Significance of Methods and Sample Volumes for *E. coli* and Total Coliform Measurements

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DISCLOSURE STATEMENT

The principal investigator (PI) for this project, Colin Fricker, CRF Consulting, is one of the inventors of Colilert-18®, a detection method for coliforms and *E. coli* in water. In this project scope of work, Dr. Fricker tested Colilert-18® compared to other USEPA approved methods and methods approved in the UK for the detection of coliforms and *E. coli* in water. The PI (Colin Fricker) and the co-PI (Analytical Services, Inc.) have stated that they did not, and do not intend to, benefit financially from the PI's relationship to the invention of Colilert-18®.

Patent information: United States Patent: Ehrenfeld, et al.; Medium for detecting target microbes in a sample (Patent number 5,610,029), March 11, 1997. The invention features a medium for the detection of target microbes in a liquefied environmental or biological sample in less than 18 hours.

Inventors: Ehrenfeld; Elizabeth (Falmouth, ME), Fricker; Colin (Reading, GB3), Townsend; David E. (Scarborough, ME)

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FOREWORD

The Water Research Foundation (Foundation) is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of consultation with subscribers and drinking water professionals. Under the umbrella of a Strategic Research 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. The Foundation also sponsors research projects through the unsolicited proposal process; the Collaborative Research, Research Applications, and Tailored Collaboration programs; and various joint research efforts with organizations such as the U.S. Environmental Protection Agency, the U.S. Bureau of Reclamation, and the Association of California Water Agencies.

This publication is a result of one of these 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 non-member 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 and consultants and manufacturers subscribe based on their annual billings. 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 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.

The selection of methodologies for the detection of coliforms and *Escherichia coli* is an important issue for all water utilities throughout the world. Comparing the performance of various procedures was the primary goal of this project. The project's intent is to assist utilities and regulatory agencies in selecting appropriate procedures for detecting coliforms and *Escherichia coli* in water supplies. Additionally, the data presented may help other researchers interested in developing procedures for comparison of performance of microbiological methods.

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Chair, Board of Trustees
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Executive Director
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EXECUTIVE SUMMARY

OBJECTIVES

The overall objectives of this project were to provide new information on the relative merits of the various procedures approved by the USEPA and the DWI for the microbiological examination of drinking water. The specific objectives of the project were:

1. Perform a literature review of microbiological methods that are being used for the detection of coliforms and *E.coli* in water,
2. Perform an initial assessment of the performance of coliform and *E.coli* methodologies which are approved for use in the US and the UK,
3. Perform in the field intensive investigations of the use of up to three methods with different sample volumes,
4. Apply selected methods for the examination of "follow up" samples after detection of coliforms by routine sampling, and
5. Round robin testing of selected methods at utilities within the US.

BACKGROUND

For many years, water microbiologists have sought improved methods for the detection of coliforms and *E. coli*, in a quest to utilize more sensitive, yet more specific tests. In the past twenty years there has been an explosion of interest in the development of methods and many laboratories have adopted procedures that rely upon the detection of expression of the enzymes β-D-galactosidase and β-D-glucuronidase for total coliforms and *E. coli* respectively. While these methods offer substantial benefits in terms of ease of use and simplicity, it remains unclear how the results generated using such methods relate to those generated using more traditional methodologies such as those based upon fermentation of lactose and growth at elevated temperatures.

The debate as to the significance of total coliform organisms with respect to public health has fuelled the fire in relation to choice of methods. Some workers believe that the newer technologies offer a more taxonomic approach to the detection of coliforms and *E. coli*, while others believe that the broader range of organisms detected by methods utilizing β-D-galactosidase means that the total coliform group has even less meaning for the indication of water safety. What is clear is that methods that utilize β-D-galactosidase for the detection of coliforms will detect a wider range of coliforms than methods that rely solely on the fermentation of lactose.

The ability to accurately, quickly and cost-effectively detect coliforms and *E. coli* is critical to water utilities in order to protect public health. This project focused on a comparison of the performance of the various methods that are approved by the U.S. Environmental Protection Agency (EPA) and the Drinking Water Inspectorate (DWI). While the ability to determine exactly which method would give the best overall performance would be an ideal achievement, the experiments described are an attempt to determine some of the performance characteristics of the various methods such that microbiologists can make more informed decisions about the methods that they use for microbiological monitoring.

The project was undertaken by a team of researchers from Analytical Services Inc, Williston, Vermont. The project was also supported by a number of microbiologists from a range of utilities in both the U.S. and the U.K.

APPROACH

The initial portion of this study involved an extensive literature review of microbiological methods used to detect the presence of coliforms and *E. coli* in drinking water. Following this initial phase, the study focused on comparing the performance of various culture media in recovering total coliforms and *E. coli* from drinking water and similar matrices. Pure cultures of accurately counted bacteria were used initially to determine differences in inherent sensitivity. Subsequent experiments utilized diluted raw water and disinfected sewage effluents to determine both sensitivity and specificity. These experiments were also used to determine the usefulness of the ISO procedure 17994 for the comparison of microbiological methods. In assessing the performance of microbiological methods, sensitivity and specificity were used in addition to other measurements such as false positive and false negative rates. After establishing some baseline information on a number of the methods, field trials were conducted using two procedures to analyze both 100 mL and 2 L samples. The differences in the number of samples found to contain coliforms using the different volumes and different methodologies were compared.

Different procedures were used to attempt to define the performance of the different media in recovering coliforms and *E. coli*. Accurately counted pure cultures of bacteria were used to investigate inherent sensitivity and some differences were seen between different media. However, this procedure actually measured the inhibitory nature of the media towards target organisms and while some useful information was produced, the performances of the methods were quite different when compared with samples containing competing flora. Competing flora have two major influences on the performance of culture media such as those used for the detection of coliforms in water. Firstly, bacteria such as *Aeromonas* and *Pseudomonas* can inhibit the growth of coliforms due to the production of bacteriocins. Secondly, the growth of competing flora can make the detection of target organisms difficult due to overgrowth.

Some effort was expended in looking at the performance of the various methodologies that use β-D-glucuronidase as a marker for *E. coli.* A distinct flaw in the design of one medium was noted.

Nine laboratories, including Analytical Services, Inc. (ASI), took part in a small round robin study to simply assess the ease of use of two procedures for analysis of 100 mL and 2 L volumes. The study was too small to look at the variation between laboratories, but did yield some interesting results with respect to the performance of the two selected methods.

During the course of the study an unusual phenomenon was encountered which had not been described previously. Essentially, the tap water supplied to the laboratory at ASI was toxic in varying degrees to bacteria, but the toxicity was neither consistent nor regular. These data were eliminated from the study to avoid bias. ASI applied for a small amount of additional funding through the Project Continuation Research Fund to investigate the cause of the toxicity.

RESULTS AND CONCLUSIONS

During the course of the literature review it became clear that while there have been an extraordinary number of studies comparing the relative merits of various media, there has been little attempt to develop a standard protocol for the comparison of microbiological methods in the United States. There is an International Standards Organization (ISO) procedure for comparing methods and whilst the basis of this procedure gives an outline of how to compare methods, some of the detail (for example the number of target bacteria present in samples) needs to be amended.

This study did not determine that any one single method was superior to all others. However, it was clear that methods that utilize detection of β-D-galactosidase for identifying the presence of coliforms will detect more coliforms (a higher concentration and a broader range) than methods that rely upon lactose fermentation. This may be the result of methodological differences and also different definitions for the coliform group. There are differing opinions as to the potential benefits of the coliform group being expanded. Furthermore, media that are incubated at elevated temperature (44-44.5^{o}C) tended to recover fewer *E. coli* than methods that rely upon detection of β-D-glucuronidase activity, and this is important for public health and safety. The methods that relied upon lactose fermentation as a phenotypic marker for the presence of total coliforms or *E. coli* tended to have more false positive "presumptive" results (i.e. organisms appeared to be the target organism on primary isolation but were subsequently shown to be a false positive) for both groups of organisms. This is of significance in dealing with positive microbiological tests since responses to finding total coliforms or *E. coli* in drinking water should be undertaken as soon as possible and should not be delayed pending the results of confirmatory tests. For total coliforms the determination of whether methods show high false negative rates depends upon the definition used for the total coliform group. Many authorities around the world now describe the total coliform group as "members of the *Enterobacteriaceae* that produce the enzyme β-D-galactosidase. That definition has been adopted by the International Standards Organization and has been used throughout this study. When using this definition, the lactose-based methods for total coliforms detect considerably fewer coliforms than the methods based upon β-D-galactosidase. For *E. coli*, the false negative rate is not so clear cut and differences between media seem to be based more upon the composition of the media rather than the phenotypic traits used to identify *E. coli*. It did appear however, that methods based upon incubation at elevated temperature detected fewer *E. coli* although this difference was not clear.

There were differences in the performance of liquid media (presence/absence tests) and also differences between different media that are based upon membrane filtration. There was no clear difference between membrane-based methods and presence-absence methods, although of the membrane-based methods, those that simultaneously detect coliforms and *E. coli* generally performed better than those that utilize two membranes to separately recover coliforms and *E. coli.*

The testing of 2 L volumes of water resulted in significantly more coliform detections when compared to 100 mL samples, which is not in itself surprising. However, the increase seen was very different in the two sites that were investigated during this study. The use of 2 L samples can be recommended for investigation of possible contamination events but examination

of this larger volume cannot be considered appropriate for routine regulatory or operational sampling with the use of currently available methods.

During the course of the work performed it became clear that some dechlorinated tap water samples had a considerable bactericidal effect. This effect was only noted in samples processed in one laboratory. Investigation of the processes used at the water treatment plant supplying the laboratory and microbiological testing demonstrated that this was due to the presence of residual polyelectrolyte (Magnifloc 572C) in the municipal supply. While this may not have a significant impact on the overall microbiological data for a water system, the effect can significantly reduce the number of bacteria in specific samples, and may be especially important if samples are not analyzed immediately. Holding samples for up to 24 hours could impact the likelihood of detecting coliforms and hence utilities should do their utmost to eliminate or minimize residual polyelectrolyte in municipal water systems and minimize hold times if polyelectrolyte is present. Somewhat counter-intuitively, the bactericidal effect was inversely proportional to concentration of the polymer, with low concentrations having the largest effect on cell recovery.

APPLICATIONS / RECOMMENDATIONS

The researchers recommend that utilities use the following criteria to select their method for both regulatory and operational monitoring; the methodology used should be sensitive, specific and accurate, simple to use and with minimal time for a confirmed result. Sensitivity and specificity address the issues of matrix effects and the effects of interfering organisms. If the method used is simple to perform, requiring minimal microbiological expertise, smaller systems will derive additional benefit. This study has provided data that indicate that methods based upon detection of activity of the two enzymes β-D-galactosidase and β-D-glucuronidase tend to be more specific, sensitive and accurate than more traditional methods based upon lactose fermentation. However, there may be matrix effects which were not studied here that could be specific to any given water source. It is crucial therefore that any method is tested for its suitability with given water sources.

Whilst the more recently developed, enzyme-based procedures cost more in materials than the traditional, lactose-based membrane filtration methods, there are many potential savings to be made when using a method that gives a more rapid and accurate result. The cost of re-sampling can be reduced with fewer false positives and the ability to act quickly and decisively based on an accurate result can lead to considerable cost benefits. The enzyme-based methods generally require less time to prepare and quality assure and this in itself can be a substantial cost saving.

It is recommended that utilities with an interest in "background flora" decouple this interest from coliform / *E. coli* monitoring, and perform heterotrophic plate counts to gather these data. Information gathered regarding background flora as a by-product of coliform / *E. coli* monitoring is likely to be both incomplete and skewed. This is because media designed to detect coliforms contain inhibitors to prevent the growth of non-target organisms and the population of non-target organisms recovered is likely to be small and a variable proportion of the heterotrophic plate count.

The researchers do not recommend increasing the volume of samples used for regulatory monitoring. However, it is recommended that utilities strongly consider the use of larger sample volumes when investigating the source of coliform organisms in water supply systems. Confirmation of positive results in these situations is required. In this study, the use of two liter (2 L) samples facilitated the detection of considerably more coliform-positive samples than did the use of 100 mL samples. It is unclear if this would lead to better public health decisions but the use of larger samples has been useful in determining the source of total coliforms in water distribution systems.

This study has demonstrated that a polymer added as a coagulation aid has bactericidal properties and its presence in treated water can lead to inaccurate microbiological results. Utilities that use such polymers should do their utmost to ensure that there is no residual polymer in the final water or, that if this is unavoidable, that they are able to demonstrate that it has no impact on microbiological data.

Overall the data generated during this study is largely in agreement with many of the other studies that have previously been reported. It is clear that methods based upon detection of the expression of β-D-galactosidase for detecting total coliforms detect more target organisms than those based upon lactose fermentation. In general, the "newer" methods for total coliforms and *E. coli* out-performed the traditional lactose-based methods which is in keeping with the majority of studies described in the literature.

CHAPTER 1
INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

INTRODUCTION

In recent years there has been much debate about the relevance of coliform organisms as indicators of the safety of drinking water as well as the differences in sensitivity and specificity of the various methods used to detect coliform bacteria in water. In particular there has been considerable discussion about the different definitions used for the description of both total coliforms and *Escherichia coli (E. coli).* The International Organization for Standardization (ISO) has recently adopted definitions that describe the organisms as possessing specific enzymes: β-D-galactosidase for total coliforms and β-D-glucuronidase for *E. coli.* Many of the methods for the detection of coliforms and *E. coli* in drinking water have been in use for many years and do not utilize these definitions, usually relying on lactose fermentation and resistance to bile salts as principal distinguishing characteristics. This means that the variety of approved methods that are available for use in the US and the UK have different targets and, therefore, comparison of their performance is difficult.

This project focused on methods that are approved for the microbiological examination of drinking water in the United States and the United Kingdom. The following methodologies were studied:

- Membrane Lauryl Sulfate Broth
- Membrane Lactose Glucuronide Agar
- Lactose Tergitol TTC Agar
- M- endo LES
- mFC
- MI Agar
- Chromocult®
- M-ColiBlue24®
- Coliscan®
- E*Colite
- ReadyCult®
- Colitag®
- Colilert®
- Colisure®
- Colilert-18®

Initial comparisons were made using all methods and selected pure cultures of organisms and these experiments were followed by comparisons using "spiked" samples of drinking water. There followed extensive study used the procedure described in the International Standard, *Water quality – Criteria for establishing equivalence between microbiological methods* (ISO 17994:2004) for the comparison of performance of quantitative microbiological methods. This was a particularly interesting aspect of the work as it clearly demonstrated that there are considerable flaws in the documented procedure and that revision is necessary. Where the different statistical approaches described in the document would gave different outcomes these

are described in this report. In addition, periods of intensive study were undertaken at two public water suppliers using two different methodologies and comparing, 100 mL and 2 L samples of water. The final aspect of the original project was to compare two methods that had performed well during the two-year project in a number of laboratories using a "round robin" format. Samples were prepared at Analytical Services, Inc., and sent to participating laboratories as "blind" samples. The results were then returned and the data analyzed at ASI.

OBJECTIVES

The overall objective of this project was to investigate the relative performance of the plethora of methods that are approved for the examination of drinking water in the United States and the United Kingdom. It was not the intent of this project to produce a "league table" demonstrating the superiority of one method over all others but rather to identify strengths and weaknesses of groups of methodologies and, where possible, individual methods themselves. Specifically the original objectives of the project were:

1. Perform a literature review of microbiological methods that are being used for the detection of coliforms and *E. coli* in water,
2. Perform an initial assessment of the performance of coliform and *E. coli* methodologies which are approved for use in the US and the UK,
3. Perform in the field intensive investigations of the use of up to three methods with different sample volumes,
4. Apply selected methods for the examination of "follow up" samples after detection of coliforms by routine sampling, and
5. Round robin testing of selected methods at utilities within the US.

During the course of the project an interesting finding emerged. Tap water from a municipal supply was shown to have toxicity towards coliform organisms and further funds were awarded to investigate the cause of the toxicity and its likely effect on coliform monitoring data. The specific objectives of this aspect of the work were:

- What part of the treatment process (or addition of what chemical) causes the bactericidal effect?
- Does the concentration of the chemical responsible have a marked effect on inactivation?
- To what degree are coliforms inactivated by these waters over the commonly used hold times for microbiological samples?

REVIEW OF THE LITERATURE

The link between consumption of contaminated drinking water and human disease is largely attributed to John Snow. When investigating the London cholera outbreak of 1854, Snow demonstrated that human cases of cholera were more frequently associated with consumption of water abstracted from the River Thames downstream of the City of London than with consumption of water abstracted upstream. This was a revelation at the time when it was commonly accepted that most all infectious diseases were spread by inhalation.

Following this discovery, Escherich, a German pediatrician, investigated the bacteria present in feces and found that "*Bacterium coli* commune" (now named *Escherichia coli*) was found in fecal material from animals and humans beings. Research in this area progressed rapidly and the Franklands started to monitor water regularly using gelatine plates and demonstrated that the use of sand filters reduced the numbers of bacteria in water substantially. Furthermore, they proposed the concept of "indicator organisms" by recognizing that individuals with enteric diseases excreted both "normal flora" and pathogens. Hence they suggested that looking for organisms "characteristic of sewage, i.e. normal flora" would be indicative of the presence of pathogens (Frankland and Frankland, 1894). These workers also suggested that drinking water should have a maximum concentration of organisms of 100 CFU/mL, a level which continues to be used as a guideline in many countries, although the US and Canada adopted a guideline level of 500 CFU/mL.

At about the same time, but independently of the Franklands, Schardinger (1892) proposed the use of what is now termed *E. coli* as an indicator of fecal contamination. This concept was based upon four criteria:

- It was abundant in animal and human fecal material
- Was absent in the environment
- Could be easily detected because of the ability to ferment glucose (later changed to lactose)
- Was easier to isolate than pathogens

These findings, along with Koch's "germ theory of disease", were the basis for the modern use of indicator organisms in the examination of drinking and food. Schardinger correctly suggested that the presence of *E. coli* in drinking water was indicative of the presence of feces and consequently of enteric pathogens.

Thus the indicator concept was born. Whilst the concept was sound, the practicalities of use were complex due to the presence of other organisms with phenotypic characteristics which were very similar to those of *E. coli*. This difficulty has remained with environmental microbiologists for over one hundred years and even today there are disagreements as to the use of indicators, their definitions and significance. The confounding organisms which were isolated and characterized at around the same time were *Aerobacter* (now *Enterobacter*), *Klebsiella* and *Citrobacter*. Since these organisms had such similar phenotypic characteristics, it was quickly understood that the microbiological methodologies available at the time would not easily distinguish between them and that it would be far simpler to group the organisms together. This was attributed to Blachstein (cited by Tallon *et al.*, 2005) who called the group "coliforms". In this initial description of "coliforms" it would appear that the definition was "taxonomic", i.e. the group was comprised of four genera. However, in practice the group was defined by the physiological characteristics of the bacteria and as methodologies were developed, the definition of "coliforms" has become very much method dependent. Whilst this work represented a major step forward in water microbiology, it also raised a number of issues many of which have yet to be adequately resolved.

It became apparent that the "coliform" group of organisms did not originate exclusively from fecal material and consequently adaptations were made to the methodology to make tests more specific. The most relevant of these was the elevation of the temperature of incubation described by Eijkman (1904). He found that using a temperature between 44°C and 46°C

inhibited the growth of many "non-fecal" coliforms whilst allowing growth of "fecal" organisms and Winslow and Walker (1907) made the important observation that *E. coli* was fecal in origin whilst many other coliforms were not. The concept of "fecal coliforms" had been developed.

As knowledge progressed it became clear that the term "fecal coliform" was a misnomer since many of the organisms which fall into this group can be of environmental origin, whilst other coliform organisms can be present in feces. Consequently, whilst the term "fecal coliform" is still used, many workers now refer to this group as "thermotolerant coliforms" which, whilst not particularly informative, is not as misleading as using the term "fecal". It has become recognized that the only true "fecal" coliform is *Escherichia coli* since this organism is almost exclusively derived from fecal material. It should be noted that there are limited reports from tropical areas that have suggested the presence of high concentrations of *E. coli* in natural waters in the absence of fecal material (Carrillo, Estrada, and Hazen, 1985; Lopez-Torres, Hazen, and Toranzos, 1987; Jimenez *et al.*, 1989). Similar findings have been reported from Australia (Power *et al*., 2005) and also in the effluent of paper mills (Gauthier and Archibald, 2001). It is not clear if the organisms recovered were in all cases actually *E. coli* or whether they may have been closely related organisms. Despite these reports, *E. coli* has become the indicator of choice, because it is the best available indicator with practical methodologies for detecting recent fecal contamination of water in most situations. However, it should be noted that there is no ideal indicator and that in some situations the presence of *E. coli* may not indicate recent fecal contamination.

The Importance of Definitions

Whilst most regulatory agencies now rely on the total coliform group and *E. coli* as indicators of water quality and fecal contamination respectively, the definitions used for these groups of organisms are not universal. Thus, "coliform" is not a taxonomic classification but rather a working definition which is method dependent. Methods that utilize fermentation of lactose as the principal criterion for recognition of coliforms will generally use a definition such as "Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35-37°C". Many definitions no longer include production of gas as a criterion since it is clearly recognized that many strains of coliforms, including some *E. coli*, are anaerogenic (LeChevallier *et al*., 1983b) although some regulatory authorities inexplicably continue to require gas production as a confirmatory test for coliforms. Most informed water microbiologists agree that an accurate definition for the total group is: "A member of the *Enterobacteriaceae* which produces the enzyme β-D-galactosidase". For practical purposes, the definition is based upon phenotypic characteristics and a working definition might be "a facultative, Gram negative organism which grows at 35°C in the presence of bile salts, is cytochrome oxidase negative and produces the enzyme β-D-galactosidase". Smith (1999) cites Standard Methods as giving three different definitions for coliforms thus:

> For the multiple tube fermentation (MTF) and presence / absence (P/A) methods the coliform "is defined as all facultative anaerobic, Gram negative, non-spore-forming, rod shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C".
>
> When the membrane filter technique is used the coliform group "is defined as those facultatively anaerobic, Gram negative, non-spore-forming, rod-shaped bacteria that develop red colonies with a metallic (golden) sheen within

> 24h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group may produce dark red, mucoid or nucleated colonies without a metallic sheen. When verified these are classified as atypical coliform colonies… Generally, pink (non-mucoid), blue, white or colorless colonies lacking sheen are considered non-coliforms by this technique".
>
> When an enzyme substrate technique is utilized the total coliform group "is defined as all bacteria possessing the enzyme β-D-galactosidase, which cleaves the chromogenic substrate, resulting in the release of the chromogen. *Escherichia coli* are defined as bacteria giving a positive coliform response and possessing the enzyme β-D-glucuronidase, which cleaves a fluorogenic substrate, resulting in the release of the fluorogen".

For *E. coli*, the definition is taxonomic and is usually defined by the nucleic acid sequence of the 16S ribosomal RNA. However, for practical purposes, again the definition is based upon phenotypic characteristics. Traditionally, *E. coli* has been defined as a member of the coliform group that is capable of growth in the presence of bile salts at 44 or 44.5^{o}C, ferments lactose and produces indole from tryptophan. Whilst this definition adequately describes most strains of *E. coli*, some strains do not ferment lactose or do not produce indole from tryptophan. Conversely, some other organisms (notably *Klebsiella oxytoca*) fulfill those criteria. In an effort to simplify physiological tests for the rapid identification of *E. coli*, many studies have investigated the use of tests for production of the enzyme β-D-glucuronidase for routine use (Feng and Hartman, 1982; Martins *et al*., 1993; Fricker *et al*., 2008). Whilst there have been differing results with respect to glucuronidase activity in *E. coli*, the most comprehensive water-related study (Fricker *et al*., 2008) has demonstrated that glucuronidase production is a much more effective test for *E. coli* isolated from water than production of indole and fermentation of lactose.

Clearly the issue of definitions is an important one. The three definitions cited by Smith are very different and in fact the third (relating to enzyme substrate methods) is incorrect. Whilst many bacteria are able to produce the enzyme β-D-galactosidase, clearly they are not all coliforms. In particular, organisms that belong to the genera *Aeromonas* and *Vibrio* may occur in water and are certainly not considered to be coliforms. However, the issue is not a simple one. Some workers believe that all coliforms are of no value as indicators whilst others consider that all coliforms together with *Aeromonas* (since some *Aeromonas* are pathogenic) should be monitored. In reality there is a lack of understanding of the value of the total coliform group as an indicator. Whilst the detection of total coliforms in drinking water in the absence of *E. coli* may not be an indication of the presence of fecal material, such a finding could well be of public health significance.

There are three major reasons for this:

1. Some members of the total coliform group survive longer than *E. coli* in drinking (and ambient) water,
2. Some members of the total coliform group are more resistant to chlorine disinfection than are *E. coli*,
3. Total coliforms as a group are more numerous in fecal material than *E. coli* alone.

Thus the value of the total coliform group as a whole should not be discarded.

Method Format

The methodology initially used for the detection of indicator bacteria in water relied upon culture of the organisms in broth and detection of gas production. Enumeration was based upon the most probable number approach first proposed by McCrady (1915). The traditional methods based upon use of multiple tubes of media to determine the concentration of organisms present has almost completely disappeared from drinking water microbiology. Whilst some modifications of the procedure are now widely used (discussed later), the acceptance of membrane filtration-based methods has become almost universal.

The first description of the efficient production of membranes for bacteriological purposes appears to be Zsigmondy and Bachmann (1918). In the 1940s, workers in Russia and Germany experimented with the use of membrane filters for detection of micro-organisms and in 1943 Mueller described the use of membrane filters in conjunction with endo broth for the detection of coliforms. Grossmann and Beling (1944) described the use of membranes for the detection of typhoid and paratyphoid organisms in drinking water using membrane filtration. By the 1950s, membrane filtration was seen as an alternative procedure to most probable number methods for water examination (Waite 1985) and a plethora of articles followed (Clark *et al*., 1952; Goetz., 1953; Yee, Krabek, and Schaufus, 1953; Laubusch, Geldreich, and Jeter, 1953; Hajna and Damon, 1954; Kabler, 1954; Laubusch, 1955). Several authors concluded that membrane filtration was a rapid and efficient procedure for the examination of drinking water (e.g. Kruse, 1952; Slanetz and Bartley, 1954). In 1957, the membrane filter technique was tentatively accepted in the US, however, it was not until 1971 that the use of membrane filters for the cultivation of coliform organisms was first included in the Standard Methods for the Examination of Water and Wastewater (APHA, 1971). Early studies on the use of membrane filters gave conflicting results. Whilst Henderson (1959) found excellent agreement between membrane filtration and the pour plate technique when determining the coliform numbers recovered from water, Geldreich and co-workers (Geldreich *et al*., 1965) found that membrane filtration recovered only 88% of the coliforms detected using pour plates. Levin and colleagues (Levin *et al*., 1961) found no agreement between the numbers generated by the two techniques. These differences can in part be explained by the different experimental procedures followed and the different media used in each study. However, in 1973 further complexity was added when it was demonstrated that the choice of brand of membrane filter significantly affected both the qualitative and quantitative recovery of coliforms (Presswood and Brown, 1973). In their study, data were evaluated by linear regression, *t*-test and coefficient of variation, although it is not stated whether the data met assumptions implicit for use of parametric statistics. Presswood and Brown (1973) proposed the method of sterilization of membranes (autoclaving vs. ethylene oxide) as a potential cause of differing results, with other possibilities including differing wetting agents, gridline ink and pH. Schaeffer *et al*. (1974) performed similar experiments to Presswood and Brown, but concluded that the use of least squares regression analysis, as employed by Presswood and Brown, was inappropriate due to the assumption that the independent variable was measured without error. Using a Type II regression analysis, Schaeffer *et al.* reported that the Gelman and Millpore membrane filters recovered fecal coliforms equally well, while Gelman membranes were superior for total coliforms.

Dutka *et al* (1974) compared autoclaved and ethylene oxide sterilized membranes from three manufacturers (Gelman, Millipore and Sartorius) for recovery of total coliforms, fecal

coliforms and other organisms. These authors reported that using mean data summaries, Gelman membranes (autoclaved) most frequently yielded the highest counts of total and fecal coliforms, however, statistically significant differences were seasonally inconsistent. For example, from samples collected during March, the Gelman membranes yielded significantly higher counts of total coliforms than any other filters, whereas no significant difference was observed between fecal coliform recoveries using Gelman and Millipore (autoclaved or ethylene oxide sterilized) membranes. In June, the authors observed no clear difference between total coliform data from Gelman, Sartorius (autoclaved) and Millipore (ethylene oxide sterilized) membranes. Fecal coliform data from the June samples revealed no difference in performance between any of the membrane types, regardless of manufacturer or sterilization procedure. It should be noted, however, that the authors elected to use membranes from various lots from each manufacturer, with the (untested) assumption that characteristics between lots from a given manufacturer would be consistent. The authors noted that their data were log transformed to "help equalize variance and provide a more normally distributed variable…" without actually stating that these assumptions were met. Dutka reported that all ethylene oxide sterilized membranes used in their study were at least six months old and speculated that evaporation of ethylene oxide may have improved the performance of these membranes beyond Pressman and Brown's observations. Subsequent work by other researchers suggests that the use of river samples and indigenous bacteria rather than pure cultures may have contributed to the differences as well. The seasonal variation reported by Dutka may have resulted in part from seasonal changes in coliform sources in the river samples. Dutka also reported observing hydrophobic areas in Sartorius membranes that reduced the filtration area, and that autoclaving Millipore and Sartorius filters caused distortion and increased fragility.

Sladek *et al*. (1975) examined sterilization method, composition, and physical structure of membranes, and reported that surface pore morphology had the greatest effect on performance. Using growth and passage data from the analysis of untreated sewage samples, the authors concluded that the optimal membrane filter for fecal coliform recovery would have surface pores of 2.4 µm diameter, with internal pores of 0.7 µm diameter. In contrast, the authors indicated that (at the time) 0.45 µm membranes typically had surface pores ranging from 1 – 2 µm in diameter. In this study, total coliform recovery was independent of surface pore size (between 1 and 3 µm). The authors examined the effect of sterilization method by randomly assigning membranes with optimal morphology into four groups (unsterilized, autoclaved, ethylene oxide treated, and gamma irradiated) and reported no significant difference in performance. Sladek *et al*. suggested that nutrient supply was a critical factor in membrane performance and was facilitated by "cradling" bacteria in the relatively large surface pores, especially at the elevated temperatures used for fecal coliform recovery, while passage was prevented by the smaller internal pores.

Subsequent research supported Sladek *et al*.'s work. Standridge (1976) used scanning electron microscopy to examine three types of filters from two manufacturers (Millipore HCWG 047S3 and HAWG 047S1 and Gelman GN-6, which were rated 0.7, 0.45 and 0.45 µm, respectively). The author documented the largest surface pore openings in the HC filters, followed by the GN-6, and reported the HA filters had considerably smaller pores requiring bacteria to be trapped on the surface of the filter rather than cradled. Noting that Presswood and Brown (1973) reported an average of approximately twice the recovery of fecal coliforms using GN-6 filters as compared to Millipore HA filters, Standridge concluded that Sladek *et al*.'s (1975) theory, that bacteria need to be immersed on nutrients for optimum recovery, was

supported and that membrane filter structure was an important component of performance. Green, Clausen and Litsky (1975) compared fecal coliform recovery from polluted water samples using a variety of types of filters, including the Millipore HC and HA filters as well as other types. The authors observed no significant difference in fecal coliform confirmation rates or variance between filters. However, they reported that differences in mean counts were significant, with the recovery ranked as Millipore HC > Gelman GN > Millipore HA. Tobin and Dutka (1977) also examined the relationship between pore size and bacterial recovery using nine types of membrane filters from five manufacturers. One tap water and two raw waters were inoculated with *E. coli* recovered from local waters. Tobin and Dutka reported bacterial recovery to be correlated with surface pore structure. They speculated that optimal recovery would be obtained using membrane filters with surface pores of approximately 3.2 µm, relatively deep pores and finer mesh below for bacterial retention.

Brodsky and Schiemann (1975) examined the effect of bacterial source on membrane filter performance and concluded it was significant. The authors compared Millipore, Sartorius, Johns-Manville and Gelman filters using dilute broth cultures from positive water samples and, subsequently, river water samples. Using broth cultures, the authors reported Johns-Manville membranes were equivalent to Sartorius for total coliforms but superior for fecal coliforms. However, using river water samples directly, Johns-Manville membranes were superior to Sartorius for total coliforms, but equivalent for fecal coliforms. The authors also reported irregular hydrophobic areas on Sartorius filters as reported by Dutka (1977) as well as limitations of growth caused by grid line markings on Johns-Manville and Millipore filters. The small data set of five replicate filtrations per membrane type (broth cultures) and eight (maximum) river samples limits the breadth of conclusions that can be drawn from this study.

McDaniels *et al*. (1987) compared hydrophobic-grid membrane filters (HG membranes) and standard membranes for total and fecal coliform recovery. These types of filters differ in structure (the gridlines on HG membranes form 1,600 separate cells in contrast to the single filtration area of standard membranes) and use (HG membranes yield an MPN result based on the number of "positive growth units" out of 1600 after incubation, rather than actual colony counts). The authors reported no statistically significant difference between the performance of HG membranes and membranes for wastewater effluent (nonchlorinated), polluted surface waters or drinking water (spiked with chlorine stressed bacteria from wastewater). The authors concluded that although equivalent in performance, the approximately three-fold higher cost associated with HG membranes for supplies and equipment limits their practical value for routine drinking water analysis, although they may be applicable to wastewater effluents or polluted water samples due to their higher counting range and restrictions of overgrowth from one grid-cell to the next by the HGMF gridlines.

Peterson (1974) discussed the relative merits of membrane filtration and the classical MPN procedures. His major findings are presented in Table 1.1.

Table 1.1
Comparative performance of fermentation tube MPN and membrane filtration procedures, after Peterson (1974)

	Fermentation Tube	Membrane Filtration
Sample size	10 mL/tube	100 mL/filter
Turbid water sample	No problem	Difficult
Media volume	50-150 mL/sample	2 mL/sample
Completion time	4-5 days	18-24 hours
Interpretation	Statistical estimate only	Direct count
False positives	Synergism or spore formers	Rare (*S.marcescens*) late lactose fermenters
False negatives	When inhibitors present	Rare (inhibitors rinsed away)
Permanent record	Not possible	Preserve cultures
Cost	Approximately $0.83/sample	Approximately $0.45/sample
Field tests	Impractical	Field kits available

Brenner and Rankin (1990) reported on a new screening test procedure using stock coliform cultures and several media types for evaluating acceptability of membrane filters. Defects previously observed included colony inhibition or enhancement along filter grid lines, atypical colony spreading, poor colony sheen formation or excessive sheen on the filter itself, brittleness and wrinkling. In this study, 142 lots of filters from 13 different manufacturers were examined and 86 lots (60.6%) were judged unacceptable. The authors reported that the most membrane filter defects were detected using *E. aerogenes*-tryptic soy agar and that poor colony sheen was best detected using *E. aerogenes*-mEndo agar.

Despite the reservations noted in early studies, membrane filtration procedures gained rapid acceptance due to their simplicity, speed, precision and enumerative results. In addition, membrane filtration procedures have an inherent ability to concentrate organisms from larger volumes of water for analysis. Some of the concerns that have been noted with respect to aspects of the membrane filtration procedure, including the filters themselves, sample matrix parameters (e.g., turbidity), interference from non-coliform bacteria, and recovery of injured organisms remain valid. However, enhanced quality control and more consistent manufacturing have largely eliminated the gross variation in recovery of coliform organisms seen earlier with different membrane batches. Nonetheless, laboratory quality control procedures should examine the performance of each new batch of membranes used in the laboratory. Unfortunately this potential source of variation is often ignored.

The presence-absence technique for the detection of total coliforms and *E. coli* was reintroduced for testing drinking water by Clark (1968) and was formally accepted as a procedure by the USEPA in 1990. Prior to acceptance, several studies showed the equivalence of the presence-absence procedure to the available membrane filtration or most probable number techniques approved by USEPA (Jacobs *et* al., 1986; Pipes *et* al., 1986; Bancroft, Nelson and Childers 1989; Rice, Geldreich, and Read, 1989). At the same time, the defined substrate technology was being developed as initially based upon a presence-absence format (Edberg and Edberg, 1988; Edberg, Allen, and Smith, 1989). Clark and El-Shaarawi (1993) subsequently compared the Colilert® test with the original lactose-based presence-absence test described by

Clark. The authors concluded that there was essentially no difference in the ability of the commercially available product to detect total coliforms and *E. coli*.

The methods approved for use in the US and UK for the examination of drinking water are shown in Table 1.2.

Table 1.2
Summary descriptions of the membrane filtration and presence/absence methods approved in the US and UK

Medium	Method	Coliform	*E. coli*	Comment	Confirmation
MLSB	Membrane filtration	Lactose fermentation. Yellow colonies	Lactose fermentation at 44°C. Yellow colonies	Samples run at two temperatures	Required
MLGA	Membrane filtration	Lactose fermentation. Yellow colonies	Cleavage of BCIG. Blue or green colonies	Single membrane	Required
Lactose tergitol TTC	Membrane filtration	Lactose fermentation. Yellow colonies	Lactose fermentation. Yellow colonies	Two membranes recommended.	Required
m-endo LES	Membrane filtration	Lactose fermentation. Sheen colonies	Not applicable	Single membrane, coliforms only	Required
mFC	Membrane filtration	Not applicable	Lactose fermentation	Single membrane. Fecal coliforms	Required
MI agar	Membrane filtration	MUgal. Fluorescent colonies	IBDG Blue or indigo colonies	Single membrane	Not required
Chromocult®	Membrane filtration	Salmon gal. Salmon colonies	X-gluc. Dark blue colonies	Single membrane	Kovacs reagent to confirm E. coli
m-ColiBlue®	Membrane filtration	Red colonies	BCIG. Blue colonies	Single membrane	Not required
Coliscan®	Membrane filtration	Red Gal. Pink/magenta	X-gluc. Purple/blue	Single membrane	Not required although Aeromonas will grow
E*Colite	Presence/absence	X-gal. Blue color	MUG. Fluorescence	Not applicable	Not required
ReadyCult®	Presence/absence	X-gal. Blue color	MUG. Fluorescence	Not applicable	Not required
Colitag®	Presence/absence	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colilert®	Presence/absence. Quantitative with Quanti-Tray®	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colilert-18®	Presence/absence Quantitative with Quanti-Tray®	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colisure®	Presence/absence Quantitative with Quanti-Tray®	CPRG Magenta	MUG Fluorescence	Not applicable	Not required

Detailed Descriptions of Media

Membrane lauryl sulphate broth (MLSB) is a selective broth for the detection of coliforms and *E. coli* in drinking water. It is the most widely used procedure in the UK for regulatory compliance. The medium utilizes lauryl sulphate as the selective agent to inhibit Gram positive organisms and lactose fermentation is detected by a color change in phenol red from pink to yellow. This color change is subject to fading after removal of membranes from the incubator. The medium is placed on absorbent pads onto which membranes are laid. Two membranes are used for isolation of total coliforms and thermotolerant coliforms and a preliminary resuscitation step of four hours incubation at 30°C followed by 37°C and 44°C for 14-20 hours is used. The beneficial nature of this resuscitation step has been confirmed by several workers (Childs and Allen, 1953; Pretorius, 1961; Burman, 1967; Rose, Geldreich, and Litsky, 1975; Dufour, Strickland, and Cabelli, 1981), whilst others have disputed its worth. Resulting yellow colonies are selected for confirmation.

Membrane lauryl glucuronide agar (MLGA) was described by Sartory and Howard (1992) as a medium allowing simultaneous detection of coliforms and *E. coli* from water samples. The medium is essentially solidified MLSB with the addition of 5 bromo-4chloro-3-indoyl-glucuronide (X-gluc) to detect the presence of β-D-glucuronidase-producing *E. coli*. Sodium pyruvate is also present to aid the recovery of damaged organisms. The medium is incubated at 30°C followed by 37°C for 4 and 14-20 hours, respectively. Yellow colonies are selected as presumptive coliforms and blue to green colonies as *E. coli*. Walter, Fricker, and Fricker (1994) demonstrated that storage of the medium for more than two weeks resulted in lower recoveries and smaller target colonies. More recently, Fricker *et al.,* (2009) showed that some strains of *E. coli* failed to produce green colonies on this medium and speculated that this was due to acid production from lactose fermentation, reducing the activity of β-D-glucuronidase.

Lactose tergitol TTC agar is a selective and differential medium for the detection of coliforms in food and water. The medium is based upon an original description by Chapman (Chapman, 1947) and uses tergitol-7 as the selective agent to inhibit the growth of Gram positive organisms and minimize the swarming of *Proteus* spp. Fermentation of lactose is detected by a change in the color of bromothymol blue. Triphenyltetrazolim chloride is rapidly reduced to formazan by the majority of coliforms with the exception of *E. coli* and *Enterobacter aerogenes* which is claimed to allow early differentiation of these organisms from other coliforms (Chapman, 1951). The medium is quoted in the European Union Directive on drinking water as the reference method for the detection of coliforms and *E. coli* in water. It is an ISO approved method (ISO 9308-1; Anon, 2000) and whilst 36°C is the primary incubation temperature, the use of 44°C has been recommended for the detection of *E. coli* (Niemela, Lee, and Fricker, 2003; Fricker *et al*. 2008). Colonies from tan to yellow color are selected for confirmation.

McCarthy, Delaney, and Grasso (1961) originally described m-Endo LES as a medium for the detection of coliforms in water, which was used as a two step process with an initial incubation on a resuscitation medium followed by incubation on the selective medium. This resuscitation procedure is cumbersome and has been shown to be unnecessary (Evans *et al.,* 1981) and consequently is not often incorporated into routine use. The selective agents are sodium desoxycholate, sodium lauryl sulphate, basic fuchsin and sodium sulfite which inhibit the growth of non-target organisms. Identification of coliforms is facilitated through the fermentation of lactose which produces aldehydes which react with the basic fuchsin-sulfite

compound present in the medium to produce a "sheen" colony. Overcrowding of membranes has been shown to lead to false positive reactions due to the synergistic production of the compounds necessary to create the sheen appearance (Schiff, Morrison, and Mayeux, 1970). However, all colonies which exhibit any degree of golden-green metallic "sheen" are considered to be presumptive coliforms.

In 1965, Geldreich (Geldreich *et al.*, 1965) described mFC agar as a medium to enumerate fecal coliforms using membrane filtration without the use of any prior enrichment. The medium uses bile salts No. 3, rosolic acid and methyl blue as selective agents together with incubation at 44.5^{o}C to inhibit non-target organisms. Fermentation of lactose is detected by a color change to the methyl blue present, producing intense blue colonies. As with all selective media, there is a balance between the inhibition of non-target organisms and the suppression of target organisms. Presswood and Strong (1978) described the use of mFC agar without rosolic acid and found a 49% increase in the recovery rate with minor effects on specificity. Despite this, mFC containing rosolic acid is still widely used.

MI agar is named after the specific substrates present in the medium and was described by Brenner *et al*. (1993). The medium contains both a fluorogen and a chromogen: the fluorogen being used to detect total coliforms and the chromogen to detect *E. coli*. Inhibitors used in the medium include sodium lauryl sulphate, sodium desoxycholate and cefsulodin; the combination of which is reported to significantly lower background growth. The amount of background growth reported was less than 5% of that seen on m-Endo plates (Brenner *et al*., 1996 a, b). Isopropyl-β-D-1 thiogalactopyranoside (IPTG) is included to stimulate β-D-galactosidase production. Coliform colonies fluoresce under ultraviolet light and *E. coli* appears blue. Based upon the data generated in several studies, there is no need to confirm the identity of either coliform or *E. coli* colonies.

Chromocult® agar is a chromogenic medium incorporating Salmon-gal and X-gluc to detect total coliforms and *E. coli* respectively. The inhibitory agent is tergitol-7 which is present at a 50% higher concentration than in lactose tergitol TTC agar.

m-ColiBlue24®, first described by Grant (1997), is a nutrient medium containing BCIG to differentiate *E. coli* (blue) from other coliforms (red). TTC is reduced by most coliforms to produce red colonies. Triton-X 114, cyclohexamide, sodium azide and erythromycin are present as inhibitors of non-target organisms. Sodium pyruvate is present to aid the recovery of damaged target cells.

Coliscan® is produced by Micrology Laboratories in Indiana (US), and claims to be suitable for incubation at any temperature between ambient and 37°C. Differentiation of coliforms and *E. coli* is achieved using Red Gal (for galactosidase) and X-gluc. No information is available on the specific inhibitors used. The manufacturers state that *Aeromonas* spp. will mimic coliforms on this medium but that its presence can be distinguished by the cytochrome oxidase test. Consequently, all coliform-like colonies require confirmation.

E*Colite, manufactured by Charm Life Sciences, is a nutritionally rich medium containing X-gal and MUG, and is used as a presence/absence test to give coliform and *E. coli* results in 28 hours. Detergents are used to inhibit non-target organisms but there are no specific details available.

ReadyCult® is manufactured by Merck, a multinational company, and is a presence/absence test that uses X-gal and MUG for detection of coliforms and *E. coli*. Lauryl sulfate is used as an inhibitor of Gram positive organisms at a concentration of 0.05 g/L. Incubation is at 35-37°C for 18-24 hours. Room temperature incubation is for 48 hours.

Samples with high background flora can give a false negative coliform result due to a lack of oxygen in the medium which prevents the choromogen from being detected. When exposed to oxygen (e.g. by removing the cap of the bottle) the chromogen becomes colored (Fricker *et al*. 2003).

Colitag® is manufactured by CPI in the US and is a presence/absence test utilizing ONPG and MUG for the detection of coliforms and *E. coli*. The detergents used for the inhibition of non-target organisms are not specified. The method incorporates a novel system of pH control. The manufacturers state that at the beginning of incubation the pH is low which is said to allow better recovery of injured *E. coli*. The manufacturers refer to this as "acid resuscitation". During subsequent incubation trimethylamine-N-oxide (TMAO) is hydrolyzed to TMA which causes the pH to rise to approximately pH 7. Experimental work within the authors' laboratories has shown that the initial pH of the medium is approximately pH 6.8. Incubation is at $35 \pm 0.5^{o}C$ for 24 ± 2 hours. .

Colilert® (Edberg and Edberg, 1988) and Colilert-18® (Fricker and Fricker, 1996a) are both methods which are based upon "defined substrate technology", a patented technology where general nutrients are limited and bacteria need to utilize the "indicator nutrients" ONPG and MUG as carbon and energy sources. This "forces" coliforms and *E. coli* to use nutrients which will indicate their presence. Detergents are not present but inhibition of non-target organisms is achieved by the judicious use of selected antibiotics combined with restricting available nutrients. The media are very similar but Colilert® requires 24 hours incubation whilst Colilert-18® requires only 18 hours. It was developed in the UK in response to a need for coliform and *E. coli* results within 18 hours.

Comparison Studies

Design of comparison studies

There are a plethora of comparison studies described in the literature, many of which relate to the comparison of different techniques with respect to their performance in recovering coliforms and *E. coli* in water. Unfortunately the wide variety of experimental designs employed in these studies means that comparison of the data presented therein is difficult. Furthermore, some studies have design faults. It is not always apparent when designing a comparison study of the possible flaws which may arise and often studies have been designed with a particular outcome in mind. For studies where the aim is to compare the performance of methodologies in recovering coliforms and *E. coli* from drinking water, the best possible data would be collected by comparing the two (or more) methods using actual drinking water samples. This would give the best understanding of how individual methods perform with naturally contaminated samples of either treated or untreated water. Unfortunately, the low occurrence of coliforms and in particular *E. coli* in these samples means that such studies are impractical. Vast numbers of samples would need to be examined in order to generate sufficient data to enable statistical analysis. Additionally, samples should not be restricted to a single geographical area if the methods are to be widely employed, since matrix effects may be substantial. Thus, studies using drinking water would need to involve several laboratories performing the tests with large numbers of samples over a long period of time in order to collect sufficient data. In comparison studies, samples which contain no target organisms are virtually meaningless, although they may

have some merit in determining false positive rates. However false positive rates can equally be determined with samples containing target organisms.

Choice of test format

Analysis of drinking water for the presence of total coliforms is performed in one of three ways, presence/absence testing where 100 mL of water is mixed with medium prior to incubation, membrane filtration, where the water is filtered through a membrane prior to incubation on a nutrient medium (quantitative), or as a most probable number (MPN) system where water is mixed with medium and partitioned into aliquots before incubation (quantitative). With the MPN system the results are used to calculate the "most probable number" of organisms present in the sample.

Bissonnette *et al.* (1977) investigated the recovery efficiency of conventional membrane filtration, pour plates, fermentation tube MPN and enrichment membrane filtration methods for enumerating coliforms in water. They reported the highest recovery using MPN fermentation tube procedures, followed by pour plates then membrane filtration. These results supported previous work by Shipe and Cameron (1954), McKee, McLaughlin, and Lesgourgues (1958), and Lin (1976). It should be noted that the physical structure of the membrane filters used in these earlier studies was not well understood and a lack of cradling/immersion may have contributed to the poor performance of these procedures. In addition, standard membrane filtration procedures included incubation at 44.5^{o}C, which Bissonnette *et al*. (1977) and Hufham (1974) noted was detrimental, although selective. Bissonnette *et* al.,(1977) also reported improved recovery when employing a two hour enrichment on rich, non-selective media prior to enumeration on selective media via membrane filtration and speculated that repair to injured cells may occur during enrichment. This further supports the work of Lin (1976) who reported that fecal coliform counts by membrane filtration generally increased with pre-enrichment incubation time. Lin reported that this two-step membrane filtration method yielded results comparable to the MPN method.

Stuart, McFeters, and Schillinger (1977) reported on a two layered medium for the recovery of injured fecal coliforms (injury-mitigating membrane filtration or IM-membrane filtration) and comparison with MTF and m-FC using chlorinated sewage effluents, laboratory chlorinated sewage samples and non-chlorinated mountain stream water. The two layered medium consisted of enriched lactose agar over enriched m-FC agar. Both layers contained glycerol and acetate to facilitate enzymatic action and thioglycolate for chlorine neutralization. In all tests, the IM-MF procedure was comparable to MTF and superior to m-FC for enumeration of fecal coliforms.

Tobin, Lomax, and Kushner (1980) compared nine brands of membrane filtration with fermentation tube MPN for total coliform enumeration from sewage contaminated drinking water. The authors reported after average ranking the membrane filters for all experiments that the top three were: Johns-Manville GA > Gelman > Millipore HC. Subsequent comparison of the best performing filters with MPN revealed no significant difference between membrane filtration and MPN in five of six tests, while membrane filtration results were significantly higher in one test. The differences in the ability of the membranes to recover injured coliforms did not appear to be easily correlated with pore diameter as previously reported with fecal coliforms and speculated that the added temperature stress of incubation at 44.5^{o}C for fecal coliforms may account for this difference. The reduced temperature used for total coliforms and

compared to fecal coliforms, as well as Tobin's use of m-Endo LES for all membrane filtration tests, may explain why their results differ from of Bissonnette *et al*. (1977) and others previously discussed.

Evans *et al.* (1981) compared species of coliforms recovered from surface and drinking water using membrane filtration and standard and modified fermentation tube MPN procedures. The authors reported varying results by procedure; each method selected for different coliforms. From surface waters, *C. freundii*, *E. coli* and *Klebsiella* were the three most frequently recovered isolates for each technique, however, the percent of each varied between methods. Membrane filtration recovered *C. freundii* predominantly (*E. coli* second), while standard and modified MPN recovered *E. coli* preferentially (second and third place recoveries varied by procedure). These results differ from those reported by Tobin and Dutka (1977) who observed that (compared to MPN) membrane filtration selected for *E. coli* and *Klebsiella* in creek water, however, *C. freundii* was not recovered at all by filtration. In addition, Evans, *et al*., were using higher quality source water (100-fold lower coliforms per mL). In drinking water, Evans, *et al*. reported *C. freundii* was the most commonly recovered isolate by each method, but lower ranked orders varied by method. The authors expressed concern that the standard MPN failed to detect *Enterobacter* spp. in chlorinated drinking water.

Hsu and Williams (1982) examined factors affecting membrane filtration performance for drinking water samples, including media preparation and storage, and pre-enrichment, and also compared membrane filtration and MPN results. Samples were collected from municipal supplies (plants and distributions systems), private supplies, and public swimming pools. Hsu and Williams reported data that indicated membrane filtration with m-Endo LES agar recovered more organisms than did MPN using LT broth as initial enrichment with confirmation in BGB broth fermentation tubes. It should be noted that the authors constructed arbitrary definitions of "agreement" between methods (meaning membrane filtration results were anywhere within the MPN range) and "preference" (if the results were not in "agreement", the preferred method was the one yielding the higher result). The authors note that these results concur with those of McCarthy *et al*. (1961), who used the same definitions of agreement when comparing MPN and membrane filtration (m-Endo LES plus pre-enrichment). Because of this, Hsu and Williams concluded pre-enrichment for m-Endo LES is not warranted. This conclusion supports the work of Evans *et al.* (1981) who also reported no benefit from resuscitation when using m-Endo LES.

Jacobs *et al.* (1986) compared membrane filtration, multiple fermentation tube and presence/absence methods for recovering total coliforms from small community drinking water systems in Vermont and New Hampshire. Of the 1483 samples tested, 23% were positive by one or more methods. Of these positive samples, the presence/absence method detected 88%, fermentation tube detected 82% and membrane filtration detected 64%. The comparatively low percent positive rate for membrane filtration is likely due in part to the use of Millipore HA filters (relatively small surface pores) and m-Endo broth pads, with subsequent verification using fermentation tubes (LTB and BGLB).

Evolution of membrane filtration media

Many researchers have investigated various media formulations for bacterial recovery efficiency and cost effectiveness. As early as the 1890s work was underway to compare the performance of media and their components for recovering indicator organisms from water. Obst (1915) compared the use of lactose peptone ox-bile and lactose bouillon because of variable

results with the former as well as the excessive cost of bile (fifty cents bought three gallons) and preparation requirements. Obst reported that lactose bouillon allowed recovery of approximately twice as much *B. coli* as bile medium, was cheaper and less labor intensive. Work aimed at improving the recovery of coliforms and in particular *E. coli* continued steadily and the importance of media components and the accuracy of temperature monitoring were noted in the late 1920s and early 1930s.

Perry and Hajna (1933) investigated the use of the Eijkman reaction in recovering *E. coli* from water. Their work revealed two extremely important factors. Firstly, it was demonstrated that variation of temperature within incubators could have a significant effect on the recovery of *E. coli* when the target temperature was 46°C and that by limiting the temperature variation to 0.5^{o}C much more consistent results were obtained. Secondly, they noted that the excess production of acid that occurred within the original Eijkman medium due to fermentation of glucose had a detrimental effect on the viability of *E. coli*. A reduction in the concentration of glucose and the addition of phosphate buffer reduced the toxic effect of the fermentation by-products. Both of these findings were significant and have helped in our understanding of some of the variations in performance of coliform and *E. coli* recovery procedures in more recent years.

The issue of incubation temperature continued to have an impact on the development of methods and in the 1940s and 1950s there was still little agreement on the optimum temperature of incubation for *E. coli*. Whilst Clegg and Sherwood (1939) had used 44°C as a test for "faecal coli", Perry and Hajna (1944) had suggested the use of 45.5^{o}C as the optimum temperature of incubation. Clark *et al*. (1957) used 43°C with a boric acid lactose broth and Geldreich *et al*. (1958) recommended 45°C. In more recent years, it has become clear that the optimum incubation temperature and the choice of growth medium are intimately related and that the presence of strongly selective agents in growth media lowers the maximum growth temperature of *E. coli* and other thermotolerant coliforms (Niemi *et al*., 2001;2003). Studies with the ISO reference procedure for the detection of coliforms and *E. coli* from water (Fricker *et al*., 2008) showed that this inter-relationship could have dramatic effects on the interpretation of results for the examination of drinking water. The study showed that incubation of the primary isolation medium (lactose tergitol TTC agar) at 36°C resulted in the recovery of *Klebsiella oxytoca* which in subsequent tests mimicked *E. coli* whilst when primary plates were incubated at 44°C the *Klebsiella* did not grow. Thus performance of the primary tests could lead to false positive *E. coli* results which would have a significant operational impact for water utilities.

The study of lactose tergitol TTC medium highlighted the whole issue of definitions of the coliform group of organisms and indeed that of *E. coli*. Media that are based upon different mechanisms for differentiation of organisms usually require a different definition of the target organism (Fricker and Fricker, 1996b). For example, methods based upon lactose fermentation for detecting coliform organisms usually define coliforms as a group of organisms that ferment lactose within 48 hours and do not produce cytochrome oxidase. However, other procedures which utilize detection of the presence of the enzyme β-D-galactosidase may define coliforms as organisms that produce the enzyme β-D-galactosidase and not the enzyme cytochrome oxidase. Similarly, many methods for the detection of *E. coli* rely on the ability to grow, ferment lactose and produce indole from tryptophan at 44°C whilst others utilise the fact that the vast majority of *E. coli* produce both of the enzymes β-D-galactosidase and β-D-glucuronidase. Clearly the use of different definitions means that different procedures will detect different groups of organisms and it is therefore not always straight forward to compare the sensitivity of two procedures based

upon their recovery of "target" organisms from water samples. Thus, definitions are one of the most important aspects to consider when examining data produced in comparison studies. However, this aspect is often overlooked and the effect of different groups of target organisms being detected by different methods is often not quantified.

Presswood and Strong (1978) reported that the elimination of rosolic acid from m-FC medium yielded higher fecal coliform recovery from a variety of water types and simplified colony counting by increasing the color contrast between fecal coliform and non-FC colonies. The authors noted that while rosolic acid suppressed non-fecal colonies at 35°C, at 44.5°C non-fecal colonies were suppressed by temperature. At the higher incubation temperature, equivalent results were obtained with or without rosolic acid. This work was later corroborated by Finch, Stiles, and Smith (1987), who examined recovery of nalidixic acid resistant *E. coli* from laboratory generated ozonated water samples. The investigators used a standard m-FC medium prepared with rosolic acid and bile salts, and a modified version without those two ingredients. Samples were incubated at 35°C and 44.5°C and significantly higher recovery of *E. coli* reported on m-FC without rosolic acid and bile salts at both temperatures.

Camper and McFeters (1979) investigated whether chlorine truly caused physiological injury to bacterial populations and, if so, whether the injury was reversible. Using *E. coli*, the authors reported that injury reproducibly occurred at 0.5 mg chlorine per liter and that such injury was reversible under incubation on non-selective media. The studies described above demonstrated the combined effects of temperature and chemicals in selecting for target organisms. Furthermore, they showed that particularly with injured cells, selective media recovered fewer organisms than non-selective media.

Grabow and du Preez (1979) compared the performance of m-Endo LES, MacConkey and Teepol media for MF recovery of total coliforms in water. The authors showed that m-Endo LES agar had the highest average count for all samples and recommended it, without enrichment, as the method of choice for enumerating coliforms from drinking water. The authors used Gelman GN-6 filters for all media, which because of the pore structure may have minimized the differences between hard media and broth saturation. Grabow and du Preez noted that the saturated pads could not be prepared in advance, tended to dry out during incubation, and were generally time consuming and inefficient. The authors did not include MPN fermentation tube methods for comparison, but noted that membrane filtration had advantages over MPN methods including more accurate and direct counts with shorter incubation times, sample processing efficiency, reduced incubator space, and ability to isolate cultures from individual colony forming units for identification. Data from chlorinated and ozonated water tests indicated Teepol and MacConkey were positive more often than m-Endo LES, however the authors noted that the difference between positive and negative was typically only a very few colony forming units, insufficient to generate statistically significant conclusions. The authors stated that the previous research had indicated MPN procedures yield higher coliform densities from chlorinated sources, but suggested the use of enrichment and membrane filtration if these sources were of interest. The authors reported that *Aeromonas hydrophila* was the most commonly identified recovered species that "conformed to the definition of total coliform bacteria", and noted that m-Endo agar was slightly more specific than the other media for *Escherichia, Citrobacter, Klebsiella,* and *Enterobacter*. The authors argue that oxidase tests should not be employed for drinking water total coliform assays and that oxidase positive genera such as *Aeromonas* should be included due to potential pathogenicity, transfer of resistance factors via plasmids, and because properly operated water treatment eliminates these organisms.

Evans *et al*. (1981) examined the impact of verification media and resuscitation on total coliform enumeration by membrane filtration (MF). Gelman GN-6 filters and m-Endo LES were used for all samples, however for resuscitated samples the MFs were placed on pads saturated with sterile lauryl tryptose broth (LTB) and incubated at 35°C for 2 hours prior to transfer to m-Endo LES. Verification media included LTB, lactose broth and m-LAC broth as primary media and brilliant green-lactose-bile broth (BGLB) and EC broth as secondary media. The authors reported verification in m-LAC broth as primary media increased accuracy and sensitivity of the MF total coliform procedure in both untreated surface water and drinking water. The authors also noted that secondary verification of isolates in BGLB or EC broth reduced verification by up to 40%, and that BGLB resulted in numerous false negatives, thus advocating a single verification procedure using m-LAC broth be further investigated. Interestingly, the authors also reported that the resuscitation step did not increase the number of typical colony forming units enumerated from surface waters or drinking waters. It is unclear why these data differ from previous work, but it may be due in part to the relatively small data set (21 untreated water samples and 120 drinking water samples) collected over a one year interval in a single geographic area (Oregon), and the authors suggested that additional work was needed.

Dufour, Strickland, and Cabelli (1981) reported on the development and testing of the mTEC method, a membrane filtration procedure to enumerate *E. coli* within 24 hours without subculturing and subsequent identification. This method included a resuscitation step (filters directly on mTEC media) at 35°C for 2 hours prior to incubation at 44.5^{o}C for 20 ± 2 hours. All countable filters were subsequently transferred to urea substrate saturated pads for urease testing and yellow colony forming units were counted after 15 minutes. The authors reported that somewhat higher recoveries were achieved with a longer (4 hr) resuscitation period, but two hours were used for convenience. The specificity of the mTEC method was greater in marine and estuarine water than in fresh waters; however, the performance in fresh water was comparable to reference methods. Varying sources of coliforms may have affected these results, however all sources were in the proximity of wastewater treatment plants and presumably received analogous coliforms. This method differed substantially from earlier methods by using a negative urease test rather than a positive indole test for differentiation. The authors noted, however, that the test performance suffered at high bacterial concentrations, and recommended that it not be employed for filters with >80 colony forming units, and also recommended evaluation of the method outside of New England.

Results from studies by Pagel *et al*. (1982) using samples collected throughout southern Ontario supported Dufour's findings. Four membrane filtration procedures using various media were compared for accuracy, specificity, counting limits and recovery comparability in six laboratories by Pagel *et al*. Media included MacConkey membrane broth, mTEC, m-FC agar and m-FC2 (m-FC agar plus resuscitation). The authors reported the best overall performance was achieved using the mTEC method, which had high accuracy with stressed and unstressed fecal coliforms, high selectivity, low false positive rates, and high counting limits.

McFeters, Cameron and LeChevallier (1982) examined the effect of diluents, media and membrane filters on recovery of injured coliform bacteria (pure cultures of *E. coli*, *K. pneumonia*, *E. aerogenes*, and *C. freundii*). The authors reported that diluents held at approximately 4°C are likely to have little effect on enumeration, whereas diluents held at room temperature and/or extended exposure times can yield lower recoveries. The addition of organic compounds to the diluents was beneficial to recovery, particularly with injured bacterial populations, and that dilutions made in peptone water and phosphate buffer were the most stable

over time. These results support previous research regarding the reduced recovery of injured coliforms on selective media and compared to non-selective media. Seemingly healthy coliforms were not affected by concentrations of the selective agent deoxycholate of up to 0.1%, whereas injured organisms were affected by concentrations as low as 0.05%. They also compared the recovery of injured and uninjured *E. coli* on Millipore HC and HA filters (2.4 and <1.0 µ pores, respectively) and, in contrast to earlier research by Sladek *et al*. (1975), reported pore morphology had no observable effect on recovery. The two studies differed in that McFeters incubated pure cultures of *E. coli* at 35°C, whereas Sladek used untreated sewage samples and incubated at 44.5^{o}C.

LeChevallier *et al.*, (1983a) reported the development of m-T7 and a new membrane filtration medium for recovery of total coliforms in drinking water. This medium was a modification of Tergitol 7 to improve selective and differential capabilities. They reported that m-T7 recovered substantially more injured coliform than did m-Endo agar or m-Endo with LTB resuscitation from drinking water, surface water and chlorinated surface water (Millipore HC filters were used for all samples). It was concluded that the difference in results was due to the negative effect of the selective agent in m-Endo agar, desoxycholate, even with enrichment, rather than atypical coliform bacteria. In a subsequent study, LeChevallier *et al.* (1986) compared enumeration of coliforms in drinking water using m-Endo LES and m-T7. This study utilized samples collected from various points within community water systems located in New England, whereas the previous work used Montana drinking water. The authors reported that m-T7 recovered 8 – 38 times more coliforms than were recovered using m-Endo LES. The authors noted that in excess of 70% of samples negative by m-Endo LES were falsely negative due to the presence of non-recovery of injured coliforms and estimated that m-Endo LES recovered less than one-tenth of the viable population of coliforms present. In contrast, Adams *et al*. (1989) observed no significant difference in performance of mT7 and m-Endo for enumeration of *E. coli* and *C. freundii* from spring water, raw settled water, and filtered water after ozonation. This study, which used Millipore HC filters and incubations of 35°C for 24 hours, was limited to two replicates of each organism/water type. The authors did not speculate on possible causes of results that differed from those previously reported using chlorine-injured coliforms.

The effect of competing flora on sheen production on m-Endo agar was investigated by Burlingame *et al*. (1984). They found that *Aeromonas hydrophila* and *Pseudomonas aeruginosa* interfered with coliform recovery at 2 CFU/mL and 30 CFU/mL, respectively, well below levels previously reported. *Bacillus* sp. and *Flavobacterium* sp. caused no interference under the test conditions.

Freier and Hartman (1987) reported the development of two media for recovery and enumeration of total coliforms and *E. coli* in 24 hr without subculturing. Along with standard media, the new media, described as lactose–monensin–MUG (m-LMM) and Tergitol-monensin-MUG (m-TMM) were tested on surface water and unchlorinated and chlorinated sewage samples. Both m-LMM and m-TMM showed higher recovery of coliforms from surface water than did m-Endo LES, although only m-TMM was significantly higher. Similarly, both m-LMM and m-TMM recovered more *E. coli* from surface water than m-FC recovered fecal coliforms, although here on m-LMM was significantly different. The authors also reported low recovery of interfering *Aeromonas* by the new media as compared to m-Endo, which reduced background interference and better reflected sanitary conditions. The authors did note that the recovery of chlorine injured coliforms on the new media was slightly below that of m-T7 media, although the

difference was not significant. The authors suggest the ability to recover *E. coli* at 35°C helps negate any need for a resuscitation step and suggested that further comparisons be made.

Calabrese and Bissonnette (1990) investigated the effect of chlorine on catalase action in *E. coli* and assessed the addition of catalase and sodium pyruvate to media for the recovery of coliforms from chlorinated samples. They reported significant increases in fecal coliform recovery on m-FC and total coliform recovery on m-T7 and m-Endo when sodium pyruvate, catalase or both were included in the media. Improved detection of total coliforms was seen by using m-Endo over m-T7 and it was suggested that this may be due to the apparent ability of m-Endo to degrade hydrogen peroxide, which is present in natural waters, particularly those containing high levels of organic mater and exposed to strong sunlight.

Alternatives to lactose fermentation

Interest in the use of enzyme substrates such as those for β-D-galactosidase or β-D-glucuronidase began as early as 1955 when Mead, Smith and Williams (1955) began experimenting with fluorogenic derivatives of glucuronides and their use in sensitive assays for the presence of β-D-glucuronidase. Further developments were made in the 1970s when several workers began to use both fluorogenic and chromogenic markers for detecting enzyme activity in coliforms and *E. coli* (Dahlen and Linde 1973; Maddocks and Greenan 1975; Kilian and Bulow 1976) largely in clinical materials. Activity in the application of enzyme-based methods to the water industry began to gather momentum in the 1980s. In 1982, Feng and Hartman added 4-methyl umbelliferyl glucuronide to lauryl tryptose broth as a means of confirming the presence of *E. coli*. They found that the procedure worked well and that 96% of *E. coli* expressed the enzyme β-glucuronidase under the conditions they used for testing. This was in close agreement with the earlier report of Kilian and Bulow (1976) who found 97% of the *E. coli* strains examined were glucuronidase positive. Other studies confirmed the utility of β-glucuronidase-based tests for detecting *E. coli* in food and water (Robinson 1984; Rice, Geldreich, and Read, 1989; Rice *et al.* 1991). A later study by Chang, Brill, and Lum (1989) in California suggested that up to 35% of *E. coli* isolates from feces failed to express the enzyme and in 1991 Clark *et al*. (also in California) reported a failure of methods for the detection of β-glucuronidase to detect *E. coli* in water samples. Clark's work compared a membrane filtration fecal coliform test with Colilert® and Coliquik (two commercially available tests for the simultaneous detection of coliforms and *E. coli*) for the detection of *E. coli* in treated and untreated water and reported no significant difference between the methods with untreated water. However, statistically significant differences were observed between both Colilert® and Coliquik as compared to mFC in treated water due to false negative results from the commercial products. Clark examined false negatives as a function of HPC concentrations and found no correlation. Clark speculated that chlorine injury may prevent *E. coli* from utilizing MUG. The authors recovered *E. coli* from six false negative Colilert® / Coliquik samples in their study and reported that after subculturing and re-inoculation, 84% of these isolates were able to utilize MUG. The explanation for these apparently conflicting data sets has not become apparent but the use of β-glucuronidase for detecting *E. coli* and of β-galactosidase for detecting coliforms has become widespread and almost universally accepted as a valid procedure. At the same time that they performed the study with *E. coli*, the same workers reported on findings with coliforms (Olson *et al*. 1991). They compared membrane filtration using m-Endo LES to both Colilert® and Coliquik for total coliform detection in samples from distributions systems, reservoirs, wells and water treatment

plant influents. Verification was in fermentation tubes using LTB and BGB, and membrane filtration results were scored in a presence/absence format to allow a simple method of comparison. Olson reported that both Colilert® and Coliquick showed greater than 94.8% agreement with membrane filtration. However, a statistically significant difference was observed between membrane filtration and Colilert®, and the authors reported that membrane filtration was superior for detecting coliforms in the waters tested, largely due to "false negative" results from Colilert®. The authors noted that the majority of coliforms detected by membrane filtration were "atypical", and emphasized the importance of including dark red mucoid colony forming units with or without nuclei in membrane filtration coliform counts.

Lewis and Mak (1989) compared membrane filtration and autoanalysis Colilert® procedures for the detection of coliforms and *E. coli* in drinking water. The authors found substantial agreement between methods, however, samples were collected from buildings and fire hydrants after flushing, and both methods reported 934 of 950 samples as negative. The two tests agreed 97% of the time on the basis of presumptive total coliform results and 98.5% of the time on the basis of verified total coliform results. Samples which produced disagreement between the two tests were most often positive by membrane filtration and negative by Colilert®. *E. coli* was detected twice by Colilert® but the medium failed to fluoresce (false negative) whilst two samples which fluoresced could not be shown to contain *E. coli* (false positive). However, the confirmation techniques used relied upon lactose fermentation and gas production and, therefore, anaerogenic coliforms and those which did not rapidly ferment lactose were excluded. Of the organisms that were recovered from Colilert® and identified as being *E. coli*, one produced fluorescence after 36 hours incubation and one failed to produce fluorescence. It is unclear if this second isolate was indeed *E. coli* or another organism that mimicked *E. coli* in traditional phenotypic tests. Both Lewis and Mak (1989), and Olson *et al*. (1991) reported difficulty recovering coliforms from "false negative" Colilert® samples. Olson calculated Poisson probabilities and concluded that negative Colilert® results due to lack of inoculation was unlikely and speculated that injury, substrate specificity/sensitivity may affect utilization of ONPG by a proportion of coliforms.

In 1988, Berg and Fiksdal had reported rapid detection of total and fecal coliforms using β-galactosidase as a marker. They suggested that a single fecal coliform cell could be detected on a membrane within six hours. This somewhat exaggerated claim certainly raised the level of interest in the use of enzymes for direct bacterial detection. This has since been shown to be misleading by other workers who have demonstrated that reliable detection of single cells of coliforms using simple fluorogenic methods requires much longer incubation periods and specialized equipment (van Poucke and Nelis 1995, van Poucke and Nelis 1997).

Nonetheless, the period of the late 1980s and early 1990s was a time of intense activity and some developments emerged which have revolutionized the way in which water microbiology is performed. In 1988, Edberg and colleagues (Edberg and Edberg 1988, Edberg, Allen, and Smith 1989) first published the development and use of "defined substrate technology" (DST); this was one of the tests used in the studies of Olson's group described above. DST is a simple concept which utilizes the concept of "indicator nutrients" (in the case of Colilert® these are ONPG and MUG) which are the major carbon and energy sources within the medium. Thus, if bacteria are to grow, they must utilize these compounds, producing a yellow color with (*E. coli*) or without (coliforms) blue fluorescence when viewed under ultraviolet light. Inhibition of competing bacteria is achieved using a "cocktail" of antibiotics, the formulation of which is proprietary and known as "Solanium". The development of the Colilert® system

coincided with increased interest (Pipes *et al.* 1986; Jacobs *et al*. 1986; Rice, Geldreich and Read 1989) in the use of a presence/absence method for detecting the presence of coliforms and *E. coli* as first described by Clark (1968). The combination of a simple presence/absence method together with a pre-dispensed medium which simultaneously detects coliforms and *E. coli* without the need for confirmation or verification of the results was extremely attractive to water utilities and many studies on the use of this type of technology have ensued.

Edberg and Edberg (1988) further compared the quantitative recovery of coliforms and *E. coli* from drinking water using Colilert® and standard USEPA procedures and showed greater sensitivity with Colilert®, although the results were not statistically significant. The authors concluded that a national evaluation of the Colilert® test showed that it: (i) was as sensitive as Standard Method's membrane filtration techniques, (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. Edberg *et al*. (1990) also used Colilert® to good effect in a most probable number (MPN) format for determining the numbers of coliforms and *E. coli* present in source water when comparing with lauryl tryptose broth. Good correlation was observed between the two methods. Conversely, Covert and colleagues from the USEPA (Covert *et al.* 1989) showed that Colilert® was significantly less sensitive than the standard ten-tube MPN procedure, and yet, incredibly, stated that the difference was "small" and therefore somehow not important. They also demonstrated false positive coliform reactions caused by *Aeromonas* spp. and false positive *E. coli* reactions caused by *Flavobacterium* spp. Petzel and Hartman (1986) had also reported positive β-glucuronidase reactions from *Flavobacterium* spp. A small study (McCarty, Standridge, and Stasiak, 1992) suggested that Colilert® gave significantly more coliform and *E. coli* isolations than did USEPA approved methods for the detection of *E. coli* in disinfected sewage effluent, adding additional information to the growing number of studies being reported.

It was around this time that work started to be undertaken in Europe on the use of Colilert® and the first paper published compared the use of the Colilert® presence/absence system with the German standard method (also a presence/absence system). Frahm *et al*. (1992) showed an agreement of 93% between the two methods and concluded that the Colilert® test was simpler to perform and gave results much more quickly than the standard procedure which requires extensive confirmation procedures. They further concluded that Colilert® offered a suitable alternative to the German standard procedure for the examination of drinking water.

Schets *et al.* (1993) painted a very different picture of Colilert® when comparing it to the Dutch standard methods for drinking water. The authors demonstrated that Colilert® gave false negative reactions particularly for *E. coli* when low numbers of target organisms were present and recorded a recovery efficiency of only 26% of *E. coli* when comparing to the Dutch standard method. However, the work was flawed and from the manuscript it is not possible to determine to what extent. The paper describes the Colilert® test as being incubated for at least 22 hours whereas the correct procedure is incubation for a minimum of 24 hours. Since the proportion of samples which fell below the required incubation time is not discernable, the Dutch results should be treated with caution. Reading tests before their minimum incubation time can lead to false negatives and must be avoided at all costs in experiments to compare the performance of different methods. Furthermore, the Dutch standard method relies upon the production of indole by colonies growing on tryptone bile agar at 44°C. There are other organisms that produce

indole from tryptophan at 44°C (Niemi *et al.* 2001, Niemi *et al.* 2003, Fricker *et al*. 2008) and it is not clear if organisms such as *Klebsiella oxytoca* will grow on bile tryptone agar at 44°C. The results presented in this paper highlight the need to be vigilant in examining the detail of the experimental protocol before drawing firm conclusions. In a subsequent paper Schets, *et al*. (2002) compared European reference methods for quantitation of coliforms and *E. coli* to alternative methods (Colilert®/Quanti-Tray®) in three Dutch laboratories. Reference methods included membrane filtration using Lactose TTC with Tergitol 7 (LTTC) with confirmation by oxidase tests (coliforms) and by indole production for *E. coli*. Membrane filtration using membrane lauryl sulphate agar (MLSA), Chromocult® (CCA) and *E. coli* direct plating (TSA/TBA) were also evaluated. Background, non target growth on LTTC was problematic for all laboratories with less observed on MLSA and minimal background growth on CCA. Colilert-18® recovered significantly more coliforms than did MLSA, LTTC or CCA. In contrast, LTTC and direct plating recovered significantly more *E. coli* than did Colilert® and CCA. The authors suggested that indole tests provide a better confirmation step for *E. coli* than does ß-glucuronidase, as only 2 - 4% of *E. coli* strains are indole-negative, while in a previous study the authors reported 14% of recovered *E. coli* strains were ß-glucuronidase negative. This has not been supported by work in other countries (Fricker *et al*. 2008, 2009).

Work was also underway in the UK during the early to mid 1990s. An initial, multicenter study, carried out under the auspices of the Standing Committee of Analysts, compared Colilert® with membrane filtration using membrane lauryl sulphate broth. The results of the study showed that Colilert® was significantly less sensitive than the membrane filtration method. However, the study was grossly flawed. In order to avoid analysis of large numbers of samples, the study used only samples which had previously been shown to contain coliforms using the membrane filter technique. This pre-selection of samples meant that only samples where the membrane filtration procedure was able to detect coliforms were used. Thus, the chance of the Colilert® procedure giving results that were equivalent or better than the membrane filter method was small. Subsequent work at Thames Water in the UK (Cowburn *et al*. 1994) showed that Colilert® was at least as specific as membrane filtration and was more sensitive, particularly for coliform organisms. The authors concluded that the increased sensitivity was due in part to the use of a liquid medium and in part to the wider range of coliform organisms being detected. These workers saw benefits to the use of enzyme substrate media, particularly with regard to increased specificity, however they noted that the specificity of all enzyme substrate-based methods was not the same (Fricker and Fricker 1996a, 1996b) and that *Aeromonas* spp., in particular were a cause of false positive coliform reactions with some methods. Whilst presence/absence methods had become accepted and widely implemented in North America, the UK still required microbiological methods for drinking water to be quantitative. The method that was being used almost universally in the UK during the mid 1990s was membrane filtration with membrane lauryl sulphate broth which could be read at 18h. The combination of the non-quantitative format (traditional multiple fermentation tube MPN procedures were too cumbersome) and the 24 h incubation period meant that Colilert® was not adopted in the UK. In 1997, Fricker *et al*., reported on the development of a new formulation of Colilert® which could allow simple and rapid quantification of coliforms and *E. coli* within 18h.

In Sweden, Eckner (1998) compared the Swedish standard methods of membrane filtration and fermentation tube MPN to Colilert® for coliform and *E. coli* recovery using raw and treated drinking water and wastewater from southern Sweden. Membrane filtration was performed using m-Endo LES with verification of oxidase negative isolates in fermentation tubes

of LTB and LTLSB. MPN was performed using three and five dilution series for drinking waters and surface water/wastewater, respectively. Colilert® was enumerated using Quanti-Tray®. Eckner reported Colilert® was more sensitive than either membrane filtration or MPN for coliform detection and equivalent for *E. coli* detection, when all samples were grouped for statistical analysis.

Since the introduction of the most recent European Drinking Water Directive, there has been an interest in the comparison of methods with lactose tergitol TTC agar which is recommended as a reference procedure. As noted earlier, Schets and co-workers (Schets *et al.*, 2002) found Colilert® to detect more coliforms but fewer *E. coli* than the reference procedure. Conversely, other European studies, some involving multiple laboratories in multiple countries (Niemela *et al.* 2003, Bonnadonna *et al.* 2006, Fricker *et al*. 2008), have not confirmed this finding. In the three studies mentioned, Colilert® recovered more coliforms and *E. coli* than the reference procedure.

Very few articles have been published on the use of other enzyme-based methods for detection of coliforms in a presence-absence format. McFeters *et al.* (1997) reported on the comparative performance of Colisure® with drinking water samples. Colisure® presence-absence medium was compared with standard reference methods for detecting low numbers of total coliform bacteria and *E. coli* in drinking water when the bacteria were subjected to chlorine stress. When Colisure® was compared with established reference methods to detect total coliforms in dilute, disinfected samples, Colisure® yielded more positive results after 24, 28, and 48 h than lauryl tryptose broth (LTB) confirmed in bile green lactose broth after 48 h. Colisure® also detected higher levels of chlorine-injured *E. coli* than LTB confirmed in EC medium with 4-methylumbelliferyl B-D-glucuronide (EC/MUG). The sensitivity and specificity of Colisure® was also evaluated and was determined to be between 96 and 100 percent on nonchlorinated samples when positive and negative tests were verified.

Membrane media containing enzyme substrates

One drawback of early membrane filtration media was the inability to recover and identify both total coliforms and *E. coli* simultaneously. Typically, these systems required the use of various incubation temperatures and media in a sequential manner, which increased the time and labor required, and delayed the results. Numerous efforts were reported to develop media that recovered total coliforms and *E. coli* with a single incubation. Shadix, Dunnigan and Rice (1993) evaluated a two-step membrane filtration procedure for *E. coli* enumeration that employed m-Endo LES for coliform isolation, with positive filters transferred to nutrient agar plus MUG for additional 2-hour incubation to identify *E. coli* by fluorescence. Shadix tested 182 pure cultures of *E. coli* recovered from environmental samples, and 91.8% fluoresced, including *E. coli* previously exposed to chlorination. Brenner *et al*., (1993) reported the development of a new medium, MI agar, for the simultaneous enumeration of total coliforms and *E. coli* from water. The medium contains MUgal for the detection of total coliforms (by fluorescence) and IBDG for *E. coli* detection (indigo colonies). They reported MI agar was statistically superior to m-Endo agar for coliform enumeration and recovered more *E. coli* than mTEC, although the differences were not statistically significant. Additionally they reported low false positive and false negative rates (both 4.3%) and specificity of 87.9% or 93.1%, depending upon how coliforms were defined (the former includes only *Escherichia, Enterobacter, Citrobacter* and *Klebsiella*). Subsequently, Brenner *et al*. (1996b) compared coliform and *E. coli* recoveries

using MI agar and the EPA-approved membrane filtration method for total coliforms and *E. coli* (m-Endo and NA-MUG) using wastewater-spiked drinking water samples. They reported that, overall, MI agar showed significantly higher recoveries of total coliforms and *E. coli*, and significantly lower recovery of background bacteria than the EPA method. Further,they reported that performance of MI agar improved with respect to the EPA method with increased injury of target organisms. In related work, Brenner *et al*. (1996a) also compared the performance of MI agar and the EPA-approved membrane filtration method (m-Endo and NA-MUG) in a collaborative study involving 19 laboratories. Membrane filters were all from a single lot of Sartorius cellulose nitrate filters, and all media were prepared in the EPA's laboratory and distributed just prior to use. A set of six tap water samples were spiked with wastewater to contain a range of *E. coli* concentrations. The MI agar recovered more total coliforms and *E. coli* than the EPA-approved method, although the differences were not significant. These results differed from the earlier (single laboratory) study, in which they reported significantly improved recoveries of coliforms and *E. coli* on MI agar as compared to the reference method. Brenner speculated that these differences may be a result of lack of media familiarity, changes in sample composition during shipment (although all samples were analyzed within 72 hrs), and/or analyst error. As in the earlier study, Brenner noted that MI agar recovered significantly less non-target bacteria than did the EPA-approved method. The MI agar was concluded to be equal to or better than the EPA-approved method.

Ciebin *et al*. (1995) modified two fecal coliform media (m-FC and m-TEC) with chromogenic substrate (BCIG) for quantitative *E. coli* detection and compared performance in sewage, lake, river and well samples. No significant difference was observed between the methods and the authors suggested that these media (FC-BCIG and TEC-BCIG) give results comparable to results from studies using MUG-based media for *E. coli* detection. The authors cite the blue-colored *E. coli* colony forming units (from BCIG) as a method advantage over MUG-based fluorescent *E. coli*. False positive results (non-*E. coli* blue colony forming units) were observed principally from *K. pneumonia, C. freundii,* and *A. hydrophila.* Low false negative results were reported from both media.

Grant (1997) reported the development of m-ColiBlue24® broth, a new membrane filtration medium for simultaneous detection of total coliforms and *E. coli*. The author evaluated its performance using a presence/absence format and reported that total coliform recovery was comparable to recovery on m-Endo, and that fecal coliform recovery was superior to recovery on mTEC in terms of specificity, sensitivity, false positive error, and undetected target error.

There have been many reports of new media or of comparisons of one medium with another but there have been very few studies which have looked at several different media simultaneously. ISO has produced a standard for comparison of quantitative microbiological methods (ISO 17994) and has also developed a "standard reference procedure" for the detection of coliforms and *E. coli* in water. Unfortunately, it is one of the least suitable methods to adopt as a reference procedure because despite its sensitivity, it is almost completely non-selective and supports the growth of a wide range of bacteria resulting in frequent overgrowth especially during side-by-side comparison studies. In situations where there is no clearly defined reference procedure or where several methods are approved then it becomes necessary to carry out large side-by-side comparison studies.

Wohlsen *et al*. (2006) reported on the evaluation of methods for enumerating coliforms from water using precise reference standards. These authors aimed to use flow cytometry-counted bacteria to compare a number of culture techniques. Whilst the study was a small one,

eight methodologies were compared albeit with only two different organisms. The results were not adequately analyzed and media and counting procedures (membrane filtration, Quanti-Trays®, MPN tubes, Petri film) were not adequately separated so that it was not clear if the media or the counting procedures were responsible for any differences. The conclusions were that two methods, Petri film and pour plates, were the "best" methods, but neither of these methods are suitable for routine use since they analyze only a small volume (usually 1 mL) of water.

Another study of considerable interest was published by Oldstadt *et al*. (2007), where ten USEPA-approved methods were compared by determining their ability to support the growth of five different coliform species and to suppress the growth of *Aeromonas*, a frequent cause of false positive coliform results. The authors concluded that there were considerable differences between the different methodologies both in terms of their ability to support the growth of target organisms and also their ability to suppress *Aeromonas*. Furthermore, there were differences observed with different waters and a suggestion was made that differing water chemistry may have had an effect. This study showed the importance of using different water types and different organisms when comparing a variety of methods. However, the authors were fortuitous in finding organisms from a relatively small group which showed differences and it is unclear how often such strains occur in naturally contaminated samples. It remains the best option to use naturally contaminated samples of drinking water but the cost and length of time required to conduct such studies makes their use almost impossible. There remains a need for established protocols to be developed for comparing both quantitative and non-quantitative procedures. Sensitivity and specificity are the two most important parameters, and these should be measured with organisms that are likely to find their way into drinking water systems. There appears to be an over emphasis on the determination of repeatability and reproducibility for microbiological methodologies without understanding how these parameters can vary with different water types and levels of contamination. It is preferable to develop robust and useful procedures for comparison of multiple microbiological methods, such that reference procedures can be developed and used to ensure progression of new techniques in performance-based methods.

CHAPTER 2
MATERIALS AND METHODS

The methods tested during this study were those approved by USEPA and the UK Drinking Water Inspectorate. Only one method (Colilert-18®) is approved by both regulatory bodies. The methods and their characteristics are listed in Table 2.1.

All media was prepared and stored according to manufacturer's instructions. Dehydrated media were stored at room temperature in the dark. Standard Operating Procedures were prepared for each method including details of media preparation. Quality certificates for each batch of each medium purchased were obtained and retained. Details of the preparation of each batch of medium were recorded in a media log with date prepared, pH, expiry date and plate sterility information.

Quality control was performed on all new lots of media received. In addition, when new media was prepared, three organisms were subcultured onto each batch (*E. coli* ATCC 11775, *E. aerogenes* ATCC 13048, and *P. aeruginosa* ATCC 10145) and incubated as per method instructions to ensure correct performance. Prepared media were stored in the dark at 2 - 8°C and discarded two weeks after preparation (Walter, Fricker, and Fricker 1994).

Table 2.1
Media tested during the study and their characteristics

Medium	Method	Coliform	*E.coli*	Comment	Confirmation
MLSB	Membrane filtration	Lactose fermentation. Yellow colonies	Lactose fermentation at 44°C. Yellow colonies	Samples run at two temperatures	Required
MLGA	Membrane filtration	Lactose fermentation. Yellow colonies	Cleavage of BCIG. Blue or green colonies	Single membrane	Required
Lactose tergitol TTC	Membrane filtration	Lactose fermentation. Yellow colonies	Lactose fermentation. Yellow colonies	Two membranes recommended.	Required
m-Endo LES	Membrane filtration	Lactose fermentation. Sheen colonies	Not applicable	Single membrane, coliforms only	Required
mFC	Membrane filtration	Not applicable	Lactose fermentation	Single membrane. Fecal coliforms	Required
MI agar	Membrane filtration	MUgal. Fluorescent colonies	IBDG Blue or indigo colonies	Single membrane	Not required
Chromocult®	Membrane filtration	Salmon gal. Salmon colonies	X-gluc. Dark blue colonies	Single membrane	Kovacs reagent to confirm *E.coli*
m-ColiBlue®	Membrane filtration	Red colonies	BCIG. Blue colonies	Single membrane	Not required
Coliscan®	Membrane filtration	Red Gal. Pink/magenta	X-gluc. Purple/blue	Single membrane	Not required although Aeromonas will grow
E*Colite	Presence/absence	X-gal. Blue color	MUG. Fluorescence	Not applicable	Not required
ReadyCult®	Presence/absence	X-gal. Blue color	MUG. Fluorescence	Not applicable	Not required
Colitag®	Presence/absence	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colilert®	Presence/absence. Quantitative with Quanti-Tray®	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colilert-18®	Presence/absence Quantitative with Quanti-Tray®	ONPG. Yellow color	MUG Fluorescence	Not applicable	Not required
Colisure®	Presence/absence Quantitative with Quanti-Tray®	CPRG Magenta	MUG Fluorescence	Not applicable	Not required

WATER SAMPLES

Each utility was asked to send at least 6 L of dechlorinated finished drinking water, 1L of raw source water, and 1 L of secondary effluent (not disinfected) from their wastewater treatment plant at least five times during the course of the study. A sampling schedule was organized with at least four utility samples expected to be received at ASI each week. Originally, twenty three U.S. utilities and three utilities in the U.K. had agreed to participate by sending letters of support for the project. One utility in the U.S. and one utility in the U.K. withdrew from the study before Phase 1 began.

Where utilities did not have access to wastewater samples, chlorine injury experiments were performed using secondary wastewater from South Burlington's Wastewater Treatment Facility.

Experiments with Bioballs™

Bioballs™ (BTF, Sydney Australia) are freeze dried water soluble balls which deliver 28 – 33 CFU of a selected organism. Each individual Bioball is prepared by counting the bacteria using flow cytometry and collecting the drop of liquid into liquid nitrogen before storing at -70°C. These reference materials are widely used in the food and pharmaceutical industries for Quality Control and Quality Assurance purposes and can also be used to determine performance characteristics of methods for specific bacteria. The organisms used in this study were *E. aerogenes* (ATCC 13048/NCTC 10006) and *E. coli* (ATCC 25922/ NCTC 12241). Bioballs were shipped on dry ice from the manufacturer and stored in a Thermo Electron Corp. chest freezer at -80 +/- 2°C.

During Phase 1 of the study, initial comparisons were made with drinking water using all of the EPA and DWI approved methods (15), using both presence/absence and quantitative procedures. A total of 22 utilities in the U.S. sent at least 5 finished drinking water samples to ASI over a 22 week period in 2007 (5/14/07 – 10/22/07). Additional samples were obtained over an 8 week period in 2008 (6/3/08 – 7/30/08) to increase the amount of *E. aerogenes* Bioball data. Studies using samples from 3 sites in the U.K. were performed in the UK during September 2007.

Study participants sent drinking water samples in cubitainers or similar containers. These samples were aliquoted into three 2 L clean containers. Each week, either *E. aerogenes* or *E. coli* was chosen to inoculate into the batch of samples received. The first 2 L aliquot was processed with no Bioballs added, the second 2 L aliquot with 1 Bioball, and the third 2 L aliquot with 2 Bioballs. Bioballs were taken from the freezer and added directly to water samples. The first sample was used to determine if there were target organisms (coliforms or *E.coli*) present in the samples. The second and third samples had theoretical concentrations of target organisms of 1.5 and 3.0 CFU/100 mL, respectively. Inoculated samples were inverted gently several times to mix well and 100 mL from each 2L aliquot was processed for each of the 15 methods.

The three quantitative DST (defined substrate test) tests, Colilert®, Colilert-18®, and Colisure® were processed by adding the contents of media blister packs (each pack contains the pre-determined amount of medium for 100 mL of water) packets directly to 100 mL of inoculated sample in 125 mL IDEXX vials, allowing media to dissolve, and sealing them in Quanti-Trays®. Three of the methods used were presence/absence formats: ReadyCult®, E*Colite, and Colitag® and were processed by adding contents of media packets to 100 mL of inoculated sample in containers provided by the manufacturers.

The remainder of the tests used membrane filtration to concentrate 100 mL inoculated samples onto a filter which was placed on each medium. Sterile, disposable 0.45 um filter

funnels (Pall Corp.) were used for all membrane filtration performed using Bioballs. All media were prepared and stored according to manufacturers' instructions (m-Endo LES, m-FC, MI, m-ColiBlue24®, Chromocult®, Coliscan® MF, MLSB, Tergitol-7, MLGA). All media were incubated and examined according to manufacturers' instructions. Incubation times and plate count results were recorded on prepared result spreadsheets.

Confirmation of target colonies (where appropriate) was performed by subculturing to nutrient agar and then testing for the ability to ferment lactose, produces the enzyme β-D-galactosidase (using ONPG broth) and production of cytochrome oxidase.

Recovery of Coliforms and *E. coli* from Raw Water using Different Media

A total of 40 raw water samples with a target count of 1 - 20 coliforms/ 100 mL were analyzed by ten quantitative methods. A total of 72 raw water samples with a target count of 1 – 10 *E. coli* / 100 mL were analyzed by the same ten quantitative methods. An initial 100 mL volume was analyzed using Colilert-18®/Quanti-Tray® and the remaining material held in the refrigerator overnight. When the results of the initial enumeration were available, the samples were diluted to give target ranges of less than 20 CFU/100 mL and for *E. coli*, approximately 5 CFU/100 mL.

Disinfection Experiments

In order to test the ability of the various methods to recover chlorine-damaged organisms, a series of experiments was performed using the protocol described in ISO 17994 for the comparison of performance of quantitative microbiological methods. The procedure has been widely used in Europe and is similar to that used in the US. Secondary sewage effluent was allowed to settle for a minimum of one hour and was then diluted 1:10 in dechlorinated tap water (10 L). Diluted samples were then placed on a magnetic stirrer and chlorine solution was then added to give an initial concentration of approximately 1.5 mg chlorine/L. Samples (1.2 L) were then removed at one-minute intervals for eight minutes. Each aliquot was then tested with Colilert-18®/Quanti-Tray® to enumerate coliforms and *E. coli*. The remaining material from each aliquot was then stored at 2-8°C overnight until the initial results were available.

After reading the results obtained using the initial Colilert-18®/Quanti-Tray® analysis, a suitable bottle was selected from each disinfection run to compare the performance of the quantitative methods. Initially, the target range for the comparisons was 20-50 CFU/100 mL as described in ISO 17994. However, experience showed that this range was far too high for dilute, disinfected wastewater and caused significant problems with reading and interpretation of membranes, particularly those methods that simultaneously detect coliforms and *E. coli* on membranes incubated at 35-37°C. Background growth was often far too extensive to enable accurate counting of target colonies. Consequently, the target range for coliforms was reduced to less than 20 CFU/100 mL and for *E. coli* to approximately 5 CFU/100 mL.

INVESTIGATION OF THE PERFORMANCE OF B-D-GLUCURONIDASE-BASED METHODS IN DETECTING GLUCURONIDASE ACTIVITY

Testing for β-D-glucuronidase activity has become the basis of many methods for the detection of *E. coli* in both food and water. Used in combination with tests for the presence of β-D-galactosidase, these tests offer a simple method for simultaneously detecting coliforms and *E. coli*. The ability of membrane lactose glucuronide agar (MLGA), Colilert-

18®, MI agar, Colitag® and Chromocult® agar to detect β-D-glucuronidase activity was tested with over 1000 isolates of *E. coli* recovered from naturally contaminated water samples.

Samples of raw water and sewage effluent that were examined for routine purposes were used in this study. All routine water samples were processed using Colilert-18® together with Quanti-Tray®. After incubation at 35°C for 18-22 h, wells that were yellow in color and fluoresced under ultraviolet light were subcultured directly onto MLGA, MI agar, Chromocult® and Coliscan® media. All media were incubated according to manufacturer's instructions.

In addition, a total of 312 blue colony forming units (so called fecal coliforms) isolated on mFC agar from diluted sewage effluent after incubation at 44°C for 24 h, were used to test the media. Cultures were checked for purity on MacConkey agar and then subcultured onto the same four media as were used above and also into Colilert-18®.

After incubation for the appropriate period, all plates were examined for reactions indicating the activity of the β-D-glucuronidase enzyme and the results recorded.

INTENSIVE SAMPLING IN FLORIDA AND CALIFORNIA

Two sites were selected, one in Florida and the other in California, on the basis that one public water system had experienced coliforms in both the finished water leaving the treatment plant and water in distribution, while the other had very rarely detected coliforms in the finished water. Sampling was undertaken four days a week for two weeks at both sites. The finished water leaving the plants was sampled on each day at hourly intervals for six hours, and a site within the distribution system was sampled hourly for four hours on each day. Two samples (2.2 L each) were taken during each sampling event. The samples were analyzed in four different ways: Coliscan® 100 mL and 2 L; and Colilert-18® 100 mL and 2 L. For the Coliscan® samples, water was concentrated by membrane filtration. For Colilert-18®, one sample was analyzed using Quanti-Tray® and for the other 1.9 L was concentrated by membrane filtration and then placed into 100 mL of the sample in which a sachet of Colilert-18® reagent had been dissolved. Samples were then incubated according to manufacturer's instructions and presumptive positive organisms confirmed using standard physiological tests.

PERFORMANCE OF TWO METHODS IN ROUND ROBIN STUDIES

Nine laboratories participated in the round robin study (eight where material had to be shipped from ASI). Each was provided with the necessary supplies (water, inoculating supplies, media, etc.) to perform Colilert® (presence/absence and quantitatively) and Coliscan® to compare the detection of total coliforms and *E. coli.* These tests were performed on 100 mL and 2 L samples of both participating laboratory's water and deionized water provided by ASI. The laboratories processed both types of water using the two methods provided – Colilert® and Coliscan® – and reported all results back to ASI. In addition, general water quality information (HPC, turbidity, etc.) was shared for quality control purposes.

To each 20 liters of ASI prepared deionized water, 3.0 g of sodium benzoate and 1.5 g of potassium phosphate were added to encourage the stability of the target organisms through shipping. A total of 4.6 L of ASI deionized water was sent to each participating laboratory consisting of two 2 L bottles and six 100 mL bottles. Each bottle was inoculated with varying concentrations of *Enterobacter aerogenes*, and *Escherichia coli*, as well as a confounding organism *Pseudomonas aeruginosa*. Table 2.2 shows the inoculation

concentrations of each bottle – two of each of the following were sent to the participants for analysis.

Table 2.2
Suspensions of bacteria used for round robin studies

	E. coli (CFU/100 mL)	*E. aerogenes* (CFU/100 mL)	*P. aeruginosa* (CFU/100 mL)
100 mL Bottle #1	20	10	20
100 mL Bottle #2	10	5	20
100 mL Bottle #3	10	5	50
2 L Bottle #1	5	5	20

For the 2L samples, each was run through a membrane filtration apparatus and analyzed using Colilert® and Coliscan®. One filter was plated onto a sterile pad containing Coliscan®. The other filter was immersed into 100 mL of the individual laboratory's DI water containing Colilert®. Of the two sets of 100 mL samples, one of each was processed using Coliscan® and the other sample was processed using Colilert®/Quanti-Tray®. Overall, one set of ASI water samples – a 2 L sample and three 100 mL samples – was processed using Colilert®/Quanti-Tray® and the remaining set was processed using Coliscan®. The samples were incubated at the appropriate temperature and time period according to the individual methods.

In addition, each participant was sent eight 10 mL vials of ASI deionized (DI) water (containing sodium benzoate and potassium phosphate) concentrates containing the same inoculation concentrations to inoculate their laboratory's water. The concentrates were sent to replicate the same volume and quantity – two 2 L and six 100 mL samples – that were sent to the participants. The laboratory added sodium thiosulfate to their water prior to inoculation and processing. The concentrates/laboratory water samples sets were processed in the same manner as the ASI water samples.

Samples were shipped via overnight priority shipping to each participant inside an insulated cooler containing 2 medium ice packs (frozen at -80°C prior to shipping). Each cooler contained two 2 L samples inside sterilized plastic Nalgene bottles, six 100 mL samples inside sterilized plastic bottles, eight 10 mL concentrates inside plastic screw-top bottles, and the necessary paperwork, media, and supplies for processing. The supplies and water samples were secured in the cooler for shipping using both bubble wrap and craft paper. Upon arrival, the temperature of the samples was taken by the participating laboratories and the samples were processed immediately. At the same time, one set of samples was packaged in accordance and the cooler retained at ASI at ambient temperature. These samples were processed the following day by a trained microbiologist at ASI in the same time frame as the shipped samples.

INVESTIGATION INTO THE BACTERICIDAL EFFECTS OF WILLISTON TAP WATER

During the course of various experiments, it was noted that Williston (Vermont) tap water had a marked bactericidal effect. Bacteria were seeded into dechlorinated tap water from various sites, deionized water and Williston tap water and the number surviving after one hour was determined. Coliform bacteria were enumerated on MacConkey agar. In initial experiments, several water samples were spiked with different coliforms that had been grown in nutrient broth and diluted in deionized water. The coliform strains used in these experiments were *E. coli*, *Klebsiella terrigena*, *Hafnia alvei* and *Enterobacter aerogenes*.

Further work was then undertaken to determine the cause of this bactericidal effect. The objectives of this work, funded as an extension to the project were:

1. Is polyelectrolyte the cause of the observed bactericidal effect?
2. Does the concentration of the chemical responsible have a marked effect on inactivation?
3. To what degree are coliforms inactivated by the chemical over the commonly used hold times for drinking water microbiological samples?

Based upon examination of the operational data at the plant together with the fact that filtration through activated carbon filters removed the bactericidal effect, it was hypothesized that the cause of cell inactivation was Superfloc C572, a polyelectrolyte used as a coagulation aid. Dilutions of the polymer were made in sterile deionized water and bacterial suspensions added. Samples were withdrawn at several time points and enumerated by membrane filtration and incubation on MacConkey agar.

STATISTICAL METHODS

The statistical approach employed for much of this study was based upon the International Standards Organization method for comparison of microbiological methods (Anon, 2004). The basic data are pairs of confirmed counts (a_i, b_i) obtained from the examination of two equal portions taken from the same vessel of a carefully mixed test sample, with one determination (count) per method. The complete design consists of a large number of similar determinations. Two methods are considered quantitatively equivalent ("not different") if the mean relative difference of the paired confirmed counts does not differ significantly from zero and the expanded uncertainty does not extend beyond the level of the stipulated maximum acceptable deviation. Internationally it has been suggested that a difference of 10% between methods should be considered significant and that value has been used in this study. ISO 17994 considers that

> "The requirements for method comparisons differ somewhat from the daily routine situation. It is useful and often necessary to pre-select or prepare special samples. Samples for method comparisons should contain enough bacteria that the likelihood of scoring a zero count is small. Samples for method comparisons should represent types that are included in the scope of both methods. Natural samples are ideal. Appropriate samples may also be prepared by dilution, spiking, or mixing of different kinds of water to achieve the desired population in a suitable density. Spiking with pure cultures should be considered the last resort. It may be appropriate to stress the microbial population of some samples by controlled application of disinfectants or by

> refrigerated storage in order to simulate situations encountered in routine laboratory practice" (ISO 2004).

During this study, virtually all samples used were "specially prepared", either by diluting raw waters or by disinfecting sewage effluent to mimic samples of non-disinfected and disinfected drinking water respectively. Where analyses were performed by 17994, tergitol TTC medium was used as the reference procedure as this is the recognized ISO reference procedure. The procedure was used to test the hypothesis that there was no more than 10% difference between the reference method (tergitol TTC) and an alternative method.

The ISO procedure offers two methods of dealing with sample pairs where one of the results is zero. Data containing zeros must be treated specially as for the analysis, the data are log transformed. The preferred option is to remove all data where one of the pair of results contains a zero. However, when dealing with data with many zero data points it may be preferable to add one to each number in a pair that contains a zero. During the analysis data were usually compared by removing pairs of results containing zeros. However, the alternative procedure was used to test if there was any difference in the statistical outcome between the two procedures. This was seen on one occasion where there were many zeros present in the data.

There were many occasions when the statistical analysis could not identify a difference between the reference procedure and the alternative method and indicated that more samples would be needed to determine if a significant difference was present. In most cases, there was insufficient time and resource to perform additional analyses. The ISO procedure, while not perfect, does offer the advantage of being able to identify if there is insufficient data to come to a conclusion about the statistical significance of the data.

For quantitative procedures, a two way Analysis of Variance (factors: sample and method) was also performed for each data set to determine if there was any significant difference between the results obtained by all of the methods.

For data sets where the methods were compared on a presence/absence basis, McNemar's test for paired samples was used. The hypothesis used for these tests was that there was no difference in the number of positive samples found with a test method when compared to a reference method.

The experiments with Bioballs™ were used to generate presence/absence data and these data were analyzed by McNemar's test. The hypothesis used was that there was no statistical difference between the proportion of samples positive for the target organisms with a test method and the reference method, which in this case was tergitol TTC agar. Data were compared for concentrations of 1.5 and 3.0 cfu/100 mL for both *E. coli* and *E. aerogenes*. The combined data for both 1.5 and 3.0 cfu/100 mL were also analyzed for each organism. The comparison-wise error rate for these experiments was 0.05 but because fourteen different methods were compared from the same data sets, the experiment-wise error was 0.05/14 which equates to 0.004. Comparisons were made both at the $p = 0.05$ and $p = 0.004$ levels.

For experiments in which the different media were compared with disinfected and non-disinfected waters the hypothesis used was that there was no difference between the performance of the different methods and the performance of the reference method (tergitol TTC) in recovering coliforms or *E. coli*. The data were compared using a two way ANOVA together with the ISO procedure described in 17994. This approach was used for most of the experiments described within this report. In order to compare the performance of 17994 and more classical statistical procedures, for the comparison of media for recovering coliforms and *E. coli* from raw water a Tukey adjustment for multiple comparisons was performed.

CHAPTER 3
RESULTS

EXPERIMENTS WITH BIOBALLS

The data generated during this study assessed various performance aspects of the different methods. Initial inherent sensitivity was determined using low numbers of specific strains of *Enterobacter aerogenes* and *E. coli.* These data were generated using Bioballs™ and assessed the ability of the various media to recover two different strains. Such comparisons do not take into account the large variation in phenotypic characteristics expressed by the large numbers of coliform strains, but give a basic understanding if there are gross differences in the ability of the media to recover members of the coliform group.

Initial studies involved the use of *E. coli* Bioballs diluted to give a theoretical concentration of 1.5 and 3.0 CFU/100 mL. The results for the presence/absence data are presented in Tables 3.1, 3.2 and 3.3. The null hypothesis for presence/absence experiments with BioBalls was that that the mean number of positive samples with any given method did not differ significantly from that of the reference procedure (lactose tergitol TTC medium) at a probability of $p = 0.05$. Comparisons were also performed at $p = 0.003$ since this is the level required when doing multiple comparisons ($p = 0.05/14$) with 15 methods in all

This work was then repeated using *E. aerogenes* Bioballs. Samples were prepared to give a theoretical concentration of 1.5 and 3.0 CFU/100 mL. The results for the presence/absence data are presented in Tables 3.4, 3.5 and 3.6. Experiments with no Bioballs added to the samples did not result in the detection of any coliform organisms.

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Table 3.1
Numbers of samples examined and found to contain *E. coli* using a theoretical concentration of 1.5 CFU/100mL. McNemar's test

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
Tergitol TTC	72	56	77.8	n/a
Colilert-18®	72	56	77.8	p = 0.92
Chromocult®	72	54	75.0	p = 0.76
m-Endo	72	51	70.8	p = 0.37
MLGA	72	51	70.8	p = 0.39
Colilert®	72	50	69.4	p = 0.31
Coliscan®	72	49	68.1	p = 0.26
Colitag®	72	47	65.3	p = 0.11
MI agar	72	46	63.9	p = 0.07
MLSB	**72**	**45**	**62.5**	**p = 0.04**
mFC	**72**	**44**	**61.1**	**p = 0.02**
ReadyCult®	**72**	**44**	**61.1**	**p = 0.04**
E*Colite	**60**	**35**	**58.3**	**p = 0.02**
Colisure®	**72**	**30**	**41.7**	**p = 0.001**
m-ColiBlue24®	**60**	**3**	**5.0**	**p < 0.001**

n/a = not applicable
Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

Table 3.2
Numbers of samples examined and found to contain *E. coli* using a theoretical concentration of 3.0 CFU/100mL. McNemar's test

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
Chromocult®	72	69	95.8	p = 0.27
Colitag®	72	67	93.1	p = 0.45
Coliscan®	72	66	91.7	p = 0.66
Tergitol TTC	72	64	88.9	n/a
Colilert®	72	64	88.9	p = 0.89
m-Endo	72	63	87.5	p = 0.89
Colilert-18®	72	63	87.5	p = 0.89
ReadyCult®	72	62	86.1	p = 0.71
MI agar	72	61	84.7	p = 0.54
MLGA	72	61	84.7	p = 0.54
mFC	72	60	83.3	p = 0.41
MLSB	72	58	80.1	p = 0.18
E*Colite	**62**	**42**	**67.7**	**p = 0.01**
Colisure®	**72**	**44**	**61.1**	**p < 0.001**
m-ColiBlue24®	**62**	**7**	**11.3**	**p < 0.001**

n/a = not applicable
Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

Table 3.3
Numbers of samples examined and found to contain *E. coli* using all data collected at theoretical concentrations of 1.5 and 3.0 CFU/100 mL. McNemar's test.

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
Chromocult®	144	123	85.4	p = 0.79
Tergitol TTC	144	120	83.3	n/a
Colilert-18®	144	119	82.6	p = 0.92
Coliscan®	144	115	79.9	p = 0.42
m-Endo	144	114	79.2	p = 0.36
Colitag®	144	114	79.2	p = 0.38
Colilert®	144	114	79.2	p = 0.40
MLGA	144	112	77.8	p = 0.26
MI agar	144	107	74.3	p =0.06
ReadyCult®	144	106	73.6	p = 0.05
m-FC	**144**	**104**	**72.2**	**p = 0.02**
MLSB	**144**	**103**	**71.5**	**p = 0.01**
E*Colite	**122**	**77**	**63.1**	**p < 0.001**
Colisure®	**144**	**74**	**51.4**	**p < 0.001**
m-ColiBlue24®	**122**	**10**	**8.2**	**p < 0.001**

n/a = not applicable
Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

Table 3.4

Numbers of samples examined and found to contain *Enterobacter aerogenes* using all data collected at a theoretical concentration of 1.5 CFU/100 mL. Data generated in the US. McNemar's test.

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
E*Colite	11	9	81.8	p = 0.77
Colilert-18®	59	46	78.0	p = 0.41
MLGA	59	44	74.6	p = 0.74
Chromocult®	59	44	74.6	p =0.75
MLSB	59	42	71.2	p = 0.92
m-Endo	59	42	71.2	p = 0.92
Tergitol TTC	59	42	71.2	n/a
Colilert®	59	41	69.5	p =0.91
Coliscan®	59	39	66.1	p = 0.59
ReadyCult®	59	38	64.4	p = 0.51
MI agar	59	38	64.4	p = 0.52
Colitag®	26	16	61.5	p = 0.21
Colisure®	**59**	**28**	**47.5**	**p = 0.01**
m-ColiBlue24®	**11**	**1**	**9.1**	**p < 0.001**

n/a = not applicable

Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

Table 3.5
Numbers of samples examined and found to contain *Enterobacter aerogenes* using all data collected at a theoretical concentration of 3.0 CFU/100 mL. Data generated in the US. McNemar's test.

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
MI agar	59	56	94.9	p = 0.11
m-Endo	59	55	93.2	p = 0.18
Colitag®	26	24	92.3	p = 0.77
Chromocult®	59	54	91.5	p = 0.31
Colilert-18®	59	52	88.1	p = 0.52
MLSB	59	52	88.1	p = 0.69
Coliscan®	59	51	86.4	p = 0.89
Colilert®	59	51	86.4	p = 0.90
ReadyCult®	59	50	84.7	p = 0.89
Tergitol TTC	59	50	84.7	n/a
MLGA	59	50	84.7	p = 0.90
E*Colite	11	9	81.8	p = 0.77
ColiSure®	59	47	80.0	p = 0.57
m-ColiBlue24®	**11**	**1**	**9.1**	**p < 0.001**

n/a = not applicable
Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

Table 3.6
Numbers of samples examined and found to contain *Enterobacter aerogenes* using all data collected at theoretical concentrations of 1.5 and 3.0 CFU/100 mL. McNemar's test.

Method	Number samples examined	Number of samples positive	Percentage of samples positive	Statistical significance
Colilert-18®	118	98	83.1	p = 0.26
Chromocult®	118	98	83.1	p = 0.35
m-Endo	118	97	82.2	p = 0.43
E*Colite	22	18	81.8	p = 0.26
MLGA	118	94	79.7	p = 0.80
MLSB	118	94	79.7	p = 0.81
MI agar	118	94	79.7	p = 0.82
Colilert®	118	92	78.0	p = 0.93
Tergitol TTC	118	92	78.0	n/a
Colitag®	52	40	76.9	p = 0.49
Coliscan®	118	90	76.3	p = 0.80
ReadyCult®	118	88	74.6	p = 0.59
Colisure®	**118**	**75**	**63.6**	**p = 0.02**
m-ColiBlue24®	**22**	**2**	**9.2**	**p < 0.001**

n/a = not applicable
Media in bold recovered significantly less organisms than the reference procedure at a probability of p = 0.05

It is clear from the results presented in Tables 3.1-3.6 that the use of pure cultures of bacteria for measuring the relative sensitivities of different methods has some merit, but that differentiation between methods is extremely sensitive to the numbers of bacteria used to perform the tests.

For *E. coli*, where very low numbers of target organisms (mean 1.5 CFU/100 mL) were used, six methods recorded significantly ($p < 0.05$) lower numbers of positive samples than did the reference procedure tergitol TTC agar: these were Colisure®, m-FC, m-ColiBlue24®,

ReadyCult®, MLSB and E*Colite. When the number of target organisms was raised to a mean of 3 CFU/ 100 mL, Colisure®, m-ColiBlue24® and E*Colite continued to detect significantly fewer positive samples but for ReadyCult®, MLSB and m-FC agar there was no significant difference. When the data from both sets of samples were combined, Colisure®, m-ColiBlue24®, mFC, MLSB and E*Colite all showed significantly fewer positive samples than the reference procedure. Colisure®, m-ColiBlue24® and E*Colite are all less inherently sensitive than the reference procedure since the differences seen between these methods and the reference procedure were < 0.003 which is the significance level required when 14 multiple comparisons are being undertaken. There is also a suggestion that at very low numbers of *E. coli*, m-FC, ReadyCult® and MLSB may result in fewer positive samples. Further, work should be undertaken to determine if these differences are indeed statistically significant. It is of interest that m-FC and MLSB are incubated at elevated temperature to help distinguish *E. coli* from other less thermotolerant coliforms.

For *Enterobacter aerogenes*, the picture was a little different but both Colisure® and m-ColiBlue24® detected significantly less positive samples than the reference procedure. For Colisure®, this difference was seen at the lower concentration of target organisms but not at 3 CFU/100 mL. However, when the data were combined the difference between the total number of positive samples was significantly less than that seen with the reference procedure. These data are of interest as they demonstrate the profound effect that using smaller numbers of bacteria can have on the overall data set. Only m-ColiBlue24® showed a statistically significant difference at the $p = 0.003$ level.

The m-ColiBlue24® and E*Colite methods did not perform well during the experiments with *E. coli* and consequently testing was discontinued as agreed with the Project Advisory Committee. There were problems with obtaining Colitag® from the manufacturers and consequently the number of samples tested was somewhat lower than for most media.

RECOVERY OF COLIFORMS AND *E. coli* USING DIFFERENT MEDIA

The second phase of experiments compared the ability of the different media to recover coliforms and *E. coli* from raw waters (non-disinfected samples) and from disinfected sewage effluent samples (chlorine stressed organisms). These comparisons took two factors into account, the sensitivity of the different media in recovering different strains of target organisms and the specificity of the media with respect to non-target organisms often found in drinking water. In addition, this phase of the work investigated the growth of non-target organisms from drinking water samples supplied by the various participating utilities.

A total of 40 raw water samples with a target count of 1 - 20 coliforms/ 100 mL were analyzed by ten quantitative methods. A total of 72 raw water samples with a target count of 1 – 10 *E. coli* / 100 mL were also analyzed by ten quantitative methods. The summary data for both experiments are shown in Table 3.7

Table 3.7
Recovery of confirmed coliforms and *E. coli* from raw water on different media.

Medium	Total number of coliforms recovered from 40 samples	Total number of *E. coli* recovered from 72 raw water samples
Colilert-18®	298	411
Colilert®	289	397
Coliscan®	282	359
Colisure®	276	353
Chromocult®	275	357
MI	266	388
MLGA	261	350
m-Endo	240	Not tested
mFC	Not tested	331
Tergitol TTC	234	336
MLSB 37°C	228	Not tested
MLSB 44°C	Not tested	363

In an effort to assess performance with low concentrations of indigenous bacteria, only data from samples in which there were no more than 20 coliform CFU detected by any of the methods were used. The fewest coliforms were recovered from raw water by MLSB medium and Colilert-18® recovered the highest number (Table 3.7). Generally, the methods that rely upon detecting the presence of the enzyme β-D-galactosidase recovered more coliforms than those that are based upon detection of lactose fermentation, which is to be expected. A two-way analysis of variance determined that there was a statistical difference between the means ($p < 0.001$). Application of ISO 17994 showed that only Colilert-18 differed significantly from the reference method, although all other differences were inconclusive, meaning that more samples would need to be tested in order to determine if the observed differences were statistically significantly more than 10%. A Tukey adjustment for multiple comparisons was applied to the ANOVA and this demonstrated that only Colilert-18® gave significantly higher recoveries than the reference method ($p = 0.01$) which was in agreement with the ISO 17994 results. The number of positive colonies/wells considered to be presumptive coliforms was method dependent (i.e. fermentation of lactose or possession of the enzyme β-D-galactosidase) and confirmation was based upon demonstrable β-D-galactosidase activity and a lack of cytochrome oxidase.

Similar experiments were performed with 72 samples of raw water for the methods used to recover *E. coli* (Table 3.7). Only data where there were no more than 10 CFU detected by any of the methods were used. Even at these levels some membranes had heavy growth, particularly

those that were incubated at 35-37°C. A two-way analysis of variance suggested that there was no statistical difference between the means (p = 0.09).

Colilert-18® recovered the highest number of *E. coli* and m-FC the lowest. No apparent difference was seen between the number of *E. coli* recovered by methods based upon detection of β-D-glucuronidase and that recovered by methods based upon incubation at elevated temperature and confirmed by fermentation of lactose and indole production. A notable exception was MLGA which surprisingly recovered slightly fewer *E. coli* than MLSB which is essentially the same medium without the addition of a β-D-glucuronidase substrate. This phenomenon was investigated later in the study. Whilst in the earlier experiments with pure cultures, MLSB detected significantly fewer *E. coli* positive samples than did the reference procedure, in this data set it actually recovered more. This may be due to the higher numbers of target organisms present in the sample but could also be explained by the wider range of *E. coli* strains encountered in this part of the study and the interaction between target and non-target flora, where the presence of background growth can significantly reduce the ability to detect target organisms.

The coliform recovery data discussed above (Table 3.7) was analyzed using statistical methods described in ISO 17994 (using the ISO reference procedure, tergitol TTC, as the reference method). The comparison was made using calculations that would detect a 10% difference between the methods. These data are shown below in Table 3.8.

Table 3.8
Statistical comparison of recovery of coliforms from raw water samples using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Results with zeros removed.

Method	Mean relative difference	Standard deviation	X_L	X_H	Result
Colilert-18®	**23.5**	**60.0**	**3.8**	**43.3**	**Higher**
Colisure®	20.2	62.5	-0.6	41.1	Inconclusive
Coliscan®	19.1	64.4	-2.4	40.6	Inconclusive
Colilert®	16.7	72.5	-7.1	40.6	Inconclusive
MLGA	15.6	57.9	-4.0	35.2	Inconclusive
Chromocult®	13.9	66.8	-9.4	37.2	Inconclusive
MI agar	12.2	50.4	-4.9	29.2	Inconclusive
MLSB	2.4	51.2	-14.9	19.8	Inconclusive
m-Endo	-6.9	77.4	-32.4	18.5	Inconclusive

The protocol described in ISO 17994 allows for either the removal of all data sets that include a zero value, or addition of one to all zeros and the corresponding value for the method being compared. Consequently, an additional statistical analysis was performed where zeroes were changed to one and the corresponding value of the method being compared, also increased by one. The data for this analysis are shown in Table 3.9.

Table 3.9
Statistical comparison of recovery of coliforms from raw water samples using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Zero values and the corresponding value in the method being compared increased by one.

Method	Mean relative difference	Standard deviation	X_L	X_H	Result
Colilert-18®	**21.6**	**65.5**	**0.6**	**42.6**	**Higher**
Coliscan®	15.8	80.9	-9.8	41.3	Inconclusive
ColiSure	15.5	68.5	-6.2	37.1	Inconclusive
MI agar	9.5	61.5	-10.0	28.9	Inconclusive
MLGA	6.5	71.7	-16.2	29.1	Inconclusive
Colilert®	2.3	90.3	-26.2	30.9	Inconclusive
Chromocult®	2.3	90.3	-26.2	30.9	Inconclusive
MLSB	-1.9	61.8	-21.4	17.7	Inconclusive
m-Endo	-4.7	89.0	-32.8	23.5	Inconclusive

Only one medium (Colilert-18®) showed a significantly higher recovery of coliforms from raw water than the reference method when the data were examined using the procedures described in ISO 17994. All other data sets were inconclusive. The reason for the large amount of inconclusive data appears to be because of the need to reduce the number of target colonies on each plate because of background growth. Very little conclusive data can be gleaned from these comparisons. The same pattern of results was seen whichever way zeros were dealt with in paired samples.

Based upon the statistical procedures applied thus far, there appears to be little statistical difference in the ability of different media to detect coliforms from raw water. However, examination of the data shows that the mean total number of coliforms recovered by methods based upon detection of the expression of β-D-galactosidase (281) was substantially higher than the mean recovery by methods based upon lactose fermentation (239). The galactosidase-based methods detected almost 20% more coliforms than the lactose-based methods. Since the galactosidase methods included both membrane filtration and liquid-based methods, it is likely that the difference is due to the difference in definitions of coliforms used by the two groups of methods. Methods based upon the detection of expression of β-D-galactosidase would be expected to detect more coliforms than methods based upon lactose fermentation, since the latter group fail to identify slow or late lactose fermenting organisms as coliforms whereas the former group would correctly identify them It cannot be definitively determined if the differences are due to the methods themselves or the difference in definition used by each type of procedure but it would appear likely that it is due largely to the difference in definitions used (i.e. lactose fermentation or possession of β-D-galactosidase activity).

The *E. coli* recovery data discussed above (Table 3.7) was also analyzed using statistical methods described in ISO 17994 (using the ISO reference procedure, tergitol TTC, as the

reference method). Again the procedure was used to detect differences greater than 10%. These data are shown below in Table 3.10.

Table 3.10
Statistical comparison of recovery of *E. coli* from raw water samples using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Results with zeros removed.

Method	Mean relative difference	Standard deviation	X_L	X_H	Result
Colilert-18®	**31.1**	**105.1**	**6.2**	**56.1**	**Higher**
MI agar	**26.3**	**92.4**	**4.5**	**48.0**	**Higher**
Colilert®	**21.9**	**85.6**	**2.3**	**41.5**	**Higher**
MLSB	14.5	71.1	-2.2	31.3	Inconclusive
Coliscan®	13.8	72.0	-3.2	30.7	Inconclusive
MLGA	11.2	76.5	-6.8	29.3	Inconclusive
ColiSure®	7.6	83.0	-12.2	30.7	Inconclusive
Chromocult®	5.0	76.9	-13.3	23.4	Inconclusive
mFC	1.0	93.8	-21.4	23.5	Inconclusive

Statistical examination of the data generated by comparing the recovery of *E. coli* by all quantitative methods indicated that three of the media tested clearly had higher recoveries than the reference procedure. These were MI agar, Colilert® and Colilert-18®. All other comparisons were inconclusive. These results were the same irrespective of which procedure was used to deal with zero counts. Again this is likely to be due to the low number of target organisms recovered. However, a two-way ANOVA suggested that there was no significant difference between the mean number of *E. coli* detected by the different methods ($p = 0.09$).

DISINFECTION EXPERIMENTS

The ability of the various methods to recover chlorine-damaged organisms was examined using diluted secondary sewage in a series of experiments using the protocol described in ISO 17994 for the comparison of performance of quantitative microbiological methods.

Substantially more data were generated for disinfected samples than for non-disinfected samples, reflecting the much higher incidence of disinfected water systems in the US and UK. The raw data are shown in the accompanying CD. Statistical comparisons of the data sets using the statistics outlined in ISO 17994 are shown in Tables 3.11-3.13. Data were compared using both methods of statistical manipulation described in ISO 17994 for total coliform organisms but for *E. coli*, because of the high proportion of zero values, only the procedure that allows for adding one to both numbers when one of the pair of values is a zero, was used.

Table 3.11
Statistical comparison of recovery of coliforms from disinfected sewage effluent using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Results with zeros removed.

Method	Mean relative difference	Standard deviation	X_L	X_H	Result
Colilert-18®	**39.6**	**104.9**	**23.2**	**56.1**	**Higher**
Coliscan®	**31.4**	**98.8**	**15.2**	**47.7**	**Higher**
ColiSure®	**30.2**	**89.9**	**15.5**	**44.8**	**Higher**
Chromocult®	**29.7**	**98.2**	**13.3**	**46.0**	**Higher**
Colilert®	**27.1**	**94.7**	**12.0**	**42.2**	**Higher**
MLGA	**22.2**	**83.8**	**8.8**	**35.5**	**Higher**
m-Endo	9.5	91.2	-4.9	23.9	Inconclusive
MI agar	7.9	91.0	-7.1	23.0	Inconclusive
MLSB	0.3	80.5	-12.7	13.4	Inconclusive

Table 3.12
Statistical comparison of recovery of coliforms from disinfected sewage effluent using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Zero values and the corresponding value in the method being compared increased by one.

Method	**Mean relative difference**	**Standard deviation**	**X_L**	**X_H**	**Result**
Colilert-18®	**59.0**	**97.6**	**45.6**	**72.4**	**Higher**
Colilert®	**36.0**	**99.8**	**21.9**	**50.0**	**Higher**
Coliscan®	**32.9**	**112.7**	**17.9**	**49.9**	**Higher**
MLGA	**30.5**	**90.7**	**17.7**	**43.2**	**Higher**
ColiSure®	**27.8**	**98.7**	**13.5**	**42.0**	**Higher**
Chromocult®	**27.4**	**108.5**	**11.8**	**43.0**	**Higher**
m-Endo	**21.1**	**93.1**	**8.4**	**33.7**	**Higher**
MI agar	10.7	101.5	-4.0	25.4	Inconclusive
MLSB	10.6	88.7	-2.1	23.4	Inconclusive

The data generated by comparing the various media for recovery of damaged (disinfected) coliforms showed that many of the media performed better than the reference procedure in terms of recovery. A two-way analysis of variance showed a highly significant difference between the means ($p < 0.0001$). MI agar and MLSB gave inconclusive results when compared to the reference procedure with both procedures described in ISO 17994 (removing zeros or adding one). However, with m-Endo the results were different depending on which

statistical manipulation was used. When all pairs of data that included a zero were deleted, the comparison between m-Endo and the reference procedure was inconclusive. When the alternative manipulation was used (adding one to each of a pair of numbers containing a zero) m-Endo was shown to give significantly higher recoveries of coliforms than the reference method. It is not clear which of these procedures is the best to use, although it is perhaps more useful to use the addition of "one" to each value in a pair that contains zeros when there are relatively large numbers of such pairs in a data set. In this particular data set 30% of the pairs of values contained one or more zeros.

The difference between MLGA and MLSB is interesting. These are essentially the same basic formulation although MLGA is agar based whereas MLSB is a liquid. The other differences are that MLGA contains a glucuronidase substrate and also contains sodium pyruvate, a compound known to aid the recovery of sublethally injured bacterial cells. It is likely that the presence of sodium pyruvate was responsible for the difference in recovery of chlorinated coliforms in these experiments. Somewhat surprisingly MI agar did not recover significantly more coliforms than the reference method when assessed using statistical procedures described in ISO 17994. The medium utilizes detection of β-D-galactosidase for recognition of coliforms. However, MI agar does seem to be particularly good at suppressing background flora and this may explain the reduced recovery of coliforms since often highly specific media can fail to recover some strains of the target organism(s). MI agar contains three inhibitors, cefsulodin, sodium lauryl sulphate and sodium desoxycholate.

Overall, the methods based upon galactosidase detection recovered approximately 22% more total coliform organisms from disinfected sewage, than those based upon lactose fermentation, which is slightly higher than the figure found with raw water.

Table 3.13
Statistical comparison of recovery of *E. coli* from disinfected sewage effluent using procedures outlined in ISO 17994. Quantitative results compared to the ISO reference procedure tergitol TTC agar. Zero values and the corresponding value in the method being compared increased by one.

Method	Mean relative difference	Standard deviation	X_L	X_H	Result
Coliscan®	**27.0**	**99.6**	**5.3**	**48.8**	**Higher**
Colilert-18®	**25.3**	**104.9**	**3.5**	**47.0**	**Higher**
Chromocult®	17.7	101.7	-3.7	39.0	Inconclusive
MLGA	11.9	90.4	-7.3	31.1	Inconclusive
Colilert®	11.6	99.3	-9.6	32.9	Inconclusive
MLSB	9.7	110.8	-14.8	34.1	Inconclusive
ColiSure®	9.5	110.2	-13.7	32.7	Inconclusive
MI agar	-0.2	97.0	-21.3	20.8	Inconclusive
mFC	-9.6	86.6	-28.8	9.7	Inconclusive

Analysis of the data using two-way ANOVA showed a significant difference between the means ($p = 0.02$) and the use of ISO 17994 statistics shows that both Colilert-18® and Coliscan® recovered significantly more *E. coli* than the reference method.

Experiments were performed with disinfected sewage effluents to try to compare the relative efficiencies of four presence/absence methods, Colilert®, ColiSure®, Colilert-18® and ReadyCult®. Unfortunately Colitag® could not be included in this comparison because at the time the manufacturers had advised us that the product was unavailable. The null hypothesis for this experiment was that there was no difference between the four methods regarding recovery of coliforms from disinfected sewage. A total of 136 data sets were obtained where at least one method was positive and least one negative. Because there is no standard reference procedure, Colilert® was chosen as the reference procedure since it was the first presence/absence method to be approved for use in the United States. The hypothesis for this comparison was that there was no difference between Colilert-18® and the other methods at a level of $p = 0.05$. The summary results are shown in Table 3.14.

Table 3.14
Positive samples detected by different presence/absence methods from disinfected sewage effluent

Medium	**Colilert®**	**Colilert-18®**	**ColiSure®**	**ReadyCult®**
Total number of positive coliform samples	75	81	71	63
X^2	n/a	0.42	0.18	1.75
probability	n/a	>0.05	>0.05	> 0.05

n/a = not applicable

No significant differences were identified between the four methods for the recovery of coliforms from disinfected water using Colilert® as the reference method. The only significant difference seen was between Colilert-18® and ReadyCult®, where when the null hypothesis was that there was no difference in the number of positive samples detected by the two methods, X^2 was 4.1 indicating that there was a significant difference.

The data from analysis of 178 samples was used to compare the efficiency of the four methods in recovering *E. coli* from disinfected water. The null hypothesis for these tests was that there was no difference between the recovery of *E. coli* with Colilert® and the other methods. The results are shown in Table 3.15.

Table 3.15.
Recovery of *E. coli* from disinfected water using four presence/absence methods.

Medium	Colilert®	Colilert-18®	ColiSure®	ReadyCult®
Total number of positive coliform samples	117	125	97	96
X^2	n/a	0.51	3.76	4.12
probability	n/a	>0.05	>0.05	< 0.05

n/a = not applicable

When compared to Colilert®, there was no significant difference in the number of samples found to contain *E. coli* with Colilert-18® or ColiSure®, but ReadyCult® recovered *E. coli* in significantly less samples at the $p = 0.05$ level although not at the $p = 0.01$ level required for a four way multiple comparison.. Colilert-18® recovered *E. coli* from significantly more samples than ColiSure® or ReadyCult® ($p < 0.01$). There was no significant difference between the numbers of samples found to contain *E. coli* with ReadyCult® and ColiSure®.

METHOD SPECIFICITY

As well as the sensitivity of each individual method, the specificity is also of considerable importance. The specificity of a microbiological method relates to which organisms are able to grow on (or in) it. Non-specific media may have two types of colonies other than the target colonies, those that mimic the target colonies and those that do not. Both are of importance. Non-target organisms that mimic target organisms can lead to misleading interpretation of the non-confirmed results. Other organisms that grow on the medium but do not mimic the target organisms can interfere by suppressing the growth of target organisms or by making their characteristics difficult to see. For this reason, the confirmation rates of each of the methods were determined using the samples from the previously described disinfection experiments. Confirmation of coliform organisms was based upon the Gram reaction, expression of β-D-galactosidase and failure to produce cytochrome oxidase. *E. coli* were defined as members of the total coliform group that express β-D-glucuronidase. By definition, organisms that produced typical *E. coli* reactions on media designed to detect the presence of β-D-galactosidase and β-D-glucuronidase activity were classified as *E. coli.* The data are presented in Tables 3.16 and 3.17.

Table 3.16
Confirmation rates for coliforms from each of ten media.

Method	Total number of colonies examined	Number of confirmed coliforms	Confirmation rate
Colilert-18®	219	214	97.7
Colilert®	226	215	95.1
Coliscan®	223	212	95.1
Chromocult®	231	215	93.1
ColiSure®	216	197	91.2
MLSB 37°C	211	188	89.1
MLGA	224	199	88.8
MI agar	216	190	87.9
m-Endo	227	192	84.6
Tergitol TTC 37°C	247	193	78.1

Methods based upon galactosidase tended to have higher confirmation rates, although the confirmation rate for these methods varied from 87.9 to 97.7%. The confirmation rates for methods based upon lactose varied from 78.1 to 88.8%. The majority of false positive total coliform reactions were due to *Aeromonas* spp. False positive reactions are undesirable because they may cause unnecessary remedial action.

Table 3.17
Verification of typical *E. coli* colonies/wells.

Method	Number of colonies/wells examined	Number confirmed	Confirmation rate (%)
Colilert-18®	214	214	100
Colilert®	219	217	99.1
MI agar	222	215	96.8
MLGA	229	221	96.5
Chromocult®	232	219	94.4
Coliscan®	223	205	91.9
Tergitol TTC 44°C	219	179	81.7
ColiSure®	225	183	81.3
mFC	217	173	79.7
MLSB 44°C	226	135	59.7

The confirmation rates for the various media varied widely. Not unexpectedly the lactose-based media generally had the lowest confirmation rates, ranging from 59.7% to 81.7%. Of the glucuronidase-based media most had confirmation rates above 90% although almost inexplicably ColiSure® had a confirmation rate of 81.3%. The majority of false positive reactions with ColiSure® appeared to be due to coculture of coliforms and a *Pseudomonas* spp. It would appear that the coliforms were giving a positive β-D-galactosidase result and some pseudomonads were naturally fluorescing. These organisms do not possess β-D-glucuronidase

As well as determining the confirmation rates for each medium, it is important to understand the frequency with which coliform organisms or *E. coli* fail to give typical reactions on the various media. While the proportion of "false negatives" will vary depending on the source of the water samples, some indication of the proportion of false negative reactions can be gained by examining "atypical" colonies or wells from a variety of water samples. In this study, the samples were obtained from a wide range of geographical areas. Atypical colonies were those that did not appear the correct color (e.g. showed no evidence of lactose fermentation) and atypical wells were those that showed signs of growth but had not changed the color of the nutrient indicator. The results of this investigation are shown in Table 3.18.

Table 3.18
Examination of "atypical" coliform colonies/wells

Method	Number of colonies/wells examined	Number confirmed as coliforms	Number not confirming	False negative rate (%)
Colilert-18®	227	4	223	1.8
Colilert®	218	6	212	2.8
ColiSure®	242	7	235	2.9
MLGA	226	10	216	4.4
Chromocult®	243	16	227	6.6
Coliscan®	229	17	212	7.4
m-Endo	235	31	204	13.2
MI agar	219	29	190	13.2
Tergitol TTC 37°C	230	39	191	17.0
MLSB	246	45	201	18.3

False negative rates varied widely, from 1.8% to 18.3%. The lactose-based methods had the highest false negative rate. This was due to the fact that many of the organisms now classified as coliforms, are slow or late lactose fermenters and do not give typical fermentation reactions within 18-24 hours. Thus many organisms that do not appear to be lactose fermenters are in fact coliforms.

Investigation of the Performance of B-D-Glucuronidase-based Methods in Detecting Glucuronidase Activity

To determine the ability of the various media based upon detection of expression of β-D-glucuronidase, an in depth study was undertaken. This was due to some of the initial data suggesting that MLGA medium may fail to detect glucuronidase activity. Presumptive *E. coli* strains were obtained either from growth in Colilert-18® or from mFC agar. The choice of Colilert-18® was based upon the fact that of the glucuronidase-based media it was the most specific and most sensitive in other tests. Strains were obtained from the two different media and then inoculated into/onto Colilert-18®, MI agar, Chromocult® and MLGA. Media that had been incubated for their recommended period of time were then examined for typical glucuronidase reactions. Tables 3.19 and 3.20 show the results of this study.

Table 3.19
Number of cultures taken from 914 Colilert-18® positive wells that failed to give a positive glucuronidase reaction on different media

Medium	Number of negative cultures	Percentage negative cultures
Chromocult®	4	0.44
Coliscan®	6	0.66
MI agar	4	0.44
MLGA	114	12.47

Table 3.20
Number of cultures taken from 251 *E.coli* isolates grown on mFC medium that failed to give a positive glucuronidase reaction on different media

Medium	Number of negative cultures	Percentage negative cultures
Chromocult®	0	0
Colilert-18®	1	0.40
Coliscan®	3	1.20
MI agar	8	3.19
MLGA	68	27.09

Clearly, there was a significant difference in the number of false negative glucuronidase reactions shown by *E. coli* on MLGA medium compared to the other methods. In earlier experiments on raw water MLGA did not perform well in recovering *E. coli* and in fact recovered less *E. coli* than MLSB which has almost the same formulation but without the glucuronidase substrate. However, in earlier experiments using Bioballs, MGLA recovered *E. coli* in more samples than MLSB. One possible explanation for this difference is that MLGA contains a high concentration of lactose (unlike the other media). When *E. coli* grows on the medium, it ferments lactose resulting in the production of acid. This acid production lowers the pH of the medium to pH 5.4 or lower. This was determined by measuring the pH of MLGA medium on which *E. coli* were growing and also by measuring the pH of *E. coli* cultures growing in MLSB (the basal medium for MLGA). If the pH optimum of the glucuronidase enzyme is higher than this (likely in living organisms) then this may inhibit the activity of the enzyme, resulting in yellow colonies rather than the green colonies that are typical of *E. coli* that are expressing β-D-glucuronidase.

In order to test the theory that the failure to detect the expression of β-D-glucuronidase in a number of strains of *E. coli* was due to low pH, an assay was developed to measure the level of β-D-glucuronidase expression under different conditions. The assay was very simple and was based on the detection of the fluorogenic substrate 4-methylumbelliferone. Esters of 4-methylumbelliferone only fluoresce when cleaved and fluorophore is released. The hydrolysis of 4- methylumbelliferone-containing substrates such as ß-4- methylumbelliferyl-glucuronide by ß-glucuronidase yields the fluorescent molecule 4- methylumbelliferone that emits light at 460 nm when excited by 365 nm light.

Further investigation into the false negative glucuronidase reactions with MLGA showed that the pH of the medium was having a marked effect on the activity of the glucuronidase enzyme in some strains of *E. coli*. The effect of pH was not consistent but in some strains the activity of the enzyme at pH 7.0 was more than twenty times the activity at pH 5.0. The pH effect was most noticeable on plates that contained larger numbers of bacteria although it was also seen on occasion with single colonies on plates. Clearly, this indicates that there is a significant potential for false negative reactions to occur with MLGA, particularly with more heavily contaminated samples.

TESTING OF TWO METHODOLOGIES AT SITES IN FLORIDA AND CALIFORNIA

Coliscan® and Colilert-18® were chosen as the two methodologies to be tested on site. The two methods were chosen on the basis of their sensitivity, specificity, ease of use (including interpretation) and the fact that they both had similar reproducibility and repeatability. Samples of 100 mL and 2 L were collected during each sampling event. A weakness in the selection was that Colilert-18® was only able to provide a presence/absence result for the 2L samples.

A total of 296 samples of water taken in California, either at the point that it left a conventional water treatment plant or within the distribution system, were examined. During each sampling event (approximately four hours) samples were collected ten minutes apart. Each sample was analyzed using 100 mL and 2 L with both Colilert-18® and Coliscan®. To process the 2L samples for Colilert-18®, a total of 1.9 L volume was filtered by membrane filtration which sometimes took ten minutes. The remaining 100 mL was used to dissolve a sachet of Colilert-18® and the membrane placed into the solution. Of these samples, only six sets of samples showed coliforms to be present. All positive results were confirmed using standard procedures as described in the methods section. No false positive results were seen in this part of the study. The results are shown in Table 3.21.

Table 3.21
Results of 6 of 296 samples taken in California that had a coliform detected by at least one procedure

Site	Colilert-18® 100 mL	Colilert-18® 2 L	Coliscan® 100 mL	Coliscan® 2 L
Distribution	Absent	Absent	1 CFU	1 CFU
Distribution	Absent	Absent	0 CFU	2 CFU
Distribution	Absent	Present	0 CFU	0 CFU
Plant effluent	Absent	Present	0 CFU	0 CFU
Plant effluent	Absent	Absent	0 CFU	1 CFU
Plant effluent	Absent	Absent	0 CFU	1 CFU

In summary, no positives were found with 100 mL samples using Colilert-18®, two using Colilert-18® on 2 L samples, one with Coliscan® on a 100 mL sample, and four using 2 L samples and Coliscan®.

In Florida, 289 sampling events took place and examination was undertaken in the same way as for the samples from California. A total of 71 samples were positive mostly from within the distribution system. All positive samples were confirmed for the presence of coliform organisms based upon the presence of β-D-galactosidase and the absence of cytochrome oxidase. No false positive results were seen. The data from the Florida sampling is included on the accompanying compact disc but due to the size of the table, is not included here in the text. The summary data for both California and Florida are shown in Table 3.22.

Table 3.22
Summary data of samples found positive for total coliform at two drinking water system sites, one in Florida and one in California.

Site	Colilert-18® 100 mL	Colilert-18® 2 L	Coliscan® 100 mL	Coliscan® 2 L
California	0/296	2/296	1/296	4/296
Florida	13/289	22/289	4/289	44/289
Total	13/585	24/585	5/585	48/585
Percentage positive	2.2%	4.1%	0.9%	8.2%

The differences between the different volumes and between methods are significant. Depending on the analytical method chosen, the percentage of positive samples ranged between 0.9% and 8.2%.

In experiments in the laboratory, Colilert-18® was more sensitive than Coliscan® and with 100 mL volumes this was also true with naturally contaminated samples, although the numbers are small. Increasing the volume examined from 100 mL to 2 L increased the rate of positive results from 2.2% to 4.1% with Colilert-18®. However, with Coliscan® the increase in volume analyzed increased the rate of positive results from 0.9% to 8.2%. Clearly, with larger volumes, Colilert-18® is not as effective in recovering coliforms and this may be due to some form of competition within the medium. As has been demonstrated on many previous occasions, increasing the volume of sample analyzed increases the likelihood of detecting coliforms. Using Coliscan®, the detection rate increased four-fold by using the larger volume of water in California and by almost an order of magnitude in Florida. Overall the increase with Coliscan® was approximately nine-fold and with Colilert-18® the rate of detection doubled when the volume examined was increased from 100 mL to 2 L.

EXAMINATION OF FOLLOW UP SAMPLES AFTER COLIFORM POSITIVE RESULTS FROM ROUTINE MONITORING

To assist in this study, several participating utilities agreed to send samples to ASI when they had coliform positive samples during routine monitoring within their distributions systems. In addition to the required 100 mL re-sample, utilities agreed to collect and ship one 10 L sample to ASI. The intent was to examine the resample water in 100 mL and 2 L aliquots using selected methