The Colilert® System for Total Coliforms and Escherichia coli

Subject Area:
Monitoring and Analysis
The Colilert® System for Total Coliforms and Escherichia coli
ADDENDUM TO

The Colilert System for Total Coliforms and Escherichia coli

The AWWA Research Foundation would like to inform you that this report was written and printed before the U.S. Environmental Protection Agency (EPA) had completed its review and decision on approval of the Colilert system for the analysis of Escherichia coli.

The final EPA decision on approval of the Colilert method is expected sometime in 1991. Water utilities and laboratories should consult with their respective regulatory agency as to the approval status of the Colilert method.
The Colilert®
System for
Total Coliforms and
Escherichia coli

Prepared by:
Stephen C. Edberg, Department of Laboratory Medicine, Yale University School of Medicine
New Haven, CT 06510
and
Frances Ludwig and Darrell B. Smith, South Central Connecticut Regional Water Authority
New Haven, CT 06511

Prepared for:
AWWA Research Foundation
6666 West Quincy Avenue
Denver, CO 80235

Published by the
AWWA Research Foundation and
American Water Works Association
DISCLAIMER

This study was funded by the American Water Works Association Research Foundation (AWWARF). AWWARF assumes no responsibility for the content of the research study reported in this publication or for the opinions or statements of fact expressed in the report. The mention of trade names for commercial products does not represent or imply the approval or endorsement of AWWARF. This report is presented solely for informational purposes.

Although the research described in this document has been funded in part by the United States Environmental Protection Agency through a Cooperative Agreement, CR811335, to AWWARF, it has not been subjected to Agency review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.
CONTENTS

LIST OF TABLES AND FIGURES ......................................................... ix

FOREWORD .................................................................................. xi

ACKNOWLEDGMENTS ..................................................................... xiii

EXECUTIVE SUMMARY ................................................................. xv

1. INTRODUCTION: THE PROBLEM .................................................. 1

2. BACKGROUND ............................................................................ 5
   Coliform Outbreak in New Haven ................................................. 5
   Conventional Water Analysis Methods .......................................... 7
   Limitations of Current Methods .................................................... 11
      Technical Limitations .............................................................. 11
      Time Required for Analysis ...................................................... 13
      Cost of Analysis ..................................................................... 14
      Quality of Information From Analysis ...................................... 14
   Need for Specialized Equipment .................................................. 15
   Production of Media .................................................................. 15
   Requirements for Trained Laboratory Microbiologists ................. 15
   Development of the Rapid Colilert System ................................. 16
      Description and Use of the Rapid Colilert System .................... 16
   Meeting the Special Needs of the Water Supply Utilities
      With the Colilert System ......................................................... 18
   Comparison of Autoanalysis Procedure With Other
      Available Water Analysis Methods ........................................... 21

3. RESEARCH PLAN ........................................................................ 23
   Production of Defined Substrate Technology Colilert ................... 23
   National Testing ........................................................................ 23
      MPN Versus MPN Comparison ................................................. 25
      Presence-Absence Comparison ............................................... 25
   Analysis of Data From National Testing and Submission
      to the USEPA as an Alternative Method .................................. 30
   Identification of Microbes Isolated From Water Samples ............ 30

4. QUALITY ASSURANCE ............................................................... 33

5. NATIONAL EVALUATION PROTOCOL ....................................... 35
   Rationale for the Study ............................................................... 35
      MPN: Current Regulations ..................................................... 35
Fecal Coliform or *E. coli* Test From Each
  Total Coliform Positive .................................... 71
  Fecal Coliform Testing ....................................... 72
  Health Risk Indicator ....................................... 73
  Twenty-four Hour Transit Time ................................ 74
  Maximum of 10°C Storage .................................... 74

LIST OF ABBREVIATONS, ACRONYMS, AND SYMBOLS .................. 77

REFERENCES .................................................. 79

APPENDICES
  A - USEPA Analytical Techniques, Coliform Bacteria; Final Rule ..... 83
  B - National Field Evaluation Publication: DST Comparison
      With P-A Techniques ......................................... 93
  C - National Field Evaluation Publication: DST Comparison
      With MTF Method ........................................... 101
TABLES AND FIGURES

Tables

3.1 Demographics of the Field Trial ........................................ 24
3.2 Species of Total Coliforms Isolated ..................................... 31

Figures

2.1 Coping with the Final Coliform Rule ....................................... 9
3.1 Data Form: EMSL Grab Sample Assay .................................. 26
3.2 Data Form: Standard Methods Versus Colilert Results ................ 27
3.3 Data Form: Bacterial Identifications ..................................... 28
3.4 Data Form: General Physical and Chemical Characteristics .......... 29
5.1 Color Reaction of the Colilert® System .................................. 38
FOREWORD

The AWWA Research Foundation is a nonprofit corporation that is dedicated to the implementation of a research effort to help local utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of grass-roots consultation with members, utility subscribers, and working professionals. Under the umbrella of a Five-Year Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection.

This publication is a result of one of those sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals.

Projects are managed closely from their inception to the final report by the Foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The Foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, through which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist local water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The Foundation's Trustees are pleased to offer this publication as a contribution toward that end.

This report on the national evaluation of the Colilert® method, which resulted in the method's subsequent approval by the U.S. Environmental Protection Agency, is a major breakthrough in the bacteriological testing of drinking water. In anticipation of the issuance of more stringent coliform monitory rules, the Colilert method was developed to fully meet these new regulations. The Colilert method's simplicity, specificity (it enumerates only coliforms and Escherichia coli), and sensitivity make it an ideal monitoring tool for the water supply industry.

Richard P. McHugh
Chairman, Board of Trustees
AWWA Research Foundation

James F. Manwaring, P.E.
Executive Director
AWWA Research Foundation
ACKNOWLEDGMENTS

We wish to thank Project Advisory Committee members Donald J. Reasoner (U.S. Environmental Protection Agency [USEPA]); Joyce Kippin (Ipswich Water), and Richard Tobin (Health and Public Welfare, Canada) for their review. The USEPA was very active in the planning and analysis of this data. We sincerely appreciate the efforts of all who were involved. Jack DeMarco and Richard McHugh, Research Foundation Trustees, were willing to look at the method with a clear eye and see how it could be useful for the water utility industry. Their guidance is very much appreciated.

The contributions of many utilities and the participation of many people were invaluable to the testing of the Colilert method. They went out and found the positives in their distribution systems, performed the tests with accuracy, and organized the results. Each of them is a coauthor of this publication.

Investigators in the National Collaborative Study include the following: Mark LeChevallier, American Water Works Service Company, Belleville, IL; Nancy Kriz and Deborah Callan, Yale University School of Medicine, New Haven, CT; Roger Ward and Dawn Calvert, California-American Water Company, Monterey, CA; Wayne Jackson and Michelle Uryc, Cobb County Marietta Water, Acworth, GA; Carol Storms and Judy Loriner, Monmouth Consolidated Water Company, Tinton Falls, NJ; Thomas Trok and Michael Burns, West-Penn Water Company, Pittsburgh, PA; Linda Hmurciak, North Andover, MA; Valerie Shinn, Washington State Department of Health, Seattle, WA; Bruce Kraus, Cincinnati Water Company, Cincinnati, OH; and Charlotte Dery, Vincent Coluccio, and Jerry Iwan, City of New York, NY.
EXECUTIVE SUMMARY

A major limitation that utility managers and public health officials face is the inability of multiple tube fermentation (MTF) and membrane filtration (MF) methods to provide specific information regarding health risks. Both MTF and MF methods are used to enumerate a group of general sentinel bacteria known as the total coliform (TC) group. The total coliforms consist of Enterobacteriaceae that produce gas as the endpoint of lactose fermentation. In addition to Enterobacteriaceae, other bacteria, such as Aeromonas, may also produce a positive total coliform test. In an effort to increase the specificity of the total coliform test, confirmed and completed procedures have been added to the screening procedure. In spite of these additional steps, it has been estimated that approximately 14 percent of total coliform tests are due to noncoliforms such as Aeromonas, other bacteria, and synergistic gas production. Furthermore, coliform detection by the MTF and MF methods may be inhibited by general noncoliform heterotrophic plate count (HPC) densities above 500 bacteria/mL (milliliter).

The MTF and MF procedures determine only the presence of total coliforms, a group of bacteria known not to be associated with any health threat. They are useful in water analysis primarily as measures of the water treatment train. Total coliform analysis has virtually no usefulness as a public health indicator to ascertain whether a fecal event has occurred in the distribution system.

In an attempt to make the total coliform test more specific, the fecal coliform (FC) procedure was developed in 1904. This test took advantage of the fact that Escherichia coli could grow at 44.5°C whereas most other total coliforms could not. Use in the field during the past 30 years has made apparent several limitations: (1) Bacteria other than those that originate in the colon will yield a positive FC test, (2) the test is quite subject to the means of performance, and (3) it is virtually impossible in the field to maintain a temperature of ± 0.2°C at 44.5°C. Deviations above and below this temperature result in significantly increased or decreased false-positives and false-negatives.

Neither the MTF nor MF total coliform or fecal coliform procedures yields a definitive endpoint analysis, i.e., bacterial species identification. Therefore, it is very difficult for public health authorities to make decisions based on these procedures.

An outbreak of fecal coliform bacteria throughout the New Haven, CT, water supply system pointedly demonstrated the limitations of the MTF and MF total coliform and fecal coliform procedures. After exhaustive investigation, it was determined that there was no cross-connection, sewage contamination, or other contamination event. In response to this biofilm occurrence, which had been noted in other utilities in the United States and abroad, a technology transfer of methods to identify Escherichia coli and total coliforms from urinary tract pathogens was undertaken. E. coli was chosen as a measure of public health; total coliforms were taken as a measure of treatment efficacy.

The Colilert® system was developed to simultaneously enumerate total coliforms and E. coli from drinking and source water without the need for confirmatory tests. No equipment other than an incubator and long-wavelength (366 nm [nanometer]) ultraviolet light is necessary. All one needs to do is add the water sample to the powdered formula in a test tube or bottle: A colorless solution results. The Colilert system was compared to the Standard Methods (SM) 10-tube MTF and the presence-absence (P-A) 100-mL format in a North American and United Kingdom (UK) evaluation. All water samples came from
distribution systems. In North America a total of 1,086 MTF tubes were positive by Standard Methods and 1,279 by Colilert (AC) most probable number (MPN). For the P-A versus P-A analysis: SM+/AC+, 302; SM+/AC-, 20; SM/AC+, 22; and SM-/AC-, 358. There was no statistical difference between confirmed Standard Methods and Colilert results in either MPN or P-A formats. Results in the UK were comparable. Use in North America and the UK for more than two years continues to demonstrate Colilert accuracy. Therefore, the test of the Colilert system showed it (1) was equivalent to confirmed Standard Methods, (2) specifically enumerated to one total coliform and E. coli/100 mL separately, in the same vessel, (3) was not subject to inhibition by heterotrophic bacteria, (4) did not require confirmatory tests, (5) was easy to inoculate, (6) was very easy to interpret, and (7) can be used equally well for large and small water systems. The USEPA has approved the Colilert system for testing both total coliforms and Escherichia coli. The cost of the Colilert system was determined by the USEPA to be one-third less than that of conventional methods.

The Colilert system also offers a number of advantages for utilities to comply with the new Total Coliform Rule. First, it is highly compatible with the P-A mode of testing, requiring only that the water sample be added to a bottle containing the powder. Second, it will eliminate unnecessary reporting of violations to the state because it specifically detects E. coli. It will also allow utilities to cope effectively with the increased resampling requirements. Further, it will allow utilities to manage the increased weekend sampling, resampling, and confirmation testing. It will also permit utilities to comply with transportation and temperature storage requirements.

In summary, the Colilert system offers utilities a cost-effective means to comply with the new Total Coliform Rule while maximizing protection of public health.
CHAPTER 1
INTRODUCTION: THE PROBLEM

Total coliforms (TC) have been the microbial indicator of water treatment efficiency for decades. Enteric bacteria of the total coliform group are present in the environment in considerably larger numbers than are pathogens such as *Salmonella* and *Shigella*. If drinking water was free of coliforms, it was presumed there would be no health risk to the consumer. However, total coliforms not associated with disease-causing enteric microorganisms are also present in the environment. The rationale for using TC as a water quality indicator originated with the difficulty in the early 1900s of isolating *Escherichia coli* (*E. coli*), the preferred indicator. It was noted that bacteria resembling *E. coli* ("coliform" means "similar to *E. coli"*) shared an important characteristic—they generally fermented lactose with the production of gas. The test for this characteristic was relatively easy to perform and was a fairly specific indicator for the presence of TC. The multiple tube fermentation (MTF) method TC procedure as we know it today was approved by the U.S. government in 1916. However, as more was learned about the TC group and bacteria in nature, it became evident that the MTF test alone was not specific enough to monitor water quality. Therefore, various confirmation and completed tests were added to enhance specificity. These additional tests not only increased the cost of the method but also created considerable delay in completing the analysis. A further limitation was that once finished, a definitive bacterial name or endpoint was not achieved. In addition, public health authorities knew that some TC (especially *Klebsiella*) were free-living in nature and that their presence was not associated with fecal pollution.

For these reasons, a better sanitary indicator of drinking water quality was needed. A more specific indicator of fecal pollution of water, including drinking water, is the enteric bacterium *Escherichia coli*. A fecal coliform test was developed in 1904 based on the observation that *E. coli* was generally more heat resistant than other coliforms. It is universally recognized that *E. coli* is present in the intestines of all warm-blooded animals and that its presence, in any numbers, in a water supply system indicates relatively recent fecal pollution. During the last decade it was found that approximately 15 percent of total coliforms, primarily *Klebsiella*, were also thermo-tolerant and would be considered fecal
coliforms. This fact compromises the usefulness of fecal coliforms as sanitary indicators because studies showed that many of these *Klebsiella* were not of colonic origin, i.e., they were environmental strains. Accordingly, *E. coli* still appeared to be the best indicator of recent and relevant contamination; however, no specific means for its detection and identification existed. Ideally, a test that could simultaneously enumerate TC for general compliance purposes and *E. coli* for immediate public health action would have the greatest appeal and application for monitoring drinking waters.

In this study, two sanitary indicators were addressed. The first was the total coliform group and the second was the bacterial species *E. coli*. "Fecal coliforms," comprising thermo-tolerant TC, have uncertain public health significance and were not considered to be useful target microbes.

Potable water is subject to microbial contamination from a variety of sources, including treatment deficiencies, cross-connections, back-siphonage, and water main failures. Advances in coagulation, sedimentation, rapid and slow filtration, and disinfection have resulted in water that is free of disease-producing microbes when it leaves a properly operated treatment plant. However, microbes may subsequently contaminate a water supply as a consequence of cross-connections, back-flow, disturbances during repairs, and back-siphonage (Hopkins et al. 1985). Bacteria may also be resident within distribution systems, a condition known as biofilm.

The two standard and widely accepted microbiological procedures for assessing drinking water quality, the multiple tube fermentation (MTF) and membrane filtration (MF) methods, were developed to detect and measure the aggregate TC population in drinking water. Through a number of general phenotypic tests that require several days, the MTF or MF protocols eventually designate the bacterial contamination as "total coliform" or "fecal coliform." These phenotypic tests do not provide specific identification of the species or genus of the organisms.

The MTF and MF methods do not provide sufficient information to the water utility to ascertain the sanitary significance of the water samples in question. The *Standard Methods* are labor intensive, require media preparation equipment, and preclude the sampling of water supplies on an ongoing, "real time" basis. In most cases, it is often days
after a contamination event has occurred that a water utility has enough information to make decisions on the sanitary significance of positive water samples.

The impetus for creating a dramatically different approach to water analysis began in 1984, when the inexplicable presence of TC bacteria was discovered in the entire distribution system of the South Central Connecticut Regional Water Authority (SCCRWA) in New Haven, CT. Because of the inadequacies of the MF and MTF methods, it was necessary to identify the bacteria to species to ascertain the public health risk and to notify the state health department and consumers, if appropriate. It was found that the TC in this outbreak comprised the species *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Citrobacter freundii*. No *E. coli* was ever isolated during the outbreak. It was felt that the system had experienced a regrowth of coliforms from biofilm present in the distribution system and that no fecal contamination event had occurred. Biofilm is common in water distribution systems, but coliform colonization is not.

During the outbreak, the need for timely and accurate laboratory information was apparent. The time delay in the analysis of a water sample, along with the lack of specificity of the available tests, provided the impetus for creating an entirely novel approach to testing drinking water. Utilizing current clinical microbiology technologies, a rapid and definitive method for enumerating TC and *E. coli* simultaneously was developed; the method was named "Colilert®."

The basic concept was to refine the clinical technology presently in use to enumerate and identify microbes from urine in the clinical laboratory into a bacteriological test that would satisfy the needs of the water supply industry. Basically, the method required merely the addition of a water sample to a vessel containing a formulation that would support only the growth of the target bacteria. The water sample could be added to the vessel containing the formulation at the time and place of sample collection and the incubation begun immediately. A color change in the inoculated vessel would indicate the presence of the target microbes—in this case, coliforms. Such a simplistic test would permit water companies to obtain results within hours rather than days. This technology offered many advantages over standard methods and was autoanalytical, i.e., it was based solely on biochemical reactions rather than phenotypic characteristics. It also required minimal labor or training.
The Colilert method was designed to achieve these major goals:

- Sensitivity to total coliform concentrations as low as 1 CFU (colony forming unit)/100 mL (milliliter).
- Definitive results in 4 to 26 hours depending on the initial TC concentration.
- Simultaneous identification of both total coliforms and *E. coli* without further testing confirmation or verification.
- Identification to species, i.e., *E. coli*.
- Lower overall cost.
- No refrigeration of prepared formula and a long shelf-life.
- Possibility of field inoculation if desired.
- Occurrence of a defined color reaction as a result of the growth and metabolic activity of the target microorganism, with no further steps necessary.
- Configuration of the final method as either a presence-absence (P-A) or MTF test.
- Suitability for use by small and large utilities.

This report describes the project on the technology transfer and evaluation of the Colilert method and its wide-ranging applications in the water supply industry.
CHAPTER 2
BACKGROUND

COLIFORM OUTBREAK IN NEW HAVEN

In August 1984, routine distribution system monitoring of treated surface and ground waters of the South Central Connecticut Regional Water Authority (SCCRWA) revealed mean total coliform counts greater than 12 CFU/100 mL. Free chlorine residuals were between 0.6 and 1.0 ppm (parts per million) in the treatment effluents from treated surface sources and between 0.2 and 0.4 ppm from the ground water. Chlorine levels of between 0.2 and 0.5 ppm were consistently present in the distribution system. The coliforms were isolated throughout the system, regardless of the original water source. Many of the isolates grew at 44.5°C and fell under the designation "fecal coliforms." Because the elevated coliform counts were found throughout the water distribution system, it was difficult to explain the continuous appearance of these bacteria as the result of a point source or an ongoing or single occurrence of fecal contamination. Examination of health records from local hospitals and the files of the State Department of Health did not reveal an increase in the incidence of infections by Salmonella, Shigella, or hepatitis A or of any diarrheal diseases.

Consultations on the coliform problem were held with the Centers for Disease Control, the principal investigator (Yale Medical School), the U.S. Environmental Protection Agency (USEPA), and the State of Connecticut. There was a consensus to identify the bacteria to species to determine whether E. coli was present. Because of the widespread nature of coliform positive water samples, it was thought that the 44.5°C fecal coliform temperature test was insufficient proof that massive fecal pollution was occurring. The Yale Clinical Microbiology Laboratory had been using a rapid method for the identification of E. coli and TC from plated urine for several years. All water samples from SCCRWA were tested by this rapid, clinically accepted methodology. There was no E. coli found in any of the water samples, and therefore an order to boil drinking water was not issued.

As a result of this outbreak, it became obvious that a method to monitor drinking water on a routine basis, at low cost, and by an uncomplicated procedure was essential for
the efficient monitoring of water supplies. In response to this urgent need, a new coliform method based on proven clinical technology was devised.

Based on the nature of the β-glucosidase system found only in *E. coli*, the mechanism of the Colilert test was established. It was found that bacteria could be identified by the way they use indicator-nutrients for growth. Testing of water samples in 1977 showed that this identification technology, called the Defined Substrate Technology (DST), could be applied to coliform analysis. However, there was no interest in this promising method for water analysis because coliform, not *E. coli*, was the bacterial indicator for drinking water. In 1984 the needs of a water utility prompted a reexamination of this technology for the analysis of drinking water. It had been used until then in the clinical field. The major difference between clinical specimens and water samples was the number of bacteria present: 1,000 to 100,000 per mL in clinical samples versus 1 per 100 mL in water.

In modifying the DST for use in drinking water analysis, the goal was to develop a test based on the hydrolyzable substrate carbon source technology that would (1) specifically enumerate 1 total coliform/100 mL in 24 hours, (2) simultaneously and specifically enumerate 1 *E. coli*/100 mL in the same sample, (3) not be subject to heterotrophic inhibition, (4) not require confirmatory tests, (5) grow injured coliforms, (6) be easy to inoculate, and (7) be very easy to interpret. The Colilert method was developed to include these attributes. Tested at each step at SCCRWA, it was refined to meet the goals.

The Colilert test is not a new medium but a new technology. A medium has traditionally been used to refer to a mixture of complex plant and animal extracts, the overall combined effect of which is to encourage the growth of specific types or groups of microorganisms. Media components provide either a narrow range of growth factors that are essential to the target organism(s) or inhibitors that suppress the growth of unwanted bacteria. The DST is different in that it provides the target bacteria with only the indicator-nutrient that they can metabolize. Simple nutrients, salts, and solanium (Access Analytical Systems, Inc., Branford, CT) make up the remainder of the formula. The resulting formula is a stable powder that can be added directly to the sample—or the sample to it. The Colilert system was designed and refined from the DST to detect 1 TC or *E. coli*/100 mL in 24 hours. The formula does not support the growth of heterotrophs. Clinical experience in exploiting the selective metabolism of target microbes provided the background for
choosing the chemicals and nutrients for the method. Because of the concern about injured coliforms, no inhibitors such as detergents were included. The formulation includes ammonium sulfate as the source of nitrogen; mineral salts; vitamin cofactors; and two hydrolyzable substrates—orthonitrophenyl-β-D-galactopyranoside (ONPG), which changes color from clear colorless to yellow upon hydrolysis by total coliforms, and 4-methylumbelliferyl-β-D-glucuronide (MUG), which produces a distinct fluorescence when hydrolyzed by \textit{E. coli}.

To use the Colilert test, all one needs to do is add water to the powder and incubate at 35°C. Growth of the target microbes begins immediately after inoculation. Depending on the number of bacteria present, a positive is revealed anywhere from 16 to 28 hours after the test begins. The test normally requires 24 hours but may be extended to 28 when the color determination is questionable at 24.

**CONVENTIONAL WATER ANALYSIS METHODS**

Coliforms were first chosen as indicators of microbial contamination of water in the 1890s because virulent waterborne pathogens such as \textit{Salmonella}, \textit{Shigella}, \textit{Vibrio}, viruses, etc., are present in water in numbers too low for efficient growth or identification (American Public Health Association 1985). Even though \textit{E. coli} was recognized as a better index of the sanitary quality of water, speciation was too inexact and cumbersome for routine water analysis. Subsequently, total coliforms were used as sentinels and surrogates for the potential presence of the agents of gastroenteritis.

In North America there are three designations of indicator bacteria. First, there are the total coliforms. This group comprises the members of the family \textit{Enterobacteriaceae} that are gram-negative bacteria that ferment lactose at 35°C in 48 hours. Recently, the definition has been modified to include or to substitute the production of β-D-galactosidase (ONPG substrate) coupled with the absence of cytochrome oxidase (American Public Health Association 1985; Bordner and Winter 1978; Covert 1985). The presence of total coliforms in drinking water is considered an overall indicator of the quality of water. They may have either an intracolonic or extracolonic existence, and their presence is considered undesirable.
The second group, the fecal coliforms, is a subgroup of the total coliform group. Fecal coliforms ferment lactose in 48 hours with the production of gas at 44.5°C. The fecal coliforms contain both intra- and extracolonic coliforms and are a better indicator of fecal pollution than are TCs, although FCs are not as relevant as *E. coli*. Under the new drinking water regulations established by the USEPA, which are scheduled to take effect December 31, 1990, *E. coli* will be considered the most specific indicator of fecal pollution of drinking water. Under the new rules, each water test (primary or retest) that is TC positive will have to be further analyzed for the presence of either fecal coliforms or *E. coli*.

The third indicator group consists of the (noncoliform) heterotrophic bacteria enumerated by the heterotrophic plate count (HPC) method. These are bacteria that are ubiquitous in treated water and can thrive in distribution systems; they are tertiary sentinels of water microbial quality. When present in large numbers, they indicate a deterioration of water quality. The species belonging to this group are diverse; their description is based on gross characteristics of growth such as pigment formation. There are no regulations governing the number of HPC bacteria in water considered to be a health hazard. However, the new drinking water regulations caution that high populations of HPC bacteria may interfere with coliform detection and invalidate coliform results of conventional methods; fortunately, Colilert is not subject to HPC interferences.

In the United States, Canada, and many other countries, there are two common methods for the analysis of coliforms in water: the multiple tube fermentation (MTF) method and the membrane filtration (MF) technique. The standard MPN MTF total coliform test requires several successive levels of analysis (Figure 2.1) (American Public Health Association 1985). The levels are known as the presumptive, confirmed, and completed stages. To perform an entire analysis requires between two and six days. As shown in Figure 2.1, the inoculated lactose broth is examined for the presence of gas for a maximum of 48 hours. If gas is present, a small amount of the media is transferred to the confirmatory broth, brilliant green lactose bile broth (BGLB). This broth may require up to 48 hours to be positive. In order to perform a completed test, an additional two to three days are required. After these manipulations, a definitive bacterial species name is not achieved, but the definitive presence of total coliform bacteria has been established.
FIGURE 2.1

Coping with the Final Coliform Rule...

Alternative Standard Methods

**Sequential Workflow**

<table>
<thead>
<tr>
<th>Time</th>
<th>Colilert</th>
<th>Sequential Workflow</th>
<th>Accelerated Workflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Collect Sample</td>
<td>Inoculate &amp; Incubate 24 hours</td>
<td>Collect Sample</td>
</tr>
<tr>
<td></td>
<td>Inoculate &amp; Incubate 24 hours</td>
<td>Read</td>
<td>Inoculate &amp; Incubate 24 hours</td>
</tr>
<tr>
<td>Day 2</td>
<td>Read E. coli Pos. (yellow and fluorescent), T.C. Pos. (presumptive)</td>
<td>T.C. Pos. (presumptive)</td>
<td>T.C. Pos. (presumptive)</td>
</tr>
<tr>
<td></td>
<td>Report to state</td>
<td>Confirm Test</td>
<td>Confirm Test</td>
</tr>
<tr>
<td></td>
<td>Collect Repeats</td>
<td>Test T.C. /E. coli</td>
<td>Collect Repeats</td>
</tr>
<tr>
<td></td>
<td>Incubate 24 hours</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24 hours</td>
</tr>
<tr>
<td>Day 3</td>
<td>Read E. coli Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Acute violation</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Collect Repeats</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24-48 hours</td>
</tr>
<tr>
<td></td>
<td>Report MCL/Acute violation or collect repeats</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td>Day 4</td>
<td>Acute violation</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24-48 hours</td>
</tr>
<tr>
<td></td>
<td>Confirm Test</td>
<td>Acute violation</td>
<td>Acute violation</td>
</tr>
<tr>
<td></td>
<td>Incubate 24-48 hours</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td>Day 5</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Pos. (confirmed) or E. coli</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Neg. (confirmed) or Infecals</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td>Day 6</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Pos. (confirmed) or E. coli</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Report MCL/Acute violation or collect repeats</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Incubate 24 hours</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Acute violation</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
</tbody>
</table>

**Accelerated Workflow**

<table>
<thead>
<tr>
<th>Time</th>
<th>Colilert</th>
<th>Sequential Workflow</th>
<th>Accelerated Workflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Collect Sample</td>
<td>Inoculate &amp; Incubate 24 hours</td>
<td>Collect Sample</td>
</tr>
<tr>
<td></td>
<td>Inoculate &amp; Incubate 24 hours</td>
<td>Read</td>
<td>Inoculate &amp; Incubate 24 hours</td>
</tr>
<tr>
<td>Day 2</td>
<td>Read E. coli Pos. (yellow and fluorescent), T.C. Pos. (presumptive)</td>
<td>T.C. Pos. (presumptive)</td>
<td>T.C. Pos. (presumptive)</td>
</tr>
<tr>
<td></td>
<td>Report to state</td>
<td>Confirm Test</td>
<td>Confirm Test</td>
</tr>
<tr>
<td></td>
<td>Collect Repeats</td>
<td>Test T.C. /E. coli</td>
<td>Collect Repeats</td>
</tr>
<tr>
<td></td>
<td>Incubate 24 hours</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24 hours</td>
</tr>
<tr>
<td>Day 3</td>
<td>Read E. coli Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Acute violation</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Collect Repeats</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24-48 hours</td>
</tr>
<tr>
<td></td>
<td>Report MCL/Acute violation or collect repeats</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td>Day 4</td>
<td>Acute violation</td>
<td>Incubate 24-48 hours</td>
<td>Incubate 24-48 hours</td>
</tr>
<tr>
<td></td>
<td>Confirm Test</td>
<td>Acute violation</td>
<td>Acute violation</td>
</tr>
<tr>
<td></td>
<td>Incubate 24-48 hours</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td>Day 5</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Pos. (confirmed) or E. coli</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td>Day 6</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Pos. (confirmed) or E. coli</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Report MCL/Acute violation or collect repeats</td>
<td>T.C. Pos. (confirmed)</td>
<td>T.C. Pos. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Incubate 24 hours</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
<tr>
<td></td>
<td>Acute violation</td>
<td>T.C. Neg. (confirmed)</td>
<td>T.C. Neg. (confirmed)</td>
</tr>
</tbody>
</table>

9
In the second coliform technique, the MF procedure, 100 mL of water is filtered through a 0.45 μm (micrometer) pore size membrane filter and the membrane transferred to selective agar (American Public Health Association 1985; Bordner and Winter 1978). The agar contains chemicals that inhibit the growth of bacteria other than coliforms. After 24 hours of incubation at 35°C, colonies that demonstrate strong lactose fermentation (green metallic sheen) are transferred to confirmatory BGLB broth (see Figure 2.1) (Grabow and Preez 1979).

The MTF assay is susceptible to interference by heterotrophic bacteria (LeChevallier et al. 1980). Noncoliform bacteria at densities higher than 500 CFU/mL can interfere with the isolation of coliform bacteria by competing for nutrients and releasing metabolites that interfere with their growth (Manja et al. 1982). This interference also occurs with the MF method. There is also evidence that heterotrophic bacteria in the piping of water distribution systems will decrease the efficiency of the isolation of coliform bacteria (Federal Register, vol. 52, November 13, 1987, p. 42224).

False-negative results can occur at the presumptive, confirmed, and completed levels of the MTF analysis. Other factors such as synergistic production of gas from lactose by noncoliforms, inability to cultivate *E. coli* that do not ferment lactose (approximately 8 percent), and the presence of lactose-fermenting noncoliforms (Geldreich et al. 1978) result in decreased specificity for both the MTF and MF tests (Seidler et al. 1981).

The fecal coliform test may be performed by either an MTF or MF method. In the MTF method, the standard lauryl tryptose broth (LTB) total coliform broth is inoculated. Fecal coliform confirmation takes place in EC (fecal coliform fermentation medium) broth at 44.5°C. Second, a plate test, m-FC (membrane fecal coliform agar), can be performed. In this test, water is filtered and the media are incubated at 44.5°C. Because the media and incubation conditions for both fecal coliforms tests do not select for the isolation of a particular species of microbe, both fecal and total coliforms, which include *E. coli*, *Klebsiella*, *Enterobacter*, and other coliforms, may yield false-positive results. Also, these methods may be inadequate for recovery of injured cells (Maxcy 1973; McFeters et al. 1986). Debilitated *E. coli* are sensitive to high incubation temperatures (McFeters et al. 1986) and their growth may be inhibited by the selective media (McFeters et al. 1980).
LIMITATIONS OF CURRENT METHODS

Technical Limitations

Competition by Heterotrophic Plate Count Bacteria

In lactose-based media, HPC organisms can compete with total coliforms for both the carbon source (lactose) and the proteins present. Because the HPC bacteria are often present in very high numbers, they can deprive the TC of food. Therefore, the total coliforms may not grow well in competition with HPC organisms, and this may result in a false-negative test for both MF and MTF methods.

False-positives by Heterotrophs

Some noncoliform heterotrophs, especially *Aeromonas*, ferment lactose and appear like total coliforms. They can also confirm in BGLB. It has been reported (Bordner and Winter 1978) that in water containing *Aeromonas*, up to 20 percent of gas positives may be false-positives due to this species. In addition, synergistic gas production by a variety of noncoliform HPC has been observed, leading to false-positive coliform results (personal communication, Nelson Moyer and Nancy Hall, Oakdale Laboratory, University of Iowa, 1989).

Low Coliform Numbers and Colony Size

Coliforms are often present in very low numbers, in the range of 1 to 4 per 100 mL. On the surface of agar, bacteria in such low numbers may not manifest themselves, particularly if the coliforms are injured. The variable quality (impurities) of the basic fuchsin dye used in m-Endo agar may adversely affect sheen production and colony size (personal communication, E. Geldreich 1987; Evans et al. 1981a).
Clogging of Membranes

The 0.45-μm filters used in the MF technique are highly susceptible to clogging by particulates. Water samples with high turbidity cannot be analyzed by this technique. Furthermore, if a utility has high turbidity in its raw water this method cannot be utilized to pretest the water before it is treated (Geldreich et al. 1972; Seidler and Evans 1982).

Aberrant Reactions in Biochemical Tests

False-negative results, indicating the absence of gas production by coliforms, may occur at the presumptive, confirmed, and completed stages of analysis. Synergistic gas production from lactose by noncoliforms can also occur. Both *E. coli* (fecal coliforms) and total coliforms may be anaerogenic and non-lactose fermenting (Hussong et al. 1981; Meadows et al. 1980; Schiff et al. 1970).

Spreading on Surfaces of Plates

*Heterotrophic* bacteria and some noncoliform *Enterobacteriaceae* (e.g., *Proteus*) can spread over the surface of agar plates and render an analysis invalid. Furthermore, under new regulations, if there are more than 200 colonies on the surface of the membrane, the analysis is invalid (Evans et al. 1981b; Evans et al. 1981c).

Growth Inhibition by Heavy Metals

Heavy metals adsorbed to entrapped particles can become concentrated in the pores of the membrane filters and adversely affect the growth of microorganisms. These heavy metals are normally present in low concentrations in water but are adsorbed onto the surface of the filters because of charge interactions. Although normally innocuous to microorganisms in the concentrations found in water effluents, local concentrations of heavy metals can become high enough to cause false-negative results (Dutka 1973).
Failure to Detect Injured Coliforms

Coliforms may be injured during the water treatment process and may fail to grow and produce the metallic sheen on m-Endo agar. It appears that the specific ingredients (surfactant) in the agar inhibit the growth of these organisms. The exact health threat of injured coliforms is unknown, but it has been shown that when they are resuscitated, they regain their virulence in animal infection models (LeChevallier et al. 1980, 1981, 1983a, 1983b; LeChevallier and McFeters 1984; Levin et al. 1961; McFeters et al. 1986).

High Rate of Unacceptable Membrane Filters

The USEPA has reported that 61 percent of membrane filters, representing all manufacturers, are unacceptable. One-third of all membranes showed two or more defects. The USEPA has recommended a quality control program to address these problems, alleging that in the past 15 years the defect problem has become worse. These defects could lead to erroneous results (Brenner and Rankin 1990).

Time Required for Analysis

Current methods require at least 24 hours of incubation for the detection of any bacterial growth and an additional 24 to 48 hours to establish or confirm the presence of total coliforms (see Figure 2.1). If fecal coliform enumeration is needed, an additional 24-48 hours of incubation is required. Inoculated media must also be incubated for 48 hours before a negative result can be reported. Each step in the MTF and MF methods requires manipulations by a technician. The subjective nature of current microbial analysis (gas formation, sheen colonies) can result in inaccuracies and increased time of analysis. With the MF and MTF methods at least 48 hours is required to establish a negative result, and up to a week is needed after a contamination event to establish a definitive answer (Reasoner et al. 1979; Reasoner and Geldreich 1985).
Cost of Analysis

The cost in supplies for a negative MF analysis is between $3.00 and $4.25. The cost in labor is approximately $2.50 (1990 figures); this labor cost includes not only the actual hands-on laboratory analysis time but also the cost of production and quality control of the medium. If there are positive colonies, the cost of a positive test, in labor and materials, averages $16.25.

The USEPA has determined that the Colilert system costs approximately one-third less than currently available methods (Federal Register, vol. 53, no. 88, May 6, 1988, p. 16348). The cost of MF and MTF analysis will be appreciably higher (perhaps double or triple) under the new coliform rule requiring fecal or \textit{E. coli} differentiation of positive samples.

Quality of Information From Analysis

The confirmed and completed conventional tests do not yield a genus or species identification. They yield a general group designation, either total coliforms or fecal coliforms. This lack of specificity does not give the water utility much help in determining the sanitary significance of coliform positive samples. Furthermore, the ultimate identification of these bacteria is based on multicomponent systems such as lactose fermentation, temperature tolerance, and production of gas. These analytical methods fail to account for the fact that many coliforms, including the fecal coliforms, neither ferment lactose nor produce gas. Currently available systems do not yield a bacterial identification, and there is no way of positively knowing if a false-negative or false-positive has occurred. This lack of a definitive answer will remain one of the major limitations of MF and MTF methods in the 1990s and beyond. Also, because the current designations "total coliform" and "fecal coliform" have no clinical meaning, their presence in drinking water is not necessarily seen as a health threat. Public health and state agencies need solid, usable, specific information to make decisions on the wholesomeness of drinking water. MTF and MF procedures do not provide this vital information.
Need for Specialized Equipment

Water analysis by the MTF and MF methods requires specific and costly lab equipment. The laboratory must produce sterile media using equipment such as autoclaves, glassware washers, pH meters, etc. Also, laboratories utilizing MF techniques must have membrane filter equipment. This requirement for specialized equipment precludes many small water companies from analyzing their own water and also requires many utilities to send their water considerable distances to central laboratories, thus increasing the time of analysis and, more importantly, leading to inaccurate analysis because the microbes in the sample may increase or decrease in number during transportation and storage.

Production of Media

Most media for water analysis must be made on site, in small batches, and refrigerated on site because of the relatively short expiration time of prepared tubes or agar plates. This limitation requires a utility to equip, maintain, and staff a facility for the production of media.

Requirements for Trained Laboratory Microbiologists

Microbial analysis of water requires a trained technologist, and subjective interpretation is required in order to complete an analysis. As seen in Figure 2.1, the flow scheme for the processing of water samples requires an individual to make judgments at several decision points.
DEVELOPMENT OF THE RAPID COLILERT SYSTEM

Description and Use of the Rapid Colilert System

Transfer of Technology

The Colilert system is a transfer of enumeration methods originally invented for the rapid identification of microorganisms in the clinical microbiology laboratory. These methods are the basis for the development of the DST for the detection of total coliforms from water.

The species of microbes of sanitary significance most commonly isolated from water and water distribution systems are the same as the species isolated from human urinary tract infections. These species include *E. coli* (the majority of the fecal coliforms) and the *Klebsiella-Enterobacter-Serratia-Citrobacter* group (with *E. coli*, comprising the total coliforms) (Bagley and Seidler 1978; Naemura et al. 1979; Woodward et al. 1979). Other microbes for which this technology is applicable and that are routinely isolated both from patients and water supplies include staphylococci, *Streptococcus faecalis*, and *Clostridium perfringens*. The rapid identification of these microorganisms is based on the assay of their constitutive enzyme systems. A constitutive enzyme is one that is always present in a given microorganism. For example, in the clinical microbiology laboratory, *E. coli* is routinely identified from colonies within 30 minutes, based on the enzymatic assay for β-D-glucuronidase. This assay has served as the basis for a rapid and economical means to process urinary tract specimens (Edberg and Trepeta 1983; Trepeta and Edberg 1984). These methods, because of their accuracy, rapidity, and low cost, have become standard in clinical laboratories throughout the country and now exist for the major groups of microorganisms.

The Colilert method employs a substrate for a constitutive enzyme in the target microbe that changes color when the enzyme is present. For each target microbe(s) there is a substrate for a specific enzyme. There is one substrate for *E. coli* and another for total coliforms. The unique feature of the technology is that the metabolic activity of the target microbe is directed toward the substrate. Microbes other than the target species cannot
grow and metabolize. Under such restrictive conditions, therefore, heterotrophic bacteria will be unable to replicate and interfere. There is no need for additional tests after a color change has been observed because the test is exclusively specific for a given organism or group of organisms.

Description of the Colilert System

The Colilert formulation can be prepared in powder form and has a shelf life, at room temperature, of at least one year. The individual who has collected the water sample needs only add 100 mL of the sample to the Colilert media-containing vessel. The Colilert vessels can be brought to the sampling site and inoculated, if desired. Incubation begins immediately; the development of a specific color indicates the presence of the target microbe(s) (see Figure 5.1, p. 38). As formulated, the two sugar dye ingredients are specific for coliforms and \( E. coli \). If quantitation of the target microbe(s) per unit volume of sample is desired, an MPN test is performed rather than a P-A analysis.

Comparison of Current Practice and Colilert System Analytical Schemes

In order to identify a microbial pathogen in a specimen, the following procedure is currently employed.

1. Current practice pathway. MF and MTF require complicated, multibranched pathways to complete an analysis (Figure 2.1, p. 9). Neither of these methods provides a specific bacterial name and is therefore of limited public health use. Moreover, there is considerable subjective interpretation all along the pathway (see Figure 2.1).

2. Rapid Colilert system pathway and formulation. The rapid Colilert formulation is in powder form. Colilert will provide a separate MPN or P-A test for both total coliforms and \( E. coli \). For a P-A test, 100 mL of the water sample can be added directly to the vessel. For an MPN, a given volume of water, usually 10 mL, is added to each of several vessels that contain the Colilert formulation. The ingredients are soluble and incubation begins immediately upon sample entry. The formulation is stable for at least a year at room temperature.
3. **Incubation.** The metabolic activity of the target microbe(s) begins as soon as the water sample is added to the Colilert formulation. Although 35°C ± 0.5°C is optimal, the total coliforms will metabolize and grow at room temperature (21°C). They will do so at approximately 50 percent of the rate seen at 35°C.

As soon as the total coliforms multiply in time, their presence is established by a color change of the specific substrates. If there is initially 1 microbe/100 mL, the time period is approximately 20 hours; if there are 100 total coliforms/100 mL, the time period is 6 to 8 hours (Edberg et al. 1988). When using the Colilert system in monitoring drinking water, incubation up to 26 hours, ± 2 hrs may be necessary to recover any slow-growing or injured coliforms. The incubation time should not be extended beyond 28 hours.

4. **Examination of the Colilert vessel.** The development of a specific color indicates the presence of the TC or *E. coli* or both. This color may develop anytime after incubation is begun. Once color is seen, no further analysis needs to be performed. For each target microbe, a specific substrate is metabolized. Each substrate exhibits its own specific color change. Therefore, within the same Colilert vessel one may detect more than one type of target microbe. For total coliforms the specific substrate is ONPG, which becomes yellow when metabolized. For *E. coli* the specific substrate is MUG, which becomes fluorescent when metabolized.

5. **Purification of the target microbe.** This step is not required in the Colilert system.

6. **Analysis of the target microbe (confirmed and completed stages).** These steps are not required by the Colilert system.

Meeting the Special Needs of the Water Supply Utilities With the Colilert System

**Technical Problems Overcome**

**Competition by heterotrophs.** Heterotrophic competition has been eliminated by the nature of the Colilert formulation, which allows only the target microbes to grow. Noncoliform bacteria (heterotrophs) cannot metabolize in the environment of the Colilert vessel and, therefore, do not interfere with or compromise the results as can occur with...
MTF and MF methods. The metabolic activity of the target microbe(s) for the enzyme substrates is intensified, thus decreasing the time of analysis.

**Clogging of membranes.** The Colilert system is not subject to clogging.

**Low coliform numbers.** The Colilert method can determine the existence of a single total coliform/100 mL within 26 hours. There are no dyes or other inhibitors to coliforms in the medium, and, therefore, when coliforms are present, they manifest themselves (Edberg et al. 1988).

**Spreading on membranes.** It is a common problem with the MF procedure that HPC can spread over the surface of a membrane and invalidate the test.

**Inhibition by heavy metals.** Because water is added to a vessel, heavy metals are not concentrated, as can be the case in MF analysis, and will not exert antibacterial action.

**Results of injured coliforms.** Because the Colilert formulation does not contain inhibitory dyes or chemicals, coliforms are not affected. Viable and injured coliforms will grow in this formulation.

**High rate of unacceptable membrane filters.** Because the Colilert system is composed of carefully controlled salts and chemicals, it undergoes rigorous quality control in the factory. The manufacturing specifications are strictly followed. In the field, the quality control procedure is simple and inexpensive, unlike for the MF procedure. The reliability and ease of quality control represent major advantages over membrane filtration.

Time of Analysis

The Colilert system forces the target microbe to direct its metabolic activities to the substrate. If one organism is present in 100 mL of water, color can be detected visually after 20 hours; if there are 100 organisms/100 mL of water, color can be detected after approximately 6 to 8 hours. No further analysis is necessary after the specific color appears. Therefore, the total time of analysis compared to MF and MTF methods is considerably reduced: 24 hours versus 2 to 3 days for a completed test.
Cost

The cost of a rapid autoanalysis by the P-A format is significantly less than the cost of MF and MTF methods in use today. The USEPA has stated, "Costs for coliform testing are expected to decrease significantly, perhaps as much as 33 percent, with the use of the Autoanalysis Colilert test. The test is simpler and less labor-intensive than existing methods. . . Testing for E. coli by the Autoanalysis Colilert method can be done at no additional cost, since it merely involves viewing a total coliform-positive culture under an ultraviolet light" (Federal Register, vol. 53, no. 88, May 6, 1988, p. 16348). Also, there is no requirement for additional laboratory space or specialized equipment. The cost of the water test is the only cost of the Colilert formulation, plus some analyst time.

Credibility of Laboratory Results

The Colilert procedure is based on the detection of stable intrinsic enzyme activity of microorganisms. The answers provided by the Colilert system are specific and definitive. For each of the target microbe(s) a specific enzyme substrate has been evaluated and chosen. Therefore, the designation of E. coli or total coliforms is as accurate as that obtained by a long battery of tests.

Equipment Required

The only equipment required for the Colilert system is a hot-air incubator and a hand-held ultraviolet (UV) light. Under field conditions the incubation can take place at room temperature, although the time of incubation is longer. Incubation at temperatures other than 35°C ± 0.5°C is not recommended for standardized testing.

Manufacture of the Colilert System

The Colilert system is available commercially. The manufacture of the Colilert system is an exacting process (see Preparation of Colilert, p. 37). Particular care must be taken to
prevent contamination of the Colilert powder during manufacture and dispensing. Even one total coliform, or \textit{E. coli}, introduced into the powder will produce a false-positive test. Total coliforms and \textit{E. coli} may be found on fingers; total coliforms can also be found in the environment. Like any item manufactured in large quantities by commercial sources specializing in a particular item, it is invariably less expensive to buy the end product than to synthesize in-house, considering the quality assurance and lot testing necessary to ensure sensitivity and specificity.

Necessity for Trained Personnel

The Colilert system does not require trained personnel. Although water testing for reporting purposes should always be supervised by knowledgeable individuals, the Colilert system permits utilities to test a wide range of source, storage, and processed water by utility professionals responsible for these areas.

Comparison of Autoanalysis Procedure With Other Available Water Analysis Methods

There are major differences between Colilert and \textit{Standard Methods} formulations. All \textit{Standard Methods} formulations except Colilert are lactose and protein based. \textit{These media are not Colilert and work in entirely different ways}. The important distinctions are:

<table>
<thead>
<tr>
<th>Distinction</th>
<th>Colilert</th>
<th>Lactose Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specifically detects and confirms 1 CFU of total coliforms and \textit{E. coli} in 100 mL of water sample in 24 hours</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Simultaneously detects and confirms total coliforms and \textit{E. coli} in the same vessel</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Eliminates competition for nutrients by noncoliform microbes and the potential inhibition of coliforms and \textit{E. coli} by noncoliform metabolic products</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Distinction</td>
<td>Colilert</td>
<td>Lactose Media</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>Not subject to noncoliform heterotrophic interference</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Buffered system that maintains optimal pH for fluorescence of metabolized MUG indicator throughout test incubation</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nationally evaluated by water utilities using USEPA protocol</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ready to use, predispensed dry formula. Up to one year stability at room temperature</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Approved for <em>E. coli</em> enumeration for drinking waters</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
CHAPTER 3
RESEARCH PLAN

In order to evaluate the Colilert system in comparison to the MTF and MF methods, a research plan was developed. The rationale leading toward this comprehensive evaluation is described below.

PRODUCTION OF DEFINED SUBSTRATE TECHNOLOGY COLILERT

The Colilert test represents a technology transfer of the DST to drinking water analysis. The test was originally constituted as a liquid formulation. However, the substrates in the liquid were not stable for more than a few days, making a national evaluation of the method, as envisioned, impossible. A number of companies supplying reagents, media, and membranes to the water utility industry were contacted to determine if they were interested in developing a stable powdered formulation. No company showed interest in making the Colilert system with any degree of reproducible quality control. There were two primary factors for this reluctance: First, the method did not have USEPA approval. Second, the water analysis media market was too small to consider anything too innovative, i.e., too speculative.

After considerable searching, a medical diagnostic company, Access Analytical Systems, Inc. (AAS), Branford, CT, expressed interest. The company’s major business was packaging medical diagnostic tests with emphasis on quality control. Accordingly, AAS was awarded the contract to make the Colilert system for the national evaluation funded by the Research Foundation. AAS agreed to stringent quality control parameters, verified by Stephen C. Edberg’s laboratory at Yale University.

NATIONAL TESTING

In order for Colilert to be used for compliance monitoring under existing drinking water rules, it had to be strictly evaluated in comparison with the other approved methods. During the design of the national evaluation, new bacteriological regulations for the analysis
of drinking water were proposed and published. These regulations were qualitative in nature and were based on the P-A concept. For this reason, the national evaluation included both MPN and P-A comparisons.

Participating utilities in the national evaluation were selected on the following criteria: (1) ability to obtain coliform positive samples from their distribution system, (2) possession of a state-certified laboratory, (3) recent evidence of proficiency testing, (4) ability to identify bacteria to species, (5) designation of an individual who would have the time and responsibility for performing the tests as prescribed in the protocol, (6) possession of an IBM PC-type computer for the recording of data, and (7) established interest in the collaborative research study.

The water utilities selected and their salient characteristics are shown in Table 3.1.

**TABLE 3.1**

Demographics of the Field Trial

<table>
<thead>
<tr>
<th>Geographical Area</th>
<th>Water Source</th>
<th>Type of Utility</th>
<th>Population Served</th>
</tr>
</thead>
<tbody>
<tr>
<td>California, New Mexico, Arizona</td>
<td>Well, ground, surface</td>
<td>Private</td>
<td>&gt;250,000</td>
</tr>
<tr>
<td>Southwestern Pennsylvania</td>
<td>Surface, river</td>
<td>Private</td>
<td>&gt;530,000</td>
</tr>
<tr>
<td>Connecticut</td>
<td>Well, ground, surface, mixed</td>
<td>Municipal</td>
<td>400,000</td>
</tr>
<tr>
<td>Northern New Jersey</td>
<td>Well, ground, surface, river</td>
<td>Private</td>
<td>250,000</td>
</tr>
<tr>
<td>Northern Georgia</td>
<td>Surface</td>
<td>Municipal</td>
<td>250,000</td>
</tr>
<tr>
<td>New England</td>
<td>Surface</td>
<td>Municipal</td>
<td>22,000</td>
</tr>
<tr>
<td>New York City</td>
<td>Surface</td>
<td>Municipal</td>
<td>8,000,000</td>
</tr>
<tr>
<td>Southern Ohio</td>
<td>River</td>
<td>Municipal</td>
<td>750,000</td>
</tr>
<tr>
<td>Washington State</td>
<td>Well, ground</td>
<td>State</td>
<td>&gt;1,000,000</td>
</tr>
</tbody>
</table>

24
MPN Versus MPN Comparison

Utilities were selected with the assistance of the American Water Works Association Research Foundation (AWWARF) to test the reproducibility of the protocol established by the USEPA’s Environmental Monitoring and Support Laboratory (EMSL). The protocol is shown in Figure 2.1 (see p. 9). The utilities and participants chosen were:

American Water Works Service Company, Belleville, IL, Mark LeChevallier
Yale University School of Medicine, New Haven, CT, Nancy Kriz and Deborah Callan
California-American Water Company, Monterey, CA, Roger Ward and Dawn Calvert
Cobb County Marietta Water, Ackworth, GA, Wayne Jackson and Michelle Uryc
Monmouth Consolidated Water Company, Tinton Falls, NJ, Carol Storms and Judy Loriner
West-Penn Water Company, Pittsburgh, PA, Thomas Trok and Michael Burns
SCCRWA, New Haven, CT, Thomas Gadish

Standardized forms for data entry were developed and provided to each utility (Figures 3.1, 3.2, 3.3, 3.4). Each participant was required to follow Standard Methods MTF test procedures.

Presence-Absence Comparison

According to the new regulations (Federal Register, vol. 54, June 29, 1989, p. 27544), a P-A concept is defined as any of the following: one tube positive in a 10-tube 10 mL/tube MTF; one confirmed colony from 100 mL of water by MF; or a positive P-A bottle with 100 mL of sample. Because of the interest in both P-A and quantitation from this national evaluation, comparisons were made with Standard Methods MF or MTF versus the Colilert as a 10-tube P-A. In addition, a number of tests were performed using the Colilert as a 100-mL P-A test.
FIGURE 3.1
Data Form: EMSL Grab Sample Assay

AWWARF/YALE COLILERT MPN vs MPN COMPARISON; EMSL GRAB SAMPLE ASSAY

<table>
<thead>
<tr>
<th>Site</th>
<th>Date</th>
<th>Sample Number</th>
<th>Analyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Auto Conf.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 Y F 2 Y 3 Y 4 Y 5 Y 6 Y 7 Y 8 Y 9 Y 10 Y</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 F 2 F 3 F 4 F 5 F 6 F 7 F 8 F 9 F 10 F</td>
</tr>
</tbody>
</table>

Comments

To: Dr. Stephen Edberg, Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut, 06510
### FIGURE 3.2
Data Form: Standard Methods Versus Colilert Results

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Standard Method Results</th>
<th>Autoanalysis Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC/100</td>
<td>Atyp/100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3.3
Data Form: Bacterial Identifications

BACTERIAL IDENTIFICATIONS

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Sample Location</th>
<th>Standard Methods Identification</th>
<th>Autoanalysis Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colony Number</td>
<td>Isolate Name</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

28
FIGURE 3.4
Data Form: General Physical and Chemical Characteristics

GENERAL PHYSICAL AND CHEMICAL CHARACTERISTICS

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Total Chlorine</th>
<th>Free Chlorine</th>
<th>NTU</th>
<th>Temp.</th>
<th>Iron</th>
<th>Mn</th>
<th>Color</th>
<th>pH</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The following sites participated in the P-A versus P-A national study:

California-American Water Company, Monterey, CA
Washington State Laboratory, Seattle, WA
West-Penn Water Company, Pittsburgh, PA
Cincinnati Water Company, Cincinnati, OH
Department of Public Works, North Andover, MA
SCCRWA, New Haven, CT
Bridgeport Hydraulic, Bridgeport, CT
Cobb County Marietta Water, Marietta, GA
Monmouth Consolidated Water Company, Tinton Falls, NJ
New York City Department of Environmental Protection (five separate sites), NY

Data entry forms were distributed to each of the participants (Figures 3.1, 3.2, 3.3, 3.4). Each participant was asked to follow the protocol scrupulously and record all data.

ANALYSIS OF DATA FROM NATIONAL TESTING AND SUBMISSION TO THE USEPA AS AN ALTERNATIVE METHOD

Data analysis was performed by the statistical methods as required by the EMSL, USEPA, Cincinnati, OH. The data and statistical analysis were used as the basis to apply for approval as an alternative test procedure for total coliforms and *E. coli*. The data were submitted to EMSL in November 1987. EMSL recommended approval of Colilert for total coliforms under the existing current coliform regulation in January 1988. Notice appeared in the Federal Register in June 1989 (vol. 54, June 29, 1989, p. 27544). EMSL further recommended approval of the *E. coli* part of the test in March 1988. Notice of approval will appear in 1991.

IDENTIFICATION OF MICROBES ISOLATED FROM WATER SAMPLES

In this study, the specificity of the Colilert system was established by first subculturing all positive tests on agar media. Each isolate was identified to bacterial species (Table 3.2). Bacteria were identified to species to determine if a yellow positive Colilert test did have a species consistent with a total coliform and if a fluorescent positive Colilert test did have an *E. coli*.
<table>
<thead>
<tr>
<th>Species</th>
<th>Percent of all Isolates Belonging to Species by Method</th>
<th>CDC groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard Methods</td>
<td>Colilert</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><em>Enterobacter agglomerans</em></td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td><em>Enterobacter species</em></td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td><em>Serratia plymuthica</em></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td><em>Serratia fonticola</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Serratia rubidaea</em></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td><em>Serratia odorifera</em></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><em>Hafnia alvei</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CDC groups</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Unidentified <em>Enterobacteriaceae</em></td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

*All isolates confirmed in BGLB.

CDC: Centers for Disease Control
CHAPTER 4
QUALITY ASSURANCE

The quality assurance guidelines described in *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association 1985) as well as in the USEPA publication *Microbiological Methods for Monitoring the Environment* (EPA 600/8-78-017) were followed. The research protocols and analysis of data were carried out under exacting requirements established by the Yale University internal audit mechanisms. All sample handling, identification, preservation, transportation, and storage were performed under accepted procedures in the field.
CHAPTER 5
NATIONAL EVALUATION PROTOCOL

RATIONALE FOR THE STUDY

MPN: Current Regulations

The most probable number (MPN) is the means used by the water supply industry for determining the number of viable target microbes in a sample based on a total estimation statistic. By taking the number of tubes positive divided by the number of total tubes inoculated, and factoring the volume of sample in each tube, one can determine a bacterial density with the probability of > 95 percent for a 10-tube test. For water distribution analysis the MPN method is performed as an MTF (multiple tube fermentation) technique. Current regulations require the use of a 5-tube, 10 mL/tube test for each water sample. Five tubes, all negative, equate to < 2.2 coliforms per 100 mL (standard 5-tube test). The current USEPA-recommended test medium is lactose broth with the appropriate inhibitors and requires 48 hours of incubation. A positive presumptive MTF test is represented by the production of gas; positive tubes must be confirmed in brilliant green lactose bile broth (Figure 2.1, p. 9). New USEPA regulations will require the testing of 100-mL samples, which may be accomplished by either 10 10-mL individual tubes or 5 20-mL fermentation tubes.

Presence-Absence: Regulations (Effective December 31, 1990)

It is generally accepted that the enumeration of very small numbers of bacteria in water samples can lead to highly variable results. Bacteria are not always uniformly distributed throughout a sample and may be maldistributed in any particular volume of the sample used for testing. In response to the inherent inability to accurately count low bacterial densities, the USEPA established the P-A concept for compliance monitoring, effective December 31, 1990. Based on the statistical and sampling work of Pipes and Christian (1984) and others, the new regulations will, depending on the population served, generally allow the occurrence of up to 5 percent positive samples from a distribution system
regardless of the total coliform density before a violation occurs. The USEPA will also require that each sample positive for total coliforms be tested for either fecal coliforms or \textit{E. coli}. If positive for either one of these indicators, the appropriate state agency must be notified. The standard volume for P-A analysis will be 100 mL. A utility may use MPN, MF, or P-A methods; all results will be reported on a P-A basis, not numerically. Average coliform densities are no longer used to establish compliance with water quality limits. Therefore, the Colilert method was tested in the P-A form.

MANUFACTURE OF COLILERT FORMULATION

The Colilert system was prepared for use as a 10-tube MPN test. The powder formula contained, per liter, the following: ammonium sulfate, 5 g (grams); manganese sulfate, 50 mg (milligrams); zinc sulfate, 50 mg; magnesium sulfate, 100 mg; sodium chloride, 5 g; calcium chloride, 50 mg; potassium dihydrogen phosphate, 900 mg; disodium hydrogen phosphate, 6.2 g; sodium sulfite, 40 mg; amphotericin B, 1 mg; orthonitrophenyl-\(\beta\)-D-galactopyranoside (ONPG), 500 mg; 4-methylumbelliferyl-\(\beta\)-D-glucuronide (MUG), 75 mg; and solanium, 500 mg. All ingredients were obtained from Sigma Chemical Company (St. Louis, MO), with the exception of solanium (AAS, Branford, CT). Hepes buffer (sodium Hepes and Hepes organic acid to yield a final pH of 7.3 ± 0.1) can be substituted for the potassium dihydrogen phosphate and disodium hydrogen phosphate (Ellgas et al. 1989).

For the P-A form of the test, each vessel received ten times the amount as a single MTF tube in analyzing 100 mL of water sample.

FORMULA AND CONVERSION FROM LIQUID

From its initial development in 1977 through testing at the SCCRWA in 1986, the DST was made as a liquid. The liquid formulation was concentrated so that the addition of a sample resulted in the appropriate overall concentration of the final mixture. The liquid form of the defined substrate technology worked extremely well; however, it was not stable--the ONPG would release its yellow color in the refrigerator after 72 hours. In addition, the MUG began to exhibit false-positive fluorescence in about the same time period. This
limitation in stability was not a problem in the research laboratory but was unsatisfactory for the national testing. Access Analytical Systems, Inc., was contracted to make a dry formulation for this study. The dry, blended powder was dispensed either into test tubes, each tube to receive 10 mL of water sample, or vessels, each vessel to receive a single 100-mL water sample. The tubes were used in the MTF format of the test and the vessels used in the P-A format (See Figure 5.1 for illustration of the test).

The procedure for making the dry powder blend must be performed with exacting care. Much of the ease of inoculation and interpretation of the Colilert test is the direct result of meticulous quality control and preparation of the parent formula. The formula is made as follows.

**Preparation of Colilert**

1. All incoming raw materials must be tested and approved for use (see 5e, below).
2. Area and equipment:
   a. The area must be environmentally controlled: temperature range of 20-22°C, relative humidity less than or equal to 40 percent.
   b. The area must be aseptically cleaned with either a 10 percent V/V (volume per volume) bleach solution or a commercial disinfectant. The area must be tested for microbial contamination for coliforms via swab testing or the equivalent.
   c. All equipment (stainless steel or glass) must also be aseptically cleaned and dried prior to use (see b, above, for procedure). The equipment should be rinsed with acetone to ensure dryness. Swab testing (as indicated above) should be undertaken prior to use.
   d. Use of plastic (or equivalent) gloves is recommended.
3. All raw materials must be reduced to a fine powder (150-200 μm) by grinding.
4. The following raw materials must be dried at 103-105°C for two hours after grinding and then allowed to cool in a dessicator or equivalent area:
   - sodium chloride
   - ammonium sulfate
   - potassium dihydrogen phosphate
FIGURE 5.1
Color Reaction of the Colilert® System.

Left bottle positive for total coliforms and *E. coli* with fluorescent color produced when exposed to UV light (bottle also yellow under white light); center bottle negative for both total coliforms and *E. coli*; right bottle positive for total coliforms with yellow color.
disodium hydrogen phosphate
calcium chloride

5. Raw material specifications:
   a. 4-methylumbelliferyl-β-D-glucuronide·2H₂O:
      • Appearance: white, fine powder.
      • Specific rotation: [α] -104°+1° (C = 0.25 in water, dissolved at 40°C for 10 min.).
      • Water content: 9.1-9.5 percent.
      • Quality assurance assay: Free 4-methylumbelliferone to be equivalent to 5, 10, 15 mg/L standards. Weigh a sample of MUG to be equivalent to 75 mg/L. All are dissolved in pH 7.8 buffer. Compare the MUG sample to the prepared standards using a long-wavelength UV light.
   b. Orthonitrophenyl-β-D-galactopyranoside:
      • Appearance: white to off-white, crystalline powder.
      • Specific rotation: [α] -67.3°+1° (C = 1 in water).
      • Quality assurance assay: Free Orthonitrophenol: 0.03 percent max. Dissolve 500 mg/L of ONPG in a pH 7.8 buffer and mix. Compare against Orthonitrophenol standards of 0.01, 0.03, and 0.05 percent at 545 nm (nanometers) with water as the blank.
   c. Amphotericin B:
      • Appearance: orange to brown, fine powder.
      • Solubility: clear, dark yellow solution at a concentration of 20 mg/mL.
   d. Solanium:
      • Appearance: white to off-white, fine powder.
      • Sterility: free of coliforms.
   e. American Chemical Society (ACS) grade chemicals:
      The following chemicals are ACS grade and are to be tested for compliance against these specifications (refer to the ACS book of specifications and test procedures): sodium chloride, ammonium sulfate, disodium hydrogen phosphate, potassium dihydrogen phosphate, magnesium sulfate·7H₂O, calcium chloride·2H₂O, zinc sulfate·2H₂O, manganese sulfate·2H₂O.
Combine all ingredients, mixing thoroughly. All chemicals should be tested for the absence of coliforms. Ensure that your sampling plan for testing is adequate. 

**NOTE:** READ WARNING LABELS ON ALL CHEMICALS BEFORE USING.

6. Weigh the quantities required and place into an aseptically clean glass or plastic container. After all ingredients have been added, mix well for 20 minutes to ensure homogeneity for batch sizes up to 500 g. Weigh 260 mg ± 25 mg per tube for MPN or 2.60 g ± 250 mg for P-A. Weighing must be done aseptically, and a sterile spatula must be used to remove the reagent powder from the container. The spatula should not be used more than twice (to prevent possible contamination). In addition, do not leave cover off powder for any prolonged period. For MPN, 10 mL of sample is added to the tube, and the tube is capped and mixed prior to incubation. For P-A, the powder is added to the vessel containing the 100-mL sample and mixed to ensure that the ingredients are dissolved. Hepes buffer has been found to be an effective substitute for the phosphate buffers (Ellgas et al. 1989). Hepes buffering will permit the formula to be used in both fresh and marine water (data on file). Note: A small quantity may remain undissolved. This will not have any effect on the test.

7. Quality control of the Colilert preparation:
   a. Appearance: White to off-white, fine, granulated powder (free flowing).
   b. pH: 7.2-7.4 at 25°C. Use a two-point calibration of pH buffer 7 and 8. Weigh 2.60 g of Colilert and dissolve in 100 mL of distilled water. Mix well and measure pH at 25°C.
   c. Moisture: < 1 percent loss on drying at 105°C. Approximately 750 mg sample can be used.
   d. Sterility: Weigh 2.60 g ± 250 mg and place into a sterile container. Add 100 mL of sterile distilled water and incubate up to 48 hours. No yellow color or fluorescence should develop. If the results are positive for yellow color or fluorescence, repeat.
   e. Performance: Using a panel of coliforms and noncoliform heterotrophs, prepare low-level concentrations of coliforms (4 and 8/100 mL in a heterotroph population of 10,000 to 15,000/mL). Verify levels by plating.

   The panel of coliforms are *E. coli*, *K. pneumonias*, *E. cloacae*, and *C. freundii*. 

40
The heterotrophs to be used are equal numbers of *Pseudomonas aeruginosa* and *Flavobacterium* spp. at concentrations of $10^4$ mL, and individually at the same concentration.

NOTE: THIS TESTING MUST BE PERFORMED ON EVERY BATCH MADE.

CONDUCT OF THE STUDY

**MPN Protocol**

The MTF protocol followed the data requirements provided by the USEPA’s EMSL for establishing equivalency between methods. The protocol used in the national evaluation was designed in conjunction with EMSL and other USEPA staff in Cincinnati (see Appendices A, B, and C). The protocol used was that described for nationwide use applications. This protocol requires that at least five geographically diverse surface and ground water supply sources be chosen. From each source, a minimum of six samples must be analyzed, representing the expected concentration range of the contaminant to be analyzed. From each sample eight replicates must be analyzed; four by the proposed alternative test (i.e., Colilert) and four by the approved test (i.e., MTF). Therefore, a minimum of 240 analyses are required. The minimum requirements described by EMSL were far exceeded in this study. All Colilert materials were supplied to all the utility participants.

The MPN Colilert test was performed as follows: Water samples were aseptically collected as described in *Standard Methods for the Examination of Water and Waste Water* (American Public Health Association 1985). All participants were advised to avoid touching or otherwise contaminating the reagent or the inside of the reaction tubes prior to the aseptic inoculation with the water sample, to adhere to good laboratory practice throughout the test procedure, and not to pipette by mouth. The 10-tube holder was labeled and each test tube was aseptically filled with water to contain 10 mL of sample. The caps were replaced and tightened and the tubes mixed vigorously to dissolve the reagents. Occasionally, high calcium salt concentrations caused a small precipitate but did not affect test results. The test tubes were incubated at 35°C ± 0.5°C for 24 hours. It was
recommended that incubation should begin within 30 minutes of initiation. Each participant was advised that incubation exceeding 28 hours should be avoided. The tubes were read at 24 hours. If a yellow color was seen, the tubes were exposed to a 366-nm, UV 4-watt lamp and observed for fluorescence.

Each tube was compared against the supplied color comparitor. If the incubated tube had a yellow color greater or equal to the comparitor, the presence of total coliforms was confirmed. Whether yellow or not, each tube was observed for fluorescence. If fluorescence was greater or equal to the comparitor, the presence of \textit{E. coli} was specifically confirmed. Samples were negative if no color was observed in 24 hours. Should a sample be so lightly yellow after 24 hours of incubation that the observer could not definitively read it relative to the positive comparitor, it was incubated for an additional four hours and read again. This same procedure applied to the observation of fluorescence. If the sample was coliform positive, the color would intensify. If the sample contained \textit{E. coli}, fluorescence would also intensify. If color did not intensify, the sample was considered negative for each analyte.

Some water samples containing humic material may have an innate color. If a water sample had a background color, it was compared against an inoculated Colilert tube and to a control blank of the same water sample.

Quality control procedures were as follows: Routine quality control was conducted on every Colilert run. The procedure was to reconstitute each of three Colilert tubes with 10 mL of sterile, distilled water. The powder was thoroughly mixed. The tubes were labeled "\textit{Escherichia coli}," "\textit{Klebsiella pneumoniae}," and "\textit{Pseudomonas aeruginosa}." A sterile inoculating loop, or needle, was touched to an 18-hour pure culture slant of each of these organisms and transferred to each of the appropriately labeled Colilert tubes. The tubes were incubated at 35°C ± 0.5°C for 24 hours. After the incubation period the \textit{E. coli} tube should be yellow and fluorescent, the \textit{Klebsiella pneumoniae} tube yellow only, and the \textit{Pseudomonas aeruginosa} tube neither yellow nor fluorescent.

**Presence-Absence Protocol**

The P-A test did not lend itself as readily to the USEPA EMSL protocol as did the MPN procedure. Therefore, a decision was made to analyze a large number of samples
from the various geographical locations; having a large P-A database would allow the USEPA to render a judgment based on chi-squared analysis concerning the equivalency of the Colilert P-A test with the *Standard Methods* P-A test. According to the new federal regulations, a P-A test may be conducted in any of three ways: (1) 100 ml in a single vessel, the vessel showing positivity indicating a positive P-A test; (2) a 10-tube MTF test with any one positive tube being a positive P-A test; and (3) a membrane filter test using a 100-mL sample with any total coliform colony indicating a positive P-A test.

The preparation, storage, and transportation of the water sample were exactly the same as described for the MTF MPN versus MPN Colilert procedure. A tube of Colilert formulation was opened and added to a 100-mL sterile, nonfluorescent borosilicate water sample bottle with an aseptic cap and seal. A 100-mL sample of water was added to the reagent in the vessel and shaken vigorously to dissolve the reagent. The reagent sample mixtures were incubated at 35°C ± 0.5°C for 24 hours. The reading and interpretation of the results followed exactly those described for the MPN procedure. Quality control procedures were also identical.

**Field Quality Assurance**

The principal investigator visited each water utility laboratory to ensure that the personnel were familiar with the requirements of the protocol and the performance of both the Colilert test and the *Standard Methods* procedures. Normally, a visit to a laboratory required a minimum of two days. On the first day the Colilert procedure was explained and a water test initiated. On the second day the water samples inoculated the previous day were interpreted and the results recorded. Also, the means to identify bacteria to species were reviewed. Some laboratories had the ability to identify bacterial isolates to species based on the API 20E® system, and others wished to transfer an aliquot of a positive Colilert tube to the surface of a trypticase soy agar slant for identification at the central reference laboratory at Yale University.
DATA COLLECTION

All data from each site were entered on the uniform data forms provided (Figures 3.1, 3.2, 3.3, 3.4, p. 26-29). The first page of the form contained the general physical and chemical characteristics of the water, including the sample number of the specimen, the total and free chlorine, turbidity, temperature, iron and manganese concentrations, color, and pH. The other pages of the form included comparison of *Standard Methods* results indicating total coliforms per 100 mL, atypical coliforms per 100 mL (if a membrane filter test was performed), and HPC (R2A medium at 35°C for 2 days) per 1 mL. In addition, the number of MPN Colilert tubes that were yellow and fluorescent was noted, and the same was done for the companion P-A test. The last page was for bacterial identifications and included the sample number, sample location, and designation from *Standard Methods* and the species name for isolates from the Colilert test.
CHAPTER 6
RESULTS OF THE NATIONAL EVALUATION

MPN RESULTS

A total of 1,086 tubes were positive by the *Standard Methods* MTF procedure, and 1,279 were positive by the Colilert test. The median number of tubes positive per 40 inoculated from an MTF sample was 24, the mean was 23.6, and the standard error was 2.0. For the Colilert test the median was 24.5, the mean 24.8, and the standard error 2.1. The results are presented in detail in Appendix C.

Statistical Analyses

The statistical analyses of the comparison of the Colilert and *Standard Methods* tests were conducted several ways. First, a comparison based on regression analysis was conducted. As Figure 2 of Appendix C shows, the *Standard Methods* MPN compared to the Colilert MPN with an $r^2$ of 0.779. Individual multiple and single regression analyses did not show significant differences between the Colilert test and *Standard Methods*. The USEPA requires that t-tests be performed on all proposed alternative test procedures. (The t-test is more suitable to chemical than biological data because the biological data are not numerical.) Accordingly, both a single- and a double-tailed t-test were done. As shown in Appendix C, there was no statistical difference between the two methods utilizing this means of analysis.

By chi-squared analysis that considers the nonparametric, noncategorical nature of the data, the two methods were also statistically indistinguishable, i.e., equivalent. First, the Pearson chi-square tests for the overall proportion of positive tubes were determined. Second, the Mantel-Haenzel test was used to compare the two methods by determining the difference between the positive tubes for each method, subtracting it from the expected number of positive tubes, and analyzing the differences. By this analysis, data from three of the utilities demonstrated that the Colilert test was more sensitive than the *Standard Methods* MPN ($p < 0.05$). There was a positive bias with slightly greater Colilert sensitivity
overall (Appendix C). This conclusion substantiates the observation in which the Colilert test found the same numbers of positive tubes as did the MTF 19 times, the Colilert was less sensitive than Standard Methods 6 times, and the Colilert was more sensitive than Standard Methods 21 times.

Another commonly used measure is the F test, a means of analyzing precision. The precision analysis revealed that the Colilert test data were significantly more precise than the MTF data from two of the five sites. The last statistical analysis used was the likelihood ratio test. This statistical analysis compares the mean bacterial density obtained from different sets of data. According to this method there was a slight bias in favor of the Colilert test at the significance level of 0.5 percent. These statistics show that the Colilert and MPN analyses produced equivalent results. Therefore, Colilert will provide the same database as will Standard Methods and can be used for compliance purposes.

Effect of HPC Bacteria on MPN Analysis

The effect of HPC bacteria on the ability of the Colilert test to produce a true-positive or true-negative result was analyzed in this national study. It has been established that HPC bacteria can affect MTF and MF test results in two ways. First, HPC populations may compete with the target total coliforms and inhibit the coliforms from growing. Second, the HPC may result in false-positive tests because they alone or synergistically may ferment lactose with the production of gas. The nature of the Colilert test should preclude either of these events. All HPC tests were divided into numerical categorical data and cross-referenced with the results of coliform testing. The HPC/mL results were divided into the following numerical categorical levels: 0-100, 101-500, 501-2,000, 2,001-5,000, 5,001-10,000, and 10,000+. Using the dBase III Plus program (Ashton-Tate, Inc.), the HPC levels were cross-referenced with the number of Standard Methods positive tubes and the number of Colilert positive tubes. It was demonstrated that the Colilert test was unaffected by any level of HPC bacteria present. It should be noted that over half of the samples tested contained more than 5,000 HPC/mL.
Specificity of the Colilert System

The establishment of the specificity of the Colilert test was extremely important; i.e., when a Colilert tube was yellow, a total coliform was present, and when fluorescent, an *E. coli* was present. As shown in Appendix B, in each case in which the Colilert was positive and *Standard Methods* negative in a P-A test, a member of the total coliform group was present. In this same analysis it was also shown that in *Standard Methods* negative and Colilert positive samples, there was no effect of HPC bacteria on the Colilert test.

Although it was not part of the protocol, researchers at one site (Monmouth) transferred positive Colilert tubes to BGLB to determine what percentage of the Colilert positive tubes confirmed. They found that approximately 90 percent of all yellow total coliform Colilert tubes produced gas in BGLB, showing a close correspondence between total coliform endpoints.

USEPA RECOMMENDATION FOR APPROVAL

Based on the results of the national evaluation, the Colilert test was recommended for approval as an alternative test procedure by the USEPA (see Appendix A). The approval memorandum stated, "We are pleased to recommend approval of the Autoanalysis Colilert test as an alternative to the currently approved MPN procedure. We also feel that the Autoanalysis Colilert test should be included in future regulations that specify approved methods for determining the presence/absence of coliform organisms in drinking water" (Appendix A, p. 86). The recommendation also stated that the Colilert MPN tests showed slightly higher indices of positive results than did *Standard Methods* and that the Colilert was not influenced by HPC bacteria. According to the memorandum, the Colilert test "provides results comparable to those obtained by the currently approved MPN method" (Appendix A, p. 86).

On June 29, 1989, the notice of approval of the Colilert system for the analysis of total coliforms from drinking water was published in the Federal Register. Approval of Colilert for the analysis of *E. coli* is in process.
CONCLUSION

The Colilert test was used to examine water samples from a wide range of geographical areas and a diverse number of water sources. It was found to yield results equivalent to *Standard Methods* in both the MPN and P-A forms. It has been recommended by the USEPA's EMSL to the Office of Drinking Water for approval as an alternative test procedure. Water utilities can use the Colilert test for compliance purposes and expect data equivalent to those of *Standard Methods* procedures. This conclusion is important for utilities that wish to use Colilert without compromising the usefulness of their historical database for monitoring seasonal finished water quality changes.

The comparison of the Colilert P-A test system with the *Standard Methods* P-A test was presented in *Applied and Environmental Microbiology* (see Appendix B). In summary, both methods agreed 94 percent of the time. There were no significant statistical differences, and, again, the specificity of the Colilert system was established by the subculture of positive tubes and finding the correct bacterial species.
CHAPTER 7
DISCUSSION AND IMPACT FOR THE UTILITY INDUSTRY

SELECTIVITY PRINCIPLES

The principle of the Colilert test is significantly different from culture media used in water analysis in the past. Conventional media utilize a broad-spectrum protein and carbohydrate sources that will support the growth of many microorganisms, including the target microorganisms. In all Standard Methods media, the bacteria have available to them the disaccharide lactose, which is not truly selective. Therefore, a wide array of microorganisms is capable of growth in MTF media and on m-Endo and other selective agars. These agars and broths are made more selective by including broad-spectrum inhibitors to certain types of microbes. For example, lactose-based MTF media contain the detergent lauryl sulfate, which inhibits many noncoliform gram-negative bacteria, and bile salts, which inhibit many gram-positives. However, these inhibitors are not uniformly selective, and some individual bacterial species may grow. Together these inhibitors favor but do not guarantee the growth of the target organisms, i.e., total coliforms. The lack of broad-spectrum inhibition is responsible for the growth of noncoliform heterotrophic bacteria and the suppression of coliform bacteria (the false-negatives that may occur). In addition, the growth of many disparate species of noncoliform heterotrophs may yield a false-positive test by the production of acid and aldehyde in the MF test (e.g., sheen colony) or acid and gas in the MTF tube.

The Colilert test utilizes a different principle. The target microorganisms are provided specific nutrients, whereas the noncoliform heterotrophs cannot utilize significant food sources that promote growth during the prescribed incubation period. Therefore, the noncoliform heterotrophic bacteria do not grow.

The indicator systems in the MTF, MF, and Colilert tests are significantly different as well. In conventional media, several secondary metabolic catabolisms must occur before a visible change is noted in the indicator. First, the target microbe must transport the substrate (e.g., lactose) through the cell membrane utilizing a specific permease system. Inside the cell, the substrate must be converted to glucose (e.g., β-D-galactosidase system),
and then the glucose must be metabolized. In most enteric bacteria, glucose is metabolized through the glycolytic cycle to pyruvate and the pyruvate is converted to an end product. For the conversion of pyruvate, enteric bacteria have several choices. Some members of the total coliform group (*Klebsiella, Enterobacter-Serratia*) utilize the butylene glycol pathway whereas others (*Escherichia-Citrobacter*) utilize mixed-acid fermentation. Generally the end product of fermentation by either pathway is acid or gas or both, the presumptive reaction in the MTF method. Because analytical methods that rely on lactose as their primary energy source require the target microbe to perform a long, complex series of biochemical steps until a measurable product such as acid or gas is produced in sufficient quantity, the analyst often observes false-negative gas producers (i.e., anaerogenic *E. coli*) or false-positive tests (i.e., acid and gas from lactose by some isolates of *Aeromonas hydrophilia*). Further confounding the conventional lactose-based tests are the many different biochemical pathways that produce gas synergistically in some mixed cultures in primary MTF tubes. Subculturing of these "gas positive" tubes to more selective broths (such as brilliant green bile) often does not "confirm". This lack of confirmation means that the individual species did not produce gas in a lactose-containing medium.

The Colilert test employs a totally different indicator mechanism. A specific hydrolyzable substrate is metabolized only by the target microbe. This one-step metabolism of the hydrolyzable substrate replaces the multistep metabolic pathway required in lactose-based *Standard Methods* media to achieve an observable endpoint, i.e., gas or acid. Because a definitive signal, or color, is released each time an indicator-nutrient molecule is hydrolyzed, color production is directly related to the growth of the target bacteria. In the application of this technology to drinking water analysis, the indicator-nutrients are ONPG for total coliforms and MUG for *E. coli*. Unlike traditional enzyme assays, which use ONPG or MUG only as substrates for constitutive enzyme tests that use cultured or purified isolates, the DST uses these compounds both as a food source and as an indicator system.

With conventional enzyme assay testing, an inoculum of several million bacteria per mL is required and physical rupture of the cells is needed to release the enzymes. This massive amount of released enzyme then hydrolyzes the specific substrate to produce the color change. In the DST, the substrate is utilized by the target organisms as a growth material. As few as one bacterium in a 100 mL water sample grows, and each time it uses
the substrate as a food source, a chromagenic molecule (chromophore) is released. At the end of an incubation period, several million organisms may be present, but they have by then already expended or largely used the substrate to produce the color reaction. Thus, in the DST the color is produced as the organism grows; whereas in the constitutive enzyme testing, the color is produced from a large mass of dead bacteria. It should be cautioned that there is not a relationship between tests in which ONPG or MUG are used as substrates in constitutive tests (e.g., Coliferm® [Millipore Corp., Waltham, MA] [Pickett et al. 1981]) and the growth-dependent Colilert test. For example, species in the genera Aeromonas, Pseudomonas, or Flavobacterium that may be positive in a constitutive ONPG or MUG enzyme assay will not be positive in the 24-hour growth-based Colilert test unless an extremely high density (> 20,000 bacteria/mL or 2,000,000 bacteria/100 mL) is present.

The inherent differences between the Colilert test and broad-spectrum, protein-sugar growth media are also responsible for the different results observed during comparison to other methods. For example, during the investigative phase of this research, lactose broth containing MUG was compared to the Colilert formula containing MUG. It was found that with natural samples, significant suppression of *E. coli* occurred; whereas in the Colilert formulation it did not occur, and the *E. coli* were allowed to grow and manifest themselves. Therefore, it is strongly recommended that lactose-based media containing MUG not be used for the direct enumeration of *E. coli* from distribution, treatment effluent, or raw water samples.

SENSITIVITY

The Colilert test was designed to detect 1 total coliform/100 mL and 1 *E. coli*/100 mL. This sensitivity was demonstrated both in the developmental laboratory studies and in the subsequent field test, where correspondence with *Standard Methods* procedures was investigated. Sensitivity did not appear to be reduced during the enumeration of injured coliforms. Previous laboratory analyses have shown that target coliforms including *E. coli*, *K. pneumoniae*, *E. cloacae*, and *C. freundii* are recoverable within the time frame of the test when injured by exposure to a dilute bleach (hypochloride) solution. There did appear to be a longer lag or accommodation phase for injured coliforms in the Colilert test, which
resulted in the production of weaker yellow color or fluorescence at the 24-hour period. Because these organisms demonstrated a weaker color it was recommended that all questionable yellow or fluorescent samples be reincubated for an additional four hours. If a total coliform is present, the organism will be in log phase growth and should manifest itself by the rapid intensification of the yellow color during the additional 4-hour incubation (i.e., from 24 hours to 28 hours). If the organism is an *E. coli*, the fluorescence will rapidly intensify. If the lack of a definitive color change was due to background yellow caused by humic or other materials, the yellow color will not increase in intensity. If the fluorescence was due to high turbidity in the water, the fluorescence will not increase in intensity. In waters with naturally high background yellow or fluorescence, it may be necessary to make a negative control by adding a small amount of household bleach to the water sample and using this as baseline negative yellow or fluorescence. In the vast majority of drinking waters, the background color should not present a problem.

**SPECIFICITY**

The specificity of the Colilert test in drinking water analysis was confirmed by the subculture protocol used by all the collaborating utilities. From each test yielding a yellow or fluorescent Colilert tube, a subculture was made to identify the bacteria to species. Each time a Colilert tube was yellow, a member of the total coliform group was recovered and identified. Therefore, yellow tubes were the result only of the growth of the target bacteria. Similarly, each time a tube was fluorescent, an *E. coli* was recovered. The specificity of the DST eliminates the need to perform confirmatory and completed tests.

In comparison, conventional methods require the transfer from either a positive MTF broth or sheen colony to a confirmatory medium such as BGLB. The transfer to a confirmatory medium requires an additional 24 to 48 hours of incubation in order to achieve a definitive result. To perform the completed test, a second transfer from BGLB must be made to a solid media such as m-Endo and a typical coliform isolated. The Colilert test is the first method in which a confirmed or completed step is not required. The specificity of the Colilert test is based on the proven fact that it does not support the growth of noncoliform HPC bacteria. During the course of the national evaluation, HPC counts as
high as 700,000/mL were noted (Appendix B, p. 99). Even so, there were no cases in which HPC interference caused a false-negative test or in which HPC digestion of the substrate caused a false-positive test.

OPTIMUM INCUBATION CONDITIONS

Because there may be many mixed HPC bacteria present in water samples, it is strongly recommended that incubation of the Colilert test sample not exceed 28 hours of incubation. The salts in the Colilert test are meant to provide optimum results during this period, and after prolonged incubation the substrate may induce the enzyme systems of noncoliform organisms to yield a false-positive test. Although this probability is not high, the maximum time frames should be strictly observed. Prolonged incubation will not increase test sensitivity and may reduce specificity. As in all biological systems, the longer a test is incubated, the more likely it is to be positive. For example, during the course of this study, incubating MTF tubes an additional 24 hours (from 48 to 72) yielded approximately a 15 percent increase in positivity. In order to correctly and reliably interpret any standardized bacteriological test, one must adhere to the defined incubation period. For example, the definition of a total coliform by the MTF method has a specified incubation period of 48 hours, and the definition of the total coliform bacterial group by the MF method has a specified time limit of 24 hours. Increased incubation of Colilert samples may result in small numbers of injured coliforms recovering at the extended incubation period.

OTHER COLILERT ATTRIBUTES

The utility participants in the national study felt the Colilert method offered several benefits to the utility laboratory. It was important that a test be developed that would not only be accurate but also "user friendly" and that would be used because of its many advantages. The participants felt that the autoanalytical nature of the method made it more practical and efficient to perform than current total coliform methods. They indicated that the interpretation of the Colilert was easier and less subjective than Standard Methods procedures. The yellow color produced by total coliforms varies from light yellow to intense
gold but is generally easily discriminated by eye. Most of the participants felt it was easier to determine whether a yellow color or fluorescence was produced than to decide whether an LTB tube was positive. By comparison, the determination of gas (tiny bubble) in a Durham tube is often subjective and is not markedly enhanced in an additional 2-4 hours as is the case with Colilert. Interpretation of color in Colilert is also not as variable as for the sheen colonies produced on m-Endo, which must be read within a particular period of time. The fact that the yellow color does not fade over time as can happen with colony sheen was also viewed as an advantage by the participants. A utility would be able to use a less-experienced individual in place of the routine microbiology technologist in times of unanticipated absence or vacation without compromising analytical credibility. Also, because no additional tests are required, an analysis does not have to extend through weekends. In addition, in contrast to the other standard methods in which weekends added two or more days to the analysis, the Colilert method would allow an individual to test water on Thursday and Friday and have someone briefly stop in on the following days to read the results, thus expanding the monitoring capabilities of a utility. The new total coliform rule will require utilities to confirm and resample within 24 hours of a positive TC, thereby ensuring weekend work. Virtually any water utility employee could accurately read and record the Colilert test results. Several of the utilities provided operators at treatment plants with training in the use of the Colilert method to monitor treated water quality on a daily basis.

A major benefit perceived by the participants is inherent in the physical configuration of the test itself. All ingredients are in powder form and are stable at room temperature for up to one year. This physical configuration is useful for several reasons. First, utilities can either make or purchase large volumes with greater economies of scale. They do not have to be as production-oriented as they are with current media, which have maximum refrigerated shelf lives of two weeks. Commercially produced Colilert should be stored in a room-temperature environment with stable humidity. The ability to stockpile Colilert for long periods is very desirable for emergencies such as coliform outbreaks or water main breaks or for public health threats such as cross-connections. A number of utilities suggested that it would be possible for organizations such as the American Red Cross to
stockpile test kits for use in natural disasters. The fact that the Colilert test is packaged in powder form makes it easily transportable into relatively inaccessible areas.

APPLICATIONS FOR SMALLER UTILITIES

A particular strength of the Colilert test noted by all participants was its use for the smaller utility that cannot use MTF or MF methods. The characteristic nature of the yellow and fluorescent endpoints makes it possible for the small utility operator to determine the presence of both total coliforms and *E. coli* from a water sample. This immediate availability of results eliminates the delay inherent in sending samples to commercial or reference laboratories. This capability would not eliminate the need for water testing by a certified laboratory for reporting purposes, but it would allow the small utility to conduct additional monitoring of water quality. A particular strength for the smaller utility that also offers advantages to the large utility is the fact that the Colilert test can be inoculated directly in the field by untrained personnel. This fact is especially important in the use of this test as a P-A configuration. Once the formula is dissolved in the water sample, the bacteria begin to grow. Although they multiply more slowly at ambient than at the recommended temperatures of 35°C ± 0.5°C, the process has begun nevertheless. Moreover, field inoculation precludes any change in the bacterial composition of the water sample during transportation and storage. It is well known that prolonged sample transit times can result in questionable or unacceptable results. Under the new regulation, the sample transit time is limited to 24 hours.

Double-strength lactose broth can also be inoculated in the field; however, the determination of a positive result from this medium is much more subjective than is the reading of a Colilert tube. Also, incubation at 35°C ± 0.5°C must occur with a double-strength lactose broth in order to achieve correct reaction conditions. Furthermore, once color forms in the Colilert tube, it is permanent; extended incubation of the MTF test can result in changes in medium that make it difficult to interpret.

Smaller utilities could expect to see their costs of total coliform analyses decrease significantly if they perform their own water tests. It has been calculated that a total coliform analysis by a commercial laboratory costs at least $18.00, which does not include
the cost of mailing. The Colilert test costs approximately $6.50 for the small utility, with no volume order discount. If the state certifying agency wishes to verify the results of the Colilert test, it could require the small utility to mail the incubated tube after it is recorded.

In summary, the participants in the study of the Colilert test perceived extremely positive benefits concerning the test's handling and use characteristics. Participants thought that as a P-A test it was virtually ideal; one need only add 100 mL of water to a vessel, mix the powder, and incubate. The handling and stability characteristics of the procedure were considered tailor-made for utility use, which is not surprising--the final configuration was developed with the utilities in mind.

**RELATIVE COSTS**

The cost of the Colilert test has been estimated to be approximately one-third less than that of *Standard Methods* procedures (Federal Register, vol. 53, no. 88, May 6, 1988, p. 16348). The cost of the ingredients is more than that of *Standard Methods*, but significant savings occur in labor and indirect costs such as autoclave time, preparation, storage, wastage, and equipment (autoclaves, glassware, pH meter, refrigerators, etc.). The production of the Colilert test entails scrupulous care. Its ease of use depends on extreme care and quality control in the production of the formula. Utilities that normally send their samples to a commercial laboratory will benefit markedly by doing their own testing.

**PRECAUTIONS**

The Colilert test has specific limitations. Although the Colilert test was not affected by the density of heterotrophic bacteria encountered during the national evaluation, there was one isolate of *Aeromonas hydrophilia* that demonstrated a positive yellow color endpoint after 32 hours in the laboratory. This period of incubation is beyond the 28 hour time frame noted for the Colilert test. The concentration of this *Aeromonas* was on the order of 20,000 *Aeromonas/mL*, considerably higher than one would expect in water. Even so, a note has been added to the instruction sheet accompanying the commercially produced Colilert test explaining that utilities with *Aeromonas* concentrations > 20,000/mL should interpret a
positive total coliform analysis with caution. If source water contains very high numbers of aeromonads and there are questions about the true nature of a positive, a subculture of the Colilert test and identification to species will confirm the result.

There were no instances of false-positive fluorescent tests. Isolates of *Pseudomonas* and *Flavobacterium* were tested to concentrations of 50,000 bacteria/mL. Although it has been reported that some species in the genus *Flavobacterium* may contain β-D-glucuronidase, none were encountered in the laboratory examination. Therefore, theoretically one might expect a false-positive fluorescent test only if there were sufficient inherent bacteria present in a sample (> 100,000-1,000,000/mL) to cause a direct enzyme reaction on the substrate.

The Colilert test should not be used as a confirmatory test for total coliforms because high *Aeromonas* concentrations may yield false-positive yellow colors. *Aeromonas* will not produce a false-positive fluorescent test.

**Procedural Cautions**

The following procedures will result in erroneous results and should never be performed.

1. Never autoclave the Colilert system prior to use. This process will destroy the reagent system, which is heat labile.
2. Avoid prolonged exposure of the inoculated Colilert system to direct sunlight. The indicator compounds may be hydrolyzed, creating a false-positive (particularly yellow) result.
3. The inoculated Colilert system should be incubated 24-28 hours at 35°C ± 0.5°C. Avoid incubation at this temperature beyond 30 hours because HPC present may overcome the suppressant systems after this time, yielding a false-positive result. Yellow color with or without fluorescence after the 30-hour incubation period should be confirmed or the sample repeated.
4. Colilert system performance characteristics do not apply to samples altered by any form of pretreatment. Pretreatment includes any method such as growth on a
membrane filter or growth in lactose-based broth in which there is a nonspecific growth enhancing step, or any prefiltration method such as filtering the sample through a membrane filter and then inoculating in the Colilert system.

a. Do not transfer colonies or cultures pregrown in any enrichment media to the Colilert system. Colonies grown in such nonspecific media may or may not be coliforms. The Colilert's suppressant reagent system may be overloaded by transferring such heavy inocula of certain very weak β-D-galactosidase-containing noncoliforms (e.g., some *Aeromonas* and *Pseudomonas*), causing a false-positive total coliform (yellow) result. Similarly, transfer of high numbers of other heterotrophs (for example, *Flavobacterium*) can cause a false-positive β-D-glucuronidase fluorescence and an inaccurate indication that *E. coli* was present (Covert et al. 1989). Although one would not normally expect to encounter such high levels of heterotrophs in a water sample, pre-enrichment could produce them.

b. Do not prefilter a sample and then place that filter in the Colilert system. The filtration step can concentrate not only coliforms but also noncoliform heterotrophs, particulates, and certain chemicals (divalent cations, heavy metals, etc.) that can overlay and suppress coliforms, adversely affecting the sensitivity of the test. Furthermore, coliform bacteria can become trapped in the filter, restricting their access to the indicator-nutrients in the Colilert reagent system and their consequent growth and detection.

5. Do not dilute samples in buffered water for addition to the Colilert system. The Colilert is already buffered, and additional buffered compounds can adversely affect the growth of the target microbes and test performance.

6. Do not store the Colilert in the refrigerator prior to subculture. Subculture from the Colilert system for either identification of colonies from agar or growth in a broth should occur the same working day on which the Colilert is positive. After 24 hours in storage, the number of coliforms or *E. coli* will decrease because the reagent system will no longer be conducive to their survival.
INJURED COLIFORMS

Laboratory testing has shown that the Colilert procedure will detect injured coliforms with at least the same frequency as *Standard Methods* tests. Injured coliforms exhibited a longer lag time than noninjured coliforms and could yield less intense colored endpoints. It has been shown in the laboratory that once the injured coliform repairs itself it grows as well as noninjured coliforms. Practically, this means that the intensity of yellow color produced at 24 hours by injured coliforms may be less than that produced by normal coliforms. Note that any color (yellow or fluorescence) produced in the Colilert greater than the comparator is positive. Therefore, it is recommended that if no color is noted at 24 hours, the results should be considered negative; if any yellow color is noted, the results should be considered positive. If the analyst is unsure of color formation, he or she should reincubate the test for an additional 2-4 hours and examine it for an increase in yellow color. This additional incubation period will account for the slightly yellow color that some samples may have due to humic material, because tubes will not increase their color intensity and be classified as negative. The Colilert test should not be considered a medium such as mT-7, which has been specifically designed to enumerate injured coliforms.

**E. COLI AND FECAL COLIFORM RELATIONSHIPS**

A significant difference between the Colilert and *Standard Methods* procedures is that the Colilert test determines the presence or absence of *E. coli* but not of fecal coliforms. The 44.5°C fecal coliform test determines heat-tolerant members of the total coliform group, which is primarily comprised of *E. coli* but which may also include thermo-tolerant *Klebsiella* and perhaps other genera. It has generally been found that *E. coli* constitutes approximately 85 percent of fecal coliforms, but this number can vary significantly. *Klebsiella pneumoniae* may or may not be of fecal origin. It is a species that is widespread, associated with vegetation, and capable of multiplying in the environment and in water distribution systems. Therefore, if *E. coli* is found by the Colilert test, the sample can be considered fecal coliform positive; however, if *E. coli* is not detected, there may still be bacterial isolates that are considered by *Standard Methods* to be fecal coliforms.
REGULATORY IMPACT

An extremely important feature of the Colilert test is the simultaneous detection of total coliforms and *E. coli*. The ability to determine the presence of *E. coli* directly from a water sample makes it more responsive to direct public health needs than any of the other methods. It is generally recognized that *E. coli* is the best single specific indicator of fecal pollution of water. The new drinking water regulations, effective December 31, 1990, will require that utilities test each positive TC sample for the presence of either fecal coliforms or *E. coli* regardless of whether it is an original test sample or a repeat. In the past, *E. coli* (indole positive, methyl red positive, Voges-Proskauer negative, citrate negative) was used as a primary sanitary indicator for water. Because of the technical difficulty in determining the presence of *E. coli*, the ability of an organism, i.e., the fecal coliform, to grow at 44.5°C was used as a surrogate indicator. Total coliforms are a secondary indicator of *E. coli*, comprising the entire group of lactose-positive *Enterobacteriaceae*. Public health authorities generally agree that the absence of *E. coli*, even when distribution water contains total coliforms originating from biofilms established on the interior surface of water mains, indicates that the finished water is acceptable for consumption. Therefore, the ability to assay simultaneously for both total coliforms and *E. coli* is an added feature for water analysis that was not previously available. It will provide utilities with an immediate measure of whether or not a water sample has been subject to fecal contamination. The utility will not have to perform fecal coliform analyses or bacterial identifications on subcultures of the primary test, as they do now. This additional confirmatory testing can delay a definitive answer for several days, an undesirable situation engendered by currently available bacteriological methods. Therefore, the Colilert test is compatible with both the current and pending regulations on several counts.

In many situations, additional water samples have to be analyzed to ascertain whether *E. coli* was present in the initial positive sample. With the Colilert method, however, this will not be necessary because the analyst knows whether total coliforms and *E. coli* were present in the same sample, satisfying regulatory requirements and public health concerns.
SUMMARY OF NATIONAL EVALUATION

In summary, the goals of the national evaluation of the Colilert test were met, including: (1) sensitivity equal to the Standard Methods MTF and P-A; (2) specific enumeration of 1 total coliform/100 mL in the 24-hour time frame; (3) simultaneous enumeration of 1 E. coli/100 mL in the same analysis; (4) imperviousness to false-positive or false-negative results by noncoliform heterotrophic bacteria; (5) no need for confirmatory tests; (6) growth of injured coliforms at least equal to that of Standard Methods m-Endo agar and MTF broths; (7) ease of inoculation; and (8) ease of interpretation.

The test's review and approval by the USEPA for current and pending drinking water monitoring requirements were as important as the project goals. This added project-related benefit--a breakthrough in bacteriological analysis--will allow utilities to meet all drinking water regulations with the least overall cost and effort.
CHAPTER 8
HANDLING CHARACTERISTICS

PHYSICAL CONFIGURATION OF THE TEST

The physical configuration of the Colilert test makes it compatible for use in both large and small utilities. Colilert is the only method that can either be added to a water sample or have a water sample added to it. The formulation can be obtained predispensed in either the MPN or P-A formats. In addition, it can also be obtained as unit-dose powder tubes that can be added to a utility's own vessel for P-A analysis.

The physical configuration of the test allows it to be used to the maximum benefit of water utilities. It is the only method in which the formulation can be added to a water sample. This characteristic is extremely important for laboratories that receive water specimens as 100-mL vessels either through the mail or delivered in mass quantity (often late in the afternoon). Rather than having to dispense water samples, the technician need only add the unit dosage of Colilert to the same vessels that have been used to collect the water. This flexibility should significantly reduce the handling of samples and allow more flexible scheduling of technician time.

USE OF THE TEST

The Colilert system is the least subjective method available and is the easiest to use and read. The yellow color and fluorescence are distinct and are not subject to the same type of subjective interpretation as is sheen or turbidity; the nature of the new technology produces a much more definite endpoint. This ease of use and interpretation should significantly help utilities in the future as it becomes more and more difficult to attract trained technologists to the field.

Moreover, the Colilert system is the first procedure that has the potential of being performed by individuals who are not trained or certified as microbiologists. It does not require any more skill to inoculate a Colilert test than it does to perform a chlorine or turbidity analysis. It is, therefore, possible for small utilities to test water in decentralized
or remote locations. Rather than risking the changes in total coliforms and \textit{E. coli} that can occur in a sample during long periods of transport to a central laboratory, the utility operator is able to perform the analysis at the utility and obtain a credible answer in 24 hours. Likewise, many large utility operators are using the Colilert test at their various treatment plants to determine plant performance. These tests are performed not for compliance purposes but for process control. This performance evaluation afforded by the Colilert method at the treatment plant not only allows the water quality manager a greater degree of control over the treatment train but also fosters a sense of "hands-on" monitoring by the individual operators. These operators feel more responsible for their finished water quality and are able to implement treatment process changes to ensure continued bacteriological quality.

Overall, the flexibility of the Colilert system allows utility managers greater liberty in establishing quality control parameters for monitoring the treatment train.

\textbf{TOTAL COLIFORMS AND \textit{E. COLI} IN ONE TEST}

The ability to analyze for both total coliforms and \textit{E. coli} in one test is extremely practical as well as beneficial for public health. Total coliforms are generally considered the best indicator of the efficacy of the treatment train process. However, \textit{E. coli} is considered the best indicator of recent fecal pollution of water, and its presence warrants immediate concern. Obtaining results for both TC and \textit{E. coli} within 24 hours, with no additional work or cost, fulfills the missions of the utility and meets the requirements of the new regulation. This simultaneous analysis ensures more timely public health decisions by both the utility and regulatory agencies.

\textbf{STORAGE}

Conventional microbiological media (broths, agars) cannot be stored for more than two weeks. Moreover, some media must be stored in a refrigerator. The utility must expend significant time and effort to manufacture and control the quality of microbiological media with short shelf lives. Utilities must dedicate laboratory space to refrigerators,
autoclaves, glassware, and other equipment that could be devoted to more cost-effective monitoring activities. The dry powder Colilert formula can be stored up to one year and does not require refrigeration. Therefore, the Colilert system may be stored in areas outside the laboratory, much like any other dry goods.

An additional benefit of the storage characteristics of the Colilert system is the price reduction achieved through volume purchases. Utilities may order in larger amounts knowing that the material can be stored for long periods of time.

The fact that the Colilert test can be stored almost anywhere allows the utility manager the further flexibility of decentralizing testing if desired. This flexibility is especially important for using the test at treatment plants to monitor treatment processes.

QUALITY CONTROL

The quality control requirements of the Colilert system and those of other microbiological media are distinctly different. Quality control of the Colilert is easy and straightforward and does not require a trained individual: For each new lot of Colilert, regardless of size, three tubes are chosen at random. The Colilert is hydrated with distilled or sterile tap water. To one tube is added a standard strain of *E. coli*, to a second a standard strain of *Klebsiella pneumoniae*, and to the third a standard strain of *Pseudomonas aeruginosa*. (These strains are available from commercial sources both as cultures and as agar chips containing bacteria that can be added directly to the Colilert tubes.) After 24 hours, the tubes are examined for typical reactions: for *E. coli*, yellow color and fluorescence; for *Klebsiella*, yellow only; and for *Pseudomonas*, neither yellow nor fluorescence. Quality control can be performed on a weekly or monthly basis, depending on how many tests are done, by simply adding a raw water source to a Colilert tube. Raw water sources generally contain *E. coli*; the tube is examined the next day for yellow color and fluorescence.

Conversely, quality control of the total coliform and fecal coliform tests by MTF or MF are much more subjective, difficult, and lengthy. The total coliform and fecal coliform tests must be evaluated for the indistinct and often subjective endpoints of gas production or colony appearance.

65
HETEROTROPHIC PLATE COUNT INTERFERENCES

The USEPA has concluded that water samples with greater than 500 HPC/mL can result in suppression of both MTF and MF procedures. In addition, MTF and MF bacteriological media lend themselves to the production of false-positive tests. For example, it has been estimated that between 5 and 20 percent of total coliform positive tests result from the noncoliform *Aeromonas hydrophilia*. The Colilert system is the most specific of the three available total coliform tests. In fact, the USEPA recommends using the Colilert system for the analysis of water with a high HPC bacterial content. When utilities use the MPN or MF methods, HPC analysis may be necessary to verify that HPC are < 500/mL. Therefore, both the MTF and MF procedures have been shown to be significantly affected by the presence of high numbers of HPC in water samples. Both false-positive and false-negative tests can result.

FIELD INOCULATION

Generally, the bacteriological analysis of drinking water occurs in centralized laboratories, partly because of the fact that most bacteriological testing is cumbersome. Also, current procedures are difficult to interpret and require a trained individual. This situation has changed with the development of the Colilert system. Field inoculation can be extremely important in investigating outbreaks and public health risks. Utilities that have to deal with an outbreak have seen their workloads escalate rapidly in short periods of time. Currently, a primary limitation to sampling is the collection and transportation and processing of specimens. In the Colilert system, standard-sized 100-mL vessels are transported to the scene of an outbreak and inoculated on site. Not only does the incubation begin sooner, but the manager performing the on-site investigation is able to collect samples as the situation dictates. The investigator of the outbreak can also sample various locations to pinpoint the sources of pollution.

Field inoculation can also decrease the time required to achieve a positive result because both total coliforms and *E. coli* grow at temperatures above 20°C. Therefore, field
inoculation is possible and can save considerable analysis time. This flexibility is especially important in the investigation of a potential waterborne outbreak.

Each box of Colilert contains a complete set of instructions and procedural cautions that should be reviewed thoroughly before field inoculation.
CHAPTER 9
THE COLILERT SYSTEM AND THE NEW COLIFORM RULE

PRESENCE-ABSENCE TESTING

The USEPA has established that the mere determination of total coliform densities is not a suitable monitoring method for establishing water quality. Total coliform densities are too variable to be reproducible or to allow public health officials to make accurate decisions. It is not possible to accurately count bacteria on the order of less than 1/mL (100/100 mL), and, more importantly, it is not possible to measure the wholesomeness of drinking water from density determinations.

TC populations often change during storage, further compounding the inaccuracies of measuring bacterial densities. The new regulations will permit a maximum 24-hour storage period at 10°C or less for transportation before analysis. It has been well established that total coliforms can die during this period. Another complication is that HPC bacteria can increase during storage, thus making MTF and MF tests less sensitive.

The greatest overall reason not to use coliform density measurements is that the USEPA, after extensive study, has found that there is no association between the numbers of total coliforms per mL and disease outbreaks. Therefore, enumerating coliform bacteria is not as important as measuring the frequency of occurrence (P-A).

The Colilert system can be used as either a quantitative MPN or P-A procedure, but a particular strength for utilities in meeting the new regulations is its compatibility in the P-A format. The Colilert formula can either be added to a water sample or have a water sample added to it. The alternative ways of determining P-A are much more cumbersome. The MTF procedure requires a 10-tube analysis (or 5-tube analysis, 20 mL/tube) or one 100-mL broth. The MF procedure requires the establishment of the presence or absence of one total coliform/100 mL. Therefore, every possible sheen colony on a plate must be tested to determine if it is a total coliform or not. Furthermore, this same colony must then be tested to determine if it is also a fecal coliform. In comparison, the ease of use and the time savings provided by the Colilert system are obvious.
INCREASED TESTING

The new regulations will require significantly increased testing, especially for the smaller utilities. First, the testing base for smaller utilities has been increased. Second, utilities will be required to perform repeat TC tests at each sample site that has shown a positive TC analysis (see the next section). According to estimates, testing will increase approximately 33 percent over current monitoring levels.

It is unlikely that utilities and state laboratories will be able to expand their personnel to deal with the increased testing need. Some large state laboratories have determined that the Colilert system is their best answer to increased labor requirements. Furthermore, microbial analysis is expected to be extended beyond the total coliform, fecal coliform, \textit{E. coli} realm to include \textit{Giardia, Cryptosporidium}, and perhaps viruses in the future. Like other breakthroughs in laboratory automation, the Colilert system will significantly boost productivity so that utilities and state agencies can keep up not only with the required increase in testing under the new rules but also with the testing of microorganisms not currently addressed.

REPEAT TESTING

Regardless of the percentage or number of TC positive samples, every water sample that shows a single total coliform will trigger a minimum of three repeat samples that must be collected within 24 hours. (From each TC positive sample either a fecal coliform or \textit{E. coli} analysis must also be made--see the next section.) One of these three samples must be from the same tap showing the total coliform, one may be within five connections upstream, and one within five connections downstream. There are no variances or exceptions to this rule. Obviously, some utilities will face a treadmill of repeat and continued analyses extending through weekends.

Of significant concern to utilities and state agencies is the potential for significantly increased weekend work. This work would occur not only on Saturday but also on Sunday because repeat samples must be collected and the analysis started within 24 hours. Few utilities or state agencies are geared to perform large-scale weekend testing.
The Colilert system can significantly alleviate the repeat testing problem in several ways. First, because Colilert is so easy to perform and interpret, any trained employee can stop by the utility or state agency laboratory for a few minutes to read Colilert tests on weekends. Most utilities have around-the-clock operators capable of performing the reading. Second, because Colilert requires no verification or confirmatory steps, this brief reading would conclude the analysis. With the MTF or MF methods, the analyst would not only have to examine closely the tubes or membrane filters but would also have to transfer any positives both to confirmatory total coliform tests (for example, BGLB) and to confirmatory fecal coliform tests (EC at 44.5°C). Then someone would have to return to the laboratory the next day to read these confirmatory tests. It has been estimated that the virtual elimination of weekend work, and its attendant overtime and personnel disruption, would more than pay for the cost of the commercially purchased Colilert formulation.

Fecal Coliform or E. coli Test from Each Total Coliform Positive

Under the new coliform rule each TC positive test, regardless of the percentage of TC positive tests in the system, must be further analyzed for either fecal coliform or E. coli. Each sheen colony that confirms as a total coliform must then be tested to determine if it is either a fecal coliform or an E. coli. Likewise, each MTF broth that shows a TC positive test must then be transferred to EC broth at 44.5°C to determine if it is a fecal coliform. The presence of a fecal coliform or E. coli is a violation and must be reported to the state.

The Colilert system, which provides the specific E. coli analysis simultaneously with the total coliform analysis, requires none of this additional work and time expenditure. Furthermore, the existence of a public health threat can be determined 24 hours sooner (Figure 2.1, p. 9). If the total coliform sample is either a fecal coliform or an E. coli and it is followed by a repeat sample that is positive for total coliforms, an acute violation is deemed to exist and public notification via electronic media must occur. As shown in Figure 2.1, utilities using MF or MTF methods experience severe problems attempting to cope with these various repeat samples, violations, and possible acute violations. The use of the Colilert system obviously streamlines the workflow while at the same time providing more
specific results with public health meaning. The Colilert system will help utilities and state agencies deal with both the repeat testing and the fecal coliform and \textit{E. coli} testing by providing specific answers in a shorter period of time, without increased labor or expenses.

**Fecal Coliform Testing**

Fecal coliform testing is an expensive, complex undertaking. The fecal coliform media must be prepared by trained individuals. Incubators must be rigidly controlled at 44.5°C ± 0.2°C. This close temperature tolerance is essential to the accurate performance of the test. Decreases as much as 0.2°C below 44°C have been shown to permit a much higher percentage of the nonfecal \textit{Klebsiella} to yield a positive test. Conversely, temperatures as little as 0.2°C above 45°C will inhibit the growth of many strains of \textit{E. coli}. It has also been found that overinoculation of fecal coliform tests will significantly increase the number of total coliforms that are demonstrated to be fecal coliforms (personal communication, Martin J. Allen, AWWARF, 1987). On repeat testing, under more carefully controlled conditions, these organisms are not thermo-tolerant. However, this overinoculation is very difficult to control in the laboratory and would vary widely among utilities.

The fecal coliform procedure is not only difficult to perform but it also provides a considerably less specific indicator of fecal pollution than does the analysis of \textit{E. coli}. It has been well established that approximately 15 percent of \textit{Klebsiella pneumoniae} are thermo-tolerant and cause a positive FC test. Many of these \textit{Klebsiella} have been shown not to have a colonic origin--they have been isolated from pristine environments with no mammalian fecal contamination. Furthermore, approximately 5 percent of \textit{Enterobacter aerogenes} and 5 percent of \textit{Enterobacter cloacae} have also been shown to be thermo-tolerant and are thus considered fecal coliforms; again, many of these organisms have been shown not to have originated in the colons of mammals. Utilities that experience a very small percentage of total coliform positives would still expect to see a number of fecal coliforms per year. For example, consider the case of a large, well-managed utility that isolates only 20 to 30 TC positive samples per year. Although this utility would be well below the 5 percent allowed under the new regulations for violation, it could experience between 3 and 5 FC positive samples per year; that is, 3 to 5 violation reports to the state per year. Conversely, \textit{E. coli}
is extremely uncommon in distribution systems. In the national evaluation of the Colilert system, only one \textit{E. coli} was isolated from all samples tested. Subsequently, in the two years in which the Colilert system has been used by state agencies and utilities, the isolation rate of \textit{E. coli} from distribution systems has been less than 0.01 percent. In other words, the ratio of fecal coliforms to \textit{E. coli} in distribution systems is on the order of $>1500:1$ (data on file until 12/92, call Project Director, Stephen C. Edberg).

Accordingly, use of the Colilert system would result in a much lower percentage of violations. This lower percentage would be in keeping with the actual presence of fecal pollutants in distribution systems. Colilert, a more specific test employing a more specific indicator, would better serve the public: Accurate reports of violations to the regulatory agencies would be cause for justified public concern over water quality, whereas continued reports of fecal coliform violations would create public apathy and loss of confidence in the utility.

HEALTH RISK INDICATOR

\textit{E. coli} is the most specific indicator of fecal pollution of drinking water because of its association only with the colons of warm-blooded mammals. It survives in water for reasonable periods of time but does not persist so long as to reduce its value as an indicator. Fecal coliforms, on the other hand, are not specific. Numerous studies have shown that some fecal coliforms such as \textit{Klebsiella pneumoniae} are found in water not associated with fecal, or colon, contamination. They have been isolated from the centers of redwood trees, pristine lakes, and other non-fecally contaminated sources. Therefore, the \textit{E. coli} test in the Colilert system serves as a more specific indicator of fecal pollution and the best protector of the public health. It is a health risk indicator that public health officials can utilize in determining whether an order to boil water should be issued. For example, there have been cases in which fecal coliforms have been associated with biofilm regrowth, which intensive study has shown not to be a fecal pollution event. \textit{E. coli} has never been isolated from the same biofilm regrowth situations. Public health officials concluded that because \textit{E. coli} was not present in these systems, there was no health risk, and therefore they did not issue orders. Officials have, however, required these utilities to monitor intensively for \textit{E. coli}.
For these reasons, public health officials and medical microbiologists are strongly in favor of *E. coli* over fecal coliforms as the fecal pollution indicator.

**TWENTY-FOUR HOUR TRANSIT TIME**

The new regulations will require that no more than a 24-hour transit be permitted from the time of collection of the sample until it is analyzed for total coliforms. This criterion will be difficult to meet for smaller utilities and rural water producers that mail water samples to laboratories for analysis. Public transportation, mail, and even courier services may not be able to provide expedited delivery of samples to meet this 24-hour limitation. Furthermore, although it is possible to use overnight delivery, the cost of such shipment would be more than the cost of the analysis. Inoculating the Colilert system at the utility and mailing it to a laboratory is an extremely attractive means of dealing with the 24-hour requirement. Some state agencies are already considering this option. Even if the Colilert test requires more than 24 hours to reach the laboratory and is positive, the laboratory can still confirm or further analyze the bacteria present in the Colilert test to establish specificity. If the test does not become positive, then the analysis is complete. Therefore, the use of the Colilert system could be very compatible with rural and small utility water testing.

**MAXIMUM OF 10°C STORAGE**

The new regulations also require that water be kept at no more than 10°C in transit to the laboratory. Obviously, this requirement will severely hamper the small utility and rural water provider, especially during summer months. In addition, utilities must provide their collection personnel with devices that maintain a consistent, controlled temperature. Maintaining the collected samples at a temperature below 10°C will be an impossible burden on many utilities. Because the Colilert system begins working as soon as the water sample is added to it, incubation and growth of the target microorganisms will occur even during sample transport. Utilities can, therefore, inoculate the Colilert system in the field and then send it to a commercial or state laboratory. If the Colilert arrives and is yellow, it can be
verified by subculture for the presence of total coliforms. If the Colilert arrives and is colorless, it can be incubated for the full time period.

Therefore, state agencies can better control and ensure the testing analysis of small and rural water supply samples than is now possible. They can interact closely with these small suppliers and utilize the measurement of total coliforms and *E. coli* in much the same way that they can utilize measurements of turbidity and chlorine.

Therefore, the possibility of utilities and state regulators having better control over their peripheral operations and small utility water providers is significantly enhanced by utilizing the Colilert system. The system can be used in tandem with chlorine testing and turbidity, other simple means of analysis that are performed by noncertified people, to best serve the public's need for safe drinking water.
### ABBREVIATIONS, ACRONYMS, AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAS</td>
<td>Access Analytical Systems</td>
</tr>
<tr>
<td>AC</td>
<td>Colilert system</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>API 20E</td>
<td>Analtab Products, Inc.</td>
</tr>
<tr>
<td>AWWARF</td>
<td>American Water Works Association Research Foundation</td>
</tr>
<tr>
<td>BGLB</td>
<td>brilliant green lactose bile broth</td>
</tr>
<tr>
<td>C</td>
<td>centigrade</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>DST</td>
<td>defined substrate technology</td>
</tr>
<tr>
<td>EC</td>
<td>fecal coliform fermentation medium</td>
</tr>
<tr>
<td>EMSL</td>
<td>Environmental Monitoring and Support Laboratory</td>
</tr>
<tr>
<td>FC</td>
<td>fecal coliform(s)</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HPC</td>
<td>heterotrophic plate count</td>
</tr>
<tr>
<td>LTB</td>
<td>lauryl tryptose broth</td>
</tr>
<tr>
<td>MF</td>
<td>membrane filtration</td>
</tr>
<tr>
<td>m-FC</td>
<td>membrane fecal coliform agar</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>MPN</td>
<td>most probable number</td>
</tr>
<tr>
<td>MTF</td>
<td>multiple tube fermentation</td>
</tr>
<tr>
<td>mT-7</td>
<td>membrane injured coliform media</td>
</tr>
<tr>
<td>MUG</td>
<td>4-methylumbelliferyl-β-D-glucuronide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>ONPG</td>
<td>orthonitrophenyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>P-A</td>
<td>presence-absence</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>$r^2$</td>
<td>random chance</td>
</tr>
<tr>
<td>R2A</td>
<td>HPC media</td>
</tr>
<tr>
<td>SCCRWA</td>
<td>South Central Connecticut Regional Water Authority</td>
</tr>
<tr>
<td>SM</td>
<td><em>Standard Methods</em></td>
</tr>
<tr>
<td>TC</td>
<td>total coliform(s)</td>
</tr>
<tr>
<td>t-test</td>
<td>comparison of means</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USEPA</td>
<td>U.S. Environmental Protection Agency</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>

**Symbols**

- $\alpha$  
- $\beta$  
- $^\circ$  
- $\mu$  

*alpha*  
*beta*  
*degree*  
*micro*
REFERENCES


APPENDIX A

USEPA ANALYTICAL TECHNIQUES.

COLIFORM BACTERIA; FINAL RULE
DATE: January 15, 1988

SUBJECT: Drinking Water Supply Alternate Test Procedure (ATP)--Action Memorandum

FROM: Robert L. Booth, Director
Environmental Monitoring and Support Laboratory - Cincinnati

TO: Michael Cook, Director
Office of Drinking Water (WH-550)

Contaminants:
Total Coliforms and Presence/Absence (P/A)

Recommendation:
Approve

Applicant:
Stephen C. Edberg
Yale University School of Medicine
Department of Laboratory Medicine
333 Cedar Street
New Haven, Connecticut 06510

Application Number:
D87-0007

Method Proposed:

The Autoanalysis Colilert Test* is being proposed for approval as an ATP for analysis of total coliforms in drinking water for the National Primary Drinking Water Regulations (NPDWR) compliance monitoring program.

Summary of the Method:

The Autoanalysis Colilert® (Colilert) test is based on the bacterial metabolism of specific indicator nutrients, o-nitrophenyl-β-d-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-d-glucuronide (MUG). The former reagent is specific for the target group, total coliforms, while the latter is metabolized only by E. coli. The sample is added directly to the reaction tube, where incubation begins immediately. After incubation for 24 hours at 35-37°C, samples containing 
coliforms at concentrations as low as one colony forming unit (CFU)/100 mL, release a yellow color. If *E. Coli* is present, the sample will fluoresce under 366 nm light. The test may be configured as either a multiple tube fermentation test (MPN) or a P/A format. Non-coliforms do not respond to this test.

**Technical Justification for Recommendation:**

Yale University has exceeded our minimum required comparability data requirement. The details of the equivalency study are described in the attached report from Yale University. Data were collected in two phases. The first compared approved multiple tube fermentation test results with those of the Colilert test configured as a multiple tube fermentation (MPN) test. The second phase compared the Colilert P/A format with currently used tests which satisfy the definition of P/A as referenced in the proposed regulations.

Statistical analyses of the data were performed using the Pearson Chi-Square and the Mantel-Haenzel tests. The reports of these analyses, dated December 9, 1987, are attached. Comparison of the approved MPN and Colilert MPN tests indicated slightly higher incidences of positive results using the Colilert Procedure. Data from the remaining two sources showed no statistically significant difference between the methods for detection rates of positive samples. The data were subsequently analyzed to determine the influence of heterotrophic plate count (HPC) on the incidence of positive results by both MPN methods. For all sources, significant associations between HPC level and the incidence of positive results were observed. The associations were similar in magnitude for both the approved and proposed Colilert methods.

Phase II data were analyzed using the same statistical techniques. There were no statistically significant differences between the incidences of positive results by the Colilert P/A test and the other proposed P.A tests.

Yale University has demonstrated that the Colilert MPN test provides results comparable to those obtained by the currently approved MPN method. However, because the test does not support the growth of heterotrophs, and can detect non-gas producing coliforms better than the currently approved MPN method, there is a tendency for the Colilert test to yield a slightly higher percentage of positive results than the approved MPN procedure. Also contributing to the higher incidence of positive results by the Colilert test is the fact that users of the Colilert test can inoculate and read results with no change in bacterial density due to transportation of samples to the laboratory. The test offers a number of other advantages, especially to the smaller utilities: the test is economical, can be performed under field conditions by relatively inexperienced personnel, and requires minimal auxiliary equipment.

Accordingly, we are pleased to recommend approval of the Autoanalysis Colilert test as an alternative to the currently approved MPN procedure. We also feel that the Autoanalysis Colilert test should be included in future regulations that specify approved methods for determining the presence/absence of coliform organisms in drinking water.
Reviewers:

Environmental Monitoring and Support Laboratory - Cincinnati

Terence Grady
Cornelius Weber
Robert Bordner
Terry Covert
Kristen Brenner
John Haines

Water Engineering Research Laboratory - Cincinnati

Edwin Geldreich, Jr.
John Hoff
Gary S. Logsdon
Donald J. Reasoner
Harry Nash
Eugene Rice
Donald Berman

Health Effects Research Laboratory - Cincinnati

Alfred J. DuFour

Office of Water

Louis Shadix

Previous Action:

No previous requests for approval of this test procedure have been received.

Attachments: (3)
As Stated
cc: All without attachments

Terence Grady
Cornelius Weber
Terry Covert
Kristen Brenner
John Haines
Edwin Geldreich, Jr.
Alfred DuFour

Gary Logsdon
Donald Reasoner
Harry Nash
Eugene Rice
Donald Berman
Robert Bordner

EMSL-CI:RLBooth:TMGrady:srg:stc,587,x7304,1/15/88 (TG 9:40)
FILE: ATPs, stc, 592

(Document retyped from original)
In response to your request for recommended methods for the detection and enumeration of *Escherichia coli* in drinking water, we have conducted a thorough search of the literature and reviewed and discussed the most promising candidate methods with the senior microbiologists at the Andrew W. Breidenbach Environmental Research Center. The criteria for an acceptable method for *E. coli* were: assurance that the method measures *E. coli*, evidence that the method has been tested in drinking water, sensitivity, specificity, selectivity, time required to complete the test, cost, and commercial availability of the media. The following multiple tube and membrane filter methods, listed with major advantages and disadvantages, are recommended at this time:

**Autoanalysis Colilert (AC) Test**

The AC test utilizes two chromogenic nutrient compounds in a defined medium to detect and measure simultaneously total coliforms and *E. coli*. The total coliform group and *E. coli* produce specific enzymes which hydrolyze the two compounds and release the chromogenic portions. *E. coli* produces the enzyme b-glucuronidase which hydrolyzes 4-methylumbelliferyl-b-D-glucuronide (MUG) contained in the medium to form 4-methylumbelliferone, which fluoresces in the unconjugated form. The test involves the addition of 10 mL sample volumes to the dry medium, incubation at 35°C for 24-28 h, and observation of a yellow color which indicates the presence of coliforms. The yellow tubes are subjected to ultraviolet light (366 nm) and fluorescence indicates the presence of *E. coli*.

The AC test has several advantages: it is rapid (24-28 h), specific (95% verification rate), sensitive (1 *E. coli*/100 mL),
has no interference with coliform growth from noncoliforms, recovers and grows injured cells, is easy to perform, is available commercially, and has a long shelf life (one year at ambient temperature).

Disadvantages are that this medium may be difficult to prepare from the basic ingredients, provides uncertain identification of weak total coliform (pale yellow) results, and requires a color comparator with unknown shelf life. In addition, the sole source of the prepared test and the dispersant, solanium, are potential drawbacks.

Multiple Tube Fermentation (MTF) Test

This test is an extension of the standard MTF test for total coliforms described in Standard Methods for the Examination of Water and Wastewater. Growth from coliform presumptive tubes is transferred to EC tubes containing MUG. The tubes are incubated at 44.5°C for 24 h. Growth, gas production, and fluorescence under ultraviolet light (366 nm) constitute a positive E. coli test.

Advantages are that this is an easy extension of the standard MTF test. The EC MUG medium is available commercially and if the laboratory is routinely performing the MTF test, growth from the presumptive test can be easily transferred into brilliant green lactose broth (BGLB) for coliform confirmation and EC MUG for E. coli determination simultaneously.

Disadvantages are that this procedure requires two incubation temperatures and 48 to 72 h from sample inoculation to completion. If prepared in the laboratory, the EC MUG medium may require pH adjustment for best fluorescence production, and turbid media may decrease fluorescence visibility.

Direct Enumeration mTEC Method

This two-step membrane filter (MF) method provides a direct count of E. coli. The water sample is filtered through the membrane, which is then placed on mTEC, a selective and differential medium. The test is incubated at 35°C for 2 h to resuscitate injured or stressed bacteria and transferred to 44.5°C for 22 hours. Following incubation, the filter is transferred to an absorbent pad saturated with urea substrate. After 15 minutes at room temperature, yellow or yellow-brown colonies are counted with the aid of a fluorescent lamp and magnification.
Advantages of the mTEC test are that it is specific for *E. coli*, provides direct counts, as differentiated from a probability index, is a 24 h test, and provides fecal coliform in addition to *E. coli* information.

Disadvantages are that the test requires additional sample volume to perform both total coliform and *E. coli* analyses, two incubation temperatures, and slightly more analysis time than the single step methods.

**Standard Coliform MF Methods Verified for E. coli**

These MF methods follow the procedures described in *Standard Methods for the Examination of Water and Wastewater* for total and fecal coliforms. Although these methods require more than one stage, laboratories concerned about biofilm or other problems routinely monitor their distribution systems using these enumeration methods. To perform the total coliform test, the water sample is filtered through the membrane which is then placed on mEndo agar and incubated at 35°C for 24 h. Typical sheen colonies are picked and transferred to lauryl sulfate broth and incubated at 35°C for 24-48 h. Growth from gas-producing tubes is then transferred to BGLB and EC MUG or inoculated into a commercially available multitest system, such as API 20E, simultaneously. The EC MUG tubes are incubated at 44.5°C for 24 h. Growth, gas production, and fluorescence under ultraviolet light (366 nm) constitute a positive *E. coli* test. The multitest system is incubated at 35°C and read for *E. coli*.

Alternatively, to perform the fecal coliform test, the water sample is filtered through the membrane, which is then placed on m-FC agar and incubated at 44.5°C for 24 ± 2 h. Blue colonies are picked and transferred to EC MUG tubes which are incubated at 44.5°C for 24 h. Growth, gas production, and fluorescence under ultraviolet light (366 nm) constitute a positive *E. coli* test. Blue colonies may also be verified in a multitest system and read for the presence of *E. coli*.

Advantages are that these tests provide actual counts as differentiated from estimated values, are specific for *E. coli* after verification, and give information on fecal coliforms in addition to *E. coli*. The EC MUG test is available commercially.

Disadvantages are that these MF tests require additional sample volume for performing both coliform and *E. coli* tests, preparation of two media, two incubation temperatures, and greater cost than single step methods. They are also time-consuming and labor intensive; the total coliform may require 72-96 h and the fecal coliform test 48 h to complete.
Two of the recommended methods, Autoanalysis Colilert, and mTEC, have been collaboratively tested and shown to be acceptable. The addition of MUG to EC medium in the MTF test and use of a MUG test or multitest system to verify typical fecal coliform colonies on m-FC agar are simply confirmatory steps for \textit{E. coli} in those methods. We are attaching copies of the supporting documents for the mTEC two step method and the use of EC medium with MUG.

It should be emphasized that these are state-of-the-art methods. Our laboratory has developed a single-step mTEC MF method which will offer significant advantages over the two-step method. However, it needs to be published and evaluated. Also, there are good possibilities for the development of a rapid MF test that will incorporate a chromogenic agent in the medium to provide a specific test for \textit{E. coli} without the need for a second test medium. We have performed successful preliminary research on this approach. We strongly urge that resources be made available for such projects to improve further useful tests for \textit{E. coli}.

Attachments:
As stated

\textbf{cc:} P. Berger  
A. Dufour  
R. Bordner  
G. Stelma  
E. Geldreich  
D. Reasoner  
D. Nash  
E. Rice  
T. Covert  
J. Haines  
K. Brenner  
L. Shadix

(Document retyped from original)
APPENDIX B

NATIONAL FIELD EVALUATION PUBLICATION:

DST COMPARISON WITH P-A TECHNIQUES

(Reprinted here with permission of the American Society for Microbiology)
National Field Evaluation of a Defined Substrate Method for the Simultaneous Detection of Total Coliforms and Escherichia coli from Drinking Water: Comparison with Presence-Absence Techniques

STEPHEN C. EDBERG, MARTIN J. ALLEN, DARRELL B. SMITH, AND THE NATIONAL COLLABORATIVE STUDY†

* Corresponding author.
† Investigators in the National Collaborative Study include the following: Mark LeChevallier, American Water Works Service Company, Belleville, IL 62220; Nancy Kriz and Deborah Callan, Yale University School of Medicine, New Haven, Connecticut 06510; Roger Ward and Dawn Calvert, California-American Water Co., Monterey, CA 93940; Linda Hmurciak, North Andover, MA 01845; Thomas Trok and Michael Burns, West-Penn Water Co., Pittsburgh, PA 15226; Valerie Shinn, Washington State Department of Health, Seattle, WA 98155; Bruce Kraus, Cincinnati Water Co., Cincinnati, OH 45228; and Charlotte Dery, Vincent Coluccio, and Jerry Iwan, City of New York, NY 10004.

Current Safe Drinking Water Act regulations require the analysis of potable water for total coliforms (15), a group of closely related bacteria in the family Enterobacteriaceae. Two quantitative methods are presently certified for this analysis, the multiple-tube fermentation (MTF) and the membrane filter (MF) techniques (1, 15). Both of these procedures need verification of first-step presumptive positives by multistep and confirmed tests. Therefore, a water analysis may require from 2 to 4 days (1, 3).

Present enumeration techniques suffer from several inherent limitations. First, estimates of coliform density from a single sample may show variability (14, 20). Second, coliform densities may significantly change from the time the sample is collected until it is processed (20). Third, the MTF method uses a 50-ml sample and is not sensitive enough to enumerate 1 total coliform per 100 ml (16). To address these shortcomings, the Environmental Protection Agency (EPA) proposed a frequency-of-occurrence monitoring approach. Known as the presence-absence (P-A) concept, this method was constituted as a presence-absence test and compared with the methods described in Standard Methods (SM) in the P-A format. Seven water utilities representing a wide geographical and hydrological spectrum participated in the evaluation. A total of 702 split drinking water samples were analyzed. Of these, 358 were negative in both tests (SM— and AC—); 302 were positive (SM+ and AC+); and 42 were mixed (SM+ and AC—, 20; AC+ and SM—, 22). The overall agreement rate was 94%.

Comparison of the SM and AC results by nonparametric statistics demonstrated no differences. Heterotrophic plate count bacteria exerted no discernible effect on the AC test. By subculture, each time the AC test was yellow, a total coliform was present; when the test was fluorescent, E. coli was isolated.

A defined substrate method was applied to drinking water to simultaneously enumerate total coliforms and total Escherichia coli directly from samples. After incubation at 35°C for 24 h, the development of yellow in an initially colorless solution was specific for total coliforms; fluorescence at 366 nm in the same tube(s) or vessel demonstrated the presence of E. coli. No confirmatory or completed steps were necessary. Known as autoanalysis colilert (AC), this method was constituted as a presence-absence test and compared with the methods described in Standard Methods (SM) in the P-A format. Seven water utilities representing a wide geological and hydrological spectrum participated in the evaluation. A total of 702 split drinking water samples were analyzed. Of these, 358 were negative in both tests (SM— and AC—); 302 were positive (SM+ and AC+); and 42 were mixed (SM+ and AC—, 20; AC+ and SM—, 22). The overall agreement rate was 94%.

Comparison of the SM and AC results by nonparametric statistics demonstrated no differences. Heterotrophic plate count bacteria exerted no discernible effect on the AC test. By subculture, each time the AC test was yellow, a total coliform was present; when the test was fluorescent, E. coli was isolated.

The overall agreement rate was 94%.
TABLE 1. Characteristics of participating water utilities

<table>
<thead>
<tr>
<th>Geographical area</th>
<th>Water source</th>
<th>Type of utility</th>
<th>Population served</th>
</tr>
</thead>
<tbody>
<tr>
<td>California, New Mexico, Arizona</td>
<td>Well, ground, surface</td>
<td>Private</td>
<td>&gt;250,000</td>
</tr>
<tr>
<td>Southwestern Pennsylvania</td>
<td>Surface, river</td>
<td>Private</td>
<td>&gt;530,000</td>
</tr>
<tr>
<td>Connecticut</td>
<td>Well, ground, surface, mixed</td>
<td>Municipal</td>
<td>400,000</td>
</tr>
<tr>
<td>New England</td>
<td>Surface</td>
<td>Municipal</td>
<td>22,000</td>
</tr>
<tr>
<td>New York City</td>
<td>Surface</td>
<td>Municipal</td>
<td>8,000,000</td>
</tr>
<tr>
<td>Southern Ohio</td>
<td>River</td>
<td>Municipal</td>
<td>750,000</td>
</tr>
<tr>
<td>Washington State</td>
<td>Well, ground</td>
<td>State</td>
<td>&gt;1,000,000</td>
</tr>
</tbody>
</table>

a 100-ml P-A test consisting of a 300-ml glass bottle with 100 ml of glutamate broth has been used for several years for the decentralized testing of distribution water (19). All available methods test only for the single component, total coliforms.

The autoanalysis Colilert (AC) method is an application of a defined substrate technology originally developed to elucidate specific species and groups of the family Enterobacteriaceae in urine samples (13). It can detect and enumerate total coliforms and E. coli simultaneously, directly from a drinking water sample. The AC method was evaluated as an enumeration most-probable-number test according to the Environmental Support and Monitoring Laboratory protocol of the EPA and found to be equivalent to currently approved methods of the EPA (11). Levels of heterotrophic bacteria as high as 7 × 10⁶/ml encountered during the study did not show interference (11).

To determine the sensitivity and specificity of the AC test in the frequency-of-occurrence format, it was constituted as a P-A test and compared with the quantitative methods described in Standard Methods (SM; 1) for the MTF, MF, and P-A tests used as P-A tests. A wide variety of geologically diverse surface and groundwater samples were tested. The P-A comparison of AC versus SM followed the guidelines of the Environmental Support and Monitoring Laboratory protocol for certification of a proposed method as an acceptable alternative (10).

MATERIALS AND METHODS

Participants and samples. Eight utilities representing seven EPA regions participated in the national evaluation (Table 1). The utilities ranged from those serving a single metropolitan area to those serving large numbers of small community water systems in three states. Water sources included deep and shallow wells, springs, rivers, and surface reservoirs. All water samples were obtained from potable distribution systems by the participating utilities; however, an effort was made to obtain water from locations most likely to yield positive samples, such as dead ends, storage reservoirs, and known problem sites. In some cases a small amount of chemically and biologically defined source water was added to a large sample of distribution water to ensure that positives were obtained. Samples were also collected during periods of distribution system flushing. These water samples were not necessarily those used for routine monitoring for regulatory purposes. Two of the participating utilities had been experiencing biofilm regrowth in their distribution systems.

Water samples were collected, transported, and stored in accordance with the guidelines described in the Handbook for Evaluating Water Bacteriological Laboratories (20). Either sterile polymethylpentene or glass flasks containing sodium thiosulfate were used to collect the samples.

AC P-A method. The AC P-A test format was either a 100-ml 10-tube most probable number test (1 tube positive denoting the presence of total coliforms in that sample) or a single vessel containing sufficient reagent to receive 100 ml of sample (Access Medical Systems, Branford, Conn.). The powdered formula was manufactured according to previously described specifications (19). Representative samples of both types of P-A tests were subject to quality control procedures described previously (19).

The AC P-A method was performed as follows. For the 10-tube method, 10 ml of water sample was added to each tube, and for the single-vessel method, 100 ml of water sample was added. In both cases the reagent powder was dissolved by agitation, producing a colorless solution. The test tubes or vessels were incubated at 35°C for 24 h. Development of yellow during incubation denoted the presence of total coliforms in either the test tube or the P-A vessel. Each positive total coliform test tube or vessel was exposed to a hand-held fluorescent (366-nm) light (Edmund Scientific Co., Barrington, N.J.). Fluorescence specifically demonstrated the presence of E. coli.

Other P-A tests. The revised rules to the Safe Drinking Water Act include the P-A concept, which allows any of three coliform methods to be considered an acceptable P-A test (16). These P-A tests included a 10-tube MTF, with one confirmed tube being a positive result; a MF, with one confirmed sheen colony being considered a positive result; and a 100-ml single fermentation tube (FT) (1). HPC. A heterotrophic plate count (HPC) was determined for each water sample by using R2A agar incubated at 35°C for 72 h (1).

Evaluation protocol. The comparison of the AC and the three SM P-A tests followed Environmental Support and Monitoring Laboratory guidelines (10). Sufficient water was collected from each location to perform simultaneous P-A tests by the AC and SM procedures. Table 2 shows the distribution of AC and SM P-A analyses performed. Each water sample was divided between a SM P-A and an AC P-A test. All positive presumptive SM tubes or sheen colonies were confirmed as total coliforms by SM procedures (20), and only these were included in the data base. To ensure that a positive result was the result of the target microbes, subcultures were made from both positive SM and AC tests, and bacteria were identified to species by the API 20E system (Analytab Products, Plainview, N.Y.) (12).

The statistics sections of the Department of Epidemiology of Yale University and the Environmental Support and Monitoring Laboratory analyzed the data. Because the data were in the hit-miss (i.e., P-A) mode, comparisons between SM and AC were made in the chi-square form. The Pearson chi-square test of association was used first. Also used were the Mantel-Haenzel test for linear association between rows and columns; the McNemar statistic, which tests whether the disagreements between methods are randomly distributed about the main diagonal; the index of agreement, i.e., the proportion of all the trials for which there are agreement (both presence or both absence); and the kappa statistic,
which is a "chance-corrected" adjustment to the index of agreement. Kappa ranges from -1 to +1; +1 indicates perfect agreement. 0 indicates no agreement, and negative values suggest less agreement than expected due to chance. The κ statistic was calculated for kappa; if this statistic is large (>2), the hypothesis that there was no agreement beyond that expected due to chance is rejected (2.10, 18).

RESULTS

Comparison of methods. A total of 702 split drinking water samples were analyzed by both methods. Of these, 322 were positive by SM and 324 were positive by AC. Conversely, 380 were negative by SM and 378 were negative by AC (Table 2). The data were further divided into those samples in which both methods were positive (SM+ and AC+), those in which both were negative (SM- and AC-), those in which SM was positive and AC was negative (SM+ and AC-), and those in which SM was negative and AC was positive (SM- and AC+). Both procedures were simultaneously positive in 302 instances, and both were simultaneously negative in 358 samples. The overall agreement rate between the two methods was 94%. SM was positive with a companion AC negative in 20 cases, and SM was negative with an AC positive in 22 cases.

Statistical analyses. The chi-square statistics were generally large. By the Pearson chi-square test, none of the individual locations showed a statistically significant difference in detection rate between the two methods. The chi-square of 2.30 at North Andover was the highest, but it still demonstrated a $P > 0.10$ (Table 3). The Mantel-Haenszel chi-square test showed that the hypothesis of zero correlation was correct. Like the related Pearson chi-square test, it did not show any statistically significant differences overall or at the individual sites. The McNemar chi-square test was used to compare the overall detection rate of positive samples between the two methods. The McNemar test was done for each site to compare the overall proportion of positive samples detected by the two methods. It compared the false-positive and false-negative rates between the two methods.

Table 2 presents kappa values and kappa values conditional on the SM result calculated to measure the agreement between the two methods for only those samples with a positive SM result. Kappa* (conditional) is similarly interpreted for those samples with a negative SM result. Table 3 presents kappa values and kappa values conditional on the SM results calculated to measure the degree of agreement between the two methods beyond chance agreement. For all but one site, overall agreement was excellent. The results for North Andover indicated chance agreement only. Kappa values conditional on the results of the SM P-A showed that for the California-American site, agreement between the two methods was excellent when SM detected a positive sample but only moderate when the SM result was negative. Conversely, the results for the South Central Connecticut Regional Water Authority site (SCCRWA) were opposite, with agreement in

TABLE 2. National field evaluation of AC and SM tests: P-A comparison

<table>
<thead>
<tr>
<th>Site</th>
<th>Total no. of samples</th>
<th>No. of each test type</th>
<th>No. of SM* tests</th>
<th>No. of AC tests (yellow)</th>
<th>Comparison of P-A results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MF</td>
<td>MTF</td>
<td>10th-nil vessel</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>California-American Water</td>
<td>196</td>
<td>165</td>
<td>31</td>
<td>33</td>
<td>163</td>
</tr>
<tr>
<td>SCCRWA</td>
<td>68</td>
<td>68</td>
<td>0</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>North Andover</td>
<td>92</td>
<td>92</td>
<td>0</td>
<td>87</td>
<td>5</td>
</tr>
<tr>
<td>Cincinnati</td>
<td>41</td>
<td>41</td>
<td>0</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>West Penn Water</td>
<td>26</td>
<td>26</td>
<td>0</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>Washington State</td>
<td>151</td>
<td>0</td>
<td>43</td>
<td>21</td>
<td>130</td>
</tr>
<tr>
<td>New York City</td>
<td>128</td>
<td>128</td>
<td>0</td>
<td>122</td>
<td>6</td>
</tr>
</tbody>
</table>

* Composite of all three SM techniques.
Table 4. Species identifications from P-A versus P-A nonagreements

<table>
<thead>
<tr>
<th>Test result</th>
<th>Site</th>
<th>SM</th>
<th>Species isolated</th>
<th>HPC/100 ml*</th>
<th>Total coliform/100 ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-/AC+</td>
<td>California-American Water</td>
<td>MTF</td>
<td><em>Enterobacter agglomerans</em></td>
<td>21,000</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Citrobacter freundii</em></td>
<td>12,000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Enterobacter cloacae</em></td>
<td>13,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Morganella morgani</em></td>
<td>17,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Citrobacter freundii</em></td>
<td>8,600</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Citrobacter freundii</em></td>
<td>24,000</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Enterobacter amnigenus</em></td>
<td>45,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>210</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-A</td>
<td><em>Citrobacter freundii</em></td>
<td>1,100</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-A</td>
<td><em>Enterobacter agglomerans</em></td>
<td>757,000</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-A</td>
<td><em>Enterobacter aerogenes</em></td>
<td>6,800</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SCCRWA</td>
<td>MF</td>
<td><em>Enterobacter cloacae</em></td>
<td>44</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>North Andover</td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Enterobacter cloacae</em></td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>TNTC</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>45,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Washington State</td>
<td>MTF</td>
<td><em>Citrobacter freundii</em></td>
<td>2,040</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Serratia fonticola</em></td>
<td>310</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Enterobacter diversus</em></td>
<td>345</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>New York City</td>
<td>MF</td>
<td><em>Escherichia coli</em></td>
<td>134</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Enterobacter agglomerans</em></td>
<td>5</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Citrobacter freundii</em></td>
<td>46</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM+/AC+</td>
<td>California-American Water</td>
<td>MTF</td>
<td><em>Citrobacter freundii</em></td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Enterobacter aerogenes</em></td>
<td>28,000</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P-A</td>
<td><em>Enterobacter agglomerans</em></td>
<td>1,000</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SCCRWA</td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>66</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Enterobacter cloacae</em></td>
<td>66</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>North Andover</td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>67,800</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>42,000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>125,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>55,000</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>7,400</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>350</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>235</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Aeromonas sp.</em></td>
<td>383,500</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella oxytoca</em></td>
<td>10,000</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>53,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella oxytoca</em></td>
<td>42,300</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>23,000</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Washington State</td>
<td>MTF</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>ca. 11,400</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTF</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>N/A</td>
<td>+</td>
</tr>
</tbody>
</table>

* NA: Not available; TNTC: too numerous to count.
+ : Positive result; number of total coliforms not determined.

Each case excellent, but stronger when SM was negative. All other sites demonstrated complete agreement.

**Characteristics of disagreements.** Table 4 shows the bacterial species identifications, HPCs, and total coliforms per 100 ml when SM and AC disagreed. In each case except one, a total coliform was isolated from the AC test. Neither the form of P-A test used nor the HPC exerted any statistically significant effect on the AC results.

Of the disagreements between SM and AC, 90% occurred when the total coliform count per 100 ml was less than 10/100 ml. The majority of these disagreements probably represented the maldistribution of bacteria within the split sample, resulting in sampling error.

A subculture was made from each of the positive AC P-A tests to yield pure cultures and isolates which could be identified to species level to determine if a total coliform was present. Total coliforms were isolated from >98% of positive AC tests (Table 5). *Klebsiella pneumoniae* was the most commonly isolated species, followed by *Enterobacter agglomerans* and *Citrobacter freundii*. *E. coli* was recovered from each fluorescent AC test. Positive AC P-A vessels demonstrated only minimal growth of heterotrophs on subculture. If a large number of heterotrophic bacteria are present in a water sample, all may not die; therefore, when an AC test is subcultured, it is necessary to examine several colonies to ensure that a total coliform is not overlooked.

**DISCUSSION**

The P-A concept is different in several major respects from the traditional quantitative water analyses. A P-A
There was also a concern that bacteria other than these tubes fluoresce. Thus, there were also no false-positive tests encountered in drinking-water samples. Moreover, unlike o-nitrophenyl-p-o-galactopyranoside tests, the defined substrate AC method does not quantify the number of coliform bacteria in a sample but only determines their presence or absence (i.e., hit or miss). It uses a statistical principle to define the minimum acceptable percentage of total coliforms isolated in the system over a particular time span (5, 23, 25). It also decreases the loss of recoverable total coliforms resulting from changes in transportation and storage (3, 5, 23, 25).

The Safe Drinking Water Act regulations propose a maximum contaminant level of no more than 5% of 100-ml samples per month containing total coliform (17). The AC test has demonstrated this level of sensitivity in the laboratory (13), in the national evaluation of the AC most-probable-number method (11), and in the current national evaluation of P-A methods. In this study there was no effect on the sensitivity of the AC method due to the presence of heterotrophic bacteria. During the course of the P-A evaluation, HPCs as high as 700,000/ml were detected. More than 25% of the 702 samples contained over 1,000 HPC per ml. The specificity of the AC test was established by subculturing positive P-A vessels and identifying the bacteria to species (Table 5). Yellow AC tests yielding total coliforms, and fluorescent AC tests yielded E. coli. These results were in keeping with the previous national most-probable-number evaluation, in which positive tests also yielded E. coli species of total coliform when yellow or E. coli when fluorescent (11). A theoretical concern about the specificity of the AC was the activity of β-galactosidase containing noncoliforms such as Aeromonas spp., which can yield false-positives in SM analyses. Unlike o-nitrophenyl-β-D-galactopyranoside tests used for species identification, which depend on bacterial inoculations of 10^3/ml and measure passive β-galactosidase, the AC test uses o-nitrophenyl-β-D-galactopyranoside as a defined substrate (13). Therefore, unless there are very large numbers of Aeromonas spp. (>10^3 to 10^5/ml present in the initial drinking-water sample, false-positive AC tests have not been found. This number of Aeromonas spp. is unlikely to be encountered in drinking-water samples but if found, should be considered a public health threat because of the association of Aeromonas spp. with waterborne disease (22). There was also a concern that bacteria other than E. coli might exhibit fluorescence. Therefore, tubes which did not produce yellow were exposed to 366-nm light. In no case did these tubes fluoresce. Thus, there were also no false-positive E. coli tests encountered during this survey.

The defined-substrate AC method was configured as a P-A test, which is compatible with the proposed Safe Drinking Water Act regulations. The results reported here show this method to be sensitive and specific for the simultaneous detection of total coliforms and E. coli in drinking water. Field testing demonstrated that statistics applicable to P-A tests in general can be used with it. It may be less costly than currently available methods (17).

### ACKNOWLEDGMENTS

We thank Ramon Lee for his helpful discussions during the course of this project. Edwin Goldreich (EPA) and Paul Berger (EPA Office of Drinking Water) are thanked for their review of the manuscript. Peter Charpentier (Yale University) and Terry Grady (EPA) are thanked for their help in the statistical analyses of the data. This study was reviewed by a project advisory committee of Joyce Kippin, Ipswich, Mass.; Richard Tobin, Health and Public Welfare, Canada; and Donald J. Reasoner, EPA.

This work was supported, in part, by the EPA through cooperative agreement CR811335 with the American Water Works Association Research Foundation.

### LITERATURE CITED


14. Evans, T. M., C. E. Waarvick, R. J. Seidler, and M. W.
APPENDIX C

NATIONAL FIELD EVALUATION PUBLICATION:

DST COMPARISON WITH MTF METHOD

(Reprinted here with permission of the American Society for Microbiology)
National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and *Escherichia coli* from Drinking Water: Comparison with the Standard Multiple Tube Fermentation Method

STEPHEN C. EDBERG,1,* MARTIN J. ALLEN,2 DARRELL B. SMITH,3 AND THE NATIONAL COLLABORATIVE STUDY†

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; American Water Works Association Research Foundation, Denver, Colorado 80235; and South Central Connecticut Regional Water Authority, New Haven, Connecticut 06511.

Received 21 January 1988/Accepted 18 March 1988

A defined substrate method was developed to simultaneously enumerate total coliforms and *Escherichia coli* from drinking waters without the need for confirmatory or completed tests. It is a new method based on technology that uses a hydrolyzable substrate as a specific indicator-nutrient for the target microbes. No equipment other than a 35°C incubator and long-wavelength (366-nm) light is necessary. To perform the test, one only has to add water to the powdered ingredients in a tube or flask. If total coliforms are present in the water sample, the solution will change from its normal colorless state (no target microbes present) to yellow. The specific presence of *E. coli* will cause the same tube to fluoresce under a longwave (366-nm) UV lamp. The test, called Autoanalysis Colilert (AC), was compared with Standard Methods—water and Wastewater 10-tube multiple tube fermentation (MTF) in a national evaluation. Five utilities, representing six U.S. Environmental Protection Agency regions, participated. All water samples came from distribution systems. Split samples from a wide variety of water sources were analyzed for the MPN-versus-MPN comparison. A total of 1,086 tubes were positive by MTF, and 1,279 were positive by AC. There was no statistical difference between MTF and AC. Species identifications from positive tubes confirmed the sensitivity of the AC. A national evaluation of the AC test showed that it: (i) was as sensitive as Standard Methods MTF, (ii) specifically enumerated 1 total coliform per 100 ml in a maximum of 24 h, (iii) simultaneously enumerated 1 *E. coli* per 100 ml in the same analysis, (iv) was not subject to false-positive or false-negative results by heterotrophic bacteria, (v) did not require confirmatory tests, (vi) grew injured coliforms, (vii) was easy to inoculate, and (viii) was very easy to interpret.

There are two standard methods for the enumeration of total coliforms from drinking water. The multiple tube fermentation (MTF) technique provides a most-probable-number (MPN) analysis after growth of total coliforms in liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technique enumerates total coliforms on the surface of agar by providing a liquid medium. The membrane filter (MF) technology originally designed to identify microbes by the analysis of their constitutive enzymes. This method uses a hydrolyzable substrate as a defined substrate for only the target microbe(s) one wishes to enumerate (S. C. Edberg and M. K. Edberg, Yale J. Biol. Med., in press). The technology is designated autoanalysis, because a color change is produced by the target microbe(s), with no need for confirmatory tests or technologist labor. To perform the test, one need only add water to the powdered formula in a tube or flask and incubate. No equipment other than an incubator and 366-nm lamp is necessary. Specific color changes denote the presence of the target microbe(s). Only the target microbes, total coliforms (yellow) and *Escherichia coli* (fluorescence), produce color changes during the test period.

In applying the autoanalysis technology to microbial water analysis, we hoped to achieve the following goals: (i) to specifically enumerate 1 total coliform per 100 ml in a

*Corresponding author.
†Investigators in the National Collaborative Study include the following: Mark LeChevallier, American Water Works Service Company, Belleville, IL 62220; Nancy Krich and Deborah Callan, Yale University School of Medicine, New Haven, CT 06510; Roger Ward and Dawn Calvert, California-American Water Company, Monterey, CA 93940; Wayne Jackson and Michelle Urce, Cobb County Marietta Water, Ackworth, GA 30101; Carol Storms and Judy Lorimer, Monmouth Consolidated Water Company, Tinton Falls, NJ 07724; and Thomas Trok and Michael Burns, West-Penn Water Company, Pittsburgh, PA 15226.
maximum of 24 h, (ii) to simultaneously, specifically enumerate 1 E. coli per 100 ml in the same test, (iii) to not be affected by heterotrophic plate count (HPC) organisms found in drinking water, (iv) to not require confirmatory tests, (v) to grow injured coliforms, (vi) to be easy to inoculate, and (vii) to be very easy to interpret.

The autoanalysis method was compared in a national evaluation with the 10-tube MTF test described in Standard Methods for the Examination of Water and Wastewater (1). The protocol explicitly followed was that of the U.S. Environmental Protection Agency (E.P.A.) Environmental Monitoring Support Laboratory for certification of an alternative method (6). Collaborative water utilities were chosen to reflect all sources of drinking water and to represent a variety of geographical locations, treatment techniques, and water quality conditions.

**TABLE 1. Characteristics of participating water utilities**

<table>
<thead>
<tr>
<th>Geographical area</th>
<th>Water source</th>
<th>Type of utility</th>
<th>Population served</th>
</tr>
</thead>
<tbody>
<tr>
<td>California, New Mexico, Arizona</td>
<td>Well, ground, surface</td>
<td>Private</td>
<td>&gt;250,000</td>
</tr>
<tr>
<td>Southwestern Pennsylvania</td>
<td>Surface, river</td>
<td>Private</td>
<td>&gt;530,000</td>
</tr>
<tr>
<td>Connecticut</td>
<td>Well, ground, surface, mixed</td>
<td>Municipal</td>
<td>400,000</td>
</tr>
<tr>
<td>Northern New Jersey</td>
<td>Well, ground, surface, river</td>
<td>Private</td>
<td>250,000</td>
</tr>
<tr>
<td>Northern Georgia</td>
<td>Surface</td>
<td>Municipal</td>
<td>250,000</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

Participants and samples. Five utilities, representing six U.S. E.P.A. regions, participated in the national evaluation (Table 1). The utilities ranged from those serving a single geographical area to those serving large numbers of small community water systems over three states. Water sources included deep and shallow wells, springs, rivers, and surface reservoirs. All water samples were obtained from distribution systems of the participating utilities; however, an effort was made to obtain water from locations most likely to yield positive samples, such as dead ends, storage reservoirs, and known problem sites. In accordance with U.S. E.P.A. Environmental Monitoring Support Laboratory guidelines, sites were permitted to mix small amounts of treatment effluent or raw water with a large volume of distribution water to achieve positive samples with natural microbial populations (6). Samples were also collected during periods of flushing. These water samples were not necessarily those used for routine monitoring for regulatory purposes. Sections of two of the utilities had been experiencing total coliform regrowth problems.

Water samples were collected, transported, and stored in strict accordance with the guidelines described by Standard Methods and the U.S. E.P.A. (6, 13). Either sterile polypropylene or glass flasks containing sodium thiosulfate were used to collect the samples.

AC test. The defined substrate method, called Autoanalysis Colilert (AC), was prepared for us as 10-tube MPN tests (Access Medical Systems, Branford, Conn.). The powder formula contained, per liter, the following: (NH4)2SO4, 5 g; Mn(SO4)2, 0.5 μg; ZnSO4, 0.5 μg; MgSO4, 100 mg; NaCl, 10 g; CaCl2, 50 mg; KH2PO4, 900 mg; Na2HPO4, 6.2 g; Na2SO4, 40 mg; amphotericin B, 1 mg; ortho-nitrophenyl-β-d-galactopyranoside (ONPG), 500 mg; 4-methylumbelliferyl-β-d-glucuronide (MUG), 75 mg; and Solanium, 50 mg. Solanium is a plant extract mixture that acts as an emulsifier.

All ingredients were obtained from Sigma Chemical Co. (St. Louis, Mo.), with the exception of Solanium (Access Medical Systems). Each test tube (13 by 100 mm) was aseptically filled and heat disinfected at 70°C for 0.5 h. The quality control parameters were based on the following criteria: the test must detect 1 CFU/100 ml in 24 h in environmental isolates of E. coli, Klebsiella pneumoniae, Enterobacter cloacae, and Citrobacter freundii and not be subject to interference by at least 10,000 CFU of Pseudomonas, Flavobacterium, and Aeromonas spp. per ml. A high proportion (1.5%) of tubes was chosen for quality control. Each of the test species was diluted to 1 CFU/100 ml, and an MPN analysis was performed. In addition, species of heterotrophs at concentrations of 10,000/ml were mixed with these bacterial concentrations in separate test tubes. Positive (yellow or yellow and fluorescent) had to be observed in 24 h.

The AC method was performed as follows: 10 ml of water was added to each tube, dissolving the powder after agitation and producing a colorless solution. The test tubes were incubated at 35°C for 24 h. Development of a yellow color after incubation indicated the presence of total coliforms in the test tube. Each positive total coliform test tube was exposed to a hand-held fluorescent (366-nm) light (Edmund Scientific Co., Barrington, N.J.) Fluorescence in the test specifically denoted the presence of E. coli. Therefore, a separate MPN analysis was obtained for total coliforms and E. coli by the 10-tube AC method. It was designed so that no confirmatory or completed tests need be performed with the AC test.

MTF test. The 10-tube MTF test was performed with each tube containing 10 ml of double-strength lauryl tryptose broth (LTB; Difco Laboratories, Detroit, Mich.). Positive tubes were confirmed in brilliant green lactose bile broth (BGLB broth; Difco) (4). The number of coliforms per 100 ml was estimated from a 10-tube MPN table (1). Tests that confirmed initial positive results were included in the data base.

HPC. An HPC was determined for each water sample according to Standard Methods using R2A agar incubated at 35°C for 48 h (1).

