BACTERIAL EXAMINATION OF WATER

The bacteriological examination of water is performed routinely by water utilities and many governmental agencies to ensure a safe supply of water for drinking, bathing, swimming and other domestic and industrial uses. The examination is intended to identify water sources which have been contaminated with potential disease-causing microorganisms. Such contamination generally occurs either directly by human or animal feces, or indirectly through improperly treated sewage or improperly functioning sewage treatment systems. The organisms of prime concern are the intestinal pathogens, particularly those that cause typhoid fever and bacillary dysentery.

Since human fecal pathogens vary in kind (viruses, bacteria, protozoa) and in number, it would be impossible to test each water sample for each pathogen. Instead, it is much easier to test for the presence of nonpathogenic intestinal organisms such as *E. coli*. *E. coli* is a normal inhabitant of the intestinal tract and is not normally found in fresh water. Therefore, if it is detected in water, it can be assumed that there has been fecal contamination of the water.

In order to determine whether water has been contaminated by fecal material, a series of tests are used to demonstrate the presence or absence of coliforms. The **coliform** group is comprised of Gram-negative, nonspore-forming, aerobic to facultatively anaerobic rods, which ferment lactose to acid and gas. Two organisms in this group include *E. coli* and *Enterobacter aerogenes*; however, the only true fecal coliform is *E. coli,* which is found only in fecal material from warm-blooded animals. The presence of this organism in a water supply is evidence of recent fecal contamination and is sufficient to order the water supply closed until tests no longer detect *E. coli.*

In this exercise, you will be testing water samples for the presence of coliforms. There will be three principal tests: the **presumptive**, **confirmed** and **completed** tests (see flow-chart).

STANDARD WATER ANALYSIS

The Presumptive Test

In the presumptive test, a series of lactose broth tubes are inoculated with measured amounts of the water sample to be tested. The series of tubes may consist of three or four groups of three, five or more tubes. The more tubes utilized, the more sensitive the test. Gas production in any one of the tubes is **presumptive** evidence of the presence of coliforms. The **most probable number** (MPN) of coliforms in 100 ml of the water sample can be estimated by the number of positive tubes (see MPN Table).

The Confirmed Test

If any of the tubes inoculated with the water sample produce gas, the water is presumed to be unsafe. However, it is possible that the formation of gas may not be due to the presence of coliforms. In order the **confirm** the presence of coliforms, it is necessary to inoculate EMB (eosin methylene blue) agar plates from a positive presumptive tube. The methylene blue in EMB agar inhibits Gram-positive organisms and allows the Gram-negative coliforms to grow. Coliforms produce colonies with dark centers. *E. coli* and *E. aerogenes* can be distinguished from one another by the size and color of the colonies. *E. coli* colonies are small and have a green metallic sheen, whereas *E. aerogenes* forms large pinkish colonies.

If only *E. coli* or if both *E. coli* and *E. aerogenes* appear on the EMB plate, the test is considered positive. If only *E. aerogenes* appears on the EMB plate, the test is considered negative. The reasons for these interpretations are that, as previously stated, *E. coli* is an indicator of fecal contamination, since it is not normally found in water or soil, whereas *E. aerogenes* is widely distributed in nature outside of the intestinal tract.

The Completed Test

The completed test is made using the organisms which grew on the **confirmed** test media. These organisms are used to inoculate a nutrient agar slant and a tube of lactose broth. After 24 hours at 37°C, the lactose broth is checked for the production of gas, and a Gram stain is made from organisms on the nutrient agar slant. If the organism is a Gram-negative, nonspore-forming rod and produces gas in the lactose tube, then it is positive that coliforms are present in the water sample.

THE MEMBRANE FILTER TECHNIQUE

In addition to the multiple-tube fermentation technique comprised of the presumptive, confirmed and completed tests, a procedure using membrane filters has now been recognized as a reliable method for the detection of coliforms in water. Parallel testing has shown that results obtained from the membrane filter technique are equivalent to those obtained by the multiple-tube tests. Standard methods for the bacteriological examination of water are detailed in *Standard Methods for the Examination of Water and Wastewater*, published by the American Public Health Association.

The membrane filter technique is a direct plating method for the detection and enumeration of the coliform group of indicator organisms. Samples of water are passed through filter discs which have pore sizes of 0.45 µm diameter. Bacteria larger than 0.45 µm will be retained directly on the surface of the filter. The filter is then placed on a selective/differential medium such as M-Endo agar. The only carbohydrate in M-Endo agar is lactose. Lactose fermenters such as *E. coli* and *Enterobacter aerogenes* which are retained on the filter will form colonies with a characteristic greenish-golden metallic sheen within 24 hours of incubation.

The size of the sample to be filtered is governed by the expected bacterial density. An ideal quantity will result in the growth of about 50 coliform colonies and not more than 200 colonies of all bacterial types. Typical coliform colonies are counted and their density is reported in terms of (total) coliforms per 100 ml of sample.

The advantages of this method over the standard multiple-tube test are: (1) higher degree of reproducibiltiy of results; (2) greater sensitivity, since larger volumes of water may be tested; and (3) shorter time for obtaining definite results. There are, however, certain limitations on the use of the membrane filter technique. Its effectiveness is decreased in water samples characterized by high turbidity, high concentrations of heavy metals or the presence of toxic materials such as phenols. Turbidity caused by the presence of algae or other interfering material may not permit examination of a sample volume large enough to yield significant results. Toxic substances, on the other hand, may cause low coliform estimates.

FIRST PERIOD

Material:

1. Nine tubes of double-strength lactose broth
2. 10, 1.0 and 0.1 ml pipets
3. Water samples

Procedure: (work in groups of four)

Presumptive Test

1. Take a water sample (dilute as instructed in some cases) and inoculate three tubes of lactose broth with 10 ml, three tubes with 1.0 ml and three tubes with 0.1 ml.
2. Incubate all tubes at 37ºC for **24 hours**.

SECOND PERIOD

Material:

1. EMB agar plates

Procedure:

Presumptive Test

1. Observe the number of tubes at each dilution that show gas production in 24 hrs. Record results.
2. Reincubate for an additional **24 hours** at 37°C.

Confirmed Test

1. Inoculate an EMB plate with material from a tube containing gas.
2. Invert and incubate the plate at 37°C for **24 hours**.

THIRD PERIOD

Material:

1. Lactose broth tubes
2. Nutrient agar slants

Procedure:

Presumptive Test

1. Observe the number of tubes at each dilution that show gas. Record results and determine the most probable number index.

Confirmed Test

1. Observe EMB agar plates. A positive confirmed test is indicated by small colonies with dark centers and a green metallic sheen (*E. coli*). Record results.

Completed Test

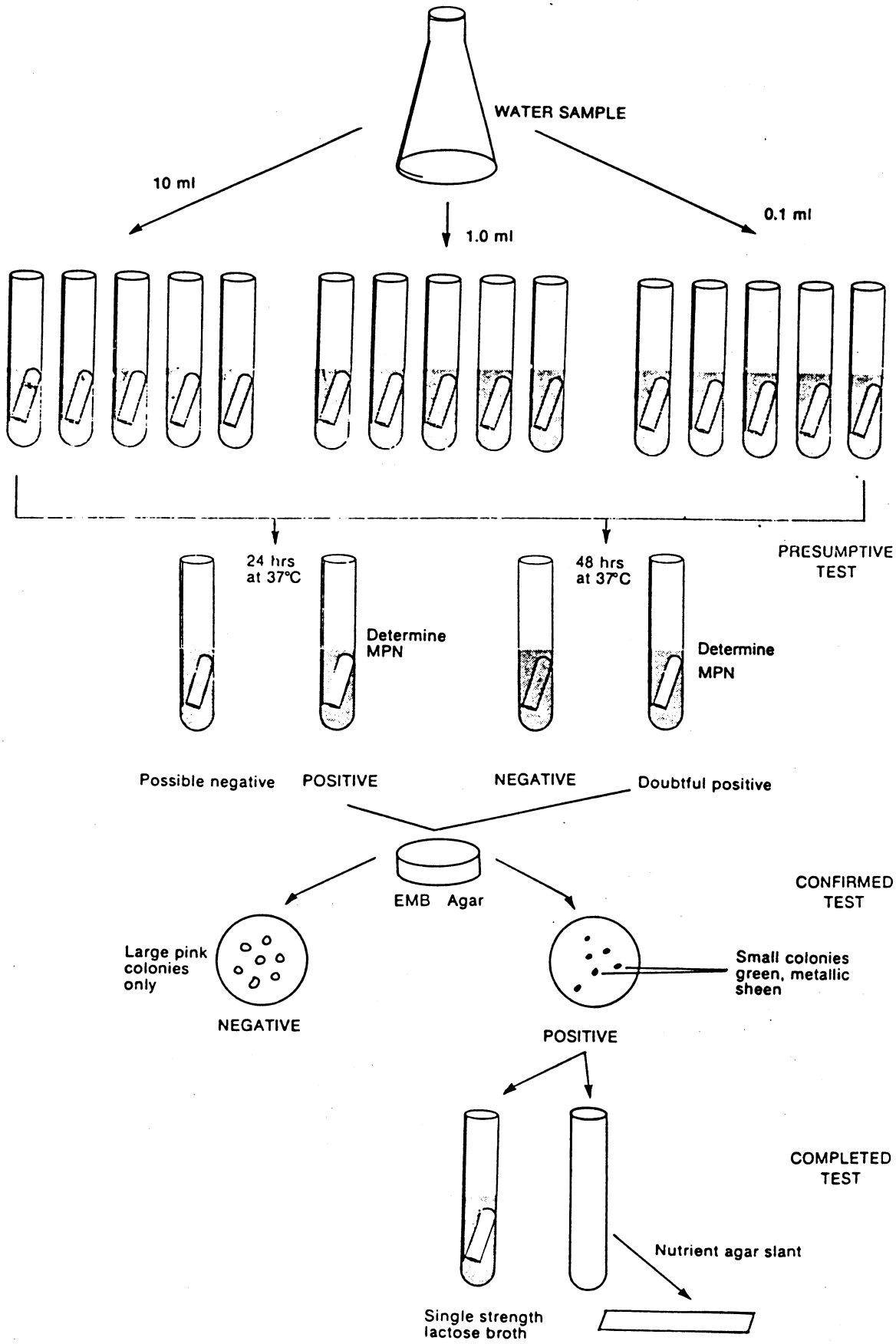
1. Inoculate a lactose broth tube and a nutrient agar slant with organisms from the EMB plate.
2. Incubate the broth tube and agar slant at 37°C for **24 hours**.

FOURTH PERIOD

Procedure:

Completed Test

1. Check for gas production in the lactose broth tube.
2. Make a Gram stain from the organisms on the nutrient agar slant.
3. Record results.


WATER SAMPLE
10 ml
1.0 ml
0.1 ml
24 hrs at 37°C
48 hrs at 37°C
PRESUMPTIVE TEST
Determine MPN
Determine MPN
Possible negative
POSITIVE
NEGATIVE
Doubtful positive
EMB Agar
CONFIRMED TEST
Large pink colonies only
NEGATIVE
Small colonies green, metallic sheen
POSITIVE
COMPLETED TEST
Nutrient agar slant
Single strength lactose broth
Gram stain

MPN DETERMINATION FROM MULTIPLE TUBE TEST

NUMBER OF TUBES GIVING POSITIVE REACTION OUT OF			MPN Index per 100 ml.	95 PERCENT CONFIDENCE LIMITS	
3 of 10 ml. each	3 of 1 ml. each	3 of 0.1 ml. each		Lower	Upper
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1,300
3	3	1	460	71	2,400
3	3	2	1,100	150	4,800

FOOD MICROBIOLOGY

The presence of microorganisms in food is beneficial in some cases and harmful in other cases. Certain microorganisms are necessary in preparing foods such as cheese, pickles, sauerkraut, yogurt and sausage. Other microorganisms, however, may be responsible for serious and sometimes fatal food poisoning and toxicity as well as food spoilage (the product smells, looks, or tastes bad).

