample subsets of treated chlorinated water immediately after filtration are shown in Table 1. As before, a high percentage (96.5%) of the coliforms were injured. The mean coliform level determined with m-Endo agar LES was less than 1.0 confirmed coliform per 100 ml, although it was much higher (5.7 confirmed coliforms per 100 ml) when enumerated with m-T7 agar. Additionally, 69.5% of the samples had positive results on m-T7 agar but failed to give any indication of coliforms on m-Endo agar LES. The total chlorine con-

centration of the water in the filter was maintained near 1.4 mg/liter. Similar results were seen in the two samples taken during and immediately after one backwash cycle of a sand filter (Table 1).

In January 1985, a distribution pipe (12 in. [30 cm] in diameter) in the Salem and Beverly system ruptured and was repaired. This break resulted in reduced chlorine levels and the occurrence of elevated numbers of coliforms in the drinking water. A summary of the resulting bacteriological data from the 2 weeks after this event are shown in Table 1. The mean coliform counts again showed a large difference between the two media and a high degree of coliform injury (97.4%). In four of these samples, the confirmed coliform count on m-T7 agar was in excess of 1,500 confirmed coliforms per 100 ml. The isolated bacteria were identified as *Klebsiella oxytoca* and *Enterobacter agglomerans*. During this time, the total (0 to 0.5 mg of chlorine per liter) and free (0 to 0.4 mg of chlorine per liter)-chlorine levels were lower than those normally observed within the system. Of particular interest are samples that were obtained in the same location 1 week after the rupture (Table 1). All 11 samples failed to produce coliform colonies on m-Endo agar LES but yielded a mean coliform count of 67.5 confirmed coliforms per 100 ml on m-T7 agar. A single sample obtained after the replacement of a distribution pipe (8 in. [20 cm] in diameter) disinfected for 24 h with 200 mg of chlorine per liter and flushed before being placed in service, showed similar results (Table 1). Here again, m-Endo agar LES failed to yield any coliform colonies, although m-T7 agar revealed 11 confirmed coliforms per 100 ml. These organisms were confirmed and identified as *E. agglomerans*.

Confirmed coliforms were identified in 27 of the samples analyzed. The majority (84%) of these isolates were *Enterobacter aerogenes*, *E. agglomerans*, and *Klebsiella pneumoniae* (Table 2). In addition, the bacteria isolated on the two media were identical with respect to organisms found and their relative abundance.

DISCUSSION

The coliform indicator concept has been useful in helping to provide safe drinking water despite its imperfections (21). Properly designed analytical programs, carefully executed to detect coliform bacteria within drinking water systems, have been of value in monitoring the effectiveness of treatment practices as well as the intrusion of contaminated water. However, within the past 20 years, the incidence of waterborne morbidity has increased steadily in the United States (17). The causes of this trend are complex and not completely understood, but excessive populations of coliforms have been associated with most of these outbreaks that have been investigated (5), suggesting that this group of bacteria can still provide useful information in many of the instances of waterborne disease (21). One important application of such information is to guide remedial action when defects of malfunctions arise in treatment and distribution systems. The majority of waterborne disease outbreaks have been caused by problems within the system or an interruption of some aspect of the treatment process (17). Therefore, it is important to optimize coliform detection by making the analysis more sensitive to maintain a commitment to high-quality drinking water.

TABLE 2. Identification of coliforms isolated from three New England drinking water systems by using m-Endo agar LES and m-T7 agar[a]

Organism	No. of isolates	% of total isolates
E. agglomerans	26	33
K. pneumoniae	21	27
E. aerogenes	19	24
E. cloacae	5	7
K. oxytoca	4	5
Citrobacter freundii	3	4

[a] Bacteria were identified from 33 of the 102 samples tested.

Recent reviews (13, 14) describe causes, implications, and methods for the enumeration of injured coliforms in drinking water, thus, those topics will not be discussed in detail here. However, the failure to detect injured coliforms in water implicated in waterborne disease outbreaks (3, 24) is an example of how injured coliforms may be of public health importance. This possibility is further supported by the recent finding by LeChevallier et al. (16) that waterborne pathogenic bacteria are more resistant to injury by chlorine than are similarly exposed coliforms. These results support the view expressed by Seidler (23) that methodological inadequacies in the enumeration of coliforms are basic to some of the dissatisfaction (7) with the indicator concept applied to the microbiological analysis of drinking water.

Available data concerning the occurrence and significance of injured coliforms in drinking water are limited. This paucity of data is caused by the relatively new concept of injury to coliform bacteria in drinking water as well as the lack of suitable commercial media to recover injured bacteria from environmental samples. However, the development of m-T7 agar (11) provided an advance in this regard, because it is both selective and differential and was formulated specifically for the enumeration of injured coliforms from drinking water. This advance paralleled efforts by others to improve the sensitivity of most-probable-number and confirmation methodologies (12, 23). A survey of 44 chlorinated drinking water samples from communities in Montana showed that m-T7 agar recovered nearly three times more coliforms than did m-Endo agar LES (11). Additionally, an earlier survey of over 200 chlorinated and unchlorinated drinking water samples from Montana and Massachusetts, by using the standard m-Endo agar LES alone and with a resuscitation step, revealed that in 31 samples containing coliforms, coliform injury levels ranged from 31 to 86% with a mean level of 43% (unpublished data). In another unpublished study, investigators at a system in Southern California examined 28 replicates of four drinking water samples with both m-Endo agar LES and m-T7 agar and found 58.5% injury in the coliforms that were detected. The present study was initiated to extend this body of knowledge by investigating the occurrence and significance of injured total coliform bacteria found in drinking water systems experiencing excessive coliform populations as well as to evaluate m-T7 agar in other geographical locations. The results show that a high percentage (86.7 to 97.4%) of the coliforms present in the three systems studied were injured (Table 1). This indicates that approximately 1/10 of the coliforms present were enumerated when the accepted method with m-Endo agar LES was used. Whether this level of cellular damage is found in all systems is uncertain, because injury results from the collective influence of chemical and physical properties of water (20) that vary markedly in different regions and systems (21). In fact, drinking water distribution samples that were similarly evaluated from a municipal system in New Jersey experiencing an occurrence of coliforms revealed little difference between enumerations with m-Endo agar LES and m-T7 agar with a mean coliform

injury level of only 13% (unpublished data). However, in all cases where we have seen this kind of comparative data from drinking water, m-T7 agar has been as effective as or superior to other media in enumerating total coliform bacteria.

Occurrences of excessive coliform populations in drinking water, termed regrowth, are being observed with increasing frequency (4, 10, 18, 22). The source of these coliforms in the distribution water is presumed, in the absence of definitive data, to be growth within a biofilm community on the pipe walls (4, 18, 22). Geldreich et al. (9) have suggested that coliforms from sand filters "seed" the distribution system and demonstrated that filters contain between 110 (deep) and 6,300 (surface) coliforms per gram of filter-bed sand. More recently (10), investigators at Springfield, Ill., proposed that chlorine-injured coliforms passed undetected into the distribution system, where they were able to recover and cause a problematic coliform occurrence. The results reported here for both filter effluents and samples taken during and immediately after a backwash cycle lend support to the first part of that scenario (Table 1). Further, it is useful to note that problem coliform occurrences in systems with surface source water usually follow major precipitation events when the water temperature is relatively warm (18, 22). The precipitation might serve to wash coliforms from the environment, where they are known to proliferate (8), into the source water along with added nutrients that allow them to grow on the filter. Chlorine and other injurious factors may damage such bacteria, making them undetectable with m-Endo media as they are released into the distribution system, where the temperature and added nutrients favor their recovery.

The results reported here address unexplained occurrences of coliforms within distribution systems (Table 1). Our findings demonstrate that a high percentage (96.5%) of the coliforms present in the chlorinated water immediately after filtration are injured and not detected with m-Endo agar LES. For that reason and because m-Endo agar LES is the accepted membrane filter medium, injured coliforms entering the distribution network are frequently not observed. That argument is further supported by the high percentage (78%) of samples with coliforms on the m-T7 agar plates that failed to yield colonies on m-Endo agar LES.

Another feature of the overall data presented in Table 1 is noteworthy. In 100 of the 102 samples examined, excluding those associated with the backwash of a sand filter, the geometric mean values of coliform enumerations with m-Endo agar LES were in compliance (0.2 to 0.9 confirmed coliform per 100 ml) with the national drinking water regulations (<1 confirmed coliform per 100 ml) (25), although they far exceeded that standard when m-T7 agar was used (5.7 to 67.5 confirmed coliforms per 100 ml) if those values were monthly averages. In such systems, therefore, the use of a more efficient medium such as m-T7 agar would afford greater sensitivity in the microbiological aspect of the routine water analysis and allow emerging problems to be detected earlier, because many such operational or intrusion difficulties are first signaled by a low level of coliforms that increases numerically until the system is no longer in compliance with the coliform limit specified in the regulations. Clearly, the operator of such a system would want to know of the impending problem as early as possible to initiate the appropriate corrective action. Hence, use of the more sensitive m-T7 agar would be preferred, because it would provide a more accurate understanding of the source of coliforms entering the distribution system as well as their location and population dynamics. This same interpretation might be extended to the finding of high percentages of samples that revealed no coliforms with m-Endo agar LES but showed positive results on m-T7 agar; these ranged from 69.5 to 100% of the samples within each data set (Table 1). Results describing the observation of injured coliforms associated with the rupture and repair of a distribution main also support the same line of reasoning (Table 1). Data from the entire 2-week period after this event again show a high degree of injury with the geometric mean for the coliforms detected with m-Endo agar LES at a level (0.9 confirmed coliform per 100 ml) that is less than the national coliform standard for drinking water (Table 1). Even more striking are the data from the samples collected 1 week after the break and repair event (Table 1). High coliform counts were seen with m-T7 agar (mean of 67.5 confirmed coliforms per 100 ml), although the samples were universally negative when enumerated with m-Endo agar LES.

The following conclusions are proposed concerning the occurrence, detection, and significance of injured coliform bacteria in the three New England municipal drinking water systems studied. (i) The coliform bacteria present in these systems were injured to the degree that accepted methods, by using m-Endo agar LES, would enumerate less than 1/10 of the viable population present. This caused 70 to 100% of the samples in the data sets examined to yield false-negative results, a finding of significance when considering presence-absence methodologies. (ii) m-T7 agar was effective in the recovery of the injured portion of the total coliform population yielding results that were 8- to 38 times greater than those with m-Endo agar LES. (iii) Significant levels of injured coliforms were undetected entering the distribution system after treatment, including filtration, and after the repair and disinfection of a broken main if m-Endo agar LES was used. These findings may not be universal but may be characteristic to some geographic regions or to particular drinking water systems where the physicochemical properties of the water induce coliform injury. In systems such as the ones described here, injured coliforms can represent the majority of the total coliform population present. Optimal enumeration of these bacteria with more sensitive media, such as m-T7 agar, provides those individuals concerned with the maintenance of high-quality drinking water a more useful and representative body of water quality information.

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PART II. GENERAL OPERATIONS

Section D Selection of Analytical Methods

This Section discusses the selection of methods for monitoring water and wastewater in response to the Laws, the microbiological standards that have been established, and the criteria that have been recommended to enforce the laws. The major problems that have developed in the application of the methods are identified and solutions are given where they are available.

1. Methodology

   1.1 National Interim Primary Drinking Water Regulations
   1.2 NPDES Guidelines
   1.3 Marine Sanitation Regulations
   1.4 Water Quality Standards
   1.5 Water Quality Criteria
   1.6 Alternate Test Procedures

2. Problems in Application

   2.1 Stressed Microorganisms
   2.2 Incomplete Recovery/ Suppression
   2.3 Interference by Turbidity
   2.4 Analysis of Ground Water
   2.5 Field Problems
   2.6 Method Modifications and Kits
   2.7 Changes in Membrane Filters and Methodology
   2.8 *Klebsiella* in Industrial Wastes

3. Recommendations for Methods in Waters and Wastewaters

1. Methodology

Test procedures have been specified and published in Federal Register for drinking water, wastewater discharges (NPDES) and vessel discharges.

1.1 National Interim Primary Drinking Water Regulations

Although the National Interim Primary Drinking Water Regulations (Title 40 CFR Part 141) state that the total coliform analyses can be performed by the membrane filter or MPN procedures, the MF procedure is preferred because large volumes of samples can be analyzed in a much shorter time, a critical factor for potable water. Samples containing excessive noncoliform populations or turbidity must be analyzed by the MPN technique. These regulations specify the testing of sample sizes of 100 ml for the MF technique and the testing of five replicate 10 or 100 ml volumes for the MPN procedure. The law directs that the samples be taken at points representative of the distribution system. The minimal schedules for the frequency of sampling are based on population and the required response is given for positive test results. A detailed description of the proposed criteria for interim certification of microbiology laboratories under

the Safe Drinking Water Act is given in Appendix B.

1.2 National Pollution Discharge Elimination System (NPDES) Guidelines

The NPDES established guidelines for analysis of pollutants under PL 92-500, Section 304 (g). The parameters and methods are described in 40 CFR Part 136, as amended (40 Code of Federal Regulations, Protection of the Environment, ch. 1 - Environmental Protection Agency, Part 136, Guidelines Establishing Test Procedures for the Analysis of Pollutants). The method must be specified and MPNs must be five tube, five dilution. See Table II-D-1.

1.3 Marine Sanitation Regulations

The regulations for marine sanitation devices (40 CFR Part 140) established performance standards and specified the analytical methods as those promulgated in 40 CFR Part 136, cited in 1.2 above.

1.4 Water Quality Standards

Water quality standards (limits) have been established by law for drinking water and certain sewage and industrial effluents. These standards and the reference sources are listed in Table II-D-2. A standard must be specified in the NPDES permit to be enforceable.

1.5 Water Quality Criteria

Water quality criteria have been recommended by the EPA for certain types of water classified according to use. These criteria are listed in Table II-D-3.