Evaluation protocol. Sufficient water was collected from each location to perform a simultaneous split sample analysis by the AC test and the MTF technique. For each water sample, the following microbiological analyses were performed: HPC, a 10-tube AC MPN analysis, and an MTF test (Fig. 1). Within the guidelines of the Handbook for Evaluating Water Bacteriological Laboratories (13), samples were prescreened for the presence of coliforms before being analyzed. The U.S. E.P.A. equivalency protocol called for four replicate analyses of each method from each sample. Accordingly, four rows of 10 LTB tubes and four rows containing 10 AC tubes were inoculated from each 1-liter water sample. LTB tubes were examined for positivity according to Standard Methods (1). Confirmation tests were also performed according to Standard Methods (1). The AC tube was examined at 24 h. Any yellow color was designated...
as positive for total coliforms; fluorescence denoted *E. coli*. From at least one positive MTF or AC tube per row, a subculture was made to ensure the presence of a total coliform by identifying the bacterial isolate(s) as to species. All bacterial identifications were performed by the API 20E system (Analytab Products, Plainview, N.Y.) (7).

**Statistical analysis.** The data were analyzed by using several statistical tests. The Statistics Section of the Department of Epidemiology of Yale University analyzed much of the data. First, by using the criteria of the U.S. E.P.A., *t* tests were performed. The *t* tests were calculated for each participating utility and for the utilities as an aggregate. The *t* tests were performed in two parts: first, by considering that the samples consisted of four replicates of 10 tubes for each utility and second, by considering each analysis to be a simple 40-tube MPN analysis (2, 12).

Second, since the *t* test is more appropriate to chemical than bacterial analysis, in which values are continuous rather than incremental, the data were also examined by chi-square analysis. Two chi-square tests were calculated: the Mantel-Haenzel and Pearson (2, 12). The Mantel-Haenzel test generates a separate 2 × 2 table from each replicate, with all statistics from all tables combined to produce a single test statistic. The Pearson test combines all samples into one 2 × 2 table (2, 12).

Third, the two methods were analyzed by regression analysis (2, 12). All statistics were generated to include and exclude all tubes, positive and negative.

**RESULTS**

**Comparison of Standard Method MTF and AC test.** (i) **Number of tubes positive.** A total of 46 distribution water samples were analyzed from the five sites. A total of 1,086 tubes were positive by MTF, and 1,279 were positive by AC. For the MTF analysis, the median number of tubes positive per 40 inoculated from a sample was 24, the mean was 23.6, and the standard error was 2.0. For the AC test, the median was 34.5, the mean was 24.8, and the standard error was 2.1.

The number of positive tubes for each of the four MPN rows for each utility is presented in Table 2. This measure allows an overall comparison between the two methods, and it permits this correspondence, regardless of the variation in an individual analysis.

(ii) **Effect of HPC on total coliform recovery.** Total HPCs ranged from 3 bacteria per ml to greater than 4,000/ml. Heterotrophic interference occurred in four fermentation test analyses. MTF tubes did not yield a coliform on subculture from these positive tubes. In each of these cases, the HPC was greater than 1,500/ml. HPC suppression of coliform growth resulted in a loss of between 17 and 24 tubes out of a possible 40 per sample (Table 3). Positive autoanalysis tubes did not demonstrate the growth of heterotrophs when samples were taken for subculture but did yield a coliform.

**Statistical analyses.** (i) *t* tests. The *t* test analysis is used by the U.S. E.P.A. to evaluate a proposed method for certification as an alternative testing procedure. *t* statistics were calculated for all sites as an aggregate and for each site individually, first excluding samples which yielded either all positive tubes or all negative tubes. There was no statistical difference in utilizing this measure between MTF and the AC, with an overall *P* value of <0.001. The *t* test was also calculated including samples that yielded either all positive tubes or all negative tubes. By paired *t* tests, which included samples yielding all positive or negative tubes, *Standard Methods* tests were inoculated from split samples to 4 rows containing 10 tubes each (see Materials and Methods).

**Note:** The number of positive tubes from the 10 tubes inoculated in that row, multiplied by the number of rows inoculated.

**Number of distribution water samples tested at the site.** Site C includes two geographically close utilities in the same U.S. E.P.A. region.

**TABLE 2. Comparison of Standard Methods and AC MPN results from split transmission water samples**

<table>
<thead>
<tr>
<th>Row</th>
<th>Site A (11)</th>
<th>Site B (11)</th>
<th>Site C (8)</th>
<th>Site D (8)</th>
<th>Site E (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>AC</td>
<td>Standard</td>
<td>AC</td>
<td>Standard</td>
</tr>
<tr>
<td></td>
<td>Methods</td>
<td></td>
<td>Methods</td>
<td></td>
<td>Methods</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>74</td>
<td>72</td>
<td>52</td>
<td>72</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>73</td>
<td>44</td>
<td>78</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>68</td>
<td>49</td>
<td>77</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>63</td>
<td>73</td>
<td>56</td>
<td>77</td>
<td>64</td>
</tr>
</tbody>
</table>

* MPN tests were inoculated from split samples to 4 rows containing 10 tubes each (see Materials and Methods).
* The number of positive tubes from the 10 tubes inoculated in that row, multiplied by the number of rows inoculated.
* Number of distribution water samples tested at the site. Site C includes two geographically close utilities in the same U.S. E.P.A. region.
Methods MPN gave a mean and standard deviation of 23.609 ± 13.881 (compared with 27.804 ± 14.120 by AC), with 46 paired observations; the t statistic was -4.196, with 45 degrees of freedom and 0.000 significance. Although the AC method appeared to yield somewhat higher MPN values (Table 2; also see Fig. 2), there was no difference between the two methods by this analysis, with a P value of <0.001.

(ii) Chi-square analyses. The two methods were also compared by two chi-square statistics. First, the Pearson chi-square test for the overall proportion of positive tubes was compared by two chi-square statistics. First, the Pearson chi-square test for the overall proportion of positive tubes was determined. By this analysis, three of the utilities demonstrated that the AC was more sensitive than the Standard Methods MTF (P > 0.05); there was a positive bias, with slightly greater AC sensitivity overall. The Mantel-Haenzel test compared the two methods by determining the difference between the positive tubes for each method and subtracting from it the expected number of positive tubes and analyzing the differences. This statistic yielded the same results as the Pearson test (Table 4).

(iii) Regression analysis. Data were analyzed in simple and multiple regression formats with the AC MPN being the x variable and Standard Methods MTF being the y variable. The two methods agreed with each other to yield an r value of 0.883 and an r² value of 0.779 (Fig. 2). The adjusted r² value was 0.774, and the standard error was 6.59 (Fig. 2). Analysis of variance demonstrated no difference between the two methods, with the F test yielding a P value of 0.001.

The beta coefficient table for the simple regression yielded a slope of 0.868, a standard error of 0.07, a standard value of 0.883, a t value of 12.47, and a P value of 0.0001. Therefore, there was no difference by regression analysis between the Standard Methods MTF and AC MPN, although some bias in favor of the AC test was evident.

(iv) Precision. For each of the two methods, assumptions of normality and homogeneity of sample variance were performed. The response analyzed was the proportion of positive samples under an arcsin transformation. For the locations meeting these requirements, an F test was used to analyze method precision. If not, Scheffé test was employed. Precision analysis revealed that the AC test was significantly more precise than MTF in two of the five sites (Table 5 courtesy of R. Freyberg and T. Grady, U.S. E.P.A. Environmental Monitoring and Support Laboratory).

(v) Likelihood ratio test. The likelihood ratio test compares the estimates of mean bacterial density obtained from different sets of data (20). If one assumes that the distribution of the number of positive responses at a given dilutional level for the MPN method is binomial, then the likelihood function may be calculated taking into account the volume of diluent, factors, etc. Calculation of the likelihood ratio (A) from the individual likelihood function L(λ) by the formula A = \frac{L(\lambda)}{L(λ)} showed a slight bias in favor of the AC method, -2 lnA significant at 0.5%.

TABLE 3. Recovery of HPC-suppressed Standard Methods tube by AC

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>No. positive</th>
<th>No. suppressed</th>
<th>No. positive by AC</th>
<th>HPC (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>11</td>
<td>17</td>
<td>28</td>
<td>&gt;3,000</td>
</tr>
<tr>
<td>22</td>
<td>17</td>
<td>22</td>
<td>39</td>
<td>&gt;4,500</td>
</tr>
<tr>
<td>30</td>
<td>22</td>
<td>18</td>
<td>40</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>42</td>
<td>2</td>
<td>24</td>
<td>26</td>
<td>&gt;2,500</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of the AC and Standard Methods MPN by regression analysis. Shown is the simple regression analysis (y = 0.868x - 0.524, with r² = 0.779). There was no difference when the comparison was made by multiple regression analysis.

TABLE 4. MPN method comparisons by paired t tests excluding samples yielding all positive or negative tubes

<table>
<thead>
<tr>
<th>Site</th>
<th>No*</th>
<th>Mean difference</th>
<th>SE</th>
<th>T</th>
<th>Probability of</th>
<th>Prob of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 1</td>
<td>Group 2 2</td>
<td>Group 1 3</td>
<td>Group 2 4</td>
<td>Group 1 5</td>
<td>Group 2 6</td>
</tr>
<tr>
<td>All</td>
<td>35</td>
<td>7.68</td>
<td>3.88</td>
<td>1.5965</td>
<td>0.90587</td>
<td>4.80</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>12.77</td>
<td>4.81</td>
<td>4.84141</td>
<td>2.26522</td>
<td>2.85</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5.95</td>
<td>2.93</td>
<td>2.59920</td>
<td>1.60107</td>
<td>2.29</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>8.31</td>
<td>2.99</td>
<td>5.41892</td>
<td>2.28604</td>
<td>1.53</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>11.74</td>
<td>7.95</td>
<td>3.35564</td>
<td>1.81269</td>
<td>3.50</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>0.63</td>
<td>0.18</td>
<td>1.25953</td>
<td>1.00181</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Number of tests, excluding all positive or negative tubes.
1 Group 1 considered the samples as one replicate of 40 tubes, whereas group 2 considered the samples as four replicates of 10 tubes.
Performance characteristics. The original performance goals for which the AC was designed were met, and additional benefits became apparent. The collaborators felt that the yellow color produced by the total coliforms and the fluorescence generated by E. coli were distinct, easy to read, and apparent within the time framework of the test (24 h). They also noted that total coliform counts greater than 25/100 ml yielded results in 16 to 18 h. Separate MPNs for total coliforms and E. coli were produced simultaneously. All participants affirmed the ease of one-step inoculation and clarity of interpretation of positive test results. They substantiated the autoanalytical nature of the test by verifying the goal that confirmatory tests need not be performed because of the specificity of the ingredients.

Bacteria isolated. Bacteria isolated from each split sample were identified as to species (Table 6). Bacteria commonly considered part of the total coliform group were found from both Standard Methods MTF and AC tubes. K. pneumoniae was the most common species isolated, followed by E. cloacae and C. freundii. There were mixed cultures of total coliforms present in both MTF and AC tubes. The higher the bacterial count in the water sample, the greater the number of species found. There did not appear to be a significant difference in the distribution of bacterial species in either method.

DISCUSSION

The AC test is based on a new technological principle in which an indicator-nutrient is an essential food source for the target microbe (Edberg and Edberg, in press). Because a chromophore is released each time an indicator-nutrient molecule is hydrolyzed, color production is directly related to the growth of the target bacteria. In the application of this technology to drinking water analysis, the indicator-nutrient is an ONPG (for total coliforms) and MUG (for E. coli). Unlike classical enzyme assays, which use ONPG or MUG only as substrates for constitutive enzyme tests, the autoanalysis technology uses these compounds both as an essential growth substance and as an indicator system (color formation and fluorescence). Therefore, there is not a direct correspondence between tests in which ONPG and MUG are used as substrates in constitutive enzyme tests (e.g., Coliform; Millipore Corp., Wallingford, Conn.) and the growth-dependent AC test. For example, species in the genera Aeromonas, Pseudomonas, or Flavobacterium that may be positive in a constitutive ONPG or MUG enzyme assay will not be positive in the 24-hour growth-based AC test, unless a high density (≥20,000 bacteria per ml) is present (Edberg and Edberg, in press).

In conventional medium, several secondary reactions must occur before a change in the indicator is visible. The target microbe must transport the substrate (e.g., lactose) through the cell membrane, transform the substrate to glucose, metabolize glucose through the glycolytic cycle to pyruvate, and then convert pyruvate to the desired end product, either acid or gas. Because conventional testing requires the microbe to go through many steps to yield a positive visible endpoint, a number of anomalous results may occur, such as false-negative gas producers (i.e., anaerobic E. coli) or false-positive tests (i.e., acid from lactose by some clones of Aeromonas hydrophila). ONPG-positive noncoliforms do not yield a positive AC test, because the formula does not support their metabolism. Therefore, the detection of the β-galactosidase system of heterotrophs will occur only at extremely high microbial concentrations (>20 to 100,000/ml). This phenomenon will be seen with approximately 10% of members of the genus Aeromonas.

The specificity of the defined substrate autoanalysis technology eliminates the need to perform confirmatory and completed tests. In conventional methodology, transfers from the original positive tube or plate must be made into BGLB broth for confirmatory testing and a second transfer must be made to solid or liquid medium for a completed test. As substantiated in this national evaluation (Table 6), the 24-h AC test is as specific as the multiple day confirmed and completed Standard Methods MTF analysis.

Species identification from positive tubes confirmed the specificity of the AC test. From positive AC tubes, members of the total coliform group were recovered. Therefore, yellow test tubes were only the result of the growth of the target microbe(s). Likewise, E. coli was routinely recovered from tubes that fluoresced.

Table 5. Precision of the two methods by source

<table>
<thead>
<tr>
<th>Location</th>
<th>F test</th>
<th>Scheffe test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobb County, Ga.</td>
<td>0.0131</td>
<td>0.2266</td>
</tr>
<tr>
<td>Monterey, Calif.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tinton Falls, N.J.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Haven, Conn.</td>
<td>0.0006</td>
<td>0.5725</td>
</tr>
</tbody>
</table>

* Standard Methods MTF was significantly less precise than the AC method.

Table 6. Species of total coliforms isolated

<table>
<thead>
<tr>
<th>Species</th>
<th>% of all isolates belonging to species by method:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard Methods</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>31</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>3</td>
</tr>
<tr>
<td>Enterobacter agglomerans</td>
<td>19</td>
</tr>
<tr>
<td>Enterobacter species</td>
<td>3</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>10</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>1</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>16</td>
</tr>
<tr>
<td>Serratia plymuthica</td>
<td>3</td>
</tr>
<tr>
<td>S. fonticola</td>
<td>1</td>
</tr>
<tr>
<td>S. rubidaea</td>
<td>3</td>
</tr>
<tr>
<td>S. odorifera</td>
<td>2</td>
</tr>
<tr>
<td>Hafnia alvei</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
</tr>
<tr>
<td>CDC groups</td>
<td>3</td>
</tr>
<tr>
<td>Unidentified Enterobacteriaceae</td>
<td>3</td>
</tr>
</tbody>
</table>

* All isolates confirmed in BGLB.
Several benefits other than those described above were noted by participating water utilities during the national evaluation. The autoanalytical nature of the method made it much more practical and efficient to perform than current coliform methods. The participants felt that interpretation of the AC test was less subjective than that of the current Standard Methods procedures. It was much easier to see whether a yellow color or fluorescence was produced than to decide whether LTB tubes were positive. The determination of gas production is less compared with that of noninjured coliforms (Edberg and Edberg, in press). Because the colors were distinct, it was possible that a moderately trained individual could interpret the AC test. Furthermore, since there were no additional tests needed, an analysis did not extend through weekends, which could result in a delay of 2 to 5 days in obtaining definitive results. Also, in contrast to other methods in which weekends added 2 or more days to the analysis, several participants said that they would be willing to test water on Friday and have someone begin it on Saturday to read the results, thus adding only 1 day to the testing regimen. Virtually any water utility employee could accurately read and record the AC test results.

Because the ingredients are in powder form and have an unrefrigerated shelf life of at least 1 year, the participants felt that they could stock a large number of tubes. This would enable the utilities to respond quickly to either changes in the water flow or emergencies.

A strength of the AC test is its applicability to use by the small utility. First, the AC test can be inoculated in the field. Once the formula is hydrated, the bacteria begin to grow, although more slowly at ambient than incubator temperature. Moreover, one avoids any change in the bacterial composition of the water sample during storage and transport. After incubation, the characteristic nature of the colors makes it possible for the small-utility operator to determine the presence of both total coliforms and E. coli. This immediate reading of results could eliminate, with state approval, the inherent delay in sending samples to commercial laboratories. Prolonging sample transit times results in questionable or unacceptable results (6). In addition, significant monetary savings can be realized by the small utility, because their only cost for bacteriological water monitoring is the cost of the test itself. If state certifying agencies require independent verification of the test, the small utility can forward the AC tube to a reference laboratory. The target microbes remain viable in the test for at least 7 days and can be analyzed by another laboratory, if required. The double-strength lactose broth (DSLB) can also be inoculated in the field. However, the determination of a positive result from DSLB is much more subjective than the reading of an AC tube. Furthermore, once color forms in the AC tube it is permanent; extended incubation of the DSLB can result in changes in the medium which make it difficult to interpret.

The cost of the AC test for a single water sample is between 20 and 100% less than Standard Methods MTF testing, depending upon whether the utility performs the analysis itself or sends it to an outside laboratory (commercial or regulatory). The participants felt that the major cost saving associated with the test lies in decreased labor and better quality control.

The AC test provides the simultaneous detection of the major fecal coliform, E. coli, for no additional cost or effort. Current Safe Water Drinking Act Regulations do not currently require the routine analysis for this species. However, the new regulations may mandate it (11). It is generally thought that E. coli is a much more specific indicator of the fecal pollution of freshwater than are total coliforms. The total coliform group is used now, because it has been technically difficult to analyze water directly for this species (5). Public health authorities generally feel that the absence of E. coli, even when distribution water has total coliforms in a biofilm regrowth situation, indicates that the finished water is acceptable for consumption (17). Therefore, the ability to simultaneously assay for both total coliforms and E. coli is an added dimension to water analysis not previously available. It will provide utilities an immediate measure of whether a sample has been subject to fecal contamination. The utility would not have to perform fecal coliform analyses or bacterial identifications on colonies after subculture of the primary test, as they do now. This additional testing could delay a definitive answer for several days, an undesirable situation engendered by the current available technology. In many cases, additional water samples have to be tested if one wished to pursue a possible pollution event further to determine whether E. coli was present in the present sample; with the AC method, this would not be necessary. The analyst would know whether total coliforms, for regulatory purposes, and E. coli, for public health purposes, were present in the same sample.

It should be noted that the AC test has specific limitations. First, while it has been found to be refractory to the density of heterotrophs encountered in the national evaluation, in the laboratory one species of bacterium, A. hydrophila, was found to yield a positive endpoint (yellow color) after 32 h of incubation at very high densities. A total of 20 Aeromonas isolates were tested, and a wide range of activity was observed; however, 2 isolates showed positive total coliform reactions at inoculation densities of 20,000/mL at 32- and 36-h incubation, respectively (Edberg and Edberg, in press). While these Aeromonas concentrations are unlikely to be encountered in drinking water, and the test should not extend beyond a 24- to 28-h incubation period, it is recommended that any AC test incubated for more than 30 h and then noted as positive should be confirmed by a BGLB broth, other Standard Methods confirmation, or species identification.

Isolates of Pseudomonas and Flavobacterium species did not demonstrate positive total coliform or E. coli tests at concentrations up to 50,000 bacteria per ml. Petzel and Hartman (22) did report Flavobacterium sp. isolates that yielded a positive constitutive enzyme MUG test from bacterial colonies. These were tested (courtesy of Eugene Rice, U.S. E.P.A.) and did not yield a positive result until densities unlikely to be found in drinking water (≥100,000 bacteria per ml) were reached (unpublished results). It appears that at very high bacterial densities, the AC test may act as an enzyme assay and become positive, not through a growth mechanism, but by direct enzyme measurement. Therefore, the AC test should not be used as a confirmatory test from either MTF broths or colonies from plates.

Second, laboratory testing has shown that injured coliforms exhibit a longer lag phase than normal coliforms in the AC test (Edberg and Edberg, in press). It was found that once the injured coliforms repaired themselves and entered log phase they grew as rapidly as normal coliforms. Practically, this meant that the intensity of yellow color produced at 24 h by injured coliforms may be less than that produced by normal coliforms. If kept between 2 and 4 h longer than
the 24-h incubation period, the injured coliforms achieved the same color level as normal total coliforms. Therefore, it is recommended that if no color is noted at 24 h, the result should be considered negative; if any yellow color is noted, the result should be considered positive. If the analyst is unsure of color formation, he or she should reincubate the test for an additional 2 to 4 h and examine it for an increase in yellow color. This additional incubation period will also account for the slightly yellow color that some waters may have due to humic material, because these tubes will not increase their color intensity and can be classified negative.

Third, although the AC determines the presence of E. coli simultaneously with total coliforms, this should not be considered the same as a fecal coliform test. E. coli makes up approximately 90% or more of fecal coliforms, but other bacteria, notably K. pneumoniae, may also fall into this category if it grows at 44.5°C in E. C. broth with the production of gas (1). Therefore, if E. coli is found by the AC test, one may consider the sample to be fecal coliform positive; however, if E. coli is not detected, there may still be fecal coliforms in the sample.

Lastly, the national evaluation of the AC test was limited by design to drinking water distribution samples. If the test is to be applied to a different water source, such as storm runoff, marine waters, waste effluents, etc., the user should first establish the efficacy of the test in the environment. In summary, the AC test can simultaneously detect total coliforms and E. coli from a water sample within 24 h. No confirmatory or completed tests need be performed. HPC bacteria do not interfere in densities likely to be encountered. All one does is add water to the tubes and incubate them for a maximum of 24 h. Interpretation of the endpoints, yellow for total coliforms and fluorescence for E. coli, is distinct. A national evaluation conducted according to U.S. E.P.A. protocol demonstrated that this test was equivalent with Standard Methods MTF. The AC test is less costly than Standard Methods MTF and requires considerably less labor. It allows small utilities to test as accurately as large utilities to test, as accurately as large.

ACKNOWLEDGMENTS
We thank Ramon Lee for his helpful discussions during the course of this project. Edwin Geldreich, U.S. E.P.A., is thanked for his review of the manuscript. This study was reviewed by a U.S. E.P.A. protocol demonstrated that this test was equivalent with Standard Methods MTF. The AC test is less costly than Standard Methods MTF and requires considerably less labor. It allows small utilities to test as accurately as large utilities to test, as accurately as large.

LITERATURE CITED
ERRATUM

National Field Evaluation of a Defined Substrate Method for the Simultaneous Enumeration of Total Coliforms and Escherichia coli from Drinking Water: Comparison with the Standard Multiple Tube Fermentation Method

STEPHEN C. EDBERG, MARTIN J. ALLEN, DARRELL B. SMITH, AND THE NATIONAL COLLABORATIVE STUDY

Department of Laboratory Medicine, Yale University School of Medicine, P. O. Box 3333, 333 Cedar Street, New Haven, Connecticut 06510; American Water Works Association Research Foundation, Denver, Colorado 80235; and South Central Connecticut Regional Water Authority, New Haven, Connecticut 06511

Volume 54, no. 6, p. 1596, column 1, paragraph 4: "Mn(SO4)2, 0.5 µg" should read "Mn(SO4)2, 50 mg"; "ZnSO4, 0.5 µg" should read "ZnSO4, 50 mg"; "Solanium, 50 mg" should read "Solanium, 500 mg".