The Use of Indicator Organisms to Assess Public Water Safety

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In memory of

Clifford C. Hach

(1919-1990)

inventor, mentor, leader and, foremost,
dedicated chemist

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SECTION 1 Introduction

Since 1855 when Snow and Budd determined that outbreaks of typhoid fever and cholera were related to water contaminated with fecal wastes,[1] the need to determine the suitability of water for drinking and bathing purposes has been recognized. To this end, simple, reliable, and rapid methods for detection and enumeration of microorganisms are necessary. Pathogens are not detected easily and routine culturing is not recommended; instead, methods have been developed to determine the presence of other fecal organisms. If these organisms are absent, bacterial pathogens are unlikely to be present. This concept of *indicator organisms* was introduced in 1892[2] and is the basis for most microbiological quality standards in water today.

Enteric (intestinal) and opportunistic diseases (which affect people when their resistance is lowered) caused by waterborne microorganisms still occur in even the most advanced nations with superior sanitation practices and sophisticated treatment and testing programs. Unfortunately, the absence of detectable indicator organisms does not effectively predict the absence of opportunistic bacteria, viruses,[3,4] parasitic protozoans such as *Giardia lamblia*,[5,6] or *Cryptosporidium,*[7] all of which may be more resistant to disinfection. Other quality parameters, such as turbidity, also must be monitored to ensure the safety of drinking water. (See Appendix D.) This monograph is limited to bacterial monitoring considerations.

SECTION 2 Indicator Organisms

Definition

Certain criteria should exist before an indicator organism can be considered reliable in predicting a health risk:

1. The organism must be exclusively of fecal origin and consistently present in fresh fecal waste.
2. It must occur in greater numbers than the associated pathogen.
3. It must be more resistant to environmental stresses and persist for a greater length of time than the pathogen.
4. It must not proliferate to any great extent in the environment.
5. Simple, reliable, and inexpensive methods should exist for the detection, enumeration, and identification of the indicator organism.

Organisms that fit these criteria include the coliform bacteria, fecal streptococci (enterococci) and the sulfite-reducing clostridia (i.e., *Clostridium perfringens*).

Gastrointestinal pathogens known to have caused outbreaks of enteric disease are largely from the systematically defined family, Enterobacteriaceae, and include *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Enterobacter* and enterotoxigenic *Escherichia coli* (*E. coli*). *Vibrio cholerae* and *Campylobacter jejuni* are two other enteric pathogens often found in contaminated water.[2] These organisms are spread by water contaminated with fecal material from humans and other warm-blooded animals.

Coliforms

In 1885, T. Escherich discovered a bacterial species that was present almost universally in human fecal material. These bacteria not only occurred in high densities but frequently were associated with the pathogenic typhoid bacillus (*Salmonella typhi*). Escherich's bacteria were named *Escherichia coli*.[3] They are part of a group of bacteria, the coliform or coli-aerogenes bacteria, of fecal origin.

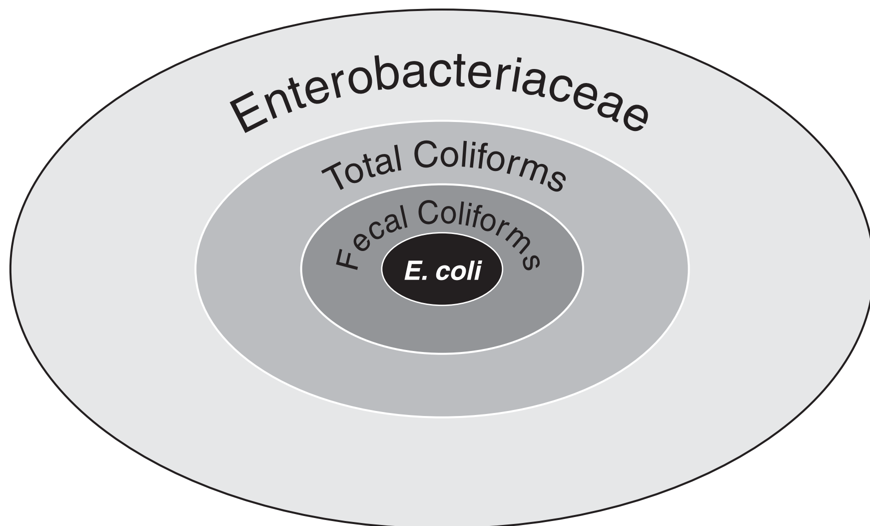
Coliform bacteria belong to the family Enterobacteriaceae but are further defined by functional characteristics rather than systematic genus and species. All of the Enterobacteriaceae are rod-shaped, non-spore-forming, and gram-negative bacteria. Certain bacteria of this family, largely belonging to the genera *Escherichia*, *Citrobacter*, *Klebsiella*, and *Enterobacter*, are capable of growth in the presence of bile salts or other surface-active agents and are cytochrome-oxidase negative. Coliforms are distinguished by the ability to ferment lactose at either 35 or 37 °C with the production of acid, gas, and aldehyde within 24 to 48 hours.

Based on these criteria, the World Health Organization has defined coliforms as any rod-shaped, non-spore-forming, gram-negative bacteria capable of growth in the presence of bile salts or other surface-active agents. Continuing, the definition states that coliforms are cytochrome-oxidase negative and able to ferment lactose at either 35 or 37 °C with the production of acid, gas, and aldehyde within 24 to 48 hours.[5] (The coliform group has been identified by genus and species based on studies performed on β-galactosidase-positive Enterobacteriaceae.[4] However, for practical purposes the functional definition is still used.)

As an indicator group, the number of viable coliform organisms present does not always correlate properly with the presence of pollution. Elevated total coliform counts can occur from the presence of *Klebsiella, Citrobacter,* and other organisms of non-fecal origin that do not necessarily reflect the health risks associated with consumption of the water. In a study done by G. McFeters, et al.,[6] the comparative survival of organisms in well water indicated that coliform bacteria have a half-life of 17 hours whereas the pathogenic *Salmonella typhi* have a half-life of 6 hours and *Vibrio cholerae* have a half-life of 7.2 hours. Data confirm the effectiveness of coliforms as indicators of the absence of these pathogenic organisms. However, other pathogenic *Salmonella* and *Shigella* organisms have half lives in the range of 16 to 27 hours. Several incidents of pathogen isolation in the absence of coliform counts or very low coliform counts have been reported. Epidemic outbreaks caused by *Salmonella typhimurium*, *Shigella flexneri* and *Shigella heidelberg* have been reported in water with very few or no detectable total coliform organisms.[1,7,8,9] Although these rare outbreaks are consistent with the McFeters data, it has been suggested that the absence of coliform counts was due to the test method used rather than being descriptive of the real condition of the sample.

The total coliform group is the most inclusive indicator classification (*Figure 1* and *Figure 2*) and contamination indicated by the presence of total coliforms is indicative of inadequate disinfection of drinking water. Therefore, in the United States and most other developed countries the microbiological quality standards for drinking water are based on the measurement of the total coliform population.

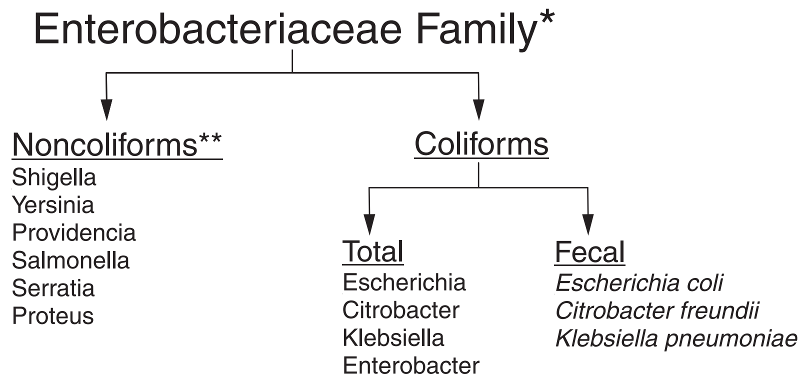
Figure 1 Relationship of Bacteria in Enterobacteriaceae Family



In 1904, Eijkman proposed gas production from glucose when incubated at 46 °C as a test for fecal coliforms.[10] This proved to be too broad since many organisms outside the genera previously defined as total coliform bacteria can produce gas from glucose. Therefore, the test was quickly modified to specify those organisms which produce gas from lactose. Additionally, the incubation temperature of the fecal test has been modified to 44.5 °C. Today the accepted definition of fecal coliform bacteria is: thermotolerant total coliform bacteria capable of growth with acid and gas production at 44.5 ± 0.2 °C. The bacteria included in this group are predominantly *E. coli*, *Citrobacter freundii* and *Klebsiella pneumoniae*.

However, some other species and strains of other genera will give positive results. The predominant genera of the Enterobacteriaceae family are listed in the coliform and noncoliform groups diagrammed in *Figure 2*. Quality standards for most natural water sources are based on fecal coliform counts.

Figure 2 Enterobacteriaceae



* Structure adapted from *Bergey's Manual of Systemic Bacteriology*, Vol. 1, edited by Noel Krieg, Williams and Wilkins, 1984.
** Represent main genera.

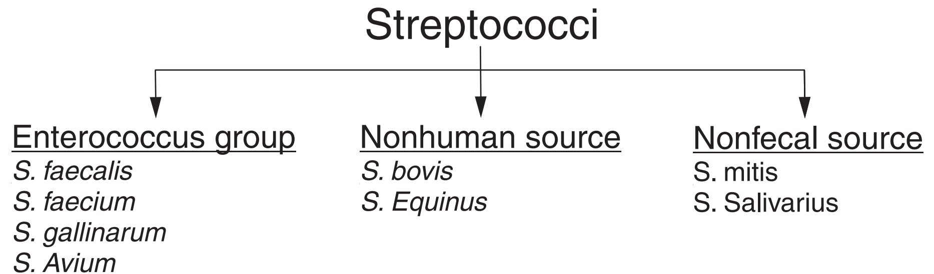
The best coliform indicator of fecal contamination from human and animal waste is *E. coli*. In human and animal feces, 90 to 100% of the coliform organisms isolated are *E. coli.* In sewage and contaminated water samples, the percentage drops to 59%.[5] For a long time, reliable and simple tests for the detection, enumeration and identification of *E. coli* did not exist. In 1986, new tests for *E. coli*, based on biochemical reactions, resulted in the promulgation of standards based on *E. coli* counts. The test proposed for recreational water quality is based on the positive reaction to urease of nearly all fecal coliforms except *E. coli*. This method promotes the growth of fecal coliform populations and subsequently employs an *in situ* urease test.[11] Another test is based on the ability of most *E. coli* strains to produce β-glucuronidase, an enzyme that hydrolyzes the glucuronosyl-o-bond of glucuronide conjugates such as 4-methylumbelliferyl-β-D-glucuronide (MUG). Among the Enterobacteriaceae, only *E. coli*, some *Shigella* and *Salmonella*[12] and a few *Yersinia*[13] produce the enzyme capable of hydrolyzing the glucuronide conjugates. Tests for the detection and enumeration of *E. coli* have been developed based on the presence of this enzyme.[12,13,14]

Fecal Streptococci (Enterococci)

J. G. Laws and F. W. Andrewes first reported the existence of streptococci (gram-positive, spherical bacteria) in the gastrointestinal tract. Subsequently these bacteria were recognized as being indicative of dangerous pollution and characteristic of sewage and animal fecal wastes.[15] However, confusion over the identity of these indicators has resulted in few standards being proposed that use them. Streptococci from the feces of humans and warm-blooded animals have been referred to as enterococci, fecal streptococci, and group D streptococci. Included in these categories are *Streptococcus bovis* and *S. equinus*, not associated with man, and *S. mitis* and *S. salivarius*, not associated with fecal sources. (See *Figure 3*.) Ideally, fecal streptococci used as indicator organisms should comprise only *S. faecalis* and *S. faecium*. Until recently, tests to identify and enumerate this more limited group of organisms were not available.[16]

Consequently, enterococci were not used as primary indicator organisms in any water quality standards until 1986 when membrane filtration methods became available. (See *Figure 9*.)