1.6 Alternate Test Procedures

The amendments to 304 (g) also provide procedures for approval of alternate methods. National approval for test methods is obtained by application to EPA through EMSL-Cincinnati while case by case approval is obtained by application through the EPA Regional Offices (40 CFR 136.4).

2. Problems in Application

Although the methods described in this Manual are judged the best available, there are difficulties in the application of methods in different geographical areas, in certain wastes and in some potable and surface waters. Additional problems can stem from the indiscriminate use of new and simplified equipment, supplies or media that have been proposed for use in these procedures.

2.1 Stressed Microorganisms

Some water and wastewater samples contain microorganisms which should reproduce but do not under the conditions of test. These organisms have been described as injured or stressed cells. The stress may be caused by temperature changes or chemical treatment such as chlorine or toxic wastes (1).

Stressed organisms are particularly important in environmental measurements because tests for bacterial indicators or pathogens can give negative responses, then recover later and multiply to produce dangerous conditions. Subsections 2.1.1 and 2.1.2 describe efforts to recover stressed microorganisms.

2.1.1 Ambient Temperature Effects

Extreme ambient temperatures stress microorganisms and reduce recovery of microbiological indicators. For example, in Alaska and other extremely cold areas, the severe change from cold stream temperature to 44.5 C temperature of incubation reduces recovery of fecal coliforms. The two-step MF test for fecal coliforms increases recoveries by use of a 2-hour acclimation on an enrichment medium at 35 C before normal incubation at 44.5 C.

In contrast, water samples from natural waters at high temperatures may include large numbers of non-coliform organisms which interfere with sheen production on MF's and with positive gas production in MPN analyses. An improved MF medium that provides greater selectivity is desirable but may not be possible without sacrificing recovery.

TABLE II-D-1

Approved Test Procedures for the Analysis of Pollutants (40 CFR 136)

Parameter per 100 ml	Method	Reference and Page No. SM[1]	USGS[2]	This Manual
Fecal Coliforms	MPN	922		Part III C, 5
	MF	937	45	Part III C, 2
Fecal Coliforms in presence of chlorine[3]	MPN	922		Part III C, 5
	MF	928, 937		Part III C, 2
Total Coliforms	MPN	916		Part III B, 4
	MF	928	35	Part III B, 2
Total Coliforms in presence of Chlorine	MPN	916		Part III B, 4
	MF	933		Part III B, 2
Fecal Streptococci	MPN	943		Part III D, 4
	MF	944	50	Part III D, 2
	Plate Count	947		Part III D, 5

[1] Standard Methods for the Examination of Water and Wastewater, 14th Edition, (1975).

[2] Slack, K. V., et.al. Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples. USGS Techniques of Water Resources Inv., Book 5, ch. A4 (1973).

[3] Since the MF technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies.

TABLE II-D-2

Water Quality Standards

Water or Wastewater	Microbiological Standards Coliforms/100 ml Total	Fecal	Reference Source
Potable Water	<5	—	PL 93-523
Chlorinated Effluents	—	200-400	PL 92-500
2° Treatment Wastes	—	200-400	40 CFR Part 133
Selected Industrial Wastes	—	200-400	PL 92-500
Leather and Tanning	—	400	40 CFR Part 425
Feed Lots	—	400	40 CFR Part 412
Meat Products	—	400	40 CFR Part 432
Beet Sugar	—	400	40 CFR Part 409
Canned Fruits and Vegetables	—	400	40 CFR Part 407
Textiles	—	400	40 CFR Part 410
Effluents from Marine Sanitation Devices with Discharges Type I	—	1000	40 CFR Part 140 and Amendments
Type II	—	200	40 CFR Part 140 and Amendments

TABLE II-D-3

Water Quality Criteria

Water or Wastewater	Statistical Measure	Microbiological Criteria Coliforms/100 ml Total	Fecal	Reference Source
Public Water Supply	log $\overline{X}$	20000	2000	A
Recreational Water:				
Primary Contact	log $\overline{X}$/30 days		200	B
	maximum/30 days, in 10% of Samples		400	B
General Contact	log $\overline{X}$/30 days		1000	B
	maximum/30 days, in 10% of Samples		2000	B
Agricultural Water	monthly $\overline{X}$	5000	1000	B
Shellfish-Raising Waters				
	Daily Median	70	14	C & D
	Highest 10% of Daily Values	230	43	

A Water Quality Criteria, EPA. March, 1973. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

B Water Quality Criteria, FWPCA, April 1,1968. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

C National Shellfish Sanitation Program Manual of Operation. U.S. Dept. of HEW, 1965. Public Health Service Publ. No. 33. Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402.

D Quality Criteria for Water, July 1976, O.W.H.M., US EPA.

2.1.2 Chlorinated Effluents and Toxic Wastes

Although thiosulfate is added to all samples suspected of containing chlorine, to neutralize its toxic effects, the membrane filter procedure yields poor recovery of coliforms from chlorinated effluents as compared to MPN recovery (1-6). A recent amendment to 40 CFR 136 added Coliform bacteria (Fecal) in the presence of chlorine, as a specific parameter and recommended analysis by the MF or MPN techniques (7). A qualifying statement appended to the method in 40 CFR Part 136 requires the five tube, five dilution MPN and states: "Since the membrane filter technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies." Therefore, the MPN procedure should be used in analysis of chlorinated effluents where the data may be challenged by legal or enforcement actions. The MF may be used currently for self-monitoring situations. (See Table II-D-1).

Proposed changes in MF materials and procedures include new membrane filter formulations, an agar overlay technique, modified media and twostep methods (1). Present modifications of the MF method have not produced recoveries of fecal coliforms from chlorinated effluents equivalent to MPN recoveries. Thorough evaluation and approval of proposed procedures by EPA are required before changes will be acceptable.

Certain types of wastes show recovery problems for total and fecal coliforms:

1. Primary and Chlorinated-Primary Waste Effluents.

2. Chlorinated-Secondary and Chlorinated-Tertiary Waste Effluents.

3. Industrial wastes containing toxic metals or phenols.

When turbidity and low recovery prevent the application of the MF technique to coliform analyses of primary and secondary effluents or industrial wastes containing toxic materials, the MPN procedure is required. However, the two-step MF procedure for total coliforms described in this Manual and in *Standard Methods* is acceptable for toxic wastes.

If the MF procedure is applied to chlorinated or toxic samples, the laboratory should require data from at least 10 samples collected over 1 week of plant processing (but not less than 5 calendar days) to show comparability of the MF to the MPN technique. See Part IV-C, 3 for details.

2.2 Incomplete Recovery/Suppression

When coliforms are present in low numbers in drinking water, high levels of non-coliforms can suppress growth or mask detection. This problem may appear as a mass of confluent growth on a membrane filter or as spots of sheen in this confluent growth. In the MPN procedure, presumptive tubes may show heavy growth with no gas bubbles, dilution skips or unusual tube combinations. When these negative presumptive tubes are transferred to BGLB, they confirm in this more restrictive medium, indicating that the coliform gas production in the Presumptive Test was suppressed by non-coliforms.

2.3 Interference by Turbidity

The tendency of bacteria to clump and adhere to particles can produce inaccurate results in the analysis of water samples. The National Interim Primary Drinking Water Regulations (NIPDWR) specify one turbidity unit as the primary maximum allowable level but permit up to five turbidity units if this level does not interfere with disinfection or microbiological analyses. Turbidity can interfere with filtration by causing a clumping of indicators or clogging of pores. The turbidity as organic solids can also provide nutrients for bacterial growth and subsequently produce higher counts. The type of particles variably affects the filtration rate; for example, clay, silt or organic debris clog more easily than sand. Background organisms may also be imbedded

in the particles and interfere with the coliform detection.

2.4 Analysis of Ground Water

Although total coliforms are a valid measure of pollution, their use as indicators in analyzing ground waters and rural community supplies may not sufficiently describe the water quality. For example, ground waters frequently contain high total counts of bacteria with no coliforms. Such waters pass Interim Drinking Water Regulations but technical judgment must conclude these are not acceptable as potable waters.

2.5 Field Problems

Assurance of data validity demands sample analyses within the shortest time interval after collection. This need requires field analyses using either a mobile laboratory or field kit equipment. Since a mobile laboratory may not be available for a survey, it is likely that at least a part of the analyses will need to be completed in an onsite facility. If the analyses can be done using membrane filtration techniques, field kits such as Millipore's Water Laboratory and MF Portable Incubator (heat sink) are particularly helpful for rapid set-up and analyses of limited samples. However, if large numbers of samples are tested per day or the survey covers more than a few days, the heat-sink incubator is impractical because of limited capacity and high cost. In such surveys, a mobile laboratory utilizing water-jacketed incubators is more practical.

2.6 Method Modifications and Kits

Commercial manufacturers continue to offer proprietory kits and method modifications to speed or simplify the procedures used in coliform and fecal coliform analyses, primarily for field use. Most of these units have not been demonstrated to produce results comparable to the official procedures. If not tested to the satisfaction of EPA, such method modifications and kits cannot be used for establishing total or fecal coliform numbers for permits under NPDES or for total coliform numbers under the Safe Drinking Water Act. The procedure required for acceptance of an alternative procedure is described in 40 CFR Parts 136.4 and 136.5, as amended.

2.7 Changes in Membrane Filters and Methodology

There is an expected pattern of changes in materials and methodology used in the manufacture of membrane filters. The changes may or may not be announced by the manufacturer. Therefore, it is important for the laboratory to monitor membrane performance as described in Section A of Quality Control in this Manual.

These changes include modification of formulations and the replacement of the 0.45 μm pore MF by a 0.7 μm retention pore MF for improved recovery. Tests by independent investigators show that several MF's give comparable recovery (5, 6, 8, 9), however, enrichment or two-temperature incubations are needed before recoveries approach the MPN values (See 2.1.2 in this Section).

This discussion of problems with new methodology and membrane materials should not be interpreted as indicating that EPA discourages new developments. Rather EPA encourages the MF supply industry to test and examine procedures, to innovate and to research. The membrane filter manufacturers should be commended and encouraged to continue their efforts toward solving problems and improving materials and techniques in water microbiology.

2.8 *Klebsiella* in Industrial Wastes

Klebsiella bacteria (part of the coliform group) multiply in certain industrial wastes, are not differentiated from fecal coliforms by MF and MPN procedures and consequently are included in the results. These recoveries have been reported in textile, paper and pulp mills and other wastes. Objections have been raised to the application of fecal coliform standards

TABLE II-D-4

Selection of Methods for Problem Samples

Problem Area	Parameter Chosen	Method of Choice
Shellfish-harvesting waters	Total coliform	MPN*
	Fecal Coliform	MPN*
Marine & Estuarine Waters	Total Coliform	MF/MPN
	Fecal Coliform	MF/MPN
Treated Industrial Wastes (non-chlorinated, non-toxic) Low Solids Wastes	Fecal Coliform	MF
Toxic Industrial Wastes (metals, phenolics) and High Solids Wastes	Fecal Coliform	MPN or alternate procedure, tested and approved**
Primary and Chlorinated-Primary Municipal/Industrial Effluent	Total Coliform	MPN or alternate procedure, tested and approved**
	Fecal Coliform	MPN or alternate procedure, tested and approved**
Chlorinated-Secondary Effluent	Total Coliform	Two-Step MF
	Fecal Coliform	MPN or alternate procedure, tested and approved**

*MPN recommended to conform with the MPN method specified for examination of shellfish.

**Requires proof of comparability under EPA's specified test regime that the alternate procedure (MF, streak plate, etc.) is valid. See This Manual, IV-C, 3.

to these wastes because *Klebsiella* originate from other than sanitary sources. However, EPA does consider large numbers of *Klebsiella, Aeromonas* and other noncoliforms as indicators of organic pollution. Further, these organisms do occur in low densities in human and animal wastes.

3. Recommendations for Methods in Waters and Wastewaters

The amended Federal Water Pollution Control Act, the Marine Protection, Research and Sanctuaries Act and the Safe Drinking Water Act require recommendations on analytical methodology. Generally, the membrane filter methods are preferred over MPN and other techniques, where proven applicable.

In Table II-D-4, problem samples are identified and the analytical method recommended for parameters of choice.

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PART III. ANALYTICAL METHODOLOGY

Section A Standard Plate Count

1. Summary of Method

The Standard Plate Count (SPC) Method is a direct quantitative measurement of the viable aerobic and facultative anaerobic bacteria in a water environment, capable of growth on the selected plating medium. An aliquot of the water sample or its dilution is pipetted into a sterile glass or plastic petri dish and a liquified, tempered agar medium added. The plate is rotated to evenly distribute the bacteria. Each colony that develops on or in the agar medium originates theoretically from one bacterial cell. Although no one set of plate count conditions can enumerate all organisms present, the Standard Plate Count Method provides the uniform technique required for comparative testing and for monitoring water quality in selected situations.

2. Scope and Application (1–6)

This simple technique is a useful tool for determining the bacterial density of potable waters and for quality control studies of water treatment processes. The Standard Plate Count provides a method for monitoring changes in the bacteriological quality of finished water throughout a distribution system, thus giving an indication of the effectiveness of chlorine in the system as well as the possible existence of cross-connections, sediment accumulations and other problems within the distribution lines. Total bacterial densities greater than 500–1000 organisms per ml may indicate coliform suppression or desensitization of quantitative tests for coliforms (1–3). The procedure may also be used to monitor quality changes in bottled water or emergency water supplies.

2.1 Theoretically, each bacterium present in a sample multiplies into a visible colony of millions of bacteria. However, no standard plate count or any other total count procedure yields the true number because not all viable bacterial cells in the water sample can reproduce under a single set of cultural conditions imposed in the test. The number and types of bacteria that develop are influenced by the time and temperature of incubation, the pH of the medium, the level of oxygen, the presence of specific nutrients in the growth medium, competition among cells for nutrients, antibiosis, predation, etc.

2.2 This procedure does not allow the more fastidious aerobes or obligate anaerobes to develop. Also, bacteria of possible importance in water such as *Crenothrix*, *Sphaerotilus*, and the actinomycetes will not develop within the incubation period specified for potable water analysis.

2.3 Clumps of organisms in the water sample which are not broken up by shaking result in underestimates of bacterial density, since an aggregate of cells will appear as one colony on the growth medium.

3. Apparatus and Materials

3.1 Incubator that maintains a stable 35 ± 0.5 C. Temperature is checked against an NBS certified thermometer or one of equivalent accuracy.

3.2 Water bath for tempering agar set at 44–46 C.

3.3 Colony Counter, Quebec darkfield model or equivalent.

3.4 Hand tally or electronic counting device (optional).

3.5 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

3.6 Petri dish containers of stainless steel or aluminum for glass petri dishes.

3.7 Thermometer certified by National Bureau of Standards or one of equivalent accuracy, with calibration chart.

3.8 Sterile TD (To Deliver) bacteriological or Mohr pipets, glass or plastic of appropriate volumes, see Part II-B, 1.8.1.

3.9 Sterile 100 mm × 15 mm petri dishes, glass or plastic.

3.10 Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volume, screw cap with neoprene rubber liner.

3.11 Bunsen/Fisher gas burner or electric incinerator.

4. Media

4.1 Sterile Plate Count Agar (Tryptone Glucose Yeast Agar) dispensed in tubes (15 to 20 ml per tube) or in bulk quantities in screw cap flasks or dilution bottles. See Part II-B, 5.1.5.

4.2 Sterile buffered dilution water, 99 ± 2 ml volumes, in screwcapped dilution bottles. See Part II-B, 7.

5. Procedure

5.1 Dilution of Sample (See Part II-C, 1.4 for details)

5.1.1 The sample is diluted to obtain final plate counts of 30–300 colonies. In this range, the plate counts are the most accurate and precise possible. Since the microbial population in the original water sample is not known beforehand, a series of dilutions must be prepared and plated to obtain a plate count within this range.

5.1.2 For most potable water samples, countable plates can be obtained by plating 1 and 0.1 ml of the undiluted sample, and 1 ml of the 1:100 sample dilution (see Figure III-A-1). Higher dilutions may be necessary with some potable waters.

5.1.3 Shake the sample vigorously about 25 times.

5.1.4 Prepare an initial 1:100 dilution by pipetting 1 ml of the sample into a 99 ml dilution water blank using a sterile 1 ml pipet (see Figure III-A-1).

5.1.5 The 1:100 dilution bottle is vigorously shaken and further dilutions made by pipetting aliquots (usually 1 ml) into additional dilution blanks. A new sterile pipet must be used for each transfer and each dilution must be thoroughly shaken before removing an aliquot for subsequent dilution.

5.1.6 When an aliquot is removed, the pipet tip should not be inserted more than 2.5 cm (1 inch) below the surface of the liquid.

5.2 Preparation of Agar

5.2.1 Melt prepared plate count agar (tryptone glucose yeast agar) by heating in boiling water or by flowing steam in an autoclave at 100 C. Do not allow the medium to remain at these high temperatures beyond the time necessary to melt it. Prepared agar should be melted once only.

5.2.2 Place melted agar in a tempering water bath maintained at a temperature of 44–46 C. Do not hold agar at this temperature

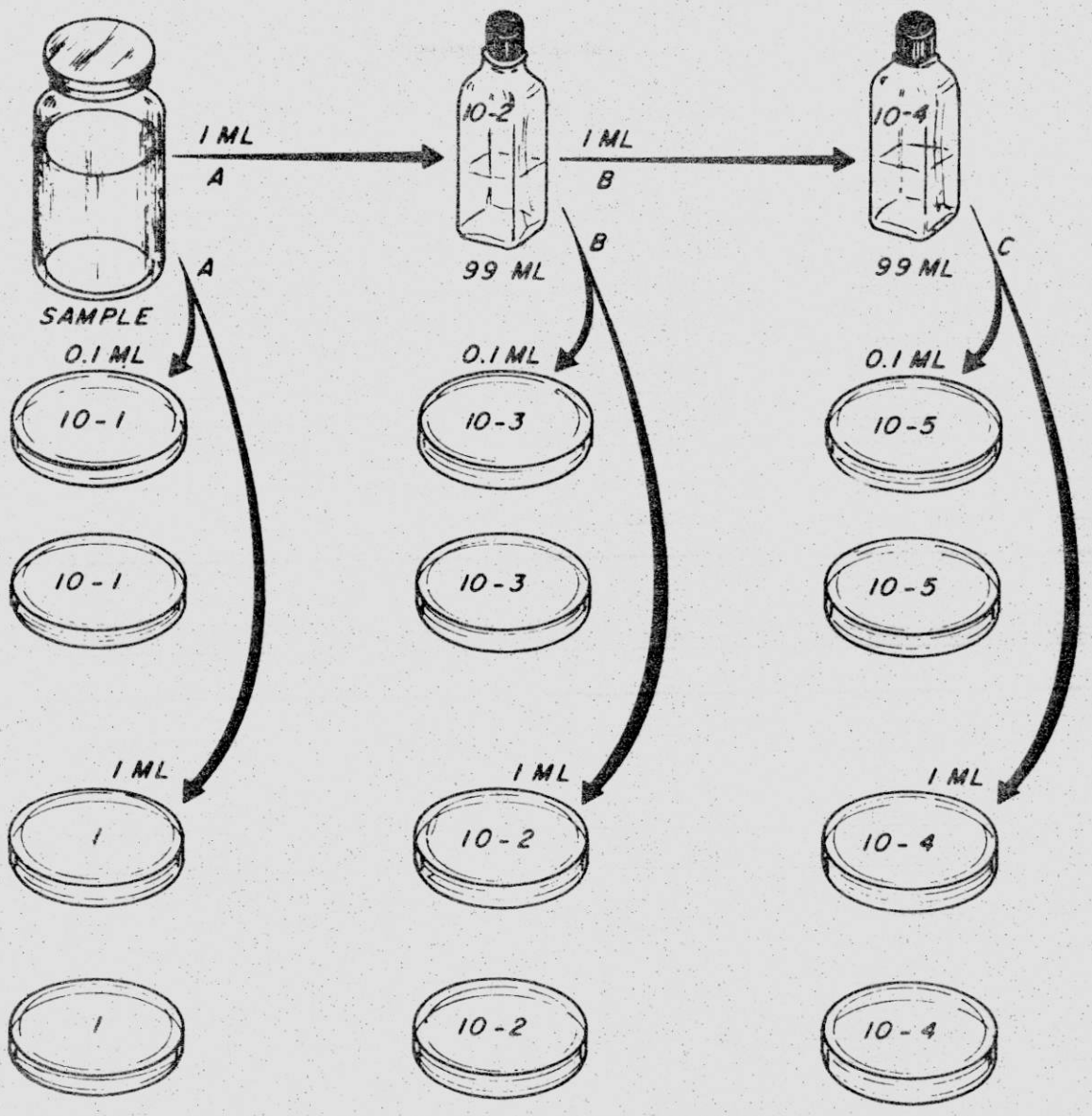

FIGURE III-A-1. Typical Dilution Series for Standard Plate Count

longer than three hours because precipitates may form which confuse the counting of colonies. Maintain a thermometer immersed in a separate bottle or flask in the water bath to monitor the temperature.

5.3 Preparation for Plating

5.3.1 Prepare at least duplicate plates for each sample or dilution tested. Mark and arrange plates in a reasonable order for use. Prepare a bench sheet or card, including sample identity, dilutions, date and other relevant information.

5.3.2 Aseptically pipet an aliquot from the appropriate dilution into the bottom of each petri dish. Use a separate sterile pipet to transfer an aliquot to each set of petri dishes for each sample or sample dilution used. Vigorously shake the undiluted sample and dilution containers before each transfer is made.

5.3.3 Pipet sample or sample dilution into marked petri dish. After delivery, touch the tip once to a dry spot in the dish.

5.3.4 To minimize bacterial density changes in the samples, do not prepare any more samples than can be diluted and plated within 20–25 minutes.

5.4 Pouring Agar Plates

5.4.1 Use the thermometer in the control bottle in the tempering bath to check the temperature of the plating medium before pouring.

5.4.2 Add not less than 12 ml (usually 12–15 ml) of the melted and cooled (44–46 C) agar medium to each petri dish containing an aliquot of the sample or its dilution. Mix the inoculated medium carefully to prevent spilling. Avoid splashing the inside of the cover. One recommended technique rotates plate five times to left, five times to the right and five times in a back and forth motion.

5.4.3 Pipet a one ml volume of sterile dilution water into a petri dish, add agar, mix and incubate with test plates. This control plate will check the sterility of pipets, agar, dilution water and petri dishes. See Part IV-C, 1.3.

5.5 Incubation of Plated Samples

5.5.1 After agar plates have hardened on a level surface (usually within 10 minutes), invert the plates and immediately incubate at 35 C.

5.5.2 Incubate tests on all water samples except bottled water at 35 $\pm$ 0.5 C for 48 $\pm$ 3 hours. Incubate the tests on bottled water at 35 $\pm$ 0.5 C for 72 $\pm$ 4 hours. The longer incubation is required to recover organisms in bottled water with longer generation times.

5.5.3 Stacks of plates should be at least 2.5 cm from adjacent stacks, the top or sides of the incubator. Do not stack plates more than four high. These precautions allow proper circulation of air to maintain uniform temperature throughout the incubator and speed equilibration.

5.6 Counting and Recording Colonies:

After the required incubation period, examine plates and select those with 30–300 colonies. Count these plates immediately. A Quebec-type colony counter equipped with a guide plate, appropriate magnification and light is recommended for use with a hand tally.

5.6.1 Electronic-assist devices are available which register colony counts with a sensing probe and automatically tabulate the total plate count.

Fully-automatic colony counters are available which count all colonies (particles) larger than a preset threshold-size. These counters scan and provide digital register and a visual image of the plate for further examination and recounting with different threshold if so desired.

Because the accuracy of automatic conters varies with the size and number of colonies per plate, the analyst should periodically compare its results with manual counts.

5.6.2 The following rules shqould be used to report the Standard Plate Count:

(a) Plates with 30 to 300 Colonies: Count all colonies and divide by the volume tested (in ml). If replicate plates from one dilution are countable (30–300), sum the counts of colonies on all plates and divide by the volumes tested (in ml) as follows:

$$\frac{\text{Sum of Colonies}}{\text{Sum of Volumes Tested, ml}} = \text{S.P. Count/ml}$$

Record the dilutions used, the number of colonies on each plate and report as the Standard Plate Count per milliliter.

If two or more consecutive dilutions are countable, independently carry each calculation of plate count to a final count per ml, then calculate the mean of these counts/ml for the reported value.

For example, if 280 and 34 colonies are counted in the 1:100 and 1:1000 dilutions of a water sample, the calculation is:

$$\frac{280}{.01} = 28{,}000/\text{ml}$$

$$\frac{34}{.001} = 34{,}000/\text{ml}$$

$$\text{Reporting Value} = \frac{28000 + 34000}{2}$$

$$= 31000 \quad \text{SPC/ml}$$

(b) All Plates with Fewer than 30 Colonies: If there are less than 30 colonies on all plates, record the actual number of colonies on the lowest dilution plated and report the count as: Estimated Standard Plate Count per milliliter. For example, if volumes of 0.1, 0.01 and 0.001 ml were plated and produced counts of 22, 2 and 0 colonies respectively, the colony count of 22 from the largest sample volume (0.1 ml) would be selected, calculated and reported as follows:

$$\frac{\text{Plate Count}}{\text{Volume Plate}} = \frac{22}{0.1} = 220$$

Count reported: Estimated Standard Plate Count, 220/ml.

(c) If 1 ml volumes of original sample produce counts < 30, actual counts are reported.

(d) Plate with No Colonies: If all plates from dilutions tested show no colonies, report the count as < 1 times the lowest dilution plated. For example, if 0.1, 0.01 and 0.001 ml volumes of sample were tested with no visible colonies developing, the lowest dilution, 0.1 ml would be used to calculate a less than (<) count as follows:

$$\frac{1}{\text{Volume Tested}} = \frac{1}{0.1} = < 10$$

Count reported: Standard Plate Count, > 10/ml.

(e) All Plates Greater than 300 Colonies: When counts per plate in the highest dilution exceed 300 colonies, compute the count by multiplying the mean count by the dilution used and report as a greater than (>), Standard Plate Count per milliliter. For example, if duplicate 1.0, 0.1 and 0.01 volumes of sample were tested with average counts of > 500, > 500 and 340 developing in the dilutions, the count would be calculated as follows:

$$\frac{\text{Plate Count}}{\text{Volume Tested}} = \frac{340}{0.01} = 34{,}000$$

or count reported as: Standard Plate Count, > 34,000/ml.

5.6.3 Count Estimations on Crowded Plates: The square divisions of the grid on the Quebec or similar colony counter can be used to estimate the numbers of bacteria per plate. With less than 10 colonies per sq cm count the colonies in 13 squares with representative distribution of colonies. Select 7 consecutive horizontal squares and 6 consecutive vertical

squares for counting. Sum the colonies in these 13 sq cm, and multiply by 5 to estimate the colonies per plate for glass plates (area of 65 sq cm) or multiply by 4.32 for plastic plates (area of 57 sq cm). With more than 10 colonies per sq cm, count 4 representative squares, average the count per sq cm, multiply by the number of sq cm/plate (usually 65 for glass plates and 57 for plastic plates) to estimate the colonies per plate. Then multiply by the reciprocal of the dilution to determine the count/ml. When bacterial counts on crowded plates are greater than 100 colonies per sq cm, report the result as Estimated Standard Plate Count greater than (>) 6,500 times the highest dilution plated.