Microbial spoilage of any food depends on the chemical composition of the food and the types of organisms with which the food comes into contact. Consider a fresh Granny Smith apple containing a high percentage of carbohydrates. If a carbohydrate-fermenting organism came in contact with the inner tissue of the apple, the organism would survive, multiply, produce acid and gas (maybe alcohol), and, at the same time, destroy the tastiness of the apple. If a proteolytic or lipolytic organism came in contact with the same apple, the microorganism may not survive for long because of the non-availability of protein or lipids, and also because of the low pH of the apple tissue.

Two physical factors involved in the rate of food spoilage are the manner in which the food is processed and the method used to preserve the food. These include cooking, salting, drying, adding microbial inhibitors, adding sugar, canning, refrigerating, freezing and irradiating.

FIRST PERIOD

Material:

1. Samples of fresh raw hamburger, raw chicken, chicken salad, oysters, fresh unwashed vegetables, fresh unwashed fruits, dried fruits, cottage cheese, or creamy salad dressings
2. One 90-ml dilution bottle of sterile saline
3. Four 9-ml dilution tubes of sterile saline
4. Six nutrient agar plates
5. 1.0 and 0.1 ml pipets
6. Glass spreader
7. 95% ethyl alcohol in glass beaker

Procedure: (work in groups of four)

1. Add 10 g of the food product to be assayed into a Waring blender jar. Add 90 ml of sterile saline and blend the mixture at high speed until a uniform slurry is formed (approximately 1 to 3 minutes). You will have made a 10^{-1} dilution of the food sample.

2. Prepare serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) by transferring **1.0** ml at each step. Be sure to mix the diluted samples before each serial transfer.

3. Transfer **0.1** ml of each of the dilutions onto nutrient agar plates.

4. Incubate all plates in an inverted position for 2 days at 37°C.

SECOND PERIOD

Material:

1. Colony counter

Procedure:

1. Count the number of colonies on each plate and record.

Results:

Type of Food	PLATE	Number of Colonies	Number of Organisms per ml
	10^{-2}		
	10^{-3}		
	10^{-4}		
	10^{-5}		
	10^{-6}		

LACTOBACILLI IN SALIVA: THE SNYDER TEST

The Snyder test is used to detect the presence of *Lactobacillus* in saliva. Only lactobacilli seem to be capable of lowering the pH enough to dissolve tooth enamel. Decalcification of the enamel plays an important role in the formation of dental caries (tooth decay). The Snyder test measures dental caries susceptibility by detecting the presence of lactobacilli in saliva.

The Snyder test medium is designed to favor growth of lactobacilli and discourage growth of most other species. This is accomplished by adjusting the pH of the medium to 4.8 and by adding glucose, a carbohydrate easily fermented by *Lactobacillus*. Lactobacilli thrive in the low pH environments and ferment the glucose, producing more acid which reduces the pH even more. The medium includes the pH indicator, bromcresol green, which is green at pH 4.8 and above, and yellow below pH 4.8.

The medium is autoclaved, cooled to just over 40°C and maintained in a water bath until needed. The molten agar is then inoculated with 0.2 ml of saliva, mixed well, and incubated at 37°C for up to 72 hours. The agar tubes are checked at 24-hour intervals for any change in color. Yellow color indicates that fermentation has taken place and is a positive result. High susceptibility to dental caries is indicated if the medium turns yellow within 24 hours. Moderate and slight susceptibility are indicated by a change within 48 and 72 hours, respectively. No change within 72 hours is considered a negative result.

FIRST PERIOD

Material:

1. Four small sterile beakers
2. Five molten Snyder agar tubes (45-50°C)
3. 1.0 ml pipets

Procedure: (work in groups of four)

1. Collect a small sample of saliva (about 0.5 ml) in a sterile beaker for each person in the group.

2. Transfer 0.2 ml of saliva to a Snyder agar tube and roll the tube between your hands until the saliva is uniformly distributed throughout the agar.

3. Allow the agar tube to cool to room temperature. Do not slant the tube.

4. Incubate each of the four Snyder tubes with an uninoculated control tube at 37°C for up to 72 hours.

5. Check the tubes at **24-hour intervals** for yellow color formation (glucose fermentation).

6. Record your results and determine your susceptibility to tooth decay. Compare your results with those from other students in your group.