Figure 3 Streptococci



Opportunistic Organisms

Often species of the family Enterobacteriaceae are opportunistic organisms not normally associated with diarrheal disease in healthy hosts.[2] These organisms are harmless in healthy hosts but are pathogenic in compromised hosts (for example, malnourished, diabetic, or immunosuppressed patients). *E. coli, Klebsiella, Enterobacter, Proteus, Provindencia,* and *Serratia marcescens,* of the Enterobacteriaceae family, are responsible for 50% of the hospital-related infections in the United States. Potable and bathing waters containing excessive numbers of other opportunistic organisms such as *Pseudomonas, Flavobacterium* and *Acinetobacter* may produce infection in compromised hosts also.

Opportunistic organisms (which require few nutrients) flourish in the pipes when disinfection levels injure, rather than kill, organisms in drinking water plants and when residual disinfectant levels are inadequate in distribution systems. Regrowth of these organisms in distribution systems has become a major problem. Coliform counts cannot be used to predict the existence of many of these bacteria. For example, *Pseudomonas aeruginosa*, often occurring in human feces in much lower numbers than coliform organisms, frequently are found in untreated water in the presence of coliforms. However, in drinking water they may occur in the absence of routinely detectable coliforms.[17,18] An examination for specific opportunistic organisms may be of value in certain circumstances; for example, specific tests for *P. aeruginosa* in the reconstitution of rehydration mixtures, such as baby food and pharmaceutical preparations, as well as in the surveillance of hospital water supplies and bottled waters.[19] An examination of total bacterial densities in distribution lines also may be important in the routine monitoring of water supply systems.

SECTION 3 Drinking Water Standards

United States Regulations

National Primary Drinking Water Regulations (NPDWR), updated most recently in 1990, apply to all public potable water systems. In some states, particularly in the Midwest, regional rural water associations also are regulated by NPDWR because they, too, operate public water systems. Generally the only drinking water not regulated is private well water, but NPDWR guidelines should be applied to well water usage as a safety precaution.

NPDWR specify a maximum contaminant level, which is the highest concentration of a particular contaminant allowed in drinking water. Maximum contaminant levels are set at levels low enough to prevent health problems.

The presence of microbiological contaminants indicates the possible presence of pollution, which can cause health problems. As in past years, drinking water is currently tested for the presence of total coliform bacteria as an indicator of recent pollution.

Information about updated drinking water regulations, effective Dec. 31, 1990, appeared in the *Federal Register* (54 FR 27543), June 29, 1989. The maximum contaminant level goal is zero total coliforms per 100 mL of sample. Compliance is based on the presence or absence of coliform rather than enumeration (as previously reported).

New regulations require a 100 mL sample to be used for each analysis. Therefore, most probable number (MPN) testing will require either ten 10-mL tubes or five 20-mL tubes or one 100-mL tube. The requirement for membrane filtration (MF) testing continues to be filtration of 100 mL of sample.

Not more than one sample per month can be total coliform-positive if less than 40 samples are examined per month. Not more than 5.0% of the samples can be total coliform-positive if 40 or more samples are examined per month.

MPN, MF and presence/absence methods may be used. However, only the presence or absence of total coliforms must be reported. All coliform-positive samples must be analyzed for either fecal coliforms or *E. coli*, and the state must be notified if a sample tests positive.

For each routine sample that is coliform-positive, three repeat samples must be analyzed in the same month. If one or fewer samples are routinely analyzed per month, four repeat samples must be analyzed. At least one repeat sample must be taken from the same tap as the original positive sample. Two of the repeat samples must be collected within five service connections of the original sample—one upstream and one downstream. When less than five routine samples normally are collected from a utility system each month, five routine samples must be collected during the month following the appearance of a coliform-positive sample.

Drinking Water Standards

World Health Organization Guidelines

Piped and Unpiped Water Supplies

Fecal coliform counts should be zero per 100 mL of sample (0/100 mL) in all water supplies, piped or unpiped, treated or untreated. Total coliform counts should be 0 per 100 mL in piped, treated water supplies with an occasional occurrence of two organisms per 100 mL being allowed. In unpiped water supplies, total coliform counts of 10 organisms per 100 mL can occur infrequently. Frequent occurrences of high coliform counts signify the need for an alternative water source, or sanitary protection of the current source. A “boil water” order is needed when emergency water supplies fail to meet a criterion of zero (0) fecal and total coliforms.

Bottled Drinking Water

The U. S. Food and Drug Administration regulates bottled drinking water as a food product in the United States. The MF method of analysis is used to determine total coliform and total aerobic plate counts. Additionally, bottled water should be treated to prevent the growth of opportunistic organisms such as *Pseudomonas aeruginosa*.

Recreational Water Quality Criteria

Criteria for recreational water quality (full body-contact waters), revised by the United States Environmental Protection Agency (USEPA) in 1986, are based on studies done by Victor Cabelli[1] and Al Dufour.[2] Data showed no conclusive evidence of a correlation between coliform levels and illnesses among bathers. Studies show a better correlation of enterococci with gastrointestinal illnesses of swimmers in both marine and fresh water, according to the USEPA. The correlation of *E. coli* with illness was equal to enterococci correlations for fresh water but not marine water. A document published by the USEPA—*Bacteriological Ambient Water Quality Criteria for Marine and Fresh Recreational Waters*[3]—specifies the following standards: for fresh water, *E. coli* count ≤126/100 mL, enterococci ≤33/100 mL; for marine water, enterococci ≤35/100 mL.

Test methods prescribed by the USEPA for the detection and enumeration of these recommended indicator organisms are explained in another USEPA report.[4] Methods for the enumeration of fecal streptococci include MPN and MF, outlined in *Standard Methods for the Examination of Water and Wastewater*, 19th edition (hereafter termed *Standard Methods*).

SECTION 4 Selected Methods for Counting Indicator and Opportunistic Organisms

Currently, three primary techniques for the routine detection and enumeration of indicator and opportunistic bacteria currently are used. They are the MPN, MF, and plate count techniques. Additionally, presence/absence tests for coliform bacteria have been approved for NPDWR reporting purposes. All methods use media designed to select for the growth and identification of specific organisms. Generally, each method also includes inhibitors, restrictive temperatures, and/or limited nutrients to restrict growth of unwanted species. In this section, a brief description of each technique is followed by an overview of some methods in current use.

Most Probable Number Tests

The MPN test uses a specified number of tubes (based on the expected population in the sample) containing a specific medium and sample water. After incubation, each tube is examined for growth of the target organism(s). The number of tubes showing growth are matched to a statistically determined table of numbers to yield the *most probable number* of organisms in the sample.

Table 1 lists these numbers (at 95% confidence limits) for a sampling series of 10 tubes containing undiluted sample. Usually, 10 tubes are used if the expected population is between 0 (zero) and 23, or 15 tubes (five of each dilution in a dilution series) if the expected population is greater than 23.

Table 1: MPN Index (95% confidence limits) 10 mL undiluted sample/tube*

Positive	Negative	MPN/100 mL
0	10	less than 1.1
1	9	1.1
2	8	2.2
3	7	3.6
4	6	5.1
5	5	6.9
6	4	9.2
7	4	12.0
8	3	16.1
9	1	23.0
10	0	more than 23

* *Standard Methods*, 19th ed.

Table 2 illustrates the MPN index for a 15-tube test—five tubes of each of three dilutions. A dilution series of 1:1 (no) dilution (five tubes), 1:10 dilution (five tubes), and 1:100 dilution (five tubes) can be used to estimate populations between <2 and 1600 organisms. The same 15-tube method can be used when the expected population is greater than 1600 organisms by using a higher series of three dilutions. (For example, 1:100-, 1:1000-, and 1:10,000-dilution series can be used to estimate populations from less than 200 to more than 160,000 per 100 mL.) The MPN method is preferred when the sample is turbid, when operator expertise may be limited, or when sufficient data to prove comparable results by MF techniques have not be gathered.

Table 2: MPN Index (95% confidence limits) five tubes each of three dilutions*

Number of tubes giving positive reactions out of:			MPN index per 100 mL**	Number of tubes giving positive reactions out of:			MPN index per 100 mL**
5 undiluted samples (dilution factor–1)	5 dilutions of 10 (dilution factor–10)	5 dilutions of 100 (dilution factor–100)		5 undiluted samples (dilution factor–1)	5 dilutions of 10 (dilution factor–10)	5 dilutions of 100 (dilution factor–100)	
0	0	0	<2	4	2	1	26
0	0	1	2	4	3	0	27
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	30
1	1	0	4	5	0	2	40
1	1	1	6	5	1	0	30
1	2	0	6	5	1	1	50
2	0	0	4	5	1	2	60
2	0	1	7	5	2	0	50
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	90
2	2	0	9	5	3	0	80
2	3	0	12	5	3	1	110
3	0	0	8	5	3	2	140
3	0	1	11	5	3	3	170
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
3	3	0	17	5	4	4	350
4	0	0	13	5	5	0	240
4	0	1	17	5	5	1	300
4	1	0	17	5	5	2	500
4	1	1	21	5	5	3	900
4	2	0	22	5	5	4	1600
4	1	2	26	5	5	5	≥1600

* *Standard Methods*, 19th ed.
** Multiply the MPN index by the dilution factor from the series used when dilutions other than 1, 10, and 100 are used.

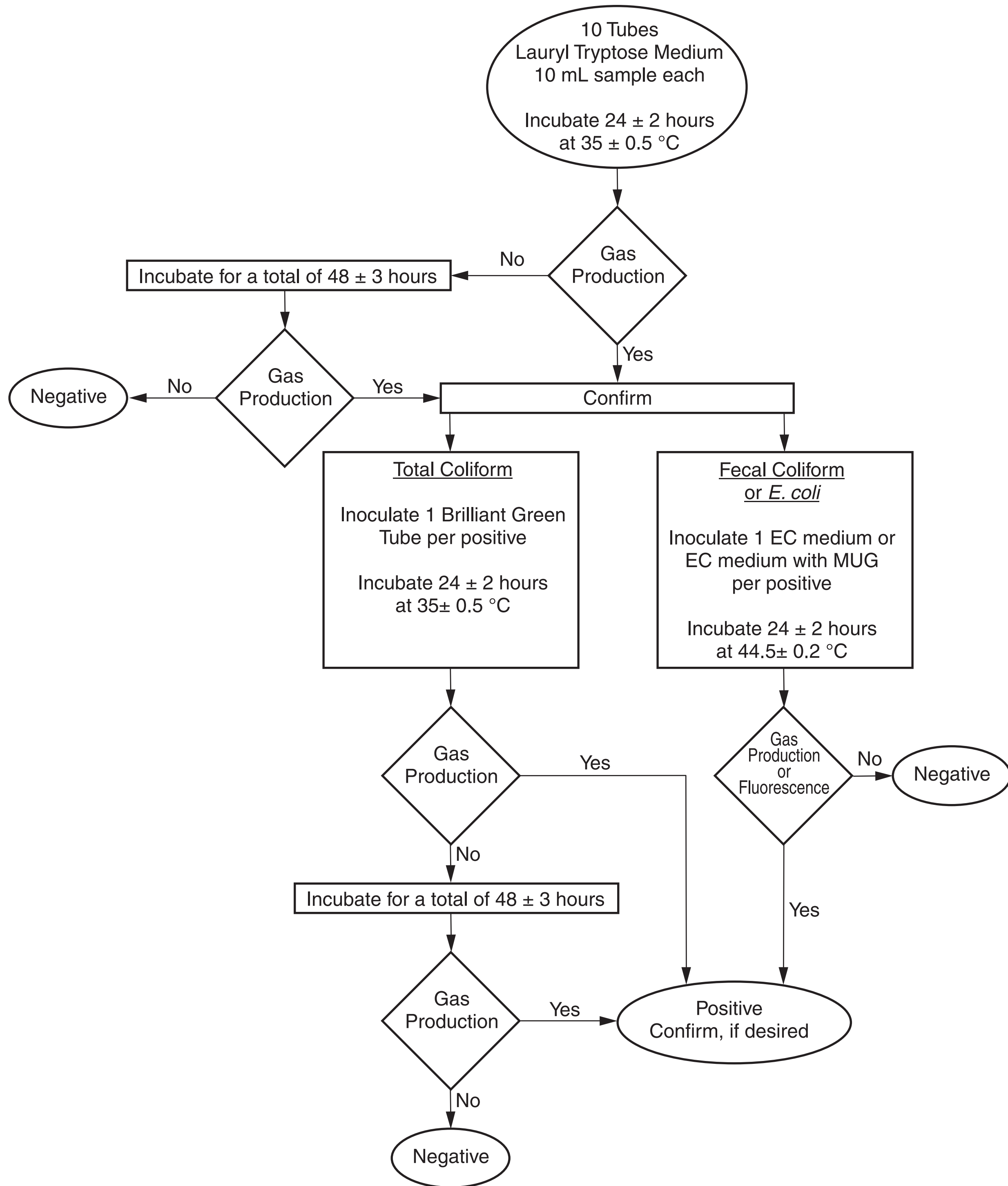
Selected Methods for Counting Indicator and Opportunistic Organisms

Total Coliforms in Potable Water

The MPN of total coliforms in potable (drinking) water is based on the World Health Organization definition of total coliforms. Presumption of the presence of coliforms is obtained when the organisms cultured produce gas (and acid) from lactose-containing medium at 35 °C in 24 to 48 hours. Confirmation of the organisms as coliforms is obtained in a second procedure when gas is produced in 24 to 48 hours from a brilliant green lactose bile broth medium. The presumptive test uses 10 tubes of an appropriate medium. The four types of presumptive media which may be used are minerals-modified glutamate medium, lauryl tryptose broth, MacConkey broth, and lactose broth.[1] The USEPA-approved procedure, found in the 19th edition of *Standard Methods*, requires either lauryl tryptose or lactose broth. However, analysts are cautioned to use lactose broth only when it has been proven not to increase the frequency of false positives or false negatives. Federal regulations also permit the use of bromcresol purple presence/absence broth. A total of 100 mL of sample is transferred to presumptive media by using a sterile transfer device. At this point the tubes are incubated. Inspections for positive reaction follow at 24 and 48 hours. Gas production constitutes a positive reaction in lauryl tryptose broth. Indicators have been used in minerals-modified glutamate medium, MacConkey broth, and presence/absence broth to signify acid production as well. A durham (inner) tube is used in some cases to collect gas. The presence of coliforms in gas-positive tubes should be confirmed by using brilliant green lactose bile broth. Growth (evidenced by increased turbidity) in the absence of gas production also should be confirmed.[2] The test is completed by following the schematic shown in *Figure 4*. An estimate of the confirmed coliform number in 100 mL of original sample is determined by using *Table 1*.

Current USEPA drinking water regulations require the analysis of each positive coliform sample to determine the presence of fecal coliforms or *E. coli*. EC medium may be used to confirm the presence of fecal coliforms, and EC medium with MUG may be used to confirm the presence of *E. coli*. The use of EC medium with MUG was promulgated in the *Federal Register* published January 8, 1991, as an acceptable method for determining the presence of *E. coli*.

Figure 4 Total Coliforms in Potable Water



Total and Fecal Coliforms in Nonpotable Water and Wastewater

The multiple-tube, decimal-dilution procedure should be used for determining MPN indices for both total and fecal coliforms in nonpotable water and wastewater. (See *Appendix A* for an explanation of dilution methods.) Five tubes each are prepared for three dilutions. The first set of five tubes is inoculated with a sample of suitable dilution. The second set is inoculated with sample that is $^{1}/_{10}$ the concentration of the sample used in the first set of tubes. The third set is $^{1}/_{100}$ the concentration of the initial sample dilution. A suitable sterile, buffered dilution water is used to make the dilutions. The total coliform procedure is the same as described in the section on *Total Coliforms in Potable Water* with the exception of the decimal dilution step. Results must be interpreted by using *Table 2*.

The USEPA-accepted method for detecting fecal coliforms uses lauryl tryptose tubes for the presumptive test and EC medium tubes for confirmation. This procedure is completed by following the schematic in *Figure 5* and the dilution chart in *Table 3*.[3] A single-step procedure using A-1 medium has been accepted by the *Standard Methods* committee. The A-1 procedure, using a 35 °C resuscitative incubation, is applicable to seawater and treated wastewater. The A-1 test is completed by following the schematic in *Figure 6*.[2] An estimate of the number of fecal coliforms in 100 mL of original sample is determined by using *Table 2*.

Figure 5 Fecal Coliforms

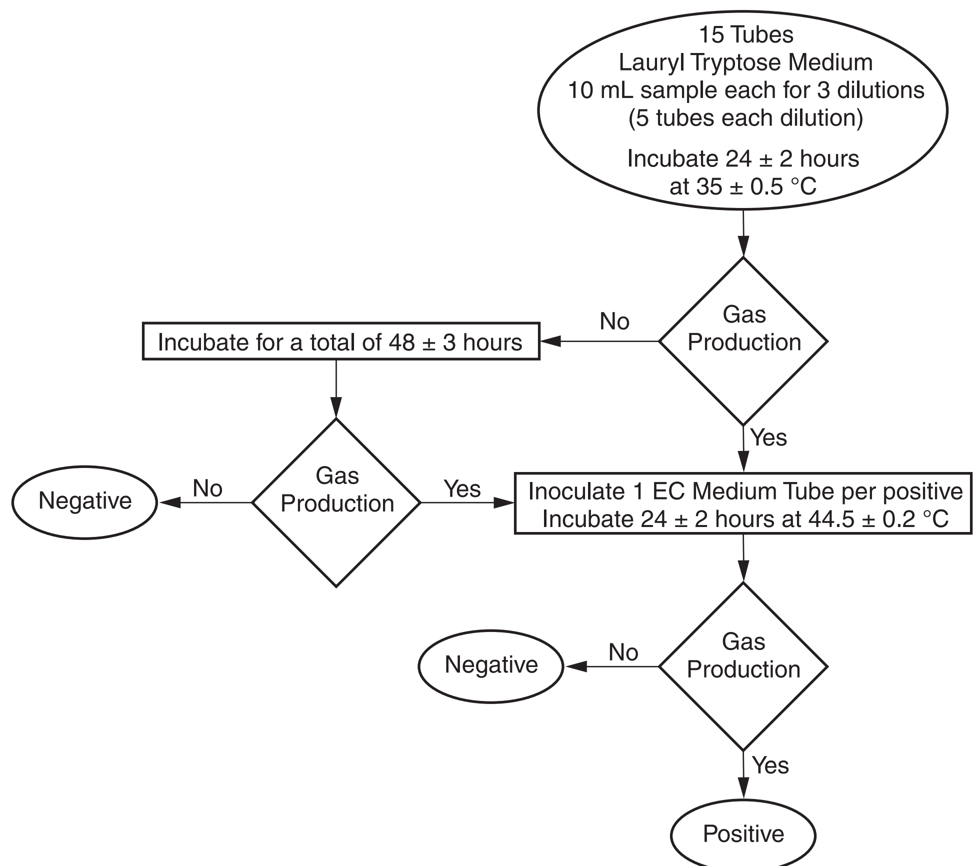


Figure 6 Fecal Coliforms—A-1 medium

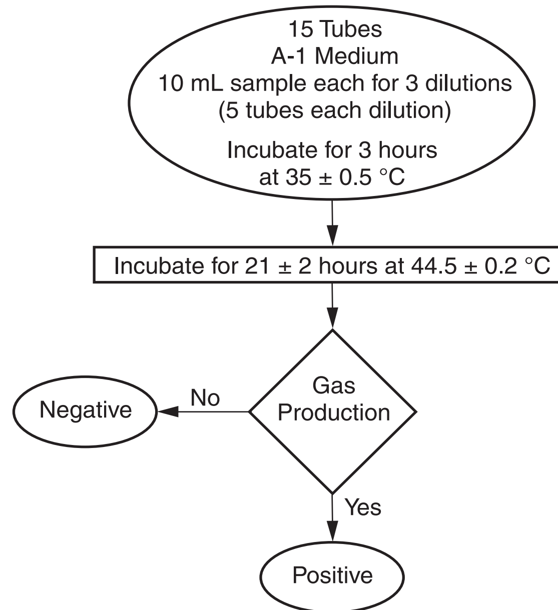


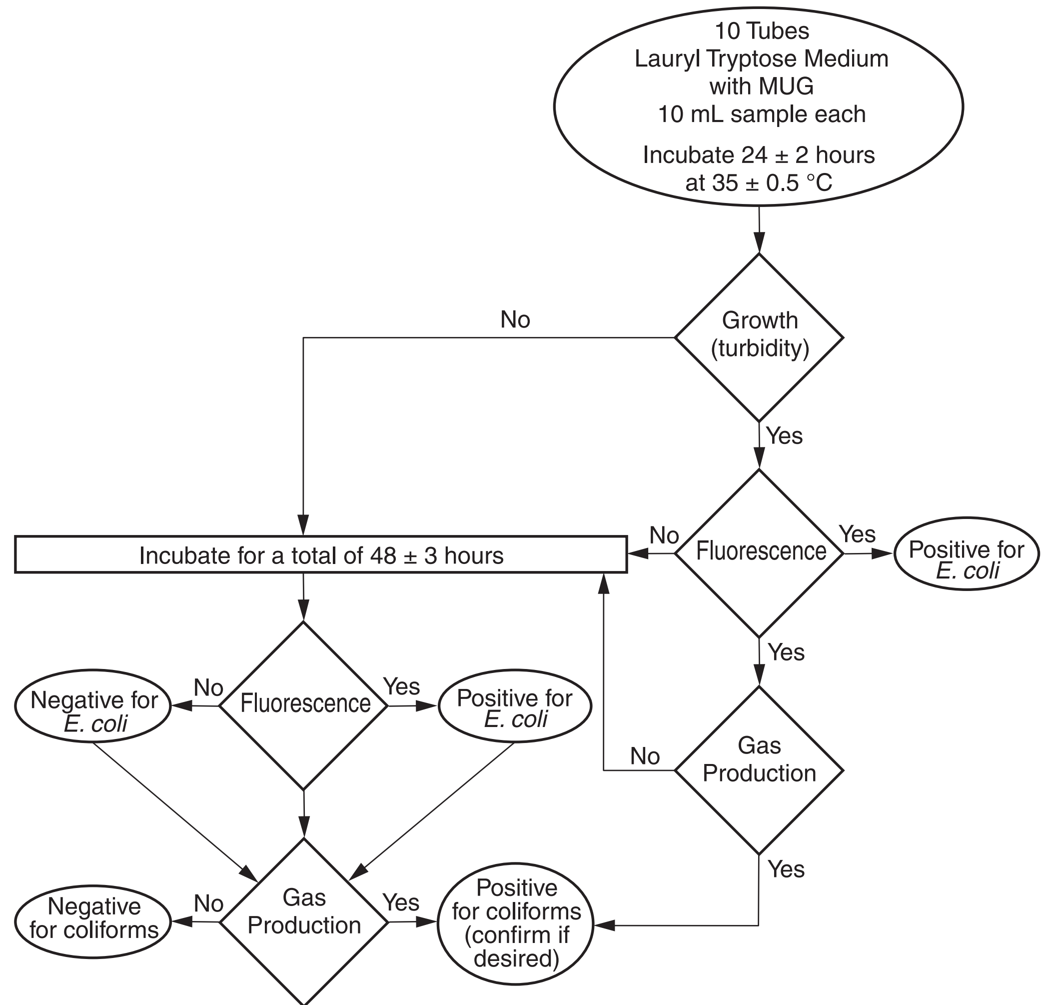
Table 3: Sample Dilutions

Dilution of 1	Fill 5 Presumptive Coliform Tubes with 10 mL of sample.
Dilution of 10	Add 11 mL of sample to a 99-mL bottle of Buffered Dilution Water. Shake well. Fill 5 Presumptive Coliform Tubes with 10 mL of diluted sample.
Dilution of 100	Add 11 mL of diluted sample (prepared in the dilution of 10 procedure) to a 99-mL bottle of Buffered Dilution Water. Shake well. Fill 5 Presumptive Coliform Tubes with 10 mL of diluted sample.

Total Coliforms and *Escherichia coli*

E. coli is recognized by the USEPA as an indicator organism that may be tested for in place of fecal coliforms. The number of presumptive *E. coli* has been determined traditionally by biochemical testing on samples that gave a positive fecal coliform test. Tubes of tryptone water are inoculated and tested for indole formation after an incubation of 24 hours. Then samples that give a positive indole test are confirmed by different biochemical tests. A more recent method of *E. coli* determination is the MUG (4-methylumbelliferyl-β-D-glucuronide) method based on the glucuronidase enzyme production of the bacteria. The test can be conducted by following the schematic in *Figure 7* or *Figure 8*. A positive test for *E. coli* is evidenced by the fluorescence of the medium when viewed under long-wave ultraviolet (UV) light. Determination of the probable number of organisms is based on the number of tubes giving a positive result.[4]

Figure 7 Total Coliforms—*Escherichia coli* Test



Fecal Streptococci

Fecal streptococci come mostly from the gastrointestinal tracts of warm-blooded animals. The ratio of fecal coliforms to fecal streptococci has been used in the past to indicate possible sources of pollution, but the value of the ratio is questioned in the 17th edition of *Standard Methods*. Variable survival rates of fecal streptococcus species, wastewater disinfection, and enumeration methods may affect the ratio of fecal coliforms to fecal streptococcus. The ratio cannot be recommended and should not be used as a means of differentiating sources of pollution.

The schematic in *Figure 8* outlines the MPN method of determining fecal streptococci.

Pseudomonas aeruginosa

The presence of *Pseudomonas aeruginosa* can be confirmed by a two-step MPN test. Fifteen tubes of asparagine broth are filled with sample (using multiple dilutions). The tubes are incubated at 35 °C for 24 to 48 hours. Test tubes showing a greenish fluorescent pigment under long-wave UV light constitute a positive presumptive test. Confirmation is done by inoculating tubes containing acetamide broth and incubating at 35 to 37 °C for 36 hours. The presence of a purple color constitutes a positive test. *Figure 9* contains the schematic for this test.[2]

Figure 8 Fecal Streptococci Test

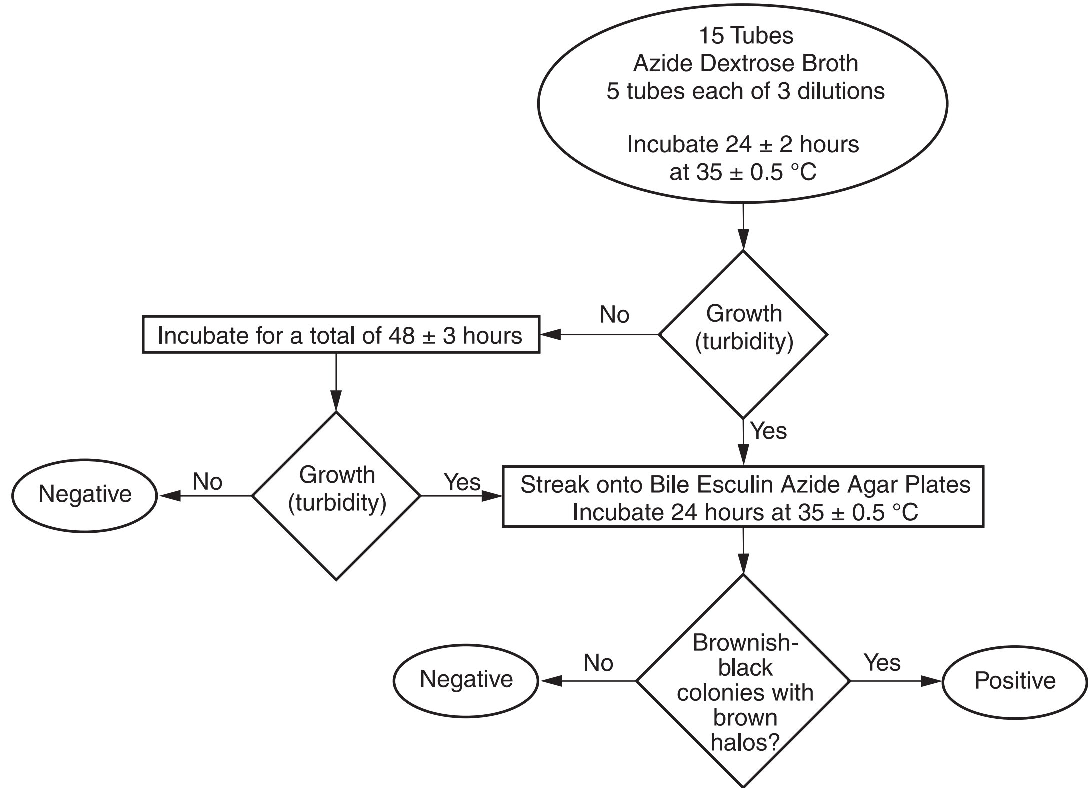
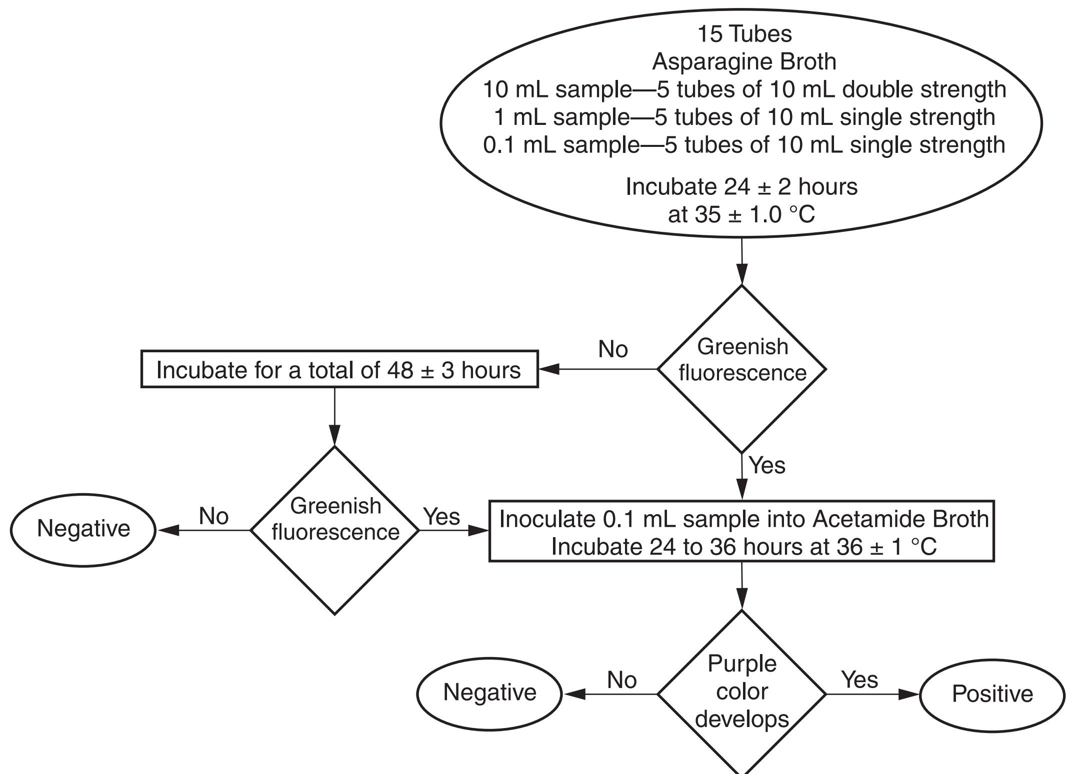


Figure 9 Multiple-Tube Technique for *Pseudomonas aeruginosa*



Membrane Filtration

The Membrane Filtration (MF) method requires filtering a sample of appropriate volume through a membrane filter of sufficiently small pore size to retain the organism(s) sought. Then the filter is placed on an appropriate agar medium, or pad saturated with an appropriate broth medium, and incubated. If the organisms sought are present, colonies will grow on the membrane filter. Colonies are examined at 10–15X magnification with a stereoscopic microscope and then identified by size, color, shape and sheen. Typical colonies are counted and the number is reported as the number of colonies per 100 mL of sample.

Total Coliform Bacteria

The standard definition of total coliform bacteria must be altered slightly when using the membrane filter method for detection and enumeration, because gas production from lactose cannot be detected on a filter surface. Therefore, when using certain types of media, such as m-Endo broth or m-Endo LES agar, it is assumed all colonies producing acid or aldehyde also produce gas. Another type of media, m-ColiBlue24®*, which was recently developed for the simultaneous detection of total coliforms and *E. coli*, relies on the presence of inhibitors in the medium which allow only total coliform bacteria to grow. The total coliform colonies are identified by a nonspecific color indicator, and *E. coli* colonies are identified by a specific enzymatic reaction.

Until recently, in the United States there were only two media, m-Endo broth or m-Endo LES agar, approved for the presumptive detection of total coliforms by MF. On Dec. 1, 1999 m-ColiBlue24® was promulgated in the Federal Register as an acceptable method for the simultaneous detection of total coliforms and *E. coli* in drinking water for monitoring under the Total Coliform Rule. Confirmation of total coliforms and *E. coli*, required with the use of m-Endo media, is unnecessary when using m-ColiBlue24®. Other countries have approved the use of lactose tergitol agar and lactose TTC (2,3,5-triphenyltetrazolium chloride) tergitol agar.[5] For both of these media, coliforms ferment lactose in the media and produce an acid-aldehyde complex. As with the m-Endo media, a specified number of suspect total coliform bacteria from wastewater or potable water samples must be transferred to liquid media for confirmation.[2]

In media like m-Endo, a fuchsin sulfite indicator is used to indicate the presence of aldehyde from the fermentation of lactose. The indicator is red in the presence of the aldehyde. A suitably prepared membrane filter is placed on m-Endo medium and incubated for 24 hours at 35 to 37 °C. (For reporting to USEPA, 35 ± 0.5 °C must be used.) Coliforms (lactose-fermenting colonies) appear red with a golden-green metallic sheen on membranes. The number of representative colonies is counted and reported as presumptive coliform organisms. For drinking water samples, atypical coliform colonies must be verified to make sure they are coliforms. Lauryl tryptose and brilliant green lactose bile broth tubes are inoculated with growth from the colonies. Gas production verifies that the suspected organisms are indeed coliforms. The procedure described in the MPN Methods section for confirmation of total coliforms is used with an additional inoculum in a single-strength lauryl tryptose broth tube. Gas production in 24 to 28 hours constitutes a positive test.

* U.S. Patent 5,650,290

Organisms often are exposed to adverse environments such as water treatment processes. When these organisms grow slowly or not at all under bacteriological testing conditions, they are called stressed, or injured, organisms. Stressed organisms are found in chlorinated effluents, saline waters, and natural waters polluted with substances such as heavy metal ions or toxic organic wastes. Sodium deoxycholate, used as an inhibitory substance in m-Endo type media, and sodium lauryl sulfate have been shown to inhibit as many as 70% of the injured coliforms.[6] Using m-Endo LES agar, Evans, et al., identified 25% of the false-negative colonies as *Citrobacter*, *Enterobacter*, *Escherichia,* or *Klebsiella* species.[7] An enrichment technique to overcome some of the problems in the detection of injured coliforms has been described in *Standard Methods.* The membrane filter is incubated on a pad saturated with lauryl tryptose broth for 1.5 hours at 35 ± 0.5 °C in a relative humidity of at least 90%. Then the membrane is transferred to a pad which has been saturated with m-Endo broth and incubated for 20 to 22 hours at 35 ± 0.5 °C. The resultant coliform colonies will have the characteristic greenish-gold sheen under 10–15X magnification. Verification is achieved by following the same procedure as the one for unstressed organisms.