5.6.4 Spreaders: Plates containing spreading colonies must be so reported on the data sheet. If spreaders exceed one-half of the total plate area, the plate is not used. Report as: No results, spreaders.

Colonies can be counted on representative portions of plates if spreading colonies constitute less than one-half of the total plate area, and the colonies are well-distributed in the remaining portion of the plate.

(a) Count each chain of colonies as a single colony.

(b) Count each spreader colony that develops as a film of growth between the agar and the petri dish bottom as one colony.

(c) Count the growth that develops in a film of water at the edge or over the surface of the agar as one colony.

(d) Adjust count for entire plate and report as: Estimated Standard Plate Count/ml.

5.6.5 Remarks on Data Sheet: Any unusual occurrences such as missed dilutions, loss of plates through breakage, contamination of equipment, materials, media, or the laboratory environment, as shown by sterility control plates, must be noted on the data sheet. Report as: Lab Accident, etc.

6. Reporting Results

Report Standard Plate Count or Estimated Standard Plate Count as colonies per ml, not per 100 ml.

Standard Plate Counts should be rounded to the number of significant figures (S.F.) obtainable in the procedure: 1 S.F. for 0–9 actual plate counts, 2 S.F. for 10–99 actual plate counts and 3 S.F. for 100–300 actual plate counts. See Part II-C, 2.8.1 of this manual.

7. Precision and Accuracy

7.1 Prescott et al (7) reported that the standard deviation of individual counts from 30–300 will vary from 0–30 percent. This plating error was 10% for plate counts within the 100–300 range. A dilution error of about 3% for each dilution stage is incurred in addition to the plating error. Large variations can be expected from high density samples such as sewage for which several dilutions are necessary.

7.2 Laboratory personnel should be able to duplicate their plate count values on the same plate within 5%, and the counts of others within 10%. If analysts' counts do not agree, review counting procedures for analyst error.

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PART III. ANALYTICAL METHODOLOGY

Section B Total Coliform Methods

This section describes the enumerative techniques for total coliform bacteria in water and wastewater. The method chosen depends upon the characteristics of the sample. The Section is divided as follows:

1. **Definition of the Coliform Group**
2. **Single-Step, Two-Step and Delayed-Incubation Membrane Filter (MF) Methods**
3. **Verification**
4. **Most Probable Number (MPN) Method**
5. **Differentiation of the Coliform Group by Further Biochemical Tests**

1. Definition of the Coliform Group

The coliform or total coliform group includes all of the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose in 24-48 hours at 35 C. The definition includes the genera: *Escherichia, Citrobacter, Enterobacter, and Klebsiella.*

2. Single-Step, Two-Step and Delayed-Incubation Membrane Filter Methods

2.1 Summary: An appropriate volume of a water sample or its dilution is passed through a membrane filter that retains the bacteria present in the sample.

In the single-step procedure the filter retaining the microorganisms is placed on M-Endo agar, LES M-Endo agar or on an absorbent pad saturated with M-Endo broth in a petri dish. The test is incubated at 35 C for 24 hours.

In the two-step enrichment procedure the filter retaining the microorganisms is placed on an absorbent pad saturated with lauryl tryptose (lauryl sulfate) broth. After incubation for 2 hours at 35 C, the filter is transferred to an absorbent pad saturated with M-Endo broth, M-Endo agar, or LES M-Endo agar, and incubated for an additional 20–22 hours at 35 C. The sheen colonies are counted under low magnification and the numbers of total coliforms are reported per 100 ml of original sample.

In the delayed-incubation procedure, the filter retaining the microorganisms is placed on an absorbent pad saturated with M-Endo preservative medium in a tight-lidded petri dish and transported from field site to the laboratory. In the laboratory, the filter is transferred to M-Endo growth medium and incubated at 35 C for 24 hours. Sheen colonies are counted as total coliforms per 100 ml.

2.2 Scope and Application: The total coliform test can be used for any type of water or wastewater, but since the development of the fecal coliform procedure there has been increasing use of this more specific test as an indicator of fecal pollution. However, the total coliform test remains the primary indicator of bacteriological quality for potable water, distribution system waters, and public water supplies because a broader measure of pollution is desired for these waters. It is also a useful measure in shellfish-raising waters.

Although the majority of water and wastewater samples can be examined for total coliforms by the single-step MF procedure, coliforms may be suppressed by high background organisms, and potable water samples may require the two-step method.

If the membrane filtration method is used to measure total coliforms in chlorinated secondary or tertiary sewage effluents the two-step enrichment procedure is required. However, it may be necessary to use the MPN method because of high solids in the wastes or toxicity from an industrial waste (see Part II-D, this Manual).

The delayed-incubation MF method is useful in survey monitoring or emergency situations when the single step coliform test cannot be performed at the sample site, or when time and temperature limits for sample storage cannot be met. The method eliminates field processing and equipment needs. Also, examination at a central laboratory permits confirmation and biochemical identification of the organisms as necessary. Consistent results have been obtained with this method using water samples from a variety of sources (1, 2). The applicability of this method for a specific water source must be determined in preliminary studies by comparison with the standard MF method.

2.3 Apparatus and Materials

2.3.1 Water jacket, air, or heat sink incubator that maintains 35 ± 0.5 C. Temperature is checked against an NBS certified thermometer or equivalent. Incubator must have humidity control if loose-lidded pertri dishes are used. See Part II-B, 1.2.

2.3.2 A binocular (dissection) microscope, with magnification of 10 or 15×, and a daylight type fluorescent lamp angled to give maximum sheen appearance.

2.3.3 Hand tally.

2.3.4 Pipet container of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Sterile 50–100 ml graduated cylinders covered with aluminum foil or kraft paper,

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper. Portable field filtration units are available.

2.3.7 Vacuum source.

2.3.8 Vacuum filter flask with appropriate tubing. Filter manifolds which hold a number of filter bases can also be used.

2.3.9 Ultraviolet sterilizer for MF filtration units (optional).

2.3.10 Safety trap flask between the filter flask and the vacuum source.

2.3.11 Forceps with smooth tips.

2.3.12 Methanol or ethanol, 95%, in small vial, for flaming forceps.

2.3.13 Bunsen/Fisher burner or electric incinerator.

2.3.14 Sterile TD bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.15 Sterile petri dishes with tight-fitting lids, 50 × 12 mm or loose-fitting lids 60 × 15 mm, glass or plastic.

2.3.16 Dilution bottles (milk dilution), pyrex, marked at 99 ml volume, screw cap with neoprene rubber liner.

2.3.17 Membrane filters, white, grid-marked, 47 mm diameter, with 0.45 µm ± 0.02 µm pore size, or other pore size, as recommended by manufacturer for water analyses.

2.3.18 Absorbent pads.

2.3.19 Inoculation loops, at least 3 mm diameter, or needles, nichrome or platinum wire, 26 B&S gauge, in suitable holder.

2.3.20 Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

2.3.21 Shipping tubes, labels, and packing materials for mailing delayed incubation plates.

2.4 Media: Media are prepared in presterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw caps.

2.4.1 M-Endo broth or agar (See Part II-B, 5.2.2).

2.4.2 LES M-Endo agar (See Part II-B, 5.2.4).

2.4.3 Lauryl tryptose broth (See Part II-B, 5.3.1).

2.4.4 Brilliant green lactose bile broth (See Part II-B, 5.3.2).

2.4.5 M-Endo holding medium (See Part II-B, 5.2.3).

2.4.6 Sodium benzoate, U.S.P., for use in the delayed incubation procedure (See Part II-B, 5.2.3).

2.4.7 Cycloheximide (Actidione – Upjohn, Kalamazoo, MI) for use as antifungal agent in delayed incubation procedure (See Part II-B, 5.2.3).

2.5 Dilution Water (See Part II-B, 7 for preparation).

2.5.1 Sterile dilution water dispensed in 99 ± 2 ml amounts in screw-capped dilution bottles.

2.5.2 Sterile dilution water prepared in 1 liter or larger volumes for wetting membranes before addition of small sample volumes and for rinsing the funnel after sample filtration.

2.6 Procedure: Refer to the general procedure in Part II-C for more complete details.

2.6.1 Single-Step Procedure

(a) Prepare the M-Endo broth, M-Endo agar or LES M-Endo agar as directed in Part II-B.

(b) Place one sterile absorbent pad in the bottom half of each petri dish. Pipet 1.8–2.0 ml M-Endo broth onto the pad to saturate it. Pour off excess broth. Alternatively, pipet 5–6 ml of melted agar into each dish (2–3 mm) and allow to harden before use. Mark dishes and bench forms with sample identities and volumes.

(c) Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times and measure the desired volume of sample into the funnel. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to the filter before adding sample.

It is desirable to filter the largest possible sample volumes for greatest accuracy. However, if past analyses of specific samples have resulted in confluent growth, "too numerous to count" membranes, or lack of sheen from excessive turbidity, additional samples should be collected and filtration volumes adjusted to provide isolated colonies from smaller volumes. See 2.7.2 in this Section for details on adjusting sample volumes for potable waters.

The suggested method for measuring sample volumes is described in Part II-C, 3.4.6.

(e) Filter sample and rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off the vacuum and remove the funnel from the filter base. Aseptically remove the membrane filter from the filter base and place grid-side up on the agar or pad.

(f) Filter samples in order of increasing sample volume, filter potable waters first.

(g) If M-Endo broth is used, place the filter on an absorbent pad saturated with the broth. Reseat the membrane, if air bubbles occur, as evidenced by non-wetted areas on the membrane. Invert dish and incubate for 24 $\pm$ 2 hours at 35 $\pm$ 0.5 C in an atmosphere with near saturated humidity.

(h) If M-Endo agar or LES M-Endo agar is used, place the inoculated filter directly on the agar surface. Reseat the membrane if bubbles occur. Invert the dish and incubate for 24 $\pm$ 2 hours at 35 $\pm$ 0.5 C in an atmosphere with near saturated humidity.

(i) If tight-lidded dishes are used, there is no requirement for near-saturated humidity.

(j) After incubation remove the dishes from the incubator and examine for sheen colonies.

(k) Proceed to 2.7 for Counting and Recording Colonies.

2.6.2 Two-Step Enrichment Procedure

(a) Place a sterile absorbent pad in the top of each petri dish.

(b) Prepare lauryl tryptose broth as directed in Part II-B. Pipet 1.8–2.0 ml lauryl tryptose broth onto the pad to saturate it. Pour off excess broth.

(c) Place a sterile membrane filter on the filter holder, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times to obtain uniform distribution of bacteria. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to filter before adding sample.

(e) Filter samples in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations. The methods of measurement and dispensation of the sample into the funnel are given in Part II-C, 3.4.6.

(f) Turn on the vacuum to filter the sample through the membrane, rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off vacuum and remove funnel from base.

(g) Remove the membrane filter aseptically from the filter base and place grid-side up on the pad in the top of the petri dish. Reseat MF if air bubbles are observed.

(h) Incubate the filter in the petri dish without inverting for 1 1/2 – 2 hours at 35 $\pm$ 0.5 C in an atmosphere of near saturated humidity. This completes the first step in the Two-Step Enrichment Procedure.

(i) Prepare M-Endo broth, M-Endo agar, or LES M-Endo agar as directed in Part II-B.

If M-Endo broth is used, place a new sterile absorbent pad in the bottom half of the dish and saturate with 1.8-2.0 ml of the M-Endo broth. Transfer the filter to the new pad. Reseat MF if air bubbles are observed. Remove the used pad and discard.

If M-Endo or LES M-Endo agar is used, pour 5-6 ml of agar into the bottom of each petri dish and allow to solidfy. The agar medium can be refrigerated for up to two weeks.

(j) Transfer the filter from the lauryl tryptose broth onto the Endo medium. Reseat if air bubbles are observed.

(k) Incubate dishes in an inverted position for an additional 20–22 hours at 35 ± 0.5 C. This completes the second step in the Two-Step Enrichment Procedure.

(l) Proceed to 2.7 Counting and Recording.

2.6.3 Delayed Incubation Procedure

(a) Prepare the M-Endo Holding Medium or LES Holding Medium as outlined in Part II-B, 5.2.3 or 5.2.5. Saturate the sterile absorbent pads with about 2.0 ml of holding broth. Pour off excess broth. Mark dishes and bench forms with sample identity and volumes.

(b) Using sterile forceps place a membrane filter on the filter base grid side up.

(c) Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

(d) Shake the sample vigorously about 25 times and measure into the funnel with the vacuum off. If the sample is < 10 ml, add 10 ml of sterile dilution water to the membrane filter before adding the sample.

(1) Select sample volumes based on previous knowledge to produce counts of 20–80 coliform colonies. See Table II-C-1.

(2) Follow the methods for sample measurement and dispensation given in Part II-C, 3.4.6

(e) Filter the sample through the membrane and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

(f) Turn off the vacuum and remove the funnel from the base of the filter unit.

(g) Aseptically remove the membrane filter from the filter base and place grid side up on an absorbent pad saturated with M-Endo Holding Medium or LES Holding Medium.

(h) Place the culture dish in shipping container and send to the examining laboratory. Coliform bacteria can be held on the holding medium for up to 72 hours with little effect on the final counts. The holding period should be kept to a minimum.

(i) At the examining laboratory remove the membrane from the holding medium, place it in another dish containing M-Endo broth or agar medium, and complete testing for coliforms as described above under 2.6.1.

2.7 Counting and Recording Colonies: After incubation, count colonies on those membrane filters containing 20–80 golden-green metallic surface sheen colonies and less than 200 total bacterial colonies. A binocular (dissection) microscope with a magnification of 10 or 15× is recommended. Count the colonies according to the general directions given in Part II-C, 3.5.

2.7.1 The following general rules are used in calculating the total coliform count per 100 ml of sample. Specific rules for analysis and counting of water supply samples are given in 2.7.2.

(a) Countable Membranes with 20–80 Sheen Colonies, and Less Than 200 Total Bacterial Colonies: Select the plate counts to be used according to the rules given in Part II-C, 3.6, and calculate the final value using the formula.

Total Coliforms/100 ml:

$$\frac{\text{No. of Total Coliform Colonies Counted}}{\text{Volume in ml of Sample Filtered}} \times 100$$

(b) Counts Greater Than the Upper Limit of 80 Colonies: All colony counts are above the recommended limits. For example, sample volumes of 1, 0.3, and 0.01 ml are filtered to

produce total coliform colony counts of TNC, 150, and 110 colonies.

Use the count from the smallest filtration volume and report as a greater than count/100 ml. In the example above:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000$$

or > 1,100,000 coliforms/100 ml.

(c) Membranes with More Than 200 Total Colonies (Coliforms plus Non-coliforms).

(1) Estimate sheen colonies if possible, calculate total coliform density as in (a) above.

Report as: Estimated Count/100 ml.

(2) If estimate of sheen colonies is not possible, report count as Too Numerous to Count (TNTC).

(d) Membranes with Confluent Growth

Report as: Confluent Growth and specify the presence or absence of sheen.

2.7.2 Special Rules for Potable Waters

(a) Countable Membranes with 0-80 Sheen Colonies, and Less than 200 Total Colonies

Count the sheen colonies per volume filtered. Calculate and report the number of Total Coliforms/100 ml.

(b) Uncountable Membranes for Potable Water Samples

If 100 ml portions of potable water samples cannot be tested because of high background counts or confluency, multiple volumes of less than 100 ml can be filtered. For example, if 60 colonies appear on the surface of one membrane through which a 50 ml portion of the sample was passed, and 50 colonies on a second membrane through which a second 50 ml portion of the sample was passed, the colonies are totaled and reported as 110 total coliforms per 100 ml.

If filtration of multiple volumes of less than 100 ml still results in confluency or high background count, the coliforms may be present but suppressed. These samples should be analyzed by the MPN Test. This MPN check should be made on at least one sample for each problem water once every three months.

(c) Membranes with Confluent Growth

For potable water samples, confluence requires resampling and retesting.

(d) Verification. Because unsatisfactory samples from public water supplies containing 5 or more coliform colonies must be verified, at least 5 colonies need to be verified for each positive sample. Reported counts are adjusted based on verification.

(e) Quality control procedures are specified by EPA under the law, and described in Appendix C in this Manual.

2.7.3 Reporting Results: Report total coliform densities per 100 ml of sample. See Figure II-C-3 for an example of a bench form for reporting results. A discussion on significant figures is given in Part II-C, 2.8.

2.8 Precision and Accuracy: There are no established precision and accuracy data available at this time.

3. Verification

Verification of total coliform colonies from M-Endo type media validates sheen as evidence of coliforms. Verification of representative numbers of colonies may be required in evidence gathering or for quality control procedures. The verification procedure follows:

3.1 Using a sterile inoculating needle, pick growth from the centers of at least 10 well-isolated sheen colonies (5 sheen colonies per plate for potable waters). Inoculate each into a tube of lauryl tryptose broth and incubate 24-48 hours at 35 C ± 0.5 C. Do not transfer exclusively into brilliant green bile lactose broth. However, colonies may be transferred to LTB and BGLB simultaneously.

3.2 At the 24 and 48 hour readings, confirm gas-positive lauryl tryptose broth tubes by inoculating a loopful of growth into brilliant green lactose bile broth and incubate for 24–48 hours at 35 ± 0.5 C. Cultures that are positive in BGLB are interpreted as verified coliform colonies (see Figure III-B-1).

3.3 If questionable sheen occurs, the worker should also verify these colonies.

4. Most Probable Number (MPN) Method

4.1 Summary: This method detects and estimates the total coliforms in water samples by the multiple fermentation tube technique. The method has three stages: the Presumptive, the Confirmed, and the Completed Tests. In the Presumptive Test, a series of lauryl tryptose broth fermentation tubes are inoculated with decimal dilutions of the sample. The formation of gas at 35 C within 48 hours constitutes a positive Presumptive Test for members of the total coliform group. However, the MPN must be carried through the Confirmed Test for valid results. In this test, inocula from positive Presumptive tubes are transferred to tubes of brilliant green lactose bile (BGLB) broth. The BGLB medium contains selective and inhibitive agents to suppress the growth of all non-coliform organisms. Gas production after incubation for 24 or 48 hours at 35 C constitutes a positive Confirmed Test and is the point at which most MPN tests are terminated. The Completed Test begins with streaking inoculum from the positive BGLB tubes onto EMB plates and incubating the plates for 24 hours at 35 C. Typical and atypical colonies are transferred into lauryl tryptose broth fermentation tubes and onto nutrient agar slants. Gas formation in the fermentation tubes and presence of gram-negative rods constitute a positive Completed Test for total coliforms. See Figure III-B-2. The MPN per 100 ml is calculated from the MPN table based upon the Confirmed or Completed test results.

4.2 Scope and Application

4.2.1 Advantages: The MPN procedure is a tube-dilution method using a nutrient-rich medium, which is less sensitive to toxicity and supports the growth of environmentally-stressed organisms. The method is applicable to the examination of total coliforms in chlorinated primary effluents and under other stressed conditions. The multiple-tube procedure is also better suited for the examination of turbid samples, muds, sediments, or sludges because particulates do not interfere visibly with the test.

4.2.2 Limitations: Certain non-coliform bacteria may suppress coliforms or act synergistically to ferment lauryl tryptose broth and yield false positive results. A significant number of false positive results can also occur in the brilliant green bile broth when chlorinated primary effluents are tested, especially when stormwater is mixed with the sewage (3). False negatives may occur with waters containing nitrates (4). False positives are more common in sediments.

4.3 Apparatus and Materials

4.3.1 Water bath or air incubator set at 35 ± 0.5 C.

4.3.2 Pipet containers of stainless steel, aluminum, or pyrex glass for glass pipets.

4.3.3 Inoculation loops, at least 3 mm diameter and needles of nichrome or platinum wire, 26 B & S gauge, in suitable holders.

4.3.4 Disposable sterile applicator sticks or plastic loops as alternatives to inoculating loops.

4.3.5 Compound microscope, oil immersion.

4.3.6 Bunsen/Fisher burner or electric incinerator unit.

4.3.7 Sterile TD Mohr or bacteriological pipets, glass or plastic, of appropriate size.

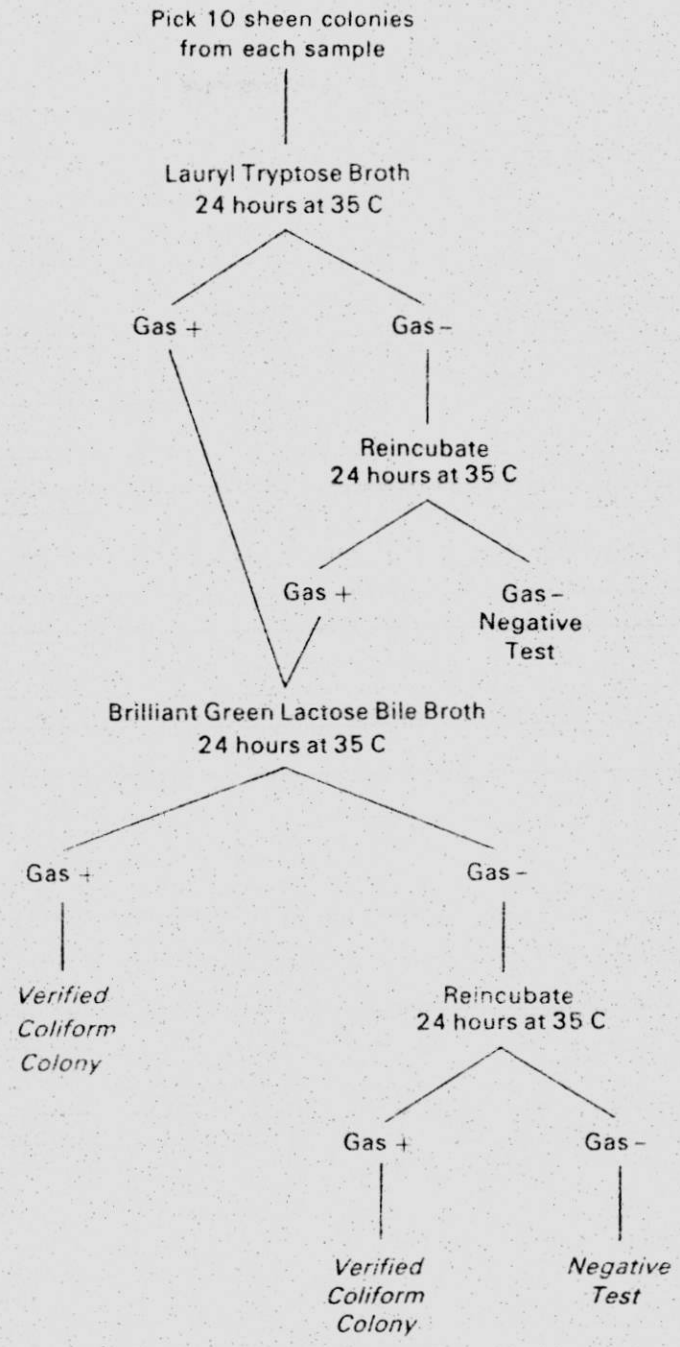

FIGURE III-B-1. Verification of Total Coliform Colonies on the Membrane Filter

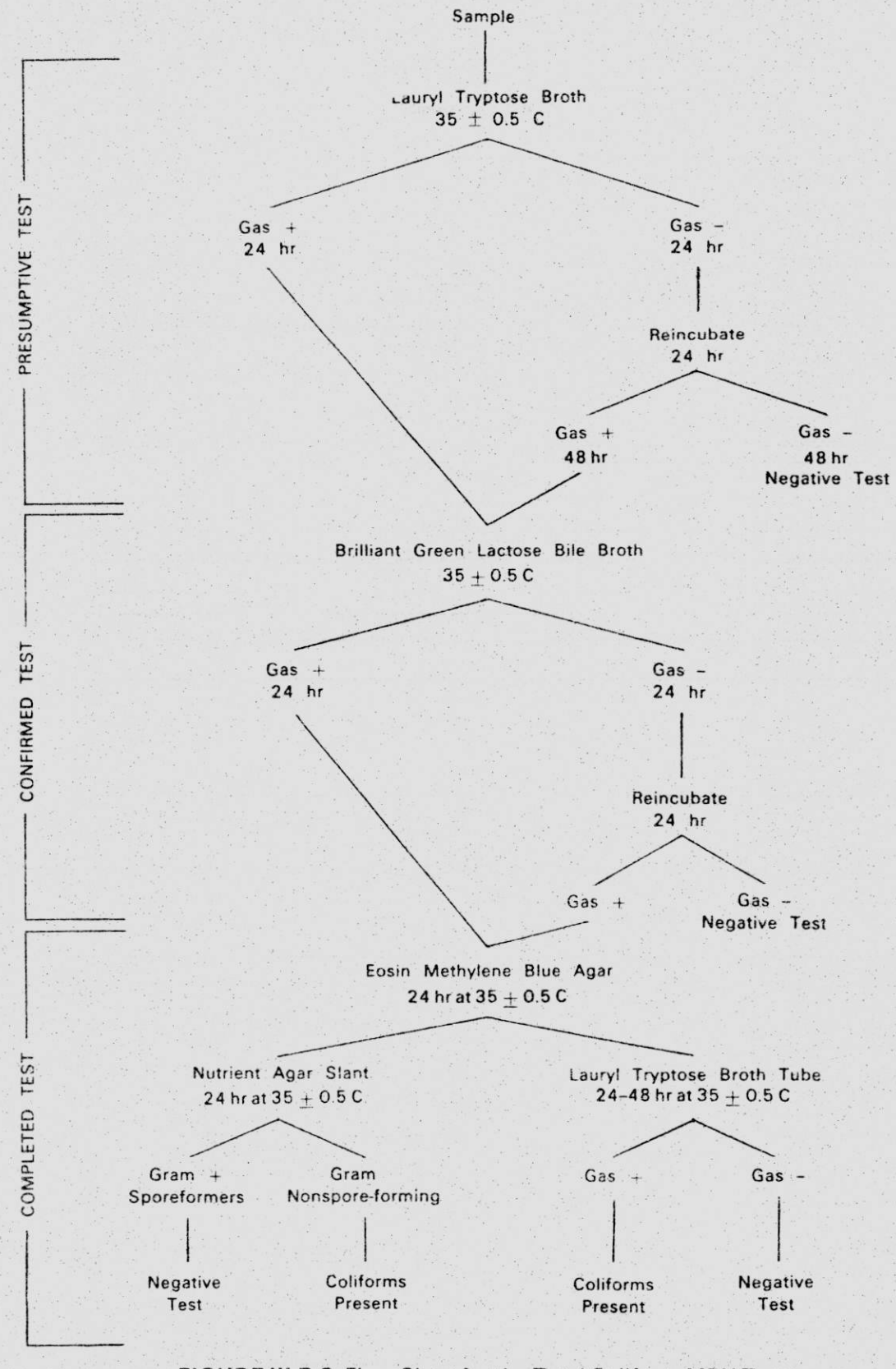

FIGURE III-B-2. Flow Chart for the Total Coliform MPN Test

4.3.8 Pyrex culture tubes, 150 × 25 mm or 150 × 20 mm, containing inverted fermentation vials, 75 × 10 mm with caps.

4.3.9 Culture tube racks to hold fifty, 25 mm diameter tubes.

4.3.10 Dilution bottles (milk dilution) pyrex glass, 99 ml volume, screw cap with neoprene rubber liners.

4.4 Media

4.4.1 Presumptive Test: Lauryl tryptose broth. See Part II-B, 5.3.1 . Lactose broth is not used because of false positive reactions.