THE CHROMOGENIC SUBSTRATE TEST

Simple one-step defined substrate tests for detecting coliforms are now available. These tests are designed to detect the presence or absence of coliform bacteria and to indicate specifically the presence or absence of *E. coli*. The Colilert® system is one example of a P-A (presence-absence) test. A water sample is added to a special medium containing **ONPG** (*o*-nitrophenyl-β-D-galacto-pyranoside) and **MUG** (4-methylumbelliferyl-β-D-glucuronide). These substrates are the major sources of carbon in Colilert®. **ONPG** is hydrolyzed by β-galactosidase, the enzme that cleaves lactose to glucose and galactose. The medium will turn yellow if coliforms, which have the ability to ferment lactose, are present. *E. coli* uses another enzyme, β-glucoronidase, to metabolize **MUG**. The modified **MUG** yields a fluorescent product that can be seen under long-wavelength ultraviolet light. Non-target organisms, i.e. non-coliforms, are both starved and suppressed in the Colilert® medium. Refer to the attached AWWA report for more information.

FIRST PERIOD

1. Add 100 ml of the water sample to a sterile, transparent, non-fluorescent bottle provided by Colilert®.

2. Tap one of the "snap packs" to ensure that all of the Colilert® reagents are in the bottom part of the pack. Pour contents of "snap pack" into the water sample. Cap and seal the bottle, and shake until the Colilert® reagents have dissolved.

3. Incubate the bottle for **24 hours at 35°C**.

SECOND PERIOD

1. Read the results at **24 hours**. Compare each result against the "comparator" provided by Colilert®.

☞ If no yellow color is observed, the test is negative.

☞ If the sample has a yellow color equal to or greater than the "comparator", the presence of coliforms of confirmed.

☞ If the sample is yellow, but lighter than the "comparator", incubate for 4 more hours (but no more than 28 hours total). If coliforms are present, the color will intensify. If the color does not intensify, coliforms are absent.

☞ If the sample developed a yellow color, check for fluorescence. If fluorescence is greater to or equal to the fluorescence of the "comparator", the presence of *E. coli* is confirmed.

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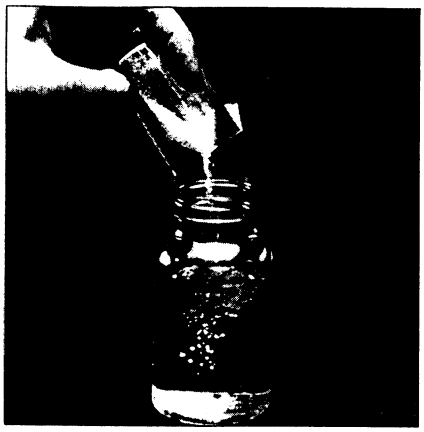
RESEARCH APPLICATIONS

PROVEN UTILITY BENEFITS

American Water Works Association RESEARCH FOUNDATION

Improving Bacterial Analysis While Lowering the Cost



Adding ready-to-use Colilert reagent to a water sample

Problem: Ensuring that drinking water is free of infectious organisms is a prime objective of the water supply community. Water purveyors have historically used microbiological analysis methods such as multiple tube fermentation and membrane filtration to measure bacteriological quality of water.

Since it is impossible to monitor for all human pathogens, these methods rely on the detection of indicator organisms, such as total or fecal coliform bacteria. Indicator tests signal when drinking water quality may be compromised.

However, *Escherichia coli* is a better indicator of contamination of public health significance. Without a simple method to speciate *E. coli*, water purveyors have had to rely on less specific, more time consuming, and more complex methods to test microbiological quality.

Solution: A technology used to identify *E. coli* and total coliform bacteria in clinical microbiology was adapted for the drinking water industry by Stephen Edberg at Yale University's School of Medicine. This technology, termed Colilert®,* was evaluated through a Research Foundation project. The project field validated Colilert's ability to simultaneously enumerate total coliforms and *E. coli* from drinking and source waters without the need for confirmatory tests.