In spite of improved recoveries with the lauryl tryptose broth enrichment technique, LeChevallier, et al.[8] reported an improved formulation of Chapman's Tergitol 7 agar (m-T7)[9] recovered 43% more coliforms than recovered by m-Endo agar and 36% more coliforms than recovered by m-Endo agar with lauryl tryptose broth resuscitation. (Appendix B contains instructions for the formulation and preparation of Chapman's Tergitol 7 agar.) Bromthymol blue and bromcresol purple are used in m-T7 to provide the characteristic yellow, acid-positive colonies and to inhibit the growth of noncoliform bacteria. Penicillin G also is added to inhibit the growth of *Staphylococcus* and *Micrococcus* species. Positive results are obtained when smooth, yellow, convex colonies are formed on the membrane after anaerobic incubation for 24 hours at 35 °C. A level of 0.5% false negatives was reported. Confirmation (gas production from lactose) was 70.6% compared to 69.6% for m-Endo agar.

The m-ColiBlue24® broth was developed at Hach Company to provide a membrane filtration method that would allow the determination of both total coliforms and *E. coli* in a single 24 hour incubation step.[10] It is a nutritive, lactose-based medium, containing specific inhibitors that selectively eliminate the growth of noncoliform bacteria, allowing only the total coliform bacteria to grow. The total coliforms become visible by reducing a non-selective dye, TTC (2,3,5-triphenyltetrazolium chloride) present in the medium. The reduction of TTC results in the formation of red colonies which can be easily seen on the membrane filter. *E. coli* are distinguishable from other total coliforms by a visible blue color which forms in these colonies. This color formation is the result of the enzymatic cleavage of a substrate, BCIG (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), by the enzyme, β-glucuronidase, which is predominantly produced by *E. coli*. The colony color remains closely associated with the colonies and does not diffuse away from target colonies, therefore, blue colonies are readily distinguishable even when background total coliform colonies are too numerous to count. This medium was specifically designed to maximize the growth rate of total coliforms and provide for optimal recovery of stressed organisms. In addition, it does not contain deoxycholate or bile acids, which have been proven to inhibit the growth of stressed coliforms.

Selected Methods for Counting Indicator and Opportunistic Organisms

Fecal Coliforms

When testing for fecal coliforms, a selective medium and an elevated incubation temperature are used to inhibit the growth of nonfecal coliforms. When Standard Methods-approved m-FC broth or agar is used, fecal coliforms growing on the membrane form an acid that reacts with aniline blue (a dye) to produce a blue color. In this test, a suitably prepared membrane filter** is placed on a pad saturated with m-FC and incubated for 24 hours at 44.5 ± 0.2 °C. The formation of blue colonies is considered a positive result. Nonfecal colonies are gray to cream-colored. However, few nonfecal colonies are observed when rosolic acid salt reagent is added as an inhibitor.

Fecal coliforms become injured by the same environmental stresses that affect total coliforms. Several procedures for the recovery of stressed fecal coliforms appear in *Standard Methods*.[2] The simplest procedures are deletion of the rosolic acid inhibitory agent from the m-FC medium, or temperature acclimation involving a five-hour incubation at 35 °C followed by 18 hours at 44.5 °C.

LeChevallier, et al.[11] reported m-T7 agar yielded 3.1 times greater recoveries of fecal coliforms than the standard m-FC agar method and averaged verification rates of 89% compared to 82.8% for m-FC. The m-T7 procedure for fecal coliforms includes pre-incubating the prepared sample at 37 °C for eight hours followed by incubation at 44.5 °C for 12 hours. Positive results are obtained when smooth, yellow, convex colonies are formed on the membrane.

USEPA regulations for drinking water, effective Dec. 31, 1990, require fecal coliform or *E. coli* confirmation of every positive total coliform sample. After 24 hours of incubation on m-Endo media, the entire surface of the membrane filter may be swabbed with a cotton swab. Then the swab is transferred to EC Medium and Brilliant Green Bile Broth for fecal coliform confirmation, or EC Medium with MUG and Brilliant Green Bile Broth for *E. coli* confirmation. After inoculation of the media, the cotton swab should be removed and the media incubated at 44.5 ± 0.2 °C and 35 ± 0.5 °C, respectively (according the Jan. 8, 1991, issue of the *Federal Register)*. Alternately, the positive total coliform membrane filter may be transferred to a plate containing Nutrient Agar with MUG and incubated for four hours at 35 ± 0.5 °C. Then colonies may be examined under a long-wave UV light. If a total coliform colony fluoresces, the colony is confirmed as *E. coli* positive.[12]

Escherichia coli

Early membrane filtration methods for the detection and enumeration of *E. coli* were based on the ability of the organism to catabolize tryptophane to indole. Then indole-positive colonies were identified by a destructive reaction with Ehrlich's reagent.[13] The destructive nature of the indole test made a more thorough examination of the colonies impossible and was therefore inherently disadvantageous. Dufour, el al.[14] proposed a test based on the assumption that the majority of the thermotolerant coliforms, except *E. coli*, are urease-positive. This method uses a medium (m-TEC) to encourage growth of coliform organisms and to differentiate *E. coli* by the use of a nondestructive *in situ* urease test. (Preparation instructions for m-TEC medium appear in Appendix B.) *E. coli* does not give the characteristic positive reaction.

** Determination of the proper sample size is important in order to grow reasonable numbers of detectable colonies. *Tables 4* and *5* list suggestions for sample sizes based on source types.

The test is done by rolling a prepared membrane filter onto the m-TEC agar and incubating for two hours at 35 °C. The the plate is transferred to a water bath at 44.5 °C for 22 to 24 hours. Then the filter is transferred aseptically to an absorbent pad saturated with urea broth. After 15 to 20 minutes at room temperature, the yellow or yellow-brown colonies are counted as presumptive *E. coli*. When using this method, recovery of *E. coli* from marine, estuarine, and fresh-water samples exceeded 90%, with 91% of the presumptively positive colonies being verified as *E. coli*. Less than 1% of the verified *E. coli* colonies reacted atypically.

This method has been recommended by the USEPA for the determination of *E. coli* in recreational waters. It also has been recommended by the American Society for Testing Material for analyses of reagent water, fresh and marine recreational waters, sewage treatment plant effluents, and shellfish-growing water. The m-TEC method requires considerable time and resources because of the need for resuscitation and the two-step verification of presumptive *E. coli.*

E. coli also may be determined from total-coliform-positive samples that have been analyzed using m-Endo broth or m-Endo LES agar. Both EC medium with MUG and Nutrient Agar with MUG are USEPA-approved for use in determining the presence of *E. coli* in drinking water after a positive total coliform result has been obtained with the m-Endo method.

One of the most convenient ways to determine the presence of *E. coli* is by using m-ColiBlue24 broth with the membrane filtration method. As mentioned in the previous section, *Total Coliform Bacteria*, this medium simultaneously detects the presence of total coliform bacteria and *E. coli* within 24 hours. With this medium *E. coli* is detected by the specific enzymatic cleavage of the compound, BCIG. This cleavage results in the formation of *E. coli* colonies that are blue in color.The medium has a high specificity and sensitivity, and needs no further confirmation.

On Dec. 1, 1999, the USEPA approved this method as a suitable method for determining the presence of total coliforms and *E. coli* in drinking water. m-ColiBlue provides the analyst with a method that is acceptable for reporting and that requires only one step, thereby saving both time and resources.

Table 4: Suggested Sample Volumes for Membrane Filter Total Coliform Test*

Water Source	Volume to be Filtered (mL)							
	100	50	10	1	0.1	0.01	0.001	0.0001
Drinking Water	X							
Swimming pools	X							
Wells, springs	X	X	X					
Lakes, reservoirs	X	X	X					
Water supply intake			X	X	X			
Bathing beaches			X	X	X			
River water				X	X	X	X	
Chlorinated sewage				X	X	X		
Raw sewage					X	X	X	X

* *Standard Methods*, 19th ed.

Table 5: Suggested Sample Volumes for Membrane Filter Fecal Coliform Test*

Water Source	Volume to be Filtered (mL)						
	100	50	10	1	0.1	0.01	0.001
Lakes, reservoirs	X	X					
Wells, springs	X	X					
Water supply intake		X	X	X			
Natural bathing waters		X	X	X			
Sewage treatment plant, secondary effluent			X	X	X		
Farm ponds, rivers				X	X	X	
Storm water runoff				X	X	X	
Raw municipal sewage					X	X	X
Feedlot runoff					X	X	X

* *Standard Methods*, 19th ed.

Fecal Streptococci

The membrane filter technique for fecal streptococci is useful for testing both fresh and marine waters. Highly turbid waters or chlorinated wastewater samples should be tested by using the multiple-tube fermentation method. Kenner, Clark, and Kabler[15] developed a media, KF Streptococcus Agar, for the enumeration of fecal streptococcus. The medium, containing bromcresol purple, produces dark red to pink colonies. Sodium azide provides the selectivity. However, a study reported by Fujioka, Ueno, and Narikawa[16] indicated tests of some nonfecal streptococci bacteria present in Hawaiian marine waters produce false positive results. Confirmation is done using brain-heart infusion agar slants. If growth is detected on the slant, a few drops of 3% hydrogen peroxide are added. The absence of bubbles constitutes a negative catalase test, indicating a probable streptococcus culture. The growth should then be transferred to a brain-heart infusion broth and a bile growth medium. Growth in both tubes constitutes confirmation of fecal streptococcus.

Enterococci

Levin, et al.[17] previously described a selective medium called m-E for the enumeration of enterococci in marine waters. This two-step approach consisted of primary isolation of the organisms on the medium followed by an *in situ* substrate test for organisms capable of hydrolyzing esculin. Nalidixic acid and sodium azide were added to a peptone, sodium chloride medium to select for gram-positive organisms. Actidione was included to inhibit the growth of fungi. The reduction of triphenyltetrazolium chloride, which colors the colonies, was used to differentiate the enterococci from other streptococci. Esculin was added to the m-E medium to induce the enzyme which catalyzes the hydrolysis of esculin in the second medium used. Prior to 1986, this test was specified by the USEPA for the determination of enterococci in marine recreational waters.

In 1986, the USEPA issued a revision of this method and proposed expanding the use of enterococci and *E. coli* as indicators for monitoring ambient water quality. The revised method, as developed by USEPA researchers, is specified in *USEPA Method 1600: Membrane Filter Test Method for Enterococci in Water*. Published as a guideline for monitoring recreational waters, this method describes a modified m-E agar, called m-EI agar, that has indoxyl-β-D-glucoside added; this

allows for the selective detection of enterococci. (Instructions for preparing m-EI Agar appear in Appendix B.) After incubation at 41 ± 0.5 °C for 24 hours, colonies with a blue halo, regardless of colony color, are counted as positive enterococci colonies. Analysis time is reduced to 24 hours and further confirmation is unnecessary.

