UNITED STATES ARMY ENVIRONMENTAL HYGIENE AGENCY

ABERDEEN PROVING GROUND, MD 21010-5422

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WATER QUALITY INFORMATION PAPER NO. 17
BACTERIOLOGICAL SURVEILLANCE OF WATER

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Water Quality Information Paper No. 17

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DEPARTMENT OF THE ARMY
U. S. ARMY ENVIRONMENTAL HYGIENE AGENCY
ABERDEEN PROVING GROUND, MARYLAND 21010-5422

REPLY TO
ATTENTION OF

HSHB-ME-WR

SUBJECT: WATER QUALITY INFORMATION PAPER NO. 17

BACTERIOLOGICAL SURVEILLANCE OF WATER

I. REFERENCES. See Enclosure 1 and 2 for a listing of military references and bibliography, respectively.

II. PURPOSE. The purpose of this information paper is to provide a general overview of current regulatory information and guidance in the bacteriological surveillance and evaluation of water quality. Microbiological contaminants of a nonbacterial nature (e.g., viruses and Giardia lamblia) will be addressed in a separate information paper.

III. GENERAL INFORMATION.

A. Development of Criteria.

1. Since 1880, the criterion for determining the microbiological quality of drinking water has been its coliform content. The first U.S. Public Health Service (USPHS) Standards for Drinking Water, established in 1914, were based on the coliform assay. Both the standards and the methods of assay have been improved since 1914, but the premise on which the standards were based is still valid (Enclosure 2, reference 1). The premise is: Since the greatest potential source of pathogenic microorganisms in water is from human waste and since each person excretes approximately $2X10^{11}$ coliforms per day, the most reliable indicator of fecal pollution of water is the presence of coliforms. While regulations governing water purity for various uses of a water resource vary from State to State, the USPHS drinking water standards have been adopted as the minimum standards for public water supplies in all States and were incorporated in the standards set by the U.S. Environmental Protection Agency (EPA) in 1975. Current EPA regulations specify the method and frequency of sampling, the analytical methods of analysis, and the maximum allowable coliform content. These procedures are described in detail in Standard Methods for the Examination of Water and Wastewater (refer to Enclosure 2, reference 2). However, EPA has recently amended the maximum contaminant level (MCL), the monitoring requirements, and analytical methodologies for total coliform bacteria (refer to Enclosure 1, reference 12). These recent changes are discussed in a later section of this paper.

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2. The control of biological pathogens is still the most significant drinking water quality goal (Enclosure 2, reference 3). Of course, disinfection using chlorine and other oxidizing species is one of the principal methods of water treatment to ensure the biological safety of water. But despite many improvements in disinfection and other types of water treatment, outbreaks of waterborne disease still occur, particularly in smaller communities. From 1971-80, there were 315 reported outbreaks of waterborne disease involving almost 78,000 cases; 50 outbreaks and 20,000 cases occurred in 1980 alone (Enclosure 2, reference 4). Major causes of outbreaks in community water systems were microbiological contamination of the distribution system and the existence of treatment deficiencies, such as inadequate filtration and interruption of disinfection (Enclosure 2, reference 3). Contamination of ground water, resulting from use without treatment or from treatment deficiencies (usually inadequate chlorine dosage or interruption of the disinfection process), was responsible for the majority of the outbreaks in noncommunity water systems (Enclosure 2, reference 3).

3. The direct measurement of many species of pathogenic organisms is extremely difficult. The density of these organisms is usually very low, even in a badly polluted water supply, and the analytical techniques used in their determination are difficult. For these reasons, indicator organisms are used to determine the presence of fecal contamination in a water supply. The most common organisms used as indicators of possible water contamination are bacteria in the coliform group of the family Enterobacteriaceae. *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, and *Enterobacter cloacae* are examples of the gram-negative, lactose-fermenting, rod-shaped bacteria. However, their occurrence, particularly in low densities, does not always mean that human fecal contamination has occurred. In actuality, the assay for total coliforms is designed to detect the presence of *E. coli*, which constitutes by far the greatest proportion of the normal intestinal flora of humans and other warmblooded animals. Constant monitoring of the water supply, using methods designed primarily to detect the presence of *E. coli*, can thus warn of potential danger due to ineffective treatment of a water supply or contamination of a water distribution system, or can detect a dangerous level of pollution by human wastes in water used for recreation. Such monitoring is designed for prevention, not detection, of an epidemic of enteric disease (Enclosure 2, reference 1).

4. It has been reported repeatedly in the literature that the presence of any type of coliform organism in drinking water is undesirable. The regulations essentially demand that a coliform-free water be distributed to consumers. The drinking water standard presently in use (approximately one coliform per

100 milliliters of sample) is, in a sense, a standard of expedience. This standard does not entirely exclude the possibility of acquiring an intestinal infection. Drinking water standards are established by the Federal or State regulatory authorities with consideration toward economically feasible treatment methods and a margin of safety to the consumer.

5. Any water consumed by the public should be safe regardless of the size of the population served, the number of days the system is operating, and whether consumers are transients or full time residents (since coliforms can cause acute illness, protection of transient users is as important as protecting nontransient users). Coliform contamination is indicative of a situation needing immediate correction. Such corrections could include flushing of mains, increased disinfection, or improvements in filtration process control. This is a situation that warrants quick action to determine the cause of the problem in order to severely limit human exposure to the pathogenic microbes for which total coliforms serve as an indicator.

B. Significance of Coliforms in Water Systems.

1. The significance of the various coliform organisms in water has been and is a subject of considerable study. All types of coliform organisms may occur in feces. Although *E. coli* nearly always will be found in fresh pollution derived from warmblooded animals, other coliform organisms may be found in fresh pollution in the absence of *E. coli*. The genera *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia* usually represent the majority of isolations made from raw and treated municipal water supplies, with *Enterobacter* being isolated most frequently and *E. coli* more readily affected by conventional water treatment than other coliforms (Enclosure 2, reference 2). The differentiation of coliform types finds one of its most practical applications in the study of unexpected coliform densities that may be explained by multiplication on or in organic materials. The presence of a large number of coliform organisms of the same type in water from a well, spring, or from a single tap on a distribution system, is suggestive of such multiplication.

2. Another area of much interest and current research is that of growth of bacteria, particularly coliforms, in distribution systems. Until recently the terms "regrowth" and "aftergrowth" were used interchangeably to refer to the unexplained increase in bacterial populations occurring between treatment and the consumer tap. Clarification of these terms has now been proposed. Regrowth organisms are those cells, and their successor cells, that originated in the source water, passed through treatment processes which may have caused injury, and recovered viability

in the distribution system. Aftergrowth describes contamination of the distribution water by bacteria detached from the system piping and not from the source water. Thus, water leaving the treatment plant organism-free will show increases in populations with passage through the distribution system (Enclosure 2, reference 5).

3. Several communities across the United States have experienced the persistent low-level occurrence of coliform bacteria in their water distribution systems (Enclosure 2, reference 3). In many cases, breakpoint chlorination was practiced at the treatment plant, and free chlorine residuals were maintained throughout the water distribution system. Coliforms in the distribution system appeared to be a result of aftergrowth rather than passage of bacteria through the treatment process. Biofilms of microorganisms isolated from the pipe network have been shown to be resistant to levels of free chlorine that are commonly found in water distribution systems (Enclosure 2, reference 3).

4. Many of the water systems reporting coliform occurrences in their distribution networks have determined that the predominant organism was a member of the Klebsiella (K.) genus. Species in this genus currently include K. pneumoniae, K. oxytoca, K. planticola, K. terrigena, K. ozaenae, and K. rhinoscleromatis, most of which have been detected in coliform contaminated public water supplies (Enclosure 2, reference 6). The Klebsiella genus is typical of most other coliform organisms in that two different origins are possible (i.e., fecal contamination or environmental surroundings). Therefore, treatment processes must be adequately monitored to prevent release of coliforms into the distribution system. On the other hand, ineffective turbidity control, inadequate disinfection, or poor maintenance of the system could lead to breakthrough and colonization by coliforms. This increases the chance that waterborne pathogens could follow the same route.

a. The Klebsiella genus normally can be controlled through adequate disinfection, but if passage into the distribution system occurs, the bacteria may be protected by particulate matter and porous pipe sediments. The organism also has the ability to produce a capsular, slime coating under adverse conditions to afford further protection against disinfectants. These mechanisms can lead to establishment of the organism in the system and result in periodic sloughing of cells into flowing water.

b. Only those Klebsiella strains that are positive by the fecal coliform test (ferment lactose with gas production at 44.5 °C) are considered to be K. pneumoniae the most opportunistic

pathogen of the genus. Many of the commercially available rapid test kits used for speciation of the Enterobacteriaceae do not take this gas production at 44.5 °C into account. Strains that yield biochemical profiles identical to that of K. pneumoniae but are negative in the fecal coliform test may be speciated based on their ability to ferment D-melizitose and grow at 10 °C (Enclosure 2, reference 6).

C. Problems in the Analysis of Coliforms.

1. Coliforms meet many of the criteria for an ideal indicator organism; however, there are some deficiencies. If there is aftergrowth/regrowth in water, some strains do not disappear but adapt to the new environment and may become part of the natural flora. Also, coliforms do not have constant characteristics; it is this property more than any other that has recently caused some water bacteriologists to question the continued use of coliforms as indicator organisms (Enclosure 2, reference 1). False-negative and false-positive test results are not uncommon. The following summarizes the objections to the continued use of coliforms (Enclosure 2, reference 7):

a. Atypical lactose reactions, a concern of bacteriologists as early as 1899, have occurred.

b. Coliforms may be suppressed by high populations of other organisms, especially in untreated ground water or where there is no free residual chlorine.

c. Coliforms do not represent a homogeneous group; it has been suggested (Dutka, 1973) that the definition of "coliform" include any organism defined by Edwards and Ewing (1972) as Enterobacteriaceae.

d. The genus Aeromonas is a common cause of false-positive results in warm weather.

e. False-negative results by strains that are unable to ferment lactose can give an unwarranted sense of security.

2. The coliform test is not without faults, and research is continuing to devise a better and more reliable testing procedure. A recent study comparing four procedures for enumerating coliforms in water, led to the conclusion that the different procedures were selective for different genera among the coliform group, and that local conditions may determine which procedures will give the best results (Enclosure 2, reference 8). Many suggestions have been made for modification of media or procedures that might make the coliform test more selective for coliforms or increase the probability of detecting coliforms if they are present (Enclosure 2, reference 1).

3. Two problems, other than those related to identification, should be mentioned. Methods and sites of sampling should be carefully controlled, and it may be necessary to compare coliform densities at different sites in natural bodies of water to determine the most critical sites for monitoring (Enclosure 2, reference 1). The depth of sampling may also be important. Dutka and Kwan (1978) reported that bacterial densities in the surface microlayer are significantly higher than they are at the below-surface depths usually sampled (Enclosure 2, reference 9). This may be particularly important in determining the safety of water for recreational use.

4. Another recent study is especially relevant to water quality in beach areas. Olson (1978) found that fecal coliforms in seawater, such as those originating from the discharge of sewage or treated effluent into the ocean, frequently do not behave typically in the standard coliform assay (Enclosure 2, reference 10). [Note: The standard coliform assay is defined as the multiple tube fermentation (MTF) method of analysis.] These "injured" coliforms grew in lactose broth but produced no gas. Further testing revealed that more than 50 percent of these false negatives were identifiable as E. coli. Beach waters that were apparently within the prescribed safety limits when tested by the standard procedure were proven to be in violation of the coliform standard when more careful testing of apparently negative samples was done (Enclosure 2, reference 1).

5. Coliforms that are exposed to chlorine but are not killed are defined as "injured" and may require special treatment. Samples to be tested by any method should, of course, be dechlorinated by the addition of sodium thiosulfate. Organisms that are not killed by chlorine may not react typically. Lin (1976) found that there was less recovery of fecal coliforms from chlorinated sewage effluents using the one-step membrane filter (MF) method of analysis rather than using the MTF method (Enclosure 2, reference 11). A 4-hour preincubation of the filters at 35 °C on phenol red lactose broth followed by incubation on Membrane-Fecal Coliform (M-FC) agar at 44.5 °C gave comparable fecal coliform counts. Interestingly, the use of M-FC agar rather than M-FC broth for incubation of the membrane filters has shown fecal coliform counts averaging twice as high as those on the broth-saturated pads (Enclosure 2, reference 11). Such variations illustrate one of the problems of coliform assays; a slight variation in technique may have very significant effects on the results.

6. The instructions in Standard Methods regarding laboratory procedures, preparation of media, handling of samples, and the choice, use, and maintenance of equipment are very specific and detailed. Obviously, it is important that they be

followed carefully. However, even when all possible care is taken, bacteria sometimes do not cooperate. Coliforms often produce colonies that do not have the typical appearance; when a method is used that relies on colony appearance, such as the MF procedure, a great deal of experience is required to decide which "atypical" colonies should be tested by inoculation into lactose broth as is recommended if no "typical" colonies are present. It is preferable to do excessive testing of possible coliforms than to rely totally on colony appearance and risk underestimating the number of coliforms present in a drinking water sample (Enclosure 2, reference 1).

D. Other Indicator Organisms.

1. Microbiological examinations of potable waters are usually conducted to determine either the presence or absence of the coliform group, and the total number of bacteria present per milliliter (mL) of sample (Enclosure 2, reference 7). In addition to the procedures for total coliforms and total plate count, methods exist to more specifically identify the origin of bacteriological contamination. Fecal coliform and fecal streptococci procedures are two commonly used analytical methodologies for this purpose. Specific testing procedures, such as fecal coliform and fecal streptococci, are recommended for drinking water when more generalized testing yields positive results (Enclosure 1, reference 5). Fecal coliform and fecal streptococcus densities may be determined by using either the MTF procedure or the MF procedure. However, because of organism survival characteristics, it is not recommended to use only the fecal streptococci procedure when investigating or determining water quality. Other fecal indicators (fecal coliforms and total coliforms) should be used concurrently (Enclosure 1, reference 5). Descriptions of the tests are provided below and the complete methodologies may be found in Standard Methods for the Examination of Water and Wastewater (Enclosure 2, reference 2).

2. Because of certain limitations of the coliform group as a general indicator of water quality, research studies have continually searched for a better indicator than the coliform group. Although no other single group of organisms has been found to be a satisfactory replacement for total coliform, it is pertinent to mention briefly the importance of these other indicator groups:

a. Fecal coliforms (defined as those organisms which develop on media incubated at 44.5 °C) have frequently been used in stream and lake pollution work, but are not suitable as the only indicator of drinking water quality because the number of fecal coliforms is considerably lower in source waters than total coliforms, making the test less sensitive (Enclosure 2, reference 7).

b. Fecal streptococci (defined as those organisms able to grow on medium containing sodium azide) have been used in water pollution work but have not proven suitable for drinking water analysis because of low recovery rates, poor agreements between various methods, and uncertainty as to their significance in water.

c. Several other organisms have been suggested as indicators but have found even less acceptance: Clostridium perfringens, Bifidobacterium, Pseudomonas, Staphylococcus (Enclosure 2, reference 7). Research studies have compared these other indicator organisms with coliforms and have suggested the substitution of other indicator organisms for the coliform group. However, none of these other indicator organisms have been found to be a superior replacement for the coliform group.

3. Detection and estimation of the coliform group of bacteria occasionally has required specific identification of species to determine the exact nature of the pollution source. In making such differential tests, be aware that all strains taxonomically assigned to the coliform group do not necessarily conform to the coliform definition used, i.e., they may not ferment lactose; or if they do, they may not produce gas. Further, not all lactose-fermenting or sheen-producing, gram-negative rods found in water are coliforms (they may be Aeromonas), and not all strains of a given species will react uniformly in a given substrate (Enclosure 2, reference 7).

a. The traditional "IMViC" (indol, methyl red, Voges-Proskauer, and citrate utilization) tests are useful for coliform differentiation but do not provide for complete identification. Additional biochemical tests may be necessary.

b. Commercial kits for identification are available and are significant alternatives to conventional media. Using these commercial materials reduces many of the required quality control activities. Prepackaged kits are simple to store and use, and give reproducible and accurate results.

E. Conclusion on Coliform Standard.

1. Good engineering and public health practices emphasize the need for using raw water of the highest possible quality, and for providing adequate sanitary survey information. Bacteriological testing or the imposed use of bacteriological standards are adjuncts, not replacements for good-quality raw water, proper water treatment, and integrity of the distribution system (Enclosure 2, reference 7).

2. In the final analysis, the testing for coliforms, while not perfect bacteriologically, is still the most reliable indicator of the possible presence of fecal contamination; and therefore, indicators of pathogens that may be present in water. The present coliform standards appear adequate to protect public health against outbreaks of bacterial diseases when raw water is obtained from a protected source, appropriately treated, and distributed in a contamination-free system. Since most water systems are deficient in one or more of these areas, frequent microbiological sampling of the water system is by far the most critical aspect in water production and distribution to consumers (Enclosure 2, reference 7).

IV. SAMPLE COLLECTION AND LABORATORY CONTROLS. The following section was extracted in part from Part 900, Microbiological Examination of Water, Standard Methods For The Examination of Water and Wastewater (Enclosure 2, reference 2). [Copyright 1985 by APHA, AWWA, and WPCF. Reproduced with permission (Reference Letter, APHA, February 22, 1989)].

A. General. Make bacteriological examinations on samples selected at representative points throughout the distribution system. Select the frequency of sampling and the location of sampling points to ensure accurate determination of the bacteriological quality of the treated water supply, which may be controlled in part by the known quality of the untreated water and thus by the need for treatment. Base the minimum number of samples to be collected and examined each month on the population served. It is important to examine repetitive samples from designated points, as well as samples from a number of widely distributed sampling points. Take samples at evenly spaced intervals. Consider daily samples collected after an unsatisfactory sample has been taken as special samples and do not count in the total number of samples examined monthly.

B. Sample Collection and Preservation.

1. Bacteriological results should be considered along with other information on the sanitary conditions of the water source. Location of sampling sites and frequency of sampling are critical factors in obtaining reliable information about bacterial pollution in any body of water (Enclosure 2, reference 2). Collect grab samples from representative locations and depths. In collecting samples directly from a river, stream, lake reservoir, spring, or shallow well, obtain samples representative of the water that is the source of the supply to the consumers. It is undesirable to take samples too near the bank or too far from the point of drawoff, or at a depth above or below the point of drawoff.

2. If the water sample is to be taken from a distribution system tap without attachments, select a tap that is supplying water from a service pipe directly connected with the main, and is not, for example, served from a cistern or storage tank. Open tap fully and let water run to waste for 2 or 3 minutes, or for a time sufficient to permit clearing the service line. In sampling from a mixing faucet, remove faucet attachments such as screen or splash guard, run hot water for 2 minutes, then cold water for 2 to 3 minutes, and collect sample.

3. If the sample is to be taken from a well fitted with a hand pump, pump water to waste for about 5 minutes before collecting sample. If the well is equipped with a mechanical pump, collect sample from a tap on the discharge. If there is no pumping machinery, collect a sample directly from the well by means of a sterilized bottle fitted with a weight at the base; take care to avoid contaminating samples by any surface scum.

4. Collect samples of bathing-beach water at locations and time of greatest bather load; and in natural bathing places, increase sampling frequency during periods of stormwater runoff in the bathing season.

5. Samples for microbiological examination should be collected in bottles that have been cleansed and rinsed carefully, given a final rinse with distilled water, and sterilized. Add a reducing agent to containers intended for the collection of water having residual chlorine or other halogen, unless they contain broth for direct planting of sample. A 0.1 mL 10 percent solution of sodium thiosulfate added to a 120 mL bottle will neutralize a sample containing about 15 mg/L residual chlorine. The examination then will indicate more accurately the true microbial content of the water at the time of sampling, since the sodium thiosulfate neutralizes residual halogen and prevents continuation of bactericidal action during sample transit.

6. Collect sufficient volume of sample to perform the required tests. Leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, preparatory to examination.

7. Collect water samples high in copper or zinc and wastewater samples high in heavy metals in sample bottles containing a chelating agent that will reduce metal toxicity. This is particularly significant when such samples are in transit for 4-hours or more. Prepare a solution of 372 mg/L of the disodium salt of ethylenediaminetetraacetic acid (EDTA) and adjust the EDTA solution to pH 6.5 before use (Enclosure 2, reference 2). Add 0.3 mL of the EDTA solution to 120-mL containers before sterilization.

8. Experience in the shipment of uniced samples by mail indicates that changes in type or numbers of bacteria during such shipment for even limited periods of time are not negligible. Therefore, refrigeration during transportation is recommended to minimize changes, particularly when ambient air temperature exceeds 13 °C.

C. Sample Storage Time.

1. Start microbiological examination of a water sample promptly after collection to avoid unpredictable changes. If samples cannot be processed within 1 hour after collection, use an iced cooler for storage during transport to the laboratory. If it is known that the results will be used in legal action, employ a special messenger to deliver samples to the laboratory within 6 hours and maintain chain of custody.

2. Hold temperature of all stream pollution samples below 10 °C during a maximum transport time of 6 hours. Refrigerate these samples upon receipt in the laboratory and process within 2 hours. When local conditions necessitate delays in delivery of samples longer than 6 hours, consider either making field examinations using field laboratory facilities located at the site of collection or using delayed-incubation procedures.

3. Unfortunately, these requirements seldom are realistic in the case of individual potable water samples sent to the laboratory by mail service, but the time elapsing between collection and examination should not exceed 30 hours. Where refrigeration of individual water samples sent by mail is not possible, a thermos-type insulated sample bottle that can be sterilized may be used. Record time and temperature of storage of all samples and consider this information in the interpretation of data.

D. Laboratory Quality Assurance.

1. The growing emphasis on water quality standards, enforcement, and monitoring has required the establishment of a quality assurance program to substantiate the validity of analytical data. A laboratory quality assurance program is the orderly application of the practices necessary to remove or reduce errors that may occur in any laboratory operation caused by personnel, equipment, supplies, sampling procedures, and analytical methodology.

2. The program must be practical and integrated. It should require only a reasonable time or it will be bypassed. When properly administered, a balanced, conscientiously applied quality assurance program will yield uniformly high-quality data without interfering with the primary analytical functions of the laboratory. Generally, 15 percent of total analyst time should be spent on different aspects of a quality assurance program.

3. The quality assurance guidelines discussed in Part 902 A of Standard Methods (Enclosure 2, reference 2) are recommended as a minimal program for a microbiology laboratory. In addition to Standard Methods, most state regulatory agencies use the Manual for the Certification of Laboratories Analyzing Drinking Water, (Enclosure 2, reference 14) to evaluate and certify laboratories for microbiological analyses of water. All laboratories that analyze public water supplies for regulatory purposes must be certified.

E. Intralaboratory Quality Control.

1. An intralaboratory quality control program is a system of agreed upon requirements and laboratory practices necessary to maintain minimal quality standards among a group of participant laboratories. Minimal standards are set for laboratory operations (personnel, facilities, equipment, supplies, data handling, and quality control). To safeguard drinking water and assure a level of data reliability, most states have approval, registration, or certification programs for laboratories that test water. These State programs provided the base for the Federal/State program for the certification of water supply laboratories developed under the Safe Drinking Water Act.

2. Criteria has been established for laboratory operations and methodology, and for onsite inspections by the certifying State agency or its surrogate to verify these minimal standards. Onsite inspections of laboratories in the present certification program has shown that the primary causes for discrepancies have been inadequate equipment, improperly prepared media, incorrect analytical procedures, and insufficiently trained personnel. Enclosure 2, references 14 and 15 provide certification requirements and guidance on equipment selection and use, sampling procedures, analytical methodologies, and quality assurance practices.

3. Analytical Quality Control Procedures.

(a) General.

(1) For MTF procedures, check sterility of media, dilution water, and glassware. Media may be checked by incubating a representative portion at the appropriate temperature and observing for growth. Sterility of glassware is confirmed by addition of 25 mL of sterile nonselective broth to a random container, incubation, and observance for growth.

(2) For MF procedures, check sterility of media, membrane filters, dilution and rinse water, and glassware and equipment once during each series of samples using sterile water as the sample.

(3) For each lot of medium, check analytical procedures by inoculating with known positive and negative control cultures for the organism(s) under test.

(4) Perform duplicate analyses on 10 percent of samples and on at least one sample per test run.

(b) Quality Control for MTF Tests.

(1) Completed test. For routine analyses, do the completed test on 10 percent of positive samples. If no positives result from potable water samples, complete at least one positive source water quarterly.

(2) Check for suppression. For public water supply samples with a history of heavy growth without gas in presumptive-phase tubes, submit such tubes to the confirmed phase to check for coliform bacteria.

(c) Quality Control for MF Procedures.

(1) Colony Verification. For each type of test conducted, verify colonies monthly from a known positive sample.

(2) Total Coliform Analyses. Verify suspected coliforms by testing for lactose fermentation or by use of rapid biochemical tests or multitest systems. For drinking water samples, verify all sheen colonies counted as coliforms when this number is <10/100 mL. When the number exceeds 10/100 mL, randomly pick and verify at least 10 colonies representative of all sheen types. If no positives result from testing drinking water samples, analyze at least one known positive source water quarterly.

V. STANDARD METHODS TEST PROCEDURES. The following section was extracted in part from Standard Methods for the Examination of Water and Wastewater (Enclosure 2, reference 2). [Copyright 1985 by APHA, AWWA, and WPCF. Reproduced with permission (Reference Letter, APHA, February 22, 1989)].

A. General Discussion.

1. The following section discusses the general procedures for making bacteriological examination of water samples based on analytical methods outlined in Standard Methods. These analytical procedures are the best currently available; however, their

limitations should be thoroughly understood. Experience has established the significance of the coliform group density as a criterion of the degree of pollution and thus of sanitary quality. The significance of the tests and the interpretation of results are well authenticated and have been used as a basis for standards to assess the bacteriological quality of water supplies. The information in this section provides an overview of the individual tests. Specific details of step by step procedures may be found in Standard Methods.

2. The standard test for the coliform group may be carried out either by the MTF method (confirmed phase or completed test) described in Section 908, Standard Methods, or by the MF method in Section 909. Each method is applicable within the limitations specified and with due consideration of the purpose of the examination.

3. It is customary to report results of the coliform test by the MTF procedure as a Most Probable Number (MPN) index. This is an index of the coliform bacteria that, more probably than any other number, would give the results shown by the laboratory examination; it is not an actual enumeration. By contrast, direct plating methods such as the MF procedure permit a direct count of coliform colonies. In both procedures, coliform density is reported conventionally as the MPN or as a count per 100 mL. Use of either procedure permits appraising the sanitary quality of water and the effectiveness of treatment processes. Because it is not necessary to provide a quantitative assessment of coliform bacteria for all samples, a qualitative Presence-Absence (P-A) test has been added.