The test uses simple equipment—an incubator and ultraviolet light (366 nm). Addition of the ready-to-use reagent to water samples in test tubes or culture bottles results in a colorless solution. The solution is incubated at 35°C for 24 hours. A yellow color indicates the presence of total coliforms, and fluorescence indicates the presence of *E. coli*.

The original research project, which consisted of a national evaluation of the method, cost $170,000 in AWWARF and in-kind funds. The research was subsequently validated by the U.S. En-

*Colilert is a registered trademark of Environetics, Inc., Branford, Conn.

TABLE 1

Cost Savings for Materials, Labor, and Quality Control*

	Utility Labs	Private Labs	Public Health Labs
Cost per Membrane Filtration Test	$4.40-$5.80	$4.10-$4.70	$5.80-$6.60
Cost per Colilert Test	$3.20-$3.50	$3.20-$3.50	$3.20-$3.50
Savings+	$1.20-$2.30	$0.90-$1.20	$2.60-$3.10

*Averages based on information provided by laboratories certified for microbiological analyses

+Depends on the number of samples per month

vironmental Protection Agency (USEPA), the American Water Works Association, many states, and numerous utilities. It is a USEPA-approved method for total coliform and *E. coli*.

Colilert is valuable for routine monitoring by all sizes of water purveyors and water quality laboratories worldwide. In fact, 46 U.S. states have approved use of the test for drinking water compliance monitoring, and approval is pending in others. Colilert is being used in several Canadian provinces where it has or is expected to receive official approval.

Because it is fast and reliable, the Colilert method is also useful in water quality emergencies following hurricanes, earthquakes, and main breaks. For example, this method was used following the San Francisco earthquake, Hurricane Andrew in Florida, and a cholera outbreak in South America. And it is used for monitoring of rural water supplies in developing countries; for testing marine water, wastewater, private well water, and recreation water; and for U.S. military operations.

Benefits: This issue of *Research Applications* provides information on potential cost savings with Colilert. These cost savings are directly related to this method's ease of use, simplicity, and accuracy.

Colilert is generally less expensive to use than membrane filtration (see Table 1). The cost savings are based on information provided by utility and water laboratories certified for microbiological analyses. These public and private laboratories represent a variety of geographical locations and number of samples processed.

Because savings depend on the number of samples processed per month, a range of values is given. The higher figures represent savings for laboratories that process 200-300 samples per month. The lower figures indicate savings for more than 400 samples per month. The larger laboratories process so many samples each month that they realize some cost savings on materials regardless of the method used.

From the data in Table 1, a conservative estimate of annual savings for the water supply community alone is $13 million. In other words, this is an annual payback of more than $77 for each $1 invested in the original research project. The payback will continue to grow annually as more water utilities and laboratories use Colilert.

Colilert provides the water supply community with an analytical tool that will allow it to better monitor the microbiological quality of drinking water in a more efficient manner, ensuring the consumer with the highest quality water possible. The test's attributes will undoubtedly find favor with the water supply community and health organizations throughout the world in years to come.

More information available from the AWWA Research Foundation:

The Colilert® System for Total Coliforms and Escherichia coli. (Order number 90576.)

Colilert: A Better Method for Ensuring Drinking Water Quality. (To borrow a copy of this 14-minute VHS video, call (303) 347-6121.)

MA-205-93-5

Examples of Where Colilert is Used

- Emergencies
  - Cholera outbreaks
  - Earthquakes
  - Hurricanes
  - Storms and coastal flooding (marine water)
- Industry
  - Aquaculture (seafood estuaries)
  - Bottled water
  - Cosmetic
  - Dairy
  - Food and beverage
  - Pharmaceutical
- Military Operations
  - Kuwait restoration
  - Operation Desert Shield
  - Operation Desert Storm
  - U.S. naval vessels
- Remote and Field Testing
  - Afghanistan
  - Caribbean
  - Central and South America
  - Malawi
  - Sierra Leone
  - South Seas
- Source and Drinking Water Testing
  - Australia
  - Canada
  - France
  - Germany
  - Italy
  - Japan
  - New Zealand
  - Spain
  - United Kingdom
  - United States
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