Pseudomonas aeruginosa

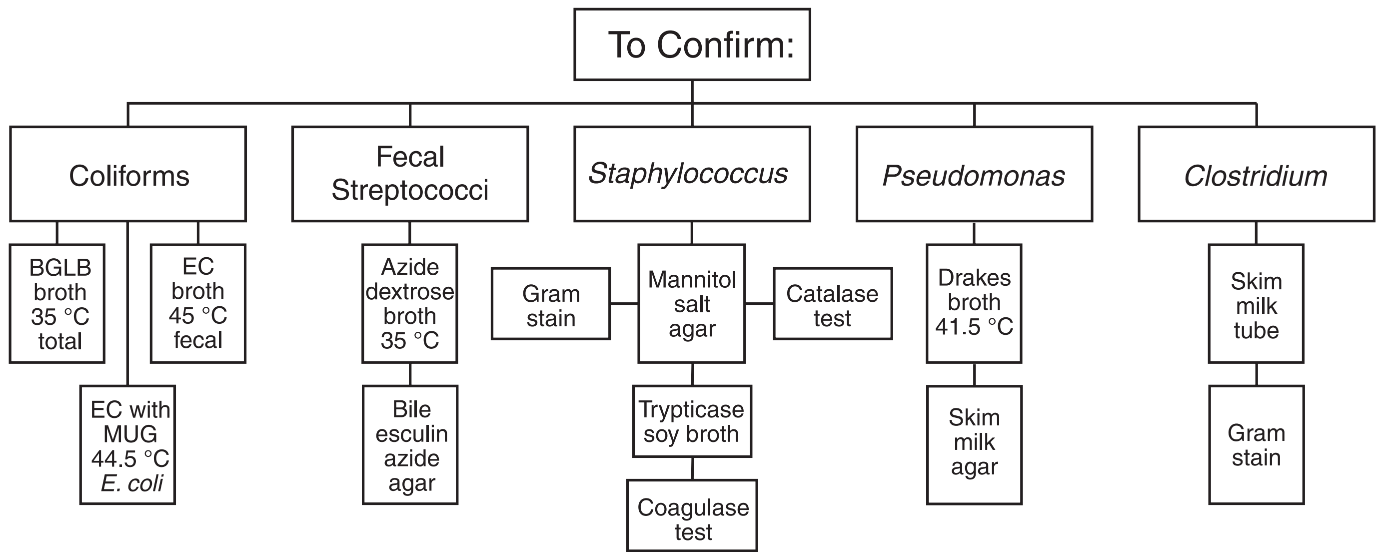
Levin, et al.,[18] have also described a membrane filtration procedure for the enumeration of *Pseudomonas aeruginosa*. Because *P. aeruginosa* can metabolize a wide variety of compounds, the medium was made selective by the inclusion of microbial inhibitors such as sulfapyridine, kanamycin, and nalidixic acid. The ferric ammonium citrate, sodium thiosulfate, and phenol red ingredients provide the characteristic tan-to-brown coloration of the *P. aeruginosa* colonies. The elevated incubation temperature of 41.5 °C inhibits the growth of most other pseudomonads. Membrane filters through which a water sample has been passed are placed on the m-PA agar plates and incubated at 41.5 °C for 48 hours. (Preparation instructions for m-PA appear in Appendix B.) Typical colonies are 0.8 to 2.2 mm in diameter, flat with light outer rims, and have brownish to greenish-black centers. The average recovery rate was 92%. Ninety-five percent of the typical colonies were verified as *P. aeruginosa* and none of the atypical colonies proved to be *P. aeruginosa*. Confirmation is done with milk agar and incubation at 35 °C for 24 hours. *P. aeruginosa* hydrolyzes casein and produces a yellowish-to-green diffusible pigment. A modification of the this method has been accepted for presumptive *P. aeruginosa* as a tentative standard method.

Presence/Absence Test

USEPA drinking water regulations, effective Dec. 31, 1990, require reporting only the presence or absence of coliforms. The maximum contaminant goal of zero total coliforms eliminates the need to enumerate coliforms in samples. USEPA representatives believe the simple presence/absence (P/A) test will help eliminate the type of errors associated with more complex enumeration techniques and record-keeping. Unlike the MPN and MF tests, the P/A test is a qualitative test giving no indication of the numbers of organisms present. P/A testing is an effective screening device when assurance of zero coliform organisms is required on a large number of samples. Only a minimal amount of analytical experience is required for the person performing P/A testing because of the simplicity of the methods. Several methods are available.

USEPA-approved methods require the use of 100 mL of sample and the bromcresol purple P/A broth described in *Standard Methods* or lauryl tryptose broth with a durham tube. The bromcresol purple P/A broth consists of lactose, lauryl tryptose, and bromcresol purple indicator. The sample is incubated for 24 to 48 hours at 35 ± 0.5 °C. A yellow coloration, indicative of acid formation from lactose fermentation, indicates a positive result. A durham tube also may be added to indicate gas production. Additional screening of the sample for fecal coliforms, *Aeromonas, Staphylococcus, Pseudomonas,* fecal streptococcus, and *Clostridium* can be done by selecting an appropriate confirmation method. *Figure 10* is an outline of operational confirmation schemes.[2] Lauryl tryptose broth is used for P/A testing simply by using the appropriate amount of media, 100 mL of sample and a durham tube for collecting gas in a bottle. Confirmation of the presence of total coliforms, fecal coliforms, or *E. coli* is performed by using the same procedures used with the lauryl tryptose MPN method.

Figure 10 Confirmation of Presence/Absence Tests



The medium, Pathoscreen™, can be used as an alternative method to coliform testing to obtain a P/A test result. This medium was developed for quick detection of common pathogens in drinking water, surface water, or recreational water. It relies on the production of hydrogen sulfide as an indication of the presence of pathogenic bacteria, such as *Salmonella*, *Proteus*, *Klebsiella*, *Citrobacter*, *Clostridium*, and *Edwardsiella*, which are commonly associated with fecal contamination. When using traditional coliform methods in tropical environments, indigenous *E. coli* may produce positive reactions when no fecal contamination exists. However, *E. coli* does not interfere with the Pathoscreen test, making it a suitable alternative for monitoring drinking water systems in developing tropical countries, remote field locations, and disaster or emergency situations. The Pathoscreen™ medium can also be used in an MPN format to provide quantitative predictions of the number of pathogens present. With either the P/A or MPN method, hydrogen sulfide production causes a black color to develop in the medium when incubated at 30 °C for 24–48 hours. Pathoscreen™ medium may also be incubated at ambient temperatures between 25 and 35 °C if an incubator is not available.

The Biological Activity Reaction Test (BART™) is another method for determining the presence of bacterial groups or algae in water. The BART™, originally developed by Drycon Bioconcepts, Inc. for the diagnosis of biofouled wells, provides an easy way to detect specific groups of microorganisms in water before excessive growth causes problems. Refer to page *37* for a list of the various types of BARTs and the group of bacteria detected by each one. Some suitable applications include the monitoring of cooling towers and heat exchangers, municipal drinking and wastewaters, private wells, hazardous waste treatment facilities, oil field drilling and refining, pools and spas, power plant utilities, process water for manufacturing, and pulp and paper plants. After the sample has been added to the BART™ tube, it is examined daily for specific indicator reactions such as color changes, turbidity, sediment, precipitation, and colored formations, such as rings and bubbles. A comparator chart is used to identify the presence or absence of bacteria. Traditional confirmation methods may be used to identify specific bacteria, if desired.

Selected Methods for Counting Indicator and Opportunistic Organisms

Plate Count Method

Heterotrophic plate count methods provide a standardized means of determining the density of aerobic and facultatively anaerobic heterotrophic bacteria in water. Plate count methods provide the best measurement of water treatment plant efficiency, regrowth in distribution lines, and general bacterial composition of source water. *Standard Methods* outlines three techniques for determining a standard plate count: pour plate, spread plate, and membrane filtration (MF). The standard plate count (also called the pour plate method) is done by pouring liquefied agar medium into petri dishes and adding sample. After the sample is mixed with the medium, the plates are set aside to solidify before they are inverted and incubated. A count is taken of the growing colonies and reported as colony-forming units (CFU) per milliliter of water sample.

The spread plate method differs from the pour plate method in that agar plates are poured and set aside to solidify before inoculation with sample. Then, the sample inoculum is spread on the surface of the plate with a bent glass rod and absorbed into the medium before incubation. The resulting colonies formed on the surface of the agar are much easier to identify and count. Three types of media are recommended for either pour plates or spread plates in *Standard Methods*: plate count agar, tryptone glucose extract agar, and R2A agar.[2]

The membrane filtration technique is best suited to testing large volumes of low-turbidity, low-count (less than 1 to 10 CFU/mL) water. Suitable media include m-TGE broth, R2A agar, and m-HPC agar. TTC (2,3,5-triphenyltetrazolium chloride), an indicator, may be added to the m-TGE broth. Colonies develop a pink-to-red color, enhancing the visibility of the bacteria, when TTC is used.

Procedural requirements for any of the media stipulate incubation of all water samples except bottled water for 48 ± 3 hours at 35 ± 0.5 °C. The incubation time for bottled water is extended to 72 hours. When using R2A agar (an extremely low-nutrient agar), all water samples should be incubated for not less than 72 hours and up to five to seven days at 35 °C or a minimum of five and preferable seven days at 20 or 28 °C.[24]

Two-sided, dual agar paddles were developed for use in screening for total aerobic bacteria, total coliforms, yeast, molds, and for monitoring the effectiveness of disinfection chemicals. For each type of paddle tester, one side of the paddle gives a nonselective total count of bacteria, while the other side tests for a more specific type of microbial contamination (see page *35*). The paddles are used by dipping them directly into a sample, or pressing the paddle onto a surface. A quantitative result is obtained by counting the colonies that have grown on the surface of the agar after the appropriate incubation.

Appendix A: Sample Dilution

Membrane Filtration Method

Sample volume requirements are based on the source of the sample and the expected bacterial population. Samples should produce between 20 and 80 colonies per membrane. Often, the original sample must be diluted in order to arrive at an appropriate sample concentration. The following procedure describes one method for preparing a dilution series.

A. If a 10-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of sample into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 10-mL sample for MF methods. Use portions of this dilution for MPN methods (1/10).

B. If a 1-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of the 10-mL dilution from *step A* into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 1-mL sample for MF methods. Use portions of this dilution for MPN methods (1/100).

C. If a 0.1-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of the 1-mL dilution from *step B* into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 0.1-mL sample for MF methods. Use portions of this dilution for MPN methods (1/1000).

D. If a 0.01-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of the 0.1-mL dilution from *step C* into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 0.01-mL sample for MF methods. Use portions of this dilution for MPN methods (1/10,000).

E. If a 0.001-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of the 0.01-mL dilution from *step D* into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 0.001-mL sample for MF methods. Use portions of this dilution for MPN methods (1/100,000).

F. If a 0.0001-mL sample is required:

Using a sterile 11-mL pipet, transfer 11 mL of the 0.001-mL dilution from *step E* into a 99-mL bottle of sterile dilution water and shake well. Filter 100 mL of this dilution to obtain the 0.0001-mL sample for MF methods. Use portions of this dilution for MPN methods (1/1,000,000).

Calculation of Results

Membrane Filtration Method

$$\text{colonies counted per 100 mL} = \frac{\text{colonies counted}}{\text{mL sample filtered}} \times 100$$

Most Probable Number Method

The number read from the 15-tube most probable number chart is multiplied by the dilution factor of the first group of five tubes.

For example, if a series of no dilution, a 1/10 dilution, and 1/100 dilution are used, the number of organisms can be taken directly from the 15-tube chart. If the sample must be diluted 1/10, 1/100, and 1/1000, the number read from the chart must be multiplied by 10.

Appendix B: Medium Formulations and Preparation

Asparagine Acetamide Broth: Media for *P. aeruginosa* by MPN

Formulation of Asparagine Broth

Asparagine, DL..3.0 g
Dipotassium hydrogen phosphate..1.0 g
Magnesium sulfate, heptahydrate..0.5 g
Distilled water..1.0 L

Preparation

Dissolve the ingredients in distilled water and adjust pH to between 6.9 and 7.2. Sterilize.

Formulation of Acetamide Broth

Acetamide..10.0 g
Sodium chloride..5.0 g
Dipotassium hydrogen phosphate..1.39 g
Potassium dihydrogen phosphate..0.73 g
Magnesium sulfate, heptahydrate..0.5 g
Phenol red..0.012 g
Distilled water..1.0 L

Preparation

Dissolve the ingredients in distilled water and adjust the pH to between 6.9 and 7.2. Sterilize.

m-EI Agar: Media for Enterococci

Formulation of m-E

Peptone..10.0 g
Sodium chloride..15.0 g
Esculin..1.0 g
Yeast extract..30.0 g
Actidione..0.050 g
Sodium azide..0.150 g
Agar..15.0 g
Distilled water..1.0 L

Additional Ingredients

Indoxyl-β-D-glucoside	0.75 g
Nalidixic acid	0.21 g
Triphenyltetrazolium chloride	0.15 g

Preparation

Dissolve the original M-E ingredients plus the indoxyl-β-D-glucoside in distilled water and autoclave for 15 minutes at 121 °C. Cool to 55–60 °C; add additional ingredients and adjust pH to 7.1 ± 0.1. Pour 3.5-mL amounts in 50-mm plates.

m-PA: Medium for *Pseudomonas aeruginosa* by MF

Formulation

	Levin's	Standard Methods
L-lysine hydrochloride	5.0 g	5.0 g
Sodium chloride	5.0 g	5.0 g
Yeast extract	2.0 g	2.0 g
Xylose	2.5 g	1.25 g
Sodium thiosulfate	6.8 g	5.0 g
Sucrose	12.5 g	12.5 g
Lactose	12.5 g	12.5 g
Phenol red	0.08 g	0.08 g
Ferric ammonium citrate	0.8 g	0.8 g
Magnesium sulfate, heptahydrate	0	1.5 g
Agar	15.0 g	15.0 g
Distilled water	1.0 L	1.0 L

Antibiotic

Sulfapyridine (Nutritional Biochemicals)	0.176 g
Kanamycin (Bristol-Myers)	0.0085 g
Nalidixic acid (Cal Chemicals)	0.037 g
Actidione (Upjohn)	0.15 g

Preparation

Dissolve all ingredients except antibiotics in distilled water. Autoclave at 121 °C for 15 minutes. Cool to 55–60 °C. Adjust pH to 7.1 ± 0.1 for Standard Methods formulation. Adjust pH to 6.5 for Levin's formulation. Add the antibiotics and mix. Pour 3-mL amounts into 50-mm plates.

m-T7: Medium for Total and Fecal Coliforms

Formulation

Protease Peptone No. 3 (Difco)	5.0 g
Yeast extract	3.0 g
Lactose	20.0 g
Tergitol 7 (25% solution)	0.4 mL
Polyoxyethylene ether W-1	5.0 g
Bromthymol blue	0.1 g
Bromcresol purple	0.1 g
Agar	15.0 g
Distilled water	1.0 L

Preparation

Dissolve all ingredients in distilled water. Autoclave at 121 °C for 15 minutes. Adjust pH to 7.4 ± 0.2. Selectivity can be enhanced with the aseptic addition of 0.1 mg/L penicillin G after autoclaving.

m-TEC: Medium for Enumerating *E. coli*

Formulation

Protease peptone No. 3 (Difco)....................5.0 g
Yeast extract....................3.0 g
Lactose....................10.0 g
Sodium chloride....................7.5 g
Dipotassium hydrogen phosphate....................3.3 g
Potassium dihydrogen phosphate....................1.0 g
Sodium lauryl sulfate....................0.2 g
Sodium desoxycholate....................0.1 g
Bromcresol purple....................0.08 g
Bromphenol red....................0.08 g
Agar....................15.0 g
Distilled water....................1.0 L

Preparation

Dissolve ingredients by stirring. Sterilize by autoclaving at 121 °C for 15 minutes. Pour 4-mL amounts into 50-mm plates. The pH of the medium is 7.3 ± 0.1

Appendices

Guide to the Use of Media

Medium	Form	Use
A-1 Medium (1, 6)	Dehydrated	MPN Method, Fecal Coliforms—Sea Water and Treated Water Samples
Azide Dextrose Broth	Dehydrated, Prepared Tubes	MPN Method, Fecal Streptococci, Presumptive
Bile Esculin Azide Agar	Dehydrated, Prepared Tubes	MPN Method, Fecal Streptococci, Confirmation
Brain-Heart Infusion Agar	Dehydrated	MF Method, Fecal Streptococci, Confirmation
Brain-Heart Infusion Broth	Dehydrated	MF Method, Fecal Streptococci, Confirmation
Brilliant Green Bile Broth (1, 2)	Dehydrated, Prepared Tubes	MPN Method, P/A Method, MF Method, Total Coliforms, Confirmation USEPA-approved Potable Water and Wastewater
EC Medium (1, 2)	Dehydrated, Prepared Tubes	MPN Method, P/A Method, MF Method, Fecal Coliforms, Confirmation, USEPA-approved
EC Medium with MUG (2)	Dehydrated, Prepared Tubes	MPN Method, P/A Method, MF Method, *E. coli,* Confirmation, USEPA-approved
EMB Agar (8)	Dehydrated	Plate Method, Gram-negative Enteric Bacteria
Esculin Iron Agar (3, 7) used with m-E Agar	Dehydrated	MF Method, Enterococci in Marine, Recreational Waters
KF Strep Agar	Dehydrated	MF Method, Fecal Streptococci
Lactose Broth (1, 2)	Dehydrated, Prepared Tubes	MPN Method, Total Coliforms, Presumptive, USEPA-approved
Lauryl Tryptose Broth (1, 2) (preferred medium)	Dehydrated, Prepared Tubes	MPN Method, Total Coliforms, Presumptive, USEPA-approved, MF Method, Confirmation
Lauryl Tryptose/MUG Broth (2)	Dehydrated, Prepared Tubes	MPN Method, Presumptive, Total Coliforms, *E. coli*
m-ColiBlue 24®	Prepared Ampules, 100-mL bottle	MF Method,Total Coliforms and *E. coli*, USEPA-approved, Potable Water (no confirmation required)
m-E Agar (3, 7), used with Esculin Iron Agar	Dehydrated	MF Method, Enterococci in Marine, Recreational Waters
m-Endo Agar	Dehydrated	MF Method, Total Coliforms, Presumptive, USEPA-approved, Potable Water
m-Endo Agar LES	Dehydrated	MF Method, Total Coliforms, Presumptive, USEPA-approved, Potable Water
m-Endo Broth (1, 4)	Dehydrated, Prepared Ampules, 100-mL bottle	MF Method, Total Coliforms, Presumptive, USEPA-approved, Potable Water
m-FC Broth (1, 3)	Dehydrated, Prepared Ampules	MF Method, Fecal Coliform, USEPA-approved
m-FC with Rosolic Acid	Prepared Ampules	MF Method, Fecal Coliform, USEPA-approved
m-Green YM Broth	Dehydrated, Prepared Ampules	MF Method, Yeast and Mold
m-HPC (Plate Count) Agar (1)	Dehydrated	Heterotrophic Plate count, MF Method, Total Bacteria
m-T7 Agar (5)	Dehydrated	MF Method, Stressed Total and Fecal Coliforms

Medium	Form	Use
m-TEC Agar (3, 11), used with urease test	Dehydrated	MF Method, *E. coli*, in Recreational Water
m-TGE Broth	Dehydrated, Prepared Ampules	MF Method, Heterotrophic Plate Count
m-TGE Broth with TTC	Prepared Ampules	MF Method, Heterotrophic Plate Count
Nutrient Agar (8)	Dehydrated	Plate Method, General Purpose
Nutrient Agar with MUG	Prepared Tubes	MF Method, *E. coli*, USEPA-approved
Plate Count Agar (1)	Dehydrated, Prepared Tubes	Pour- and Spread-Plate Methods, Heterotrophic Plate Count, Total Bacteria, USEPA-approved
Presence/Absence Broth (1, 9)	Dehydrated, Prepared Ampules and Bottles	Presence/Absence Method, Total Coliforms, USEPA-approved
Presence/Absence Broth (1, 9) with MUG	Prepared Ampules and Bottles	Presence/Absence Method, Total Coliforms, and *E. coli*
PathoScreen™	Powder Pillows	Presence/Absence Method, for Hydrogen Sulfide-Producing Pathogens, Potable Water, Surface Water, Recreational Water
R2A Agar (1, 10)	Dehydrated	Heterotrophic Plate Count, MF, Pour- and Spread-Plate Methods, Total Bacteria
Violet Red Bile Agar	Dehydrated	Coliform in Foods Method

Guide for Selected Media

Total Count Plates

Medium	Use	Hach Cat. No
EMB Agar	Gram-negative enteric bacteria	Dehydrated: 21777-26
Nutrient Agar	General purpose	Dehydrated: 21792-26
Plate Count Agar	Total Bacteria	Tubed: 24067-20 Dehydrated: 21778-26
R2A Agar	Total Bacteria	Dehydrated: 22810-34
Paddle Testers	Total Bacteria/Total Coliforms	Pkg/10: 26109-10
Paddle Testers	Total Bacteria/Yeast and Mold	Pkg/10: 26108-10
Paddle Testers	Total Bacteria/Disinfection Control	Pkg/10: 26195-10

Membrane Filtration Methods

Bacteria Group: Coliforms

Medium	Use	Hach Cat. No
EC Medium	Confirmed Fecal Coliforms, USEPA-approved	Tubed: 14104-15 Dehydrated: 14103-26
EC Medium with MUG	Confirmed *E. coli,* USEPA-approved	Tubed: 22824-15 Tubed, without durham tubes: 24715-15
Brilliant Green Bile Broth	Confirmed Total Coliform, USEPA-approved	Tubed: 322-15 Dehydrated: 159-26
Lauryl Tryptose Broth	Confirmed Total Coliform, USEPA-approved	Tubed (single strength): 21623-15
m-ColiBlue 24®	Confirmed Total Coliform and *E. coli*, USEPA-approved	Ampules:glass, 26084-20 plastic, 26084-10 100-mL bottle, 26084-42
m-Endo Broth	Presumptive Total Coliform, USEPA-approved	Ampules: glass, 23735-20 plastic, 23735-10 100-mL bottle, 23735-42 Dehydrated: 14623-26
m-FC Broth	Fecal Coliform, USEPA-approved	Ampules: glass, 23732-20 plastic, 23732-10 Dehydrated: 14624-26
m-FC Broth with Rosolic Acid	Fecal Coliform, USEPA-approved	Ampules: glass, 24285-20 plastic, 24285-10
m-TEC Agar, used with urease test	Presumptive *E. coli*, Recreational Waters	Dehydrated: 22811-26
Nutrient Agar with MUG	Confirmed, E. coli, USEPA-approved	Tubed: 24373-06

Bacteria Group: Fecal Streptococci and Enterococci

Medium	Use	Hach Cat. No
m-E Agar, used with Esculin Iron Agar	Presumptive Enterococci, Marine and Recreational Waters	Dehydrated m-E: 22812-26 Dehydrated Esculin: 22813-26
KF Streptococci Agar	Fecal Streptococci	Dehydrated: 14853-34
m-TGE Broth	Heterotrophic Plate Count	Ampules: glass, 23738-20 plastic, 23738-10 Dehydrated: 24264-00
m-TGE with TTC Indicator Broth	Heterotrophic Plate Count	Ampules: glass, 24284-20 plastic, 24284-10
m-HPC Agar, used with glycerol	Heterotrophic Plate Count	Dehydrated: 22807-34
R2A Agar	Heterotrophic Plate Count	Dehydrated: 22810-34

Bacteria Group: Yeast and Mold

Medium	Use	Hach Cat. No
m-Green YM Broth	Yeast and Mold	Ampules: glass, 24283-20 plastic, 24283-10 Dehydrated: 24282-00

Most Probable Number Method

Bacteria Group: Coliforms

Medium	Use	Hach Cat. No
A-1 Medium	Fecal Test, Sea Water, Treated Waters	Dehydrated: 23099-34
Brilliant Green Bile Broth	Confirmed Test, USEPA-approved	Tubed: 322-15 Dehydrated: 159-26, 159-34
EC Medium	Fecal Test, USEPA-approved	Tubed: 14104-15 Dehydrated: 14103-26, 14103-34
EC/MUG Medium	*E. coli*, USEPA-approved	Tubed: 22824-15, 24715-15 Dehydrated: 23101-26, 23101-34
Lactose Broth	Presumptive Test, USEPA-approved	Tubed: 21013-15 Dehydrated: 196-26, 196-34
Lauryl Tryptose Broth	Presumptive Test, USEPA-approved	Tubed, concentrated: 21014-15 Tubed, single strength: 21623-15 Dehydrated: 197-26,197-34
LT/MUG Broth	Presumptive Test, Total Coliform, *E. coli*, Confirmed, AOAC*-approved	Tubed: 21821-15 Dehydrated: 22557-26, 22557-34

Bacteria Group: Fecal Streptococci (Strep)

Medium	Use	Hach Cat. No
Azide Dextrose Broth	Presumptive Test	Tubed: 26478-15 Dehydrated:
Bile Esculin Azide Agar	Confirmed Test	Tubed: 24069-20 Dehydrated:

Bacteria Group: *Pseudomonas aeruginosa*

Medium	Use		Hach Cat. No
Asparagine Broth	Presumptive Test, Medium prepared from:		
		Asparagine:	23100-26
		K_2HPO_4:	7080-34
		$MgSO_4$–$7H_2O$:	6088-34
Acetamide Broth	Confirmed Test, Medium prepared from:		
		Acetamide:	[Not available from Hach]
		NaCl:	182-01
		K_2HPO_4:	7080-34
		KH_2PO_4	170-01H
		$MgSO_4$–$7H_2O$:	6088-34
		Phenol Red:	267-22

* Association of Official Analytical Chemists

Serial Dilution Method

Medium	Use	Hach Cat. No
Total Bacteria Medium	Total Bacterial Count	Tubed: 22777-00

Presence/Absence Method

Medium	Use	Hach Cat. No
PathoScreen™ Medium	Hydrogen Sulfide-Producing Pathogens	P/A Pillows, 26106-96 MPN Pillows, 26107-96
Presence/Absence Broth	Total Coliforms	Ampule, pkg/25: 24949-25 Bottle, disposable: pkg/12: 23232-12 pkg/50: 23232-50 Dehydrated: 22809-34
Presence/Absence Broth with MUG	Total Coliforms and *E. coli*	Ampule: 24955-25 Bottle, disposable: pkg/12: 24016-12 pkg/50: 24016-50

Biological Activity Reaction Tests (BARTS)

Medium	Use	Hach Cat. No
IRB	Iron-related Bacteria	pkg/9: 24323-09 pkg/27: 24323-27
SRB	Sulfate-reducing Bacteria	pkg/9: 24234-09 pkg/27: 24234-27
SLYM	Slime-forming Bacteria	pkg/9: 24325-09 pkg/27: 24325-27
HAB	Heterotrophic Aerobic Bacteria	pkg/9: 24904-09 pkg/27: 24904-27
FLOR	Fluorescent Pseudomonads	pkg/9: 24326-09
ALG	Blue-green Algae	pkg/9: 24327-09 pkg/27: 24327-27
POOL	Pool and Spa	pkg/9: 24784-09
DN	Denitrifying Bacteria	pkg/9: 26193-09
N	Nitrifying Bacteria	pkg/7: 26194-07
Combination Pack	Contains IRB, SRB, and SLYM	pkg/9: (3 of each): 24348-09

Medium Ingredients: Chemicals and Extracts

Ingredient	Use	Hach Cat. No
Acetamide, 100 g	*Pseudomonas* Test	*
Acridine Orange, 2-mL ampules	Direct count methods (Acridine Orange Direct Count [AODC] Method)	23756-20
Agar, 500 g	Gelling agent in culture media	23250-34
Asparagine, 100 g	*Pseudomonas* Test	23100-26
Beef Extract	Total Bacteria Test	*
Bromcresol Purple, 5 g	pH indicator	361-22
DAPI (4’6-diamino-2-phenylindole)	Epifluorescent dye	*
Dechlorinating Reagent Powder Pillows pkg/100	For dechlorinating samples	14363-69
Esculin Iron Agar, 100 g	Used with m-E Agar	22813-26
Glycerol	Used with m-HPC Agar	*
Glutaraldehyde Powder Pillows	Fixative for preserving samples for direct count method (AODC)	*
Hydrogen Peroxide, 3%, 29 mL	Confirmation fecal streptococci	24062-20
Immersion Oil, self-contained dropping bottle, 35 mL	Nonfluorescing for oil immersion microscopy	23875-00
INT (p-iodonitrotetrazolium violet), 1 g	Epifluorescent dye	24073-21
Isopropyl Alcohol	Used in direct count method (AODC)	*
Lactose, 500 g	Nutrient used in media	23251-34
Magnesium Chloride, 500 g	Dilution water	6114-34
Magnesium Sulfate, 500 g	Various media	6088-34
Monensin	Used in bacteriological media	*
MUG, 1 g, 5 g	Used with media for *E. coli*	21844-21 21844-22
Nalidixic Acid, 25 g	Used in *Pseudomonas* media	24071-24
Oxgall, 100 g	Fecal strep confirmation, used with Brain–Heart Infusion Broth	24059-26
Oxgall Solution, 10%	Selective ingredient for culture media	*
Peptone, 113 g	Total Bacteria Broth, Dilution Water	21429-34
Phenol Red, 5 g	Acetamide Broth Pseudomonas Test Urea Substrate—m-TEC Test for *E. coli*	267-22
Phosphate Buffer Solution Pillows, pkg/50	Sterile rinse	23758-66
Potassium Phosphate, Dibasic, 500 g	Various media	7080-34
Potassium Phosphate, Monobasic, 454 g	Various media, dilution water	170-01
Rosolic Acid, 1 g	Increases selectivity of m-FC	21629-21
Tergitol 7	Used in m-T7 media for total coliform	*
Tryptone	Nitrogen source in culture media	*
TTC	Indicator used to enhance visibility of colonies in m-TGE Broth and Esculin Iron Agar	*
TTC Solution, 1%, 100 mL	Indicator used to enhance visibility of colonies in m-TGE Broth and Esculin Iron Agar	24060-42
Urea, 100 g	Urea Test for m-TEC Presumptive *E. coli*	11237-26
Xylene	Used in direct count method (AODC)	*
Yeast Extract, 500 g	Growth stimulant in culture media	22324-34

* Not available from Hach Company

Appendix C: Collection, Storage and Transport of Water Samples

Procedures followed when collecting water samples for subsequent bacteriological examination should be consistent with prescribed guidelines. Failure to collect and transport sample properly will cause meaningless results. The following guidelines were established by the World Health Organization:[1]

- Sample so as to ensure seasonal variances are detected and results are representative of the sample source.
- Use sterile vessels with securely fitted closures for sample collection and storage. Sterilize with an autoclave or with a hot air oven. Pre-sterilized, disposable bottles and bags are available.
- Collect a sufficient volume of sample. This means at least 200 mL for most analyses.
- Avoid contaminating the sample during collection.
- Treat samples to destroy chlorine residual. (Generally, sodium thiosulfate, sterilized within the collection vessel, is used for this purpose.) Transport immediately after collection.
- Mark the collection vessels with a description of the sample.
- Place collection vessels in strong boxes for transport. Mark the boxes clearly for proper handling during transport. Samples should arrive at the testing site no more than 24 hours after collection. In warm climate,s the samples must be packed in a freezing mixture to maintain the sample temperature between 4 and 10 °C. USEPA guidelines recommend a maximum storage time of 30 hours and a temperature of 1 to 4 °C.

Appendix D: Disinfection of Bacteriologically Contaminated Waters

Chlorine has been used for the disinfection of bacteriologically contaminated waters for many years. More recently the use of other disinfectants, such as bromine and iodine for recreational waters, ozone, chloramines, and chlorine dioxide, and has become common. Although this report is limited to the use of chlorine as a disinfectant, the principles explained are applicable to other disinfectants.

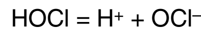
Factors influencing the effectiveness of chlorine as a disinfectant are concentration (C), contact time (T), pH, temperature, and the presence of interfering substances.[1] The measurement of chlorine concentration in a water source is of little significance as an indicator of health risk if the factors of pH and contact time are not known. Turbidity of the water sourcemay also affect the bactericidal activity of the halogen.

Effect of pH on Bactericidal Activity

Chlorine gas (Cl_2) hydrolyzes almost immediately according to the following equation:

$$Cl_2 + H_2O = HOCl + H^+ + Cl^-$$

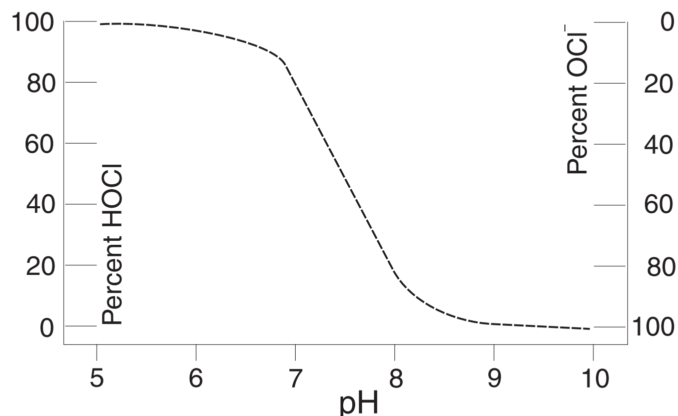
Hypochlorous acid (HOCl), the chief product of this hydrolysis, exists at equilibrium according to the following equation:



$$HOCl = H^+ + OCl^-$$

The equilibrium relationship between hypochlorous acid and hypochlorite ion (OCl^-) is dependent upon the pH (or H^+ concentration) of the water. *Figure 11* shows the pH dependence of the species.

Figure 11 pH Dependence



Research has shown that hypochlorous acid is 80 times more effective than the hypochlorite ion in the inactivation of *E. coli* and 150 times more effective for cysts of *Entamoeba histolytica*.[2] The microbiocidal properties of hypochlorous acid probably can be attributed to its ability to penetrate cell walls. The ease of penetration is due to the low molecular weight and electrical neutrality of hypochlorous acid. The negatively charged hypochlorite ion is unable to diffuse through cell walls and is, therefore, a very poor disinfectant.[3]

Effect of Interfering Substances

Particulate matter in water (turbidity) creates physical interferences which may decrease the effectiveness of chlorination. Turbidity may be caused by either inorganic or organic particles; however, inorganic particles appear to be of little consequence. Various chemical substances, such as organic and inorganic forms of nitrogen, hydrogen sulfide, iron, and manganese, react with chlorine in water, consuming the chemical and rendering it ineffective as a bactericide. This creates what is called a chlorine demand in water. The effective concentration of chlorine required to disinfect water is the chlorine demand plus the necessary germicidal concentration.

A study by Edward Katy[4] revealed a strong correlation between turbidity and chlorine demand. LeChevallier, et al.,[5] showed that a chlorine dose of 5 mg/L with one hour of contact time resulted in a 99.5% reduction in coliforms in waters with a turbidity of 5 NTU. However, under the similar conditions but with turbidities of 8 to 13 NTU, the reduction was only 20%.

Contact Time

The time required for chlorine to disinfect a water source is dependent upon the concentration of the chlorine, pH, temperature, and turbidity of the water. A useful relationship for determining the disinfection requirement is the ***CT***, defined as the effective concentration of chlorine in mg/L times the contact time. This relationship indicates that a lower concentration of chlorine may be used as the initial dose if the contact time is longer. If the contact time is very short, high dose of chlorine must be used. The ***CT*** is dependent upon pH, temperature and turbidity.[1] However, guidelines for 99% inactivation of viruses and bacteria in ground waters and protozoan inactivation in surface waters have been determined. These ***CT*** values are guidelines only and should be adjusted for the dependent factors. For inactivation of viruses and bacteria, a ***CT*** of 15 to 30 is minimal if the pH is between 7 and 8. For inactivation of protozoan activity, a minimum ***CT*** of 100 to 150 must be achieved.[2]

The contact time (***T***, the time in minutes between dosing and usage) should be determined. Then the dose of chlorine required for disinfection is determined by selecting the limiting ***CT*** parameter—and applying that value ***CT*** to the equation:

$$\text{dosage} = (CT/T) + \text{chlorine demand}$$

Temperature

The CT relationship is related inversely to the water temperature. Thus, as the temperature increases, the chlorine dosage required to inactivate the microbiological organisms decrease.[1] The temperature relationship may be invalid for bacteria that thrive at elevated temperatures.

Determination of Proper Disinfection

So long as the contact time is sufficient to ensure minimum CT values, proper disinfection can also be determined by measuring the free chlorine residual, turbidity, and pH. However, microbiological monitoring should be performed to ensure disinfection efficiency. When the pH is greater than 8.0 or the turbidity is greater than 6 NTU, microbiological monitoring is essential to confirm appropriate disinfection.[1]

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