4. The MF test, which involves a direct plating for detection and estimation of coliform densities, is as effective as the MTF for detecting bacteria of the coliform group. Modification of procedural details, particularly of the culture medium, has made the MF test results comparable with those given by the MTF procedure. Although there are limitations in the application of the MF procedure, it is equivalent when used with strict adherence to these limitations and to specified technical details.

5. Coliform group bacteria present in the gut and feces of warmblooded animals generally include organisms capable of producing gas from lactose in a suitable culture medium at 44.5 $\pm$ 0.2 °C. Inasmuch as coliform organisms from other sources often cannot produce gas under these conditions, this criterion is used to define the fecal component of the coliform group. Both the MTF procedure and the MF procedure have been modified to incorporate incubation in confirmatory tests at 44.5 °C to provide estimates of the density of fecal organisms, as defined. Procedures for

fecal coliforms also include a 24-hour MTF test using A-1 medium and a 7-hour rapid method. This differentiation yields valuable information concerning the possible source of pollution in water, and especially its remoteness, because the nonfecal members of the coliform group may be expected to survive longer than the fecal members in the unfavorable environment provided by the water.

6. The fecal streptococci are valuable pollution indicators in the study of rivers, streams, lakes, and marine systems, especially when used with the fecal coliform bacteria. Periodic verification of isolates is necessary to ensure adequate medium performance. Further identification of species will eliminate organisms of little sanitary significance while verifying human and animal sources of pollution. Approved methods include: the MTF procedure for fresh but not saline water, raw wastewater and chlorinated wastewater effluent samples; the MF procedure for fresh and saline water samples but not for highly turbid waters and chlorinated wastewater; and fecal streptococcal plate count (tentative) for those samples containing few fecal streptococci and significant turbidity.

7. The heterotrophic plate count (HPC) may be determined by pour plate, spread plate, or MF procedure. It provides an approximate enumeration of total numbers of bacteria multiplying at 35 °C that may yield useful information about water quality and may provide supporting data on the significance of coliform test results. The HPC is useful in judging the efficiency of various treatment processes and may have significant application as an in-plant control test. It also is valuable for checking quality of finished water in a distribution system as an indicator of microbial regrowth and sediment buildup in slow-flow sections and dead ends.

B. MTF Method.

1. Test Quantities. When drinking water is analyzed to determine if the quality meets the standards of the EPA, use five fermentation tubes, each containing 10 mL of sample. If 100 mL sample portions are inoculated, use five culture bottles. Because treated drinking water should contain no coliforms per 100 mL, water plant laboratories should consider using 10 fermentation tubes, each containing 10 mL or 100 mL sample portions, to obtain a better statistical estimate. Use the confirmed phase when examining all drinking waters, natural waters, and effluents. The completed test is the reference standard to be applied to selected samples on a seasonal basis.

2. Potable Waters. For the routine examination of most potable water supplies, particularly those that are disinfected, the object of the coliform test is to determine compliance with EPA standards as a measure of either the efficiency of treatment plant operation or the possibility of bacterial contamination. A high proportion of coliform occurrences in a distribution system are attributed not to treatment failure at the plant or the well source, but to bacterial aftergrowth/regrowth in the mains. Because it is difficult to distinguish between regrowth coliforms and new contamination, all coliform occurrences should be assumed to signal new contamination unless otherwise demonstrated. The assessment of potability generally is based on knowledge of the sanitary condition of the supply as determined by bacteriological monitoring. It is expected that more than 95 percent of all samples examined will yield negative results. An occasional positive result, unless repeated from the same sampling point (resampling), usually is of limited significance but should not be ignored. An increase in the number of positive samples over a period of time or an abrupt increase in a short period of time indicates a change in the quality of the water, the significance of which should be determined by a sanitary survey. Necessary corrections, based on the sanitary survey, should be made promptly and verified for effectiveness by additional bacteriological testing.

3. Nonpotable Waters. The object of the examination of nonpotable water generally is to estimate the density of bacterial contamination, determine a source of pollution, enforce water quality standards, or trace the survival of microorganisms. Each objective requires a numerical value for reporting results. The MTF procedure may be used to obtain statistically valid MPN estimates of coliform density. Generally, the log average or median value of the results of a number of samples will yield a value in which the effect of sample-to-sample variation is minimized. The MF procedure may prove the better procedure to accomplish this objective.

4. Standard Total Coliform MTF Tests.

a. Presumptive Phase. The first step of the MTF test is inoculation of a selected quantity of the sample into a series of test tubes containing lauryl tryptose broth. Incubation follows. Formation of gas in any amount in the inverted tubes or vials within 48 $\pm$ 3 hours constitutes a positive presumptive reaction. Confirm presumptive tubes with heavy growth and no visible gas from drinking and recreational waters in brilliant green lactose bile broth to check for coliform suppression. An arbitrary limit of 48 hours for observation may exclude from consideration occasional members of the coliform group that form gas very slowly.

b. Confirmed Phase. Submit all primary fermentation tubes showing any amount of gas within 24 hours of incubation to the confirmed phase. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile broth fermentation tube at any time within 48 ± 3 hours constitutes a positive confirmed phase. Do not use the presumptive phase without confirmation of positive tubes; use the confirmed phase as a minimum for all samples.

c. Completed Test. Use the completed test on positive confirmed tubes to establish definitively the presence of coliform bacteria and to provide quality control data. Double confirmation into brilliant green lactose bile broth for total coliforms and EC broth for fecal coliforms may be used. Consider positive EC broth elevated temperature (44.5 °C) results as a positive completed test response. Parallel positive brilliant green lactose bile broth cultures with negative EC broth cultures indicate the presence of nonfecal coliforms and must be submitted to the completed test procedure to obtain an MPN test value. Apply the completed test in the examination of samples of unknown quality, samples yielding unexpected results, samples with high noncoliform counts, and for quality control purposes. If the completed test is not applied to all samples, apply it to at least 10 percent of positive nonpotable water samples on a seasonal basis and all unsatisfactory potable water samples to establish, beyond reasonable doubt, the results from the confirmed phase, in determining sanitary quality of water supplies. Analyze, by the completed tests, repeat samples of finished water from the same location that consistently show three or more positive 10-mL portions in the confirmed phase.

5. Fecal Coliform MPN Procedure.

a. Elevated-temperature tests for the separation of organisms of the coliform group into those of fecal origin and those derived from nonfecal sources are available. Modifications in technical procedures, standardization of methods, and detailed studies of members of the coliform group found in the feces of various warmblooded animals compared with those from other environmental sources have established the value of a fecal coliform determination. The test can be performed by one of the MTF procedures or by MF procedures. The procedure using EC medium yields adequate information about the source of the coliform group (fecal or nonfecal) when used as a confirmatory test. Do not use it for direct isolation of coliforms from water because prior enrichment in a presumptive medium for optimum recovery of fecal coliforms is required. The procedure using A-1 broth is a single-step method not requiring confirmation.

b. The fecal coliform test (EC medium) is applicable to investigations of stream pollution, raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. The procedure is not recommended as a substitute for the coliform test in the examination of potable waters, because no coliform bacteria of any kind should be tolerated in a treated water.

6. Estimation of Bacterial Density.

a. Results of the examination of replicate tubes and dilutions are reported in terms of the MPN. This number based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. Theoretical considerations and large-scale replicate determinations indicate that this estimate tends to be greater than the actual mean density and the disparity tends to diminish with increasing numbers of tubes in each dilution examined.

b. The precision of any single test depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examination shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in all or a majority of the tubes. The numerical value of the estimation of the bacterial content is determined by the dilution that shows both positive and negative results. The number of portions scheduled, especially in the critical dilution, will be governed by the desired precision of the result. Adequate sample shaking to produce a random dispersion of bacteria in the test portions is another factor that affects accuracy of analysis. The MPN tables are based on the assumption of a Poison distribution of random dispersion; however, if the sample is not adequately shaken before the portions are removed or if clumping of bacterial cells occurs, the MPN value will be an underestimate of the actual bacterial density.

c. After completion of incubation in the confirmed or completed phases of the MTF test, examine the fermentation tubes for evidence of growth and gas formation. Record the number of positive tubes in the five tube or ten tube test, or the combination of positives if the three dilution procedure is used. Compare the results to the tables provided in Standard Methods to determine the MPN index. The five or ten tube method is most often used for potable water.

7. P-A Coliform Test.

a. The P-A test for the coliform group is a simple modification of the MTF procedure. Simplification, by the use of one large test portion (100 mL) in a single culture bottle to

obtain qualitative information on the presence or absence of coliforms, is justified on the theory that no coliforms should be present in 100 mL of a drinking water sample. The P-A test also provides the optional opportunity for further screening of the culture to isolate other indicators (fecal coliform, Aeromonas, Staphylococcus, Pseudomonas, fecal streptococcus, and Clostridium) on the same qualitative basis. Additional advantages include the possibility of examining a larger number of samples per unit of time and comparative studies with the MF procedure indicate that the P-A test may maximize coliform detection in samples containing many organisms that could overgrow coliform colonies and cause problems in differentiation.

b. The P-A test is intended for use on routine sample submissions collected from distribution systems or water treatment plants. Initially, examine samples by a combination of either the MF method or MTF method, as well as the P-A test. After it is established that quantitative methods usually give negative results for coliforms, the P-A test alone may be used. When sample locations produce a positive P-A result for coliforms, examine subsequent repeat samples by a quantitative procedure until negative results are obtained from two consecutive samples. Analyze infrequently collected samples such as those from private wells by MTF method or by the MF method.

c. Detection of fecal coliforms, fecal streptococci, and other indicator bacteria by the P-A test may be achieved by appropriate selection of confirmatory media and incubation time and temperature as outlined in Figure 908:2 of Standard Methods. Water treatment and other adverse environmental conditions often place great stress on indicator bacteria, resulting in an extended lag phase before logarithmic growth takes place. Extending the P-A test incubation period to 72 or 96 hours often will allow isolation of other indicator organism. Isolations of indicator bacteria after the usual 48 hour incubation period will not be used for regulatory purposes, but will allow the laboratory to advise water treatment plants of potential adverse water quality problems.

C. MF Method.

1. General. The MF method is highly reproducible, can be used to test relatively large volumes of sample, and yields definite results more rapidly than the MTF procedure although it has limitations in testing waters high in turbidity and in noncoliform bacteria. The MF method is extremely useful in monitoring drinking water emergencies and in the examination of a variety of natural waters. However, when the MF method has not been used previously, it is desirable to conduct parallel tests with the MTF method to demonstrate applicability. Statistical comparisons of results obtained by the MTF method and the MF method have shown that the MF method is more precise.

2. Definition of Coliforms. As applied to the MF method, the coliform group may be redefined as comprising all aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that produce a dark colony with a metallic sheen within 24 hours at 35 °C on an Endo-type medium containing lactose.

3. Limitations of Procedure. Turbidity caused by the presence of algae or other interfering material may not permit testing of a sample volume sufficient to yield significant results. Low coliform estimates may be caused by the presence of high numbers of noncoliforms or of toxic substances. The MF method is applicable to examination of saline waters, but not wastewaters that have received only primary treatment followed by chlorination because of turbidity in high volume samples or wastewaters containing toxic metals or toxic organics such as phenols. For total coliforms in chlorinated secondary or tertiary effluents, use the two-step procedure listed in Section 909A of Standard Methods. For fecal coliforms, use the MTF procedure in Section 908C of Standard Methods if the results are to be used in enforcement actions. A modified MF procedure for fecal coliforms may be used if parallel testing with the MTF procedure shows significant agreement.

4. Test Quantities. The standard volume to be filtered for drinking water samples is 100 mL. This may be distributed among multiple membranes if necessary. Because treated drinking water should contain no coliforms per 100 mL, water plant laboratories should consider testing a 1 liter (L) sample of finished water, provided that particulates are not present to interfere with filtration or development of discrete colonies. In such situations, divide the sample into four portions of 250 mL for analysis and total any coliforms on the cultures into a single report on 1 L sample examined. Smaller or larger samples may be used for other waters or special analyses.

5. Standard Total Coliform MF Procedure.

a. All organisms that produce a colony with a golden-green metallic sheen within a 24 hour incubation period on a suitable medium are considered members of the coliform group. The sheen may cover the entire colony or may appear only in a central area or on the periphery. The coliform group thus defined is based on the production of aldehydes from the fermentation of lactose. While this biochemical characteristic is part of the metabolic pathway of gas production in the MTF test, some variations may be observed among coliform strains. However, this slight difference in indicator definition is not considered to change its sanitary significance, particularly if suitable studies have been conducted to establish the relationship between results obtained by the MF and those obtained by the standard MTF procedure.

b. Verify sheen colonies to avoid false positive results, especially for drinking water samples. Verification of all colonies is preferred. For coliform counts of more than 5/100 mL, verify a minimum of five colonies included in the direct count. Coliforms occasionally may produce atypical colonies. Experience in recognizing such growth may be gained by verifying all types of sheen and nonsheen colonies.

c. Generally, an enrichment procedure may improve the assessment of the quality of drinking water. However, this step may be eliminated in the routine examination of drinking water where the repeated determinations have shown that adequate results are obtained by a single-step MF procedure. Enrichment usually is not necessary in the examination of nonpotable water or sewage. Verify all samples of drinking water giving positive results.

d. The volume of the sample to be filtered will be governed by expected bacterial density, which in drinking water samples will be limited only by the degree of turbidity or by the noncoliform growth on the medium. The drinking water standard is based on a filtered volume of 100 mL. Appropriate volumes for other water sources may be determined from Table 909:1 in Standard Methods. Various volumes are used according to anticipated degrees of contamination. Overcrowding of the filter membrane with colony growth is to be avoided. Ideally, the selected sample volume will yield no more than 50 coliform colonies and no more than 200 colonies of all types.

e. Coliform Verification. Typical sheen colonies may be produced occasionally by noncoliform organisms. Verify by a test for lactose fermentation, or by using alternative procedures involving either the rapid (4 hour) test of two key biochemical reactions or multitest system for speciation (See Section 902A.4d and 909A.5f of Standard Methods).

f. Coliform Density.

(1) Report coliform density as (total) coliforms/100 mL. If several dilutions have been filtered for a sample, select those dilutions with membrane filters yielding 20 to 80 coliform colonies and not more than 200 colonies of all types of growth per membrane. Compute the count using the formula:

$$\text{Coliform colonies/100 mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}}$$

(2) With water of good quality, the occurrence of coliforms generally will be minimal. Therefore, count all coliform colonies (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density. If confluent growth occurs, that is, growth covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with (or without) coliforms."

(3) If the total number of bacterial colonies, coliforms plus noncoliforms, exceeds 200 per membrane, or if the colonies are not distinct enough for accurate counting, report results as "too numerous to count" (TNTC). The presence of coliforms in such cultures showing no sheen may be indicated by placing the entire membrane filter culture into a sterile tube of brilliant green lactose bile broth. As an alternative, brush the entire surface with a sterile loop or applicator stick and inoculate this growth to the tube of brilliant green lactose bile broth. If gas is produced from this culture within 48 hours at 35 ± 0.5 °C, conclude that coliforms are present. In any case, request a new sample and select more appropriate volumes to be filtered per membrane, remembering that the standard drinking water portion is 100 mL.

(4) For nonpotable waters it may be practical to split the 100 mL volume among several filters. For example, filter 50 mL portions through two membranes or 25 mL portions through four membranes. In this case, total the coliform counts for all filters and report as number per 100 mL.

g. Although the statistical reliability of the method is greater than that of the MPN procedure, membrane counts really are not absolute numbers. Table 909:II in Standard Methods illustrates the 95 percent confidence limits for MF coliform results. These values assume that bacteria are distributed randomly.

6. Delayed-Incubation Total Coliform Procedure.

a. Modification of the standard MF method permits membrane shipment or transport after filtration to a distant laboratory for incubation and completion of the test. This delayed-incubation test may be used where it is impractical to apply conventional procedures. It also may be used: where it is not possible to maintain the desired sample temperature during transport; when the elapsed time between sample collection and analysis would exceed the approved time limit; where the sampling location is remote from laboratory services; and when it is necessary to monitor streams for water quality or pollution control activities by a standardized procedure; or for other reasons that prevent analysis of the sample at or near the sample site.

b. Data secured by the delayed-incubation test have yielded results consistent with those from the immediate standard test in independent studies of samples from both fresh and salt waters. Determine the applicability of the delayed-incubation test for a specific water source by comparing with the results of test procedures using conventional methods.

c. To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on the transport medium, and ship to the laboratory. Complete the coliform determination in the laboratory by transferring the membrane to standard M-Endo or LES Endo medium, incubating at 35 ± 0.5 °C for 20 to 22 hours, and counting typical coliform colonies that develop. Transport media are designed to keep coliform organisms viable and generally do not permit visible growth during the time of transit. Bacteriostatic agents in holding/preservative media suppress growth of microorganisms en route but allow normal coliform growth after transfer to a fresh medium.

d. The delayed-incubation test follows the methods outlined for the total coliform MF procedure, except as indicated in Section 909B of Standard Methods.

7. Fecal Coliform MF Procedure.

a. Fecal coliform bacterial densities may be determined either by the MTF procedure or by the MF procedure. If the MF procedure is used for chlorinated effluents, demonstrate that it gives comparable information to that obtainable by the MTF test before accepting it as an alternative. The MF procedure uses an enriched lactose medium and incubation temperature of 44.5 ± 0.2 °C for selectivity and gives 93 percent accuracy in differentiating between coliforms from warmblooded animals and those from other sources. Because incubation temperature is critical, submerge MF cultures in a water bath for incubation at the elevated temperature or use an appropriate, accurate solid heat sink incubator. Air incubation is undesirable because of heat layering within the chamber and the slow recovery of temperature each time the incubator is opened during daily operations.

b. Select the volume of water sample to be examined in accordance with the information in Table 909:III of Standard Methods. When the bacterial density of the sample is unknown, filter several decimal volumes to establish fecal coliform density. Estimate volume expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively. Use sample volumes that will yield counts between 20 and 60 fecal coliform colonies per membrane.

c. Colonies produced by fecal coliform colonies on M-FC medium are various shades of blue. Nonfecal coliform colonies are grey to cream-colored. Normally, few nonfecal coliform colonies will be observed on M-FC medium because of the selective action of the elevated temperature and addition of the rosolic acid salt reagent.

d. Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 fecal coliform colonies. This colony density range is more restrictive than the 20 to 80 total coliform range because of larger colony size on M-FC medium.

8. Delayed-Incubation Fecal Coliform Procedure.

a. This delayed-incubation procedure is comparable to the delayed-incubation total coliform procedure. It eliminates the need for a field water bath incubator and frees the field investigator from the time-consuming task of counting colonies. Examination at a central laboratory, rather than in the field, permits colony confirmation and complete biochemical identification of the organisms, as necessary.

b. Results obtained by this method have been consistent with results from the immediate standard test under various laboratory and field use conditions. However, determine the applicability of this test for a specific water source by comparison with the standard MF test, especially for saline waters, chlorinated wastewaters, and waters containing toxic substances. Use the delayed-incubation test only when the standard immediate fecal coliform test cannot be performed.

c. To conduct the delayed-incubation test filter the sample in the field immediately after collection, place filter on M-VFC holding medium, and ship to the laboratory. Complete the fecal coliform test in the laboratory by transferring filter to M-FC medium, incubating at 44.5 °C for 24 $\pm$ 2 hours, and counting the fecal coliform colonies. Membrane filters can be held for up to 3 days on M-VFC holding medium with little effect on the fecal coliform counts.

9. Klebsiella MF Procedure.

a. Klebsiella, a genus included in the coliform group as defined herein, may be associated with coliform regrowth in water supply distribution systems and is often a major component of the coliform population in paper mill, textile, and other industrial wastes. The normal coliform population in human and other warmblooded animal feces may contain 30 to 40 percent Klebsiella strains. Approximately 4 percent of bacterial pneumonia cases

and 18 percent of urinary tract infections are caused by pathogenic strains of Klebsiella most often K. pneumoniae. Environmental sources, such as vegetation, paper mills, textile production, sugar cane, and farm produce, contribute 71 to 88 percent of all Klebsiella in the total coliform population. Klebsiella occasionally may become established in the sediments of water supply distribution networks as a result of inadequate source water protection, unsatisfactory treatment protocols, or changes in the integrity of the pipe environment.

b. Although this test is not routinely performed in water monitoring, in some instances it may be useful as an aid in identification of the source of contamination. Rapid quantitation may be achieved in an MF procedure by modifying either M-FC or MacConkey agar base through substitution of inositol for lactose and adding carbenicillin. This method reduces the necessity for biochemical testing of pure strains. Preliminary verification of differentiated colonies is recommended.

c. Klebsiella colonies on modified M-FC agar (M-FCIC agar) are blue or bluish-grey. Most atypical colonies are brown or brownish. Occasional false positive occurrences are caused by Enterobacter species. Klebsiella colonies on modified MacConkey agar (MCIC agar) are pink to red. Background colonies are yellow except for rare occurrences of carbenicillin-resistant Serratia marcescens strains that may produce a pink pigmentation.

d. Verify Klebsiella colonies from the first set of samples from ambient waters and effluents and when Klebsiella is suspected in water supply distribution systems. Verify a minimum of five typical colonies by transferring growth from a colony or pure culture to a commercial multitest system for gram-negative speciation. Key tests for Klebsiella are citrate (positive), indole (negative), motility (negative), lysine decarboxylase (negative), ornithine decarboxylase (negative), and urease (positive). Klebsiella of nonfecal origin can be identified by correlation of indole production and liquefaction of pectin with the ability to grow at 10 °C and a negative fecal coliform response.

D. Tests for the Fecal Streptococcus Group.

1. When the term fecal streptococcus is used as an indicator of fecal contamination, the following species and subspecies are implied: S. faecalis, S. faecalis subsp. liquefaciens, S. faecalis subsp. zymogenes, S. faecium, S. bovis, and S. equinus. The term "enterococcus group" has been substituted erroneously for the fecal streptococcus group. The former group excludes S. bovis, S. equinus, and S. avium organisms. Consequently, media that are specific for the enterococcus group are restrictive and may not indicate the full extent of contamination by streptococci derived from fecal sources.

2. Assays for fecal streptococci may provide valuable supplementary data on the bacteriological quality of lakes, streams and estuaries. Because of limited survival in the environment, it is not recommended to use only fecal streptococci when determining water quality. In combination with fecal coliform data, data on fecal streptococci may provide more specific information about pollution sources because certain fecal streptococci are host-specific. Biochemical characterization or speciation may provide source information.

a. A predominance of S. bovis and S. equinus would indicate pollution due to the excrement of nonhuman, warmblooded animals. Investigations have demonstrated high numbers of these species associated with pollution involving meat processing plants, dairy wastes, and feedlot and farmland runoff. The S. bovis and S. equinus species have limited or short survival times outside of their natural habitat; thus, their presence in water indicates very recent contamination.

b. The group S. faecalis subsp. liquefaciens is not restricted to the intestines of humans and animals. It has been found associated with vegetation, insects, and certain types of soils. This is detrimental, especially for indicating low-density fecal contamination, because when the count is below 100 fecal streptococci/100 mL this organism generally predominates. Because media currently in use do not exclude such strains selectively, use of fecal streptococcus limits for recreational water based on densities below 100 organisms/100mL must be assumed unreliable unless confirmed by concurrent fecal coliform testing. Biochemical characterization of fecal streptococci also may eliminate the possibility of predominance of S. faecalis subsp. liquefaciens.

c. Fecal coliform/fecal streptococcus (FC/FS) ratios may provide information on possible sources of pollution. Estimated per capita contributions of fecal coliforms and fecal streptococci for animals were used to develop FC/FS ratios. A ratio greater than 4.1 is considered indicative of pollution derived from domestic wastes composed of human excrement, whereas ratios less than 0.7 suggest pollution due to nonhuman sources. Ratios between 0.7 and 4.4 usually indicate wastes of mixed human and animal sources. Speciation will provide more definitive information on potential sources. To minimize misinterpretation of ratios, take the following precautions:

(1) Measure sample pH because streptococcal densities can be altered significantly if water pH is above 9.0 or below 4.0;

(2) Sample as close as possible to the pollution source because fecal streptococci have relatively short lives outside the animal host. Points downstream where travel time from pollution source exceeds 24 hours will provide erroneous ratios;

(3) Inspect sources(s) of pollution when various pollution sources are present because ratios may yield deceptive assessments;

(4) Carefully use ratios for samples from marine waters, bays, and estuaries, because ratios may be of little value in differentiating between human and nonhuman sources; and

(5) Do not use ratios when fecal streptococcus counts are below 100/100 mL.

3. The MTF test and the MF test for fecal streptococci are very similar to the tests for total coliforms with the substitution of appropriate media.

E. HPC Plate Count Method.

1. The 16th edition of Standard Methods introduced the first major procedural change in what had been called the standard plate count (SPC). The SPC attempted to provide a single standardized means of determining the density of aerobic and facultative anaerobic heterotrophic bacteria in water. Because it is impossible to recover all viable bacteria present in a water sample with a single procedure, new methods have been developed to improve recovery of the heterotrophic bacteria population. Three alternative methods and two new media are presented. Any measurement is still empirical because bacteria occur singly or in pairs, chains, clusters, or packets, and no single method, growth medium, or set of physical conditions can satisfy the physiological requirements of all bacteria in the water sample. Whichever procedure is used, the HPC is the best available measure of water treatment plant efficiency, aftergrowth in transmission lines, and general bacterial composition of source water.

2. The pour plate method, formerly known as the SPC, has several disadvantages that limit recovery of the maximum number of organisms irrespective of medium and incubation temperatures and the time used; tempered medium at 44 to 46 °C may cause heat shock to stressed bacteria and the nutritionally rich medium may decrease recovery of starved bacteria. The alternative methods have attempted to overcome these liabilities.

3. The spread plate method eliminates heat shock caused by tempered agar. Additionally, all colonies will be on the agar surface where they can be seen and counted readily and can be distinguished easily from particulates or air bubbles. This procedure may require less time and space than the pour plate method but is limited by the small sample volume that can be used: 0.1 to 0.5 mL depending on absorbancy of the agar. As in the pour plate method, spreaders also may be a problem.

4. The MF method permits testing large volumes of low-turbidity waters. It may require the least analytical time. Disadvantages include problems with spreaders, contamination if plates are prepared and stored for too long, and varible recovery dependent on quality of membrane filter used.

5. The HPC procedure counts both bacterial pathogens and innocuous bacteria; there is no way to know whether the bacteria counted are pathogens or innocuous, or the proportion of each (50 FR 46955). Therefore, it is impossible to specify a scientifically rational maximum contaminant level goal (MCLG), and to set any particular HPC level (other than at zero) at which no adverse health effects occur. Drinking water with any given HPC level might contain numerous, few, or no pathogens.

VI. CURRENT AND NEW TOTAL COLIFORM REGULATIONS.

A. Current Total Coliform Regulations.

1. Regulations for microbiological monitoring of drinking water are part of the National Primary Drinking Water Regulations (NPDWR), Title 40, Code of Federal Regulations, Part 141. Presently, regulatory compliance from a microbiological standpoint is based on the level of total coliforms in the water.

2. The NPDWR contains subparts specifying MCLs, monitoring and analytical requirements, and reporting and public notification requirements. Major components are summarized below.

a. If the MF method of analysis is used, the MCL for total coliforms is 1 per 100 mL as an arithmetic mean for all samples during the month and 4 per 100 mL for individual samples. Not more than 5 percent of the monthly samples may exceed the 4 per 100 mL limit. For the MTF procedure using 10 mL sample portions, no more than 10 percent of all tubes examined in the month may be positive and not more than 5 percent of all samples may have three of more positive tubes.

b. Monitoring is based on the population served by the system. The table in 40 CFR 141 specifies the number of samples to be collected monthly proportionate to the population. Samples are to be collected from locations designated to be representative of conditions in the distribution system.

c. When the MCL is exceeded for an individual sample point, a check sample must be collected daily from that point. This procedure must continue until results are negative for 2 consecutive days.

d. If the presence of coliforms is confirmed by check samples for a particular sampling point, the water supplier shall notify the State within 48 hours.

B. Status of Final Rule.

1. The proposed total coliform rule of the NPDWR was published in the Federal Register (FR) during November 1987 (Enclosure 1, reference 11). In May 1988, additions/changes to the proposal and an extension of the public comment period was published in the FR. The final rule was promulgated on 29 June 1989 (Enclosure 1, reference 12).

2. The new total coliform rule will become effective 18 months from promulgation or 31 December 1990. Until that time, the current total coliform monitoring requirements remain in effect. The following section provides highlights of the new total coliform rule. Several of the requirements are very different from those currently in effect.

C. Summary of Final Rule.

1. MCL for Total Coliforms.

a. The MCL will be based on the presence or absence of total coliforms in the sample, rather than an estimate of coliform density.

b. Determination of whether the MCL is exceeded will be based on the number of samples tested and the number found positive during the month.

(1) If fewer than 40 samples/month are analyzed within a system, no more than one sample/month can be coliform-positive.

(2) If at least 40 samples/month are analyzed within a sytem, no more than 5.0 percent of the samples can be coliform-positive. Notice that the 5.0 percent is taken to one decimal place in order to determine the MCL.

2. Monitoring Frequency for Total Coliforms.

a. The frequency that a system must monitor for total coliforms is based on the population served and is shown in Tables 1 and 2.

b. Systems using unfiltered surface water, regardless of population served, must collect and analyze one coliform sample from near the first service connection each day the turbidity of the source water exceeds one Nephelometric Turbidity Unit (NTU).

c. Individual states must approve the sample site plan for each public water system.

3. Response to a Coliform-Positive Sample.

a. A set of repeat samples (Table 1) must be collected for each total coliform-positive routine sample. At least one repeat sample must be from the same tap as the original positive and the others must be within five service connections of the original to include an upstream and a downstream tap. Repeat samples must be collected within 24 hours after notification of the original positive result. If any repeat samples are total coliform-positive, additional repeat samples are required until an MCL has been violated and the State has been notified. Continued monitoring will be accomplished at the discretion of State's regulatory authorities.

b. When total coliforms are detected in any original or repeat samples, and the sample is not invalidated by the State, the system must collect a minimum of five routine samples the next month the system is in operation. This is in addition to the repeat sampling requirement noted in paragraph a, above.

c. Invalidation of a sample by the State may occur if the laboratory acknowledges analytical error or written documentation reflects that the coliform-positive result was due to a local circumstance and not an indication of distribution system quality. Samples that are invalidated do not count toward the minimum monitoring frequency.

TABLE 1. COLIFORM MONITORING REQUIREMENTS - SYSTEMS SERVING < 4900

System Size	No. Samples	No. Repeats	Response to Positive Additional Monitoring
NCWS	quarterly	4	5/mo for 1 add mo
25-1000	monthly*	4	5/mo for 1 add mo
1001-2500	2/month	3	5/mo for 1 add mo
2501-3300	3/month	3	5/mo for 1 add mo
3301-4100	4/month	3	5/mo for 1 add mo
4101-4900	5/month	3	NA
>4900	Table 2	3	NA

* For exceptions, see Table 2.
NCWS - Noncommunity water systems.
NA - Not applicable

TABLE 2. COLIFORM MONITORING FREQUENCY BY POPULATION SERVED

Population Served	Minimum Number Samples/Month*
25 - 1,000	1†
1,001 - 2,500	2
2,501 - 3,300	3
3,301 - 4,100	4
4,101 - 4,900	5
4,901 - 5,800	6
5,801 - 6,700	7
6,701 - 7,600	8
7,601 - 8,500	9
8,501 - 12,900	10
12,901 - 17,200	15
17,201 - 21,500	20
21,501 - 25,000	25
25,001 - 33,000	30
33,001 - 41,000	40
41,001 - 50,000	50
50,001 - 59,000	60
59,001 - 70,000	70
70,001 - 83,000	80
83,001 - 96,000	90
96,001 - 130,000	100
130,001 - 220,000	120

Listing abbreviated based on average effective population of most military installations.

* A noncommunity water system shall monitor in each calendar quarter during which the system provides water to the public. If such a system uses unfiltered surface water, it must sample at the same frequency as a community water system, except that in no case shall it be reduced to less than once per month.

† Based on a history of no coliform contamination and on a sanitary survey every 5 years by the State showing the water system to be supplied solely by a protected ground water source and free of sanitary defects, a community public water system serving 25 to 1,000 persons may reduce this sampling frequency with the written permission of the State, except that in no case shall it be reduced to less than once per quarter.

4. Tests for Fecal Coliforms.

a. Total coliform-positive samples must be analyzed for either fecal coliforms or E. coli. If a total coliform-positive sample is also shown to be fecal coliform or E. coli-positive, the system must notify the State of this no later than the end of the next business day.

b. A fecal coliform or E. coli-positive repeat sample, or a fecal coliform or E. coli-positive original sample followed by a total coliform-positive repeat, is considered an acute violation of the MCL for total coliforms. This occurrence would require public notification by electronic media within 72 hours.

c. Invalidation of a total coliform-positive sample also invalidates any subsequent fecal testing on the same sample.

5. Heterotrophic Bacteria.

a. The EPA is concerned that heterotrophic bacteria, if present in the distribution system, could interfere with the analysis for total coliforms. Therefore, the analyst shall visually inspect the coliform media to determine if interference has occurred. If a coliform sample produces a turbid culture in the absence of gas production using the MTF method, produces a turbid culture in the absence of an acid reaction with the P-A test, or produces either a confluent growth or a colony number that is TNTC using the MF procedure, the sample is invalid (unless total coliforms are detected). The water purveyor must, within 24 hours of receiving the result, collect another sample from the same location as the original and analyze for total coliforms. The EPA recommends using media less prone to interference by the heterotrophic bacteria in such cases.

b. The second sample should be counted toward total coliform compliance calculations unless it, too, demonstrates interference by the heterotrophic bacteria. The system must continue to resample within 24 hours until a valid result is obtained.

6. Variances and Exemptions. No variances or exemptions will be allowed to the total coliform rule.

D. Final Rule MCLG. When EPA proposes an MCL for a contaminant, it must simultaneously propose an MCLG. The MCLG is that level at which there would be no anticipated adverse health impact, allowing for a margin of safety. It is not an enforceable level. Total coliforms, which include the fecal

coliforms, are usually not pathogenic in themselves. However, their presence in drinking water indicates the potential presence of fecal pathogens, as shown by their frequent association with waterborne disease outbreaks. For these reasons, EPA feels the appropriate MCLG must be 0.

E. Final Rule MCL.

1. P-A Concept.

a. The EPA has decided to base the coliform MCL on their presence or absence rather than on an estimation of coliform density because data in the literature do not demonstrate a quantitative relationship between coliform densities and either pathogenic density or the potential for a waterborne disease outbreak. This P-A concept, not to be confused with the P-A analytical test described in paragraph VB7, has several advantages:

(1) Unlike the uncertainties associated with estimates of coliform density of a sample, it is easy to determine the presence or absence of coliforms;

(2) The sample transit time is less critical, because any decrease in coliform density between sample collection and analysis will seldom result in complete die-out of all coliforms in that sample; and

(3) This change eliminates the data truncation implicit in the analytical methodology as a calculation difficulty - e.g., the 5-tube MTF procedure is not sufficiently sensitive to allow estimation of densities less than 2.2 or greater than 16 coliforms/100 mL.

b. The EPA also has recognized some shortcomings associated with the P-A concept. High coliform levels may, on occasion, signal the occurrence of high pathogenic densities. In addition, the current regulations have been in use for decades and State officials and the public water system operators are familiar with them.

2. Monthly MCL.

a. As mentioned in paragraph VIC1b above, the number of coliform-positive samples may not exceed 5.0 percent of the total samples analyzed from a system when the system collects 40 or more samples per month. Systems in which fewer than 40 samples are analyzed are allowed one positive.

b. The system must report a violation of the total coliform MCL no later than the close of the next business day after learning of the violation.

F. Sanitary Survey Requirements.

1. States must determine the vulnerability of systems to coliform contamination as a condition for reduced monitoring. The new coliform rule will require that the State, or an agent acceptable to the State, conduct sanitary surveys and that the State determine whether the results are satisfactory. Guidance for conducting a sanitary survey is provided in the EPA's Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources (EPA, in press).

2. All systems collecting fewer than 5 samples per month must have periodic sanitary surveys as shown in Table 3. Community water systems must complete an initial survey by 29 June 1994 and noncommunity systems by 29 June 1999. As stated in the newly promulgated surface water treatment rule (Enclosure 1, reference 13), systems using unfiltered surface water must perform an annual on-site inspection [40 CFR 141.71(b)]. However, the on-site inspection is not equivalent to the sanitary survey. Therefore, additional criteria may need to be met to satisfy the coliform rule requirements.

G. Sampling Sites.

1. The current regulation states that "... samples shall be taken at points which are representative of the conditions within the distribution system" [40 CFR 141.21(a)]. This suggests, but does not specify, sampling throughout the water distribution system.

2. The EPA intended in its proposal that sampling sites be varied over a year in a manner, so that the use of the same sites is confined to a short period of time, e.g., 1 or 2 months. In addition, new sampling sites would be selected every year. The intention was that all isolated sections of the distribution system would be sampled periodically. In the final rule, the State will now have the final authority to approve the system sampling plan. The sampling program should be designed so that there is no place in the distribution system where microbiological contamination could persist indefinitely with little

chance of detection. Larger systems tend to maintain the same sample sites in order to build historical records on coliform incidence. These sites are often selected as being key locations within the distribution systems.

TABLE 3. FREQUENCY OF SANITARY SURVEYS
SYSTEMS COLLECTING FEWER THAN 5 SAMPLES PER MONTH

Type System	Sanitary Survey within (yrs) Initial	Subsequent
Community water systems		
Filtered surface water	5	5
Unfiltered surface water	*	3/5*
Undisinfected ground water	5	5
Disinfected ground water	5	5
Nontransient noncommunity system	5	5
Other noncommunity systems		
Filtered surface water	10	5
Unfiltered surface water	*	3/5*
Undisinfected ground water	10	5
Disinfected ground water	10	10

* Sanitary surveys shall be performed at a frequency specified by 40 CFR 141, Subpart H.

H. Repeat Samples.

1. The EPA will require a set of repeat samples for every positive coliform sample, to evaluate the significance of a coliform-positive occurrence. The number of repeat samples required is scaled according to how many are normally collected each month (see Table 1). The EPA believes a set of repeat samples is appropriate because it would allow the system to determine quickly whether a serious contamination problem exists and whether an MCL has been exceeded. Negative repeat samples

could give some assurance that the contamination is not extensive or has been eliminated. Moreover, negative repeat samples provide confidence to a small system that a much smaller percentage of its samples are coliform-positive. Coliform-positive repeat samples would indicate a serious continuing problem.

2. Repeat samples differ from additional routine samples which must be collected during the following month in response to a total coliform-positive sample. The repeat samples must be collected within five service connections of the original positive sample while the routine samples may be collected at any distribution system site consistent with the approved sampling plan.

I. Analytical Methodology for Total Coliforms.

1. The current EPA regulations (Enclosure 1, reference 8) specify the use of either the MTF method or the MF method, as described in the 16th edition of Standard Methods (Enclosure 2, reference 2), to quantify the presence of coliforms in drinking water.

2. The analysis of coliforms under this new regulation may be conducted using the 10-tube MTF test, the MF test, or the P-A test using a standard 100 mL volume of sample. The protocol for each of these tests is provided in Standard Methods, Parts 908, 908A and 908B (pp. 870-878); 909 and 909A (pp. 886-894); and 908E (pp. 882-884), respectively.

3. A fourth methodology will be allowed under the final coliform rule. The Minimal Media ONPG-MUG (MMO-MUG) test (marketed as the Autoanalysis Colilert System) detects the presence of total coliforms and *E. coli* simultaneously in the same culture vessel.

a. For this method, the water sample is added to the culture vessel containing a predispensed medium and incubated at 35 °C for 24 hours. If coliforms are present in the water sample, an enzyme reaction will occur, producing a yellow color. Similarly, an enzyme unique to *E. coli* will produce a fluorescence detectable under ultraviolet light. Noncoliform bacteria cannot metabolize the indicator nutrients and therefore do not interfere with the test.

b. The EPA has conducted extensive studies that verified this method as comparable to the MTF and MF methods. Subsequent to promulgation of the final total coliform rule, EPA has approved the MMO-MUG for use under the current regulations (Enclosure 1, reference 14). There are now three approved methods to enumerate total coliforms in drinking water. The MMO-MUG media is available in either the MPN or P-A formats.

4. Regardless of which analytical method is used under the new rule, the determination of coliform density would not be required for MCL compliance; only the presence or absence of coliforms in the samples would be reportable to the State (or EPA). The new coliform rule, however, would not preclude a system from either enumerating or characterizing coliforms or other organisms.

J. Reporting, Public Notification, and Record Maintenance.

1. The current requirements for reporting, public notification, and record maintenance may be found in 40 CFR 141.31, 141.32, and 141.33, respectively.

2. The final total coliform rule requires that the State be notified of a violation of an MCL by the end of the next business day after the violation is discovered. Also, if a coliform monitoring requirement, e.g., a sample siting plan or collection of required repeat samples, is violated, the system must notify the State within 10 days.

3. The public notification regulations require that notices of MCL violations include a description of adverse health effects. The presence of fecal coliforms/*E. coli* in treated water is cause for grave concern and may pose an acute risk to human health, because when fecal coliforms are detected, it is likely that human pathogens are present. For this reason, EPA believes that more urgent public notice language is needed when fecal coliforms are detected, compared to that for total coliforms, even though the detection of fecal coliforms may only constitute a violation of the monthly MCL for total coliforms. The EPA has revised the mandatory language to be used by the system to notify consumers of the presence of fecal coliforms/*E. coli*. The notice should read as follows:

"The United States Environmental Protection Agency sets drinking water standards and has determined that the presence of fecal coliforms or E. coli is a serious health concern. Fecal coliforms and E.coli are generally not harmful themselves, but their presence in drinking water is serious because they usually are associated with sewage or animal wastes. The presence of these bacteria in drinking water is generally a result of a problem with water treatment or the pipes which distribute the water, and indicates that the water may be contaminated with organisms that can cause disease. Disease symptoms may include diarrhea, cramps, nausea, and possibly jaundice, and associated headaches and fatigue. These symptoms, however, are not just associated with disease-causing organisms in drinking water, but also may by caused by a number of factors other than your drinking water. EPA has set an enforceable drinking water standard for fecal coliforms and E. coli to reduce the risk of these adverse health effects. Under this standard all drinking water samples must be free of these bacteria. Drinking water which meets this standard is associated with little or none of this risk and should be considered safe. State and local health authorities recommend that consumers take the following precautions: [To be inserted by the public water systems, according to instructions from State of local authorities]."

4. The requirements for record maintenance remain unchanged by the final total coliform rule. Records pertaining to coliform monitoring of drinking water must be maintained for 5 years.

VII. TECHNICAL ASSISTANCE. Requests for services should be directed through appropriate channels of the requesting activity to Commander, U.S. Army Environmental Hygiene Agency, ATTN: HSHB-ME-W, Aberdeen Proving Ground, MD 21010-5422, with an information copy furnished to the Commander, U.S. Army Health Services Command, ATTN: HSCL-P, Fort Sam Houston, TX 78234-6000.

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3 Encls

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ABBREVIATIONS

CFR	Code of Federal Regulations
cm	centimeter
E. Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
EPA	U.S. Environmental Protection Agency
FC	fecal coliform
FR	Federal Register
FS	fecal streptococci
HPC	Heterotrophic Plate Count
IMViC	indol, methyl red, Voges-Proskauer, and citrate utilization
K.	Klebsiella
L	liter
MCL	Maximum Contaminant Level
MCLG	Maximum Contaminant Level-Goal
M-FC	Membrane-Fecal Coliform
MF	Membrane Filter Method
mg/L	milligram per liter
mL	milliliter
MPN	Most Probable Number
MTF	Multiple-Tube Fermentation Method
M-VFC	Membrane-Fecal Coliform holding medium
NTU	Nephelometric Turbidity Unit
NPDWR	National Primary Drinking Water Regulations
P-A	Presence-Absence Test
pH	negative logarithm of hydrogen ion
SPC	Standard Plate Count
TNTC	Too Numerous To Count
USAEHA	U.S. Army Environmental Hygiene Agency
USPHS	U.S. Public Health Service