4.4.2 Confirmed Test: Brilliant green bile broth. (See Part II-B, 5.3.2).

4.4.3 Completed Test:

(a) Eosin methylene blue agar (see Part II-B, 5.3.3).

(b) Nutrient agar or plate count agar slants (see Part II-B, 5.1.1 and 5.1.5).

4.5 Dilution Water: Sterile dilution water dispensed in 99 ± 2 ml amounts preferably in screw-capped bottles. (See Part II-B, 7).

4.6 Procedure: Part II-C describes the general MPN procedure in detail.

4.6.1 Prepare the media for Presumptive, Confirmed or Completed Tests selected. (See Part II-B, 5.3).

4.6.2 Presumptive Test (See Figure III-B-2): To begin the Presumptive Test, arrange fermentation tubes of lauryl tryptose broth in rows of 5 tubes each in the tube rack. Select sample volumes and clearly label each bank of tubes to identify the sample and volume inoculated.

(a) For potable waters, five portions of 10 ml each or five portions of 100 ml each are used.

(b) For relatively-unpolluted waters the sample volumes for the five rows might be 100, 10, 1, 0.1 and 0.01 ml, respectively; the latter two volumes delivered as dilutions of original sample.

(c) For known polluted waters the initial sample inoculations might be 0.1, 0.01, 0.001, 0.0001, and 0.00001 ml of original sample delivered as dilutions into successive rows each containing five replicate volumes. This series of sample volumes will yield determinate results from a low of 200 to a high of 16,000,000 organisms per 100 ml.

(d) Shake the sample and dilutions vigorously about 25 times. Inoculate each 5-tube row with replicate sample volumes in increasing decimal dilutions and incubate at 35 C ± 0.5 C.

(e) After 24 ± 2 hours incubation at 35 C, gently agitate the tubes in the rack and examine the tubes for gas. Any amount of gas constitutes a positive test. If there is no gas production in the tubes, reincubate for an additional 24 hours and reexamine for gas. Positive Presumption tubes are submitted directly to the Confirmed Test. Results are recorded on laboratory bench forms.

(f) If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

(g) If The Presumptive Test tubes are gas-negative after 48 ± 3 hours, they are discarded and the results recorded as negative Presumptive Tests. Positive Presumptive tubes are verified by the Confirmed Test.

(h) If the fecal colifrom test is to be run, (Part III-C), the analyst can inoculate growth from positive Presumptive Test tubes into EC medium at the same time as he inoculates the Confirmed Test Medium.

4.6.3 Confirmed Test (See Figure III-B-2)

(a) Carefully shake each positive Presumptive tube. With a sterile 3 mm loop or a sterile

applicator stick, transfer growth from each tube to BGLB. Gently agitate the tubes to mix the inoculum and incubate at 35 ± 0.5 C.

(b) After 24 ± 2 hours incubation at 35 C examine the tubes for gas. Any amount of gas in BGLB constitutes a positive Confirmed Test. If there is no gas production in the tubes (negative test) reincubate tubes for an additional 24 hours. Record the gas-positive and gas-negative tubes. Hold the positive tubes for the Completed Test if required for quality control or for checks on questionable reactions.

(c) After 48 ± 3 hours reexamine the Confirmed Test Tubes. Record the positive and negative tube results. Discard the negative tubes and hold the positive tubes for the Completed Test if required as in (b) above.

(d) In routine practice most sample analyses are terminated at the end of the Confirmed Test. However, the Confirmed Test data should be verified by carrying 5% of Confirmed Tests with a minimum of one sample per test run through the Completed Test.

(e) For certification of water supply laboratories, the MPN test is carried to completion (except for gram stain) on 10 percent of positive confirmed samples and at least one sample quarterly.

4.6.4 Completed Test (See Figure III-B-2)

Positive Confirmed Test cultures may be subjected to final Completed Test identification through application of further biochemical and culture tests, as follows:

(a) Streak one or more EMB agar plates from each positive BGLB tube. Incubate the plates at 35 ± 0.5 C for 24 ± 2 hours.

(b) Transfer one or more well-isolated typical colonies (nucleated with or without a metallic sheen) to lauryl tryptose broth fermentation tubes and to nutrient or plate count agar slants. Incubate the slants for 24 ± 2 or 48 ± 3 hours at 35 ± 0.5 C. If no typical colonies are present, pick and inoculate at least two atypical (pink, mucoid and unnucleated) colonies into lauryl tryptose fermentation tubes and incubate tubes for up to 48 ± 3 hours.

(c) The formation of gas in any amount in the fermentation tubes and presence of gram negative rods constitute a positive Completed Test for total coliforms.

4.6.5 Special Considerations for Potable Waters

Sample Size – For potable waters the standard sample shall be five times the standard portion which is either 10 milliliters or 100 milliliters as described in 40 CFR 141 (5).

Confirmation – If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

Completion — In water supply laboratories, 10% of all samples and at least one sample quarterly must be carried to completion but no gram stain of cultures is required.

4.7 Calculations: The results of the Confirmed or Completed Test may be obtained from the MPN table based on the number of positive tubes in each dilution. See Part II-C, 4.9 for details on calculation of MPN results.

4.7.1 Table II-C-4 illustrates the MPN index and 95% Confidence Limits for combinations of positive and negative results when five 10 ml, five 1.0 ml, and five 0.1 ml volumes of sample are tested.

4.7.2 Table II-C-5 provides the MPN indices and limits for the five tube, single volumes used for potable water supplies.

4.7.3 When the series of decimal dilutions is other than those in the tables select the MPN value from Table II-C-4 and calculate according to the following formula:

$$\text{MPN (From Table)} \times \frac{10}{\text{Largest Volume Tested}} = \text{MPN/100 ml}$$

4.8 Reporting Results: Report the MPN values per 100 ml of sample. See an example of a report form in Figures III-D-2 and III-D-3.

4.9 Precision and Accuracy: The precision of the MPN value increases with increased numbers of replicates tested. A five tube, five dilution MPN is recommended for natural and waste waters. Only a five tube, single volume series is required for potable waters.

5. Differentiation of the Coliform Group by by Further Biochemical Tests

5.1 Summary: The differentiation of the members of the coliform group into genera and species is based on additional biochemical and cultural tests (see Table III-B-1). These tests require specific training for valid results.

5.2 Apparatus and Materials

5.2.1 Incubator set at 35 ± 0.5 C.

5.2.2 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

5.2.3 Inoculation loop, 3 mm diameter and needle.

5.2.4 Bunsen/Fisher type burner or electric incinerator.

5.2.5 Sterile TD Mohr and bacteriological pipets, glass or plastic, of appropriate volumes.

5.2.6 Graduates, 25 - 500 ml.

5.2.7 Test tubes, 100 × 13 mm or 150 × 20 mm with caps, in racks.

5.2.8 Reagents

(a) Indole Test Reagent: Dissolve 5 grams para-dimethylamino benzaldehyde in 75 ml isoamyl (or normal amyl) alcohol, ACS grade, and slowly add 25 ml conc HCl. The reagent should be yellow and have a pH below 6.0. If the final reagent is dark in color it should be discarded.

Some brands are not satisfactory and others become unsatisfactory after aging. Both amyl alcohol and benzaldehyde compound should be purchased in as small amounts as will be consistent with the volume of work anticipated. Store the reagent in the dark in a brown bottle with a glass stopper.

(b) Methyl Red Test Reagent: Dissolve 0.1 gram methyl red in 300 ml of 95% ethyl alcohol and dilute to 500 ml with distilled water.

(c) Voges-Proskauer Test Reagents

(1) Naphthol solution: Dissolve 5 grams purified alphanaphthol (melting point 92.5 C or higher) in 100 ml absolute ethyl alcohol. This solution must be freshly prepared each day.

(2) Potassium hydroxide solution: Dissolve 40 grams KOH in 100 ml distilled water.

(d) Oxidase Test Reagents

(1) Reagent A: Weigh out 1 gram alphanapthol and dissolve in 100 ml of 95% ethanol.

(2) Reagent B: Weigh out 1 gram paraaminodimethylaniline HCl (or oxylate) and dissolve in 100 ml of distilled water. Prepare frequently and store in refrigerator.

5.3 Media

5.3.1 Tryptophane broth for demonstrating indole production in the Indole Test. (See Part II-B, 5.1.9 (a) for preparation).

5.3.2 MR-VP broth (buffered glucose) to demonstrate acid production by methyl red color change in the Methyl Red Test and to demonstrate acetyl methyl carbinol production in the Voges-Proskauer test. (See Part II-B, 5.1.9 (b) for preparation).

5.3.3 Simmon's Citrate Agar to demonstrate utilization of citrate as a sole source of carbon. (See Part II-B, 5.1.9 (c) for preparation).

TABLE III-B-1

Differentiation of the Coliform and Related Organisms Based Upon Biochemical Reactions

Bacterium	Tests								
	Indole	Methyl Red	Voges-Proskauer	Citrate	Cytochrome Oxidase	Ornithine Decarboxylase	Lysine Decarboxylase	Arginine Dehydrolase	Motility
Escherichia coli	+	+	–	–	–	V	V	V	±
Citrobacter freundii	–	+	–	+	–	V	V	V	+
Enterobacter aerogenes	–	–	+	+	–	+	+	–	+
Klebsiella	±	–	+	+	–	–	+	–	–
Pseudomonas	–	–	–	+	+	–	–	–(+)[1]	+
Aeromonas	+	–	+	+	+	+	–	+	+

V = variable

( )[1] = reaction of *P. aeruginosa*

5.3.4 Nutrient agar slant for oxidase test. (See Part II-B, 5.1.1 for preparation).

5.3.5 Decarboxylase medium base containing lysine HCl, arginine HCl or ornithine HCl to demonstrate utilization of the specific amino acids. (See Part II-B, 5.5.14 for preparation).

5.3.6 Motility test medium (Edwards and Ewing). (See Section II-B, 5.1.10 for preparation).

5.3.7 Multitest Systems (optional to Single Test Series)

(a) API Enteric 20 (Analytab Products, Inc.).

(b) Enterotube (Roche Diagnostics).

(c) Inolex (Inolex Biomedical Division of Wilson Pharmaceutical and Chemical Corp.).

(d) Minitek (Baltimore Biological Laboratories, Bioquest).

(e) Pathotec Test Strips (General Diagnostics Division of Warner-Lambert Company).

(f) r/b Enteric Differential System (Diagnostic Research, Inc.).

5.4 Procedure

5.4.1 Biochemical tests should always be performed along with positive and negative controls. See Table IV-A-5.

5.4.2 Indole Test

(a) Inoculate a pure culture into 5 ml of tryptophane broth.

(b) Incubate the tryptophane broth at 35 ± 0.5 C for 24 ± 2 hours and mix well.

(c) Add 0.2–0.3 ml test reagent to the 24 hour culture, shake and allow the mixture to stand for 10 minutes. Observe and record the results.

(d) A dark red color in the amyl alcohol layer on top of the culture is a positive indole test; the original color of the reagent, a negative test. An orange color may indicate the presence of skatole and is reported as a ± reaction.

5.4.3 Methyl Red Test

(a) Inoculate a pure culture into 10 ml of buffered glucose broth.

(b) Incubate for 5 days at 35 C.

(c) To 5 ml of the five day culture, add 5 drops of methyl red indicator.

(d) A distinct red color is positive and distinct yellow, negative. Orange color is dubious, may indicate a mixed culture and should be repeated.

5.4.4 Voges Proskauer Test: This procedure detects the production of acetyl methyl carbinol which in the presence of alphanapthol and potassium hydroxide develops a reddish color.

(a) Use a pure culture to inoculate 10 ml of buffered glucose broth or 5 ml of salt peptone glucose broth or use the previously inoculated buffered glucose broth from the Methyl Red Test.

(b) Incubate the inoculated salt peptone glucose broth or the buffered glucose broth at 35 ± 0.5 C for 48 hours.

(c) Add 0.6 ml naphthol solution and 0.2 ml KOH solution to 1 ml of the 48 hour salt peptone or buffered glucose broth culture in a separate clean test tube. Shake vigorously for 10 seconds and allow the mixture to stand for 2–4 hours.

(d) Observe the results and record. A pink to crimson color is a positive test. Do not read after 4 hours. A negative test may develop a copper or faint brown color.

5.4.5 Citrate Test

(a) Lightly inoculate a pure culture into a tube of Simmon's Citrate Agar, using a needle to stab, then streak the medium. Be careful not to carry over any nutrient material.

(b) Incubate at 35 C for 48 hours.

(c) Examine agar tube for growth and color change. A distinct Prussian blue color in the presence of growth indicates a positive test; no color change is a negative test.

5.4.6 Cytochrome Oxidase Test (Indophenol): The cytochrome oxidase test can be done with commercially-prepared paper strips or on a nutrient agar slant as follows:

(a) Inoculate nutrient agar slant and incubate at 35 C for 18–24 hours. Older cultures should not be used.

(b) Add 2–3 drops of reagent A and reagent B to the slant, tilt to mix and read reaction within 2 minutes.

(c) Strong positive reaction (blue color slant or paper strip) occurs in 30 seconds. Ignore weak reactions that occur after 2 minutes.

5.4.7 Decarboxylase Tests (lysine, arginine and ornithine)

(a) The complete decarboxylase test series requires tubes of each of the amino acids and a control tube containing no amino acids.

(b) Inoculate each tube lightly.

(c) Add sufficient sterile mineral oil to the broths to make 3–4 mm layers on the surface and tighten the screw caps.

(d) Incubate for 18–24 hours at 35 C and read. Negative reactions should be reincubated up to 4 days.

(e) Positive reactions are purple and negative reactions are yellow. Read the control tube without amino acid first; it must be yellow for the reactions of the other tubes to be valid. Positive purple tubes must have growth as evidenced by turbidity because uninoculated tubes are also purple; nonfermenters may remain alkaline throughout incubation.

5.4.8 Motility Test

(a) Stab-inoculate the center of the tube of Motility Test Medium to at least half depth.

(b) Incubate tubes 24–48 hours at 35 C.

(c) Examine tubes for growth. If negative, reincubate at room temperature for 5 more days.

(d) Non-motile organisms grow only along the line of inoculation. Motile organisms grow outward from the line of inoculation and spread throughout the medium producing a cloudy appearance.

(e) Addition of 2, 3, 5 triphenyl tetrazolium chloride (TTC) will aid recognition of motility. Growth of microorganisms reduces TTC and produces red color along the line of growth.

5.4.9 Additional Biochemical Tests: If other biochemical tests are necessary to further identify enteric bacteria, for example specific carbohydrate fermentation, see the Table III-E-5, Biochemical Characteristics of Enterobacteriaceae.

5.4.10 Multitest Systems: Multitest systems are available which use tubes containing agar media that provide numerous biochemical tests, plastic units containing a series of dehydrated media, media-impregnated discs and reagent-impregnated paper strips. Some of the systems use numerical codes to aid identification. Others provide computerized identification of bacteria. A number of independent investigators have compared one or more multitest systems with conventional or traditional biochemical tests. Some of the earlier systems have been improved. Most of the recent studies report the correct identification of high percentages of isolates. The systems are described in Part III-E, 5.6.

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PART III. ANALYTICAL METHODOLOGY

Section C Fecal Coliform Methods

The direct membrane filter (MF), the delayed-incubation MF and the multiple-tube, most probable number (MPN) methods can be used to enumerate fecal coliforms in water and wastewater. For a general description of the fundamental laboratory techniques refer to Part II-C. The method chosen depends upon the characteristics of the sample. The Section is divided as follows:

1. **Definition of the Fecal Coliform Group**
2. **Direct Membrane Filter (MF) Method**
3. **Delayed-Incubation Membrane Filter Method**
4. **Verification**
5. **Most Probable Number (MPN) Method**

1. Definition of the Fecal Coliform Group

1.1 The fecal coliforms are part of the total coliform group. They are defined as gram-negative nonspore-forming rods that ferment lactose in 24 $\pm$ 2 hours at 44.5 $\pm$ 0.2 C with the production of gas in a multiple-tube procedure or produce acidity with blue colonies in a membrane filter procedure.

1.2 The major species in the fecal coliform group is *Escherichia coli*, a species indicative of fecal pollution and the possible presence of enteric pathogens.

2. Direct Membrane Filter (MF) Method

2.1 Summary: An appropriate volume of a water sample or its dilution is passed through a membrane filter that retains the bacteria present in the sample. The filter containing the microorganisms is placed on an absorbent pad saturated with M-FC broth or on M-FC agar in a petri dish. The dish is incubated at 44.5 C for 24 hours. After incubation, the typical blue colonies are counted under low magnification and the number of fecal coliforms is reported per 100 ml of original sample.

2.2 Scope and Application

2.2.1 Advantages: The results of the MF test are obtained in 24 hours. Up to 72 hours are required for the multiple-tube fermentation method. The M-FC method provides direct enumeration of the fecal coliform group without enrichment or subsequent testing. Over 93% of the blue colonies that develop in this test using M-FC medium at the elevated temperature of 44.5 C $\pm$ 0.2 C are reported to be fecal coliforms (1). The test is applicable to the examination of lakes and reservoirs, wells and springs, public water supplies, natural bathing waters, secondary non-chlorinated effluents from sewage treatment plants, farm ponds, stormwater runoff, raw municipal sewage, and feedlot runoff. The MF test has been used with varied success in marine waters.

2.2.2 Limitations: Recent data (2, 3) indicate that the single-step MF fecal coliform procedure may produce lower results than those obtained with the fecal coliform

multiple-tube procedure, particularly for chlorinated effluents. Since chlorination stresses fecal coliforms and significantly reduces recovery, this method should not be used with chlorinated wastewater. Disinfection and toxic materials such as metals, phenols, acids or caustics also affect recovery of fecal coliforms on the membrane filter. Any decision to use this test for stressed microorganisms requires parallel MF/MPN evaluation based on the procedure described in Part IV-C, 3.

Recently-proposed solutions to problems of lower recovery (2, 4, 5, 6) include the use of two-step incubation, two-step incubation overlay and/or enrichment techniques and modification of membrane filter structures.

2.3 Apparatus and Materials

2.3.1 Water bath, aluminum heat sink, or other incubator that maintains a stable 44.5 ± 0.2 C. Temperature is checked against an NBS certified thermometer or one of equivalent accuracy.

2.3.2 Binocular (dissecting type) microscope, with magnification of 10–15× and daylight-type fluorescent lamp.

2.3.3 Hand tally.

2.3.4 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Graduated cylinders, covered with aluminum foil or kraft paper before sterilization.

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil and kraft paper.

2.3.7 Vacuum source.

2.3.8 Vacuum filter flask, with appropriate tubing. Filter manifolds which hold a number of filter bases can also be used.

2.3.9 Safety trap flask between the filter flask and the vacuum source.

2.3.10 Forceps with smooth tips.

2.3.11 Ethanol, 95% or methanol, in small vial, for sterilizing forceps.

2.3.12 Bunsen/Fisher burner or electric incinerator.

2.3.13 Sterile TD bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.14 Sterile petri dishes, 50 × 12 mm plastic with tight-fitting lids.

2.3.15 Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volume, screw-cap with neoprene rubber liner.

2.3.16 Membrane filters, white, grid marked, 47 mm diameter, 0.45 ± 0.02 μm pore size or other pore size recommended by manufacturer for water analyses. The Millipore HC MF, not the HA, is recommended.

2.3.17 Absorbent pads.

2.3.18 Water-proof plastic bags.

2.3.19 Inoculation loops, 3 mm diameter, or needle of nichrome or platinum wire, 26 B&S gauge, in suitable holder.

2.3.20 Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

2.3.21 Ultraviolet sterilizer for MF filtration units (optional).

2.4 Media

2.4.1 M-FC broth or agar prepared in presterilized erlenmeyer flasks (See Part II-B, 5.2.1).

2.4.2 Lauryl tryptose broth prepared in 10 ml volumes in fermentation tubes (see Part II-B, 5.3.1) for verification.

2.4.3 EC medium prepared in 10 ml volumes in fermentation tubes (see Part II-B, 5.3.4) for verification.

2.5 Dilution Water (See Part II,B, 7 for preparation).

2.5.1 Sterile buffered dilution water or peptone water dispensed in 99 ± 2 ml amounts in screw-capped dilution bottles.

2.5.2 Sterile buffered water or peptone water prepared in 500 ml or larger volumes for wetting membranes before addition of the sample, and for rinsing the funnel after sample filtration.

2.6 Procedure: The general membrane filter procedure is described in detail in Part II-C.

2.6.1 Prepare the M-FC broth or agar medium as outlined in Part II-B, 5.2.1. Saturate the sterile absorbent pads with about 2.0 ml of broth or add 5–6 ml of M-FC agar to the bottom of each 50 × 12 mm petri dish (to a depth of 2–3 mm). Pour off excess liquid from broth-saturated pads. Mark dishes and bench forms with sample identity and sample volumes.

2.6.2 Using a sterile forceps place a sterile membrane filter on the filter base, grid side up.

Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

2.6.3 Shake the sample vigorously about 25 times and measure the sample into the funnel with the vacuum off. If sample volume is < 10 ml, add 10 ml of sterile dilution water to the filter before adding the sample.

2.6.4 Sample volumes for fecal coliform enumeration in different waters and wastewaters are suggested in Table III-C-1. These volumes should provide the recommended count of 20–60 colonies on a membrane filter. Fecal coliform levels are generally lower than total coliform densities in the same sample; therefore larger volumes are sampled.

2.6.5 Do not filter less than 1.0 ml of undiluted sample.

2.6.6 Filter the sample and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

2.6.7 Turn off the vacuum and remove the funnel from the filter base.

2.6.8 Aseptically remove the membrane filter from the filter base. Place the filter, grid side up, on the absorbent pad saturated with M-FC Broth or on M-FC agar, using a rolling motion to prevent air bubbles.

2.6.9 Incubate the petri dishes for 24 ± 2 hours at 44.5 ± 0.2 C in sealed waterproof plastic bags submerged (with the petri dishes inverted) in a waterbath, or without plastic bag in a heat-sink incubator. MF cultures should be placed in incubator within 30 minutes of filtration.

2.6.10 After 24 hours remove dishes from the incubator and examine for blue colonies.

2.7 Counting and Recording Colonies: Select those plates with 20-60 blue (sometimes greenish-blue) colonies. Non-fecal colonies are gray, buff or colorless and are not counted. Pinpoint blue colonies should be counted and confirmed. The colonies are counted using a microscope of 10-15× and a fluorescent lamp. Use of hand lens or other simple optical devices of lower magnification make difficult the identification and differentiation of typical and atypical blue colonies.

2.7.1 The general counting rules are given in Part II-C, 3.5. The following rules are used in calculating the fecal coliform count per 100 ml of sample:

(a) Countable Membranes with 20–60 Blue Colonies. Count all blue colonies using the formula:

$$\frac{\text{No. of Fecal Coliform Colonies Counted}}{\text{Volume in ml of Sample Filtered}} \times 100 = \text{fecal coliform count/100 ml}$$

TABLE III-C-1

Suggested Range of Sample Volumes for Fecal Coliform Tests
Using the Membrane Filter Method

Sample Source	100	30	10	3	1	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	0.0001
Swimming Pools	X												
Wells, Springs	X	X											
Lakes, Reservoirs	X	X	X										
Water Supply Intakes			X	X	X	X	X						
Bathing Beaches			X	X	X	X	X						
River Water					X	X	X	X	X				
Chlorinated Sewage Effluent					X	X	X	X	X				
Raw Sewage									X	X	X	X	X

For example, if 40 colonies are counted after the filtration of 50 ml of sample, the calculation is:

$$\frac{40}{50} \times 100 = 80 \text{ fecal coliforms/100 ml.}$$

(b) Countable Membranes With Less Than 20 Blue Colonies. Report as: Estimated Count/100 ml and specify the reason.

(c) Membranes With No Colonies. Report the count as: Less than (calculated value)/100 ml, based upon the largest single volume filtered.

For example, if 10, 3 and 1 ml are filtered and all plates show zero counts, select the largest volume, apply the general formula and report the count as a < (less than) value:

$$\frac{1}{10} \times 100 = 10$$

or < 10 fecal coliforms/100 ml.

(d) Countable Membranes With More Than 60 Blue Colonies. Calculate count from highest dilution and report as a > value.

(e) Uncountable Membranes With More Than 60 Colonies. Use 60 colonies as the basis of calculation with the smallest filtration volume, e.g., 0.01 ml:

$$\frac{60}{0.01} \times 100 = 600{,}000$$

Report as: > 600,000 fecal coliforms/100 ml.

2.7.2 Reporting Results. Report fecal coliform densities per 100 ml. See discussion on significant figures in Part II-C, 2.8.1.

2.8 Precision and Accuracy

2.8.1 Ninety-three percent of the blue colonies that develop on M-FC medium at the elevated temperature of 44.5 ± 0.2 C were verified as fecal coliform (1).

2.8.2 Laboratory personnel should be able to duplicate their own colony counts on the same plate within 5%, and the counts of other analysts on the same plate within 10%.

3. Delayed-Incubation M(;brane Filter (MF) Method

3.1 Summary: Bacteria are retained on 0.45 μm filters after passage of selected sample volumes through the filters. The filters are placed on M-VFC broth (a minimum rowth medium) and transported from field sites to the laboratory. In the laboratory, the filters are transferred to the M-FC medium and incubated at 44.5 C for 24 hours. Blue colonies are counted as fecal coliforms.

3.2 Scope and Application

3.2.1 Advantages: The delayed incubation MF method is useful in survey monitoring or emergency situations when the standard fecal coliform test cannot be performed at the sample site, or when time and temperature limits for sample storage cannot be met. The method eliminates field processing and equipment needs. Also, examination at a central laboratory permits confirmation and biochemical identification of the organisms as necessary. Consistent results have been obtained with this method using water samples from a variety of sources (7).

3.2.2 Limitations: The applicability of this method for a specific water source must be determined in preliminary studies by comparison with the standard MF method. For example, limited testing has indicated that the delayed-incubation method is not as effective in saline waters (7).

3.3 Apparatus and Materials

3.3.1 Water bath, aluminum heat sink, or equivalent incubator that maintains a 44.5 ± 0.2 C temperature.

3.3.2 Binocular (dissection) microscope, with magnification 10 or 15×, binocular, wide-field type. A microscope lamp producing

diffuse daylight from cool white fluorescent lamps.

3.3.3 Hand tally.

3.3.4 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

3.3.5 Graduated cylinders, covered with aluminum foil or kraft paper before sterilization.

3.3.6 Sterile unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel wrapped with aluminum foil or kraft paper.

3.3.7 Vacuum source.

3.3.8 Filter flask to hold filter base, with appropriate tubing. Filter manifold to hold a number of filter bases can also be used. In the field, portable field kits are also used.

3.3.9 Safety trap flask between the filtering flask and the vacuum source.

3.3.10 Forceps with smooth tip.

3.3.11 Ethanol, 95% or methanol, in small vial, for sterilizing forceps.

3.3.12 Bunsen/Fisher type burner.

3.3.13 Sterile TD bacteriological or Mohr pipets, glass or plastic, in appropriate volumes.

3.3.14 Sterile petri dishes, 50 × 12 mm plastic with tight-fitting lids.

3.3.15 Dilution bottles (milk dilution), pyrex glass, 99 ml volume, screw-caps with neoprene rubber liners.

3.3.16 Membrane filters, white, grid marked, 47 mm in diameter, 0.45 ± 0.02 μm pore size, or other pore size recommended by the manufacturer for water analyses. The Millipore HC MF, not the HA is recommended.

3.3.17 Shipping tubes, labels, and packing materials for mailing delayed incubation plates.

3.3.18 Ultraviolet sterilizer for MF filtration units (optional).

3.4 Media: The following media are prepared in pre-sterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw-caps:

3.4.1 M-VFC holding media (see Part II-B, 5.2.6).

3.4.2 M-FC broth or agar (see Part II-B, 5.2.1).

3.5 Dilution Water

3.5.1 Sterile dilution water dispensed in 99 ± 2 ml volumes in screw-capped bottles.

3.5.2 Sterile dilution water prepared in large volumes for wetting membranes before the addition of the sample, and for rinsing the funnel after sample filtration.

3.6 Procedure: The general membrane filter procedure is described in detail in Part II-C.

3.6.1 Prepare the M-VFC holding medium as outlined in Part II-B, 5.2.6. Saturate the sterile absorbent pads with about 2.0 ml of M-VFC broth. Pour off excess broth. Mark dishes and bench forms with sample identity and volumes.

3.6.2 Using sterile forceps place a membrane filter on the filter base grid side up.

3.6.3 Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

3.6.4 Shake the sample vigorously about 25 times and measure into the funnel with the vacuum off. If the sample is < 10 ml, add 10 ml of sterile dilution water to the membrane filter before adding the sample.

(a) Sample volumes for fecal coliform enumeration in different waters and wastewaters are suggested in Table III-C-1. These volumes should produce membrane filters with a recommended count of 20–60 colonies.

(b) Follow the methods for sample measurement and dispensation given in Part II-C, 3.4.6.

3.6.5 Filter the sample through the membrane and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

3.6.6 Turn off the vacuum and remove the funnel from the base of the filter unit.

3.6.7 Aseptically remove the membrane filter from the filter base and place grid side up on an absorbent pad saturated with VFC medium.

3.6.8 Place the culture dish in shipping container and send to the examining laboratory. Fecal coliform bacteria can be held on the VFC holding medium for up to 72 hours with little effect on the final counts. The holding period should be kept to a minimum.

3.6.9 At the examining laboratory remove the membrane from the holding medium, place it in another dish containing M-FC broth or agar medium, and complete testing for fecal coliforms as described above under 2.6.

3.7 Counting and Recording Colonies: After the required incubation select those plates with 20-60 blue (sometimes greenish-blue) colonies. Gray to cream colored colonies are not counted. Pin-point blue colonies are not counted unless confirmed. The colonies are enumerated using a binocular microscope with a magnification of 10 or 15×.

Refer to 2.7.1, for rules used in reporting the fecal coliform MF counts.

3.8 Reporting Results: Record densities as fecal coliforms per 100 ml. Refer to Part II-C, 2.8, for discussions on the use of significant figures and rounding off values.

3.9 Precision and Accuracy: As reported in 2.8, this Section.

4. Verification

Verification of the membrane filter test for fecal coliforms establishes the validity of colony differentiation by blue color and provides supporting evidence of colony interpretation. The verification procedure corresponds to the fecal coliform MPN (EC Medium) test.

4.1 Pick from the centers of at least 10 well-isolated blue colonies. Inoculate into lauryl tryptose broth and incubate 24-48 hours at 35 ± 0.5 C.

4.2 Confirm gas-positive lauryl tryptose broth tubes at 24 and 48 hours by inoculating a loopful of growth into EC tubes and incubating for 24 hours at 44.5 ± 0.2 C. Cultures that produce gas in EC tubes are interpreted as verified fecal coliform colonies (see Figure III-C-1).

4.3 A percent verification can be determined for any colony-validation test:

$$\frac{\text{No. of colonies meeting verification test}}{\text{No. of colonies subjected to verification}} \times 100 = \text{Percent verification}$$

Example: Twenty blue colonies on M-FC medium were subjected to verification studies shown in Figure III-C-1. Eighteen of these colonies proved to be fecal coliforms according to provisions of the test:

$$\text{Percent verification} = \frac{18}{20} \times 100 = 90\%$$

4.4 A percent verification figure can be applied to the direct test results to determine the verified fecal coliform count per 100 ml:

$$\frac{\text{Percent verification}}{100} \times \text{count per 100 ml} = \text{Verified fecal coliform count}$$

Example: For a given sample, by the M-FC test, the fecal coliform count was found to be

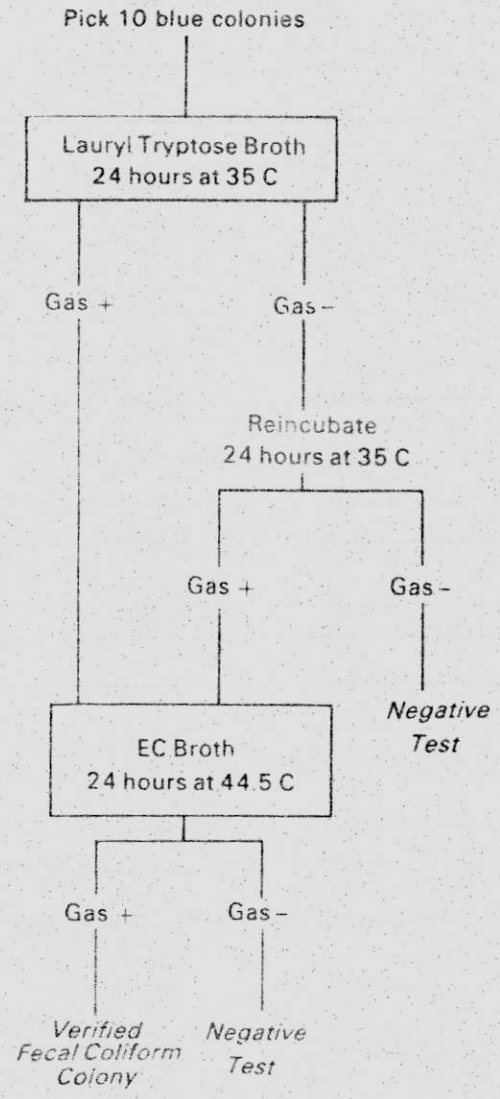

FIGURE III-C-1. Verification of Fecal Coliform Colonies on the Membrane Filter

42,000 organisms per 100 ml. Supplemental studies on selected colonies showed 92% verification.

$$\text{Verified fecal coliform count} = \frac{92}{100} \times 42{,}000 = 38{,}640$$

Rounding off = 39,000 fecal coliforms per 100 ml

The worker is cautioned not to apply percentage of verification determined on one sample to other samples.

5. Most Probable Number (MPN) Method

5.1 Summary: Culture from positive tubes of the lauryl tryptose broth (same as presumptive MPN Method, Part III-B) is inoculated into EC Broth and incubated at 44.5 C for 24 hours (see Figure III-C-2). Formation of gas in any quantity in the inverted vial is a positive reaction confirming fecal coliforms. Fecal coliform densities are calculated from the MPN table on the basis of the positive EC tubes (8).

5.2 Apparatus and Materials

5.2.1 Incubator that maintains 35 ± 0.5 C.

5.2.2 Water bath or equivalent incubator that maintains a 44.5 ± 0.2 C temperature.

5.2.3 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

5.2.4 Inoculation loop, 3 mm diameter and needle of nichrome or platinum wire, 26 B & S gauge, in suitable holder. Sterile applicator sticks are a suitable alternative.

5.2.5 Sterile pipets T.D., Mohr or bacteriological, glass or plastic, of appropriate size.

5.2.6 Dilution bottles (milk dilution), pyrex, 99 ml volume, screw-cap with neoprene liners.

5.2.7 Bunsen or Fisher-type burner or electric incinerator unit.

5.2.8 Pyrex test tubes, 150 × 20 mm, containing inverted fermentation vials, 75 × 10 mm, with caps.

5.2.9 Culture tube racks to hold fifty, 25 mm diameter tubes.

5.3 Media

5.3.1 Lauryl tryptose broth (same as total coliform Presumptive Test medium) prepared in 10 ml volumes in appropriate concentration for sample volumes used. (Part II-B, 5.3.1).

5.3.2 EC medium prepared in 10 ml volumes in fermentation tubes (Part II-B, 5.3.4).

5.4 Dilution Water: Sterile buffered or peptone dilution water dispensed in 99 ± 2 ml volumes in screw-capped bottles.

5.5 Procedure: Part II-C describes in detail the general MPN procedure. See Figure III-C-2.

5.5.1 Prepare the total coliform Presumptive Test medium, (lauryl tryptose broth) and EC medium. Clearly mark each bank of tubes, identifying the sample and the volume inoculated.

5.5.2 Inoculate the Presumptive Test medium with appropriate quantities of sample following the Presumptive Test total coliform procedure, (Part III-B).

5.5.3 Gently shake the Presumptive tube. Using a sterile inoculating loop or a sterile wooden applicator, transfer inocula from positive Presumptive Test tubes at 24 and 48 hours to EC confirmatory tubes. Gently shake the rack of inoculated EC tubes to insure mixing of inoculum with medium.

5.5.4 Incubate inoculated EC tubes at 44.5 ± 0.2 C for 24 ± 2 hours. Tubes must be placed in the incubator within 30 minutes after inoculation. The water depth in the water bath incubator must come to the top level of the culture medium in the tubes.

5.5.5 The presence of gas in any quantity in the EC confirmatory fermentation tubes af-

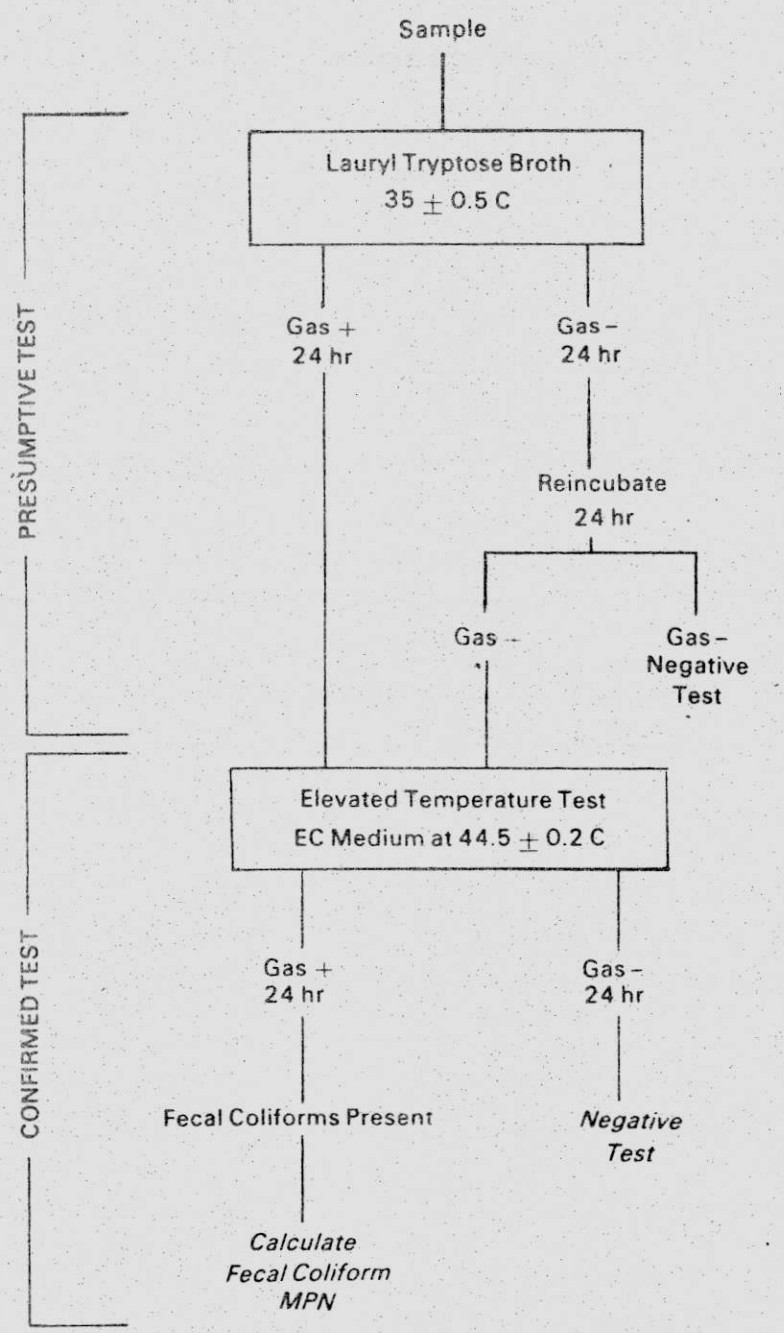

FIGURE III-C-2. Flow Chart for the Fecal Coliform MPN Tests.

ter 24 ± 2 hours constitutes a positive test for fecal coliforms.

5.6 Calculations

5.6.1 Calculate fecal coliform densities on the basis of the number of positive EC fermentation tubes, using the table of most probable numbers (MPN).

5.6.2 The MPN results are computed from three dilutions that include the highest dilution with all positive tubes and the next two higher dilutions. For example, if five 10 ml, five 1.0 ml, and five 0.1 ml sample portions are inoculated initially into Presumptive Test medium, and positive EC confirmatory results are obtained from five of the 10 ml portions, three of the 1.0 ml portions, and none of the 0.1 ml portions, the coded result of the test is 5-3-0. The code is located in the MPN Table II-C-4, and the MPN per 100 ml is recorded. See Part II-C, 4.9 for rules on selection of significant dilutions.

5.7 Reporting Results: Report the fecal coliform MPN values per 100 ml of sample.

5.8 Precision and Accuracy: The precisions of the MPN counts are given as confidence limits in the MPN tables. Note that the precision of the MPN value increases with increased numbers of replicates per sample tested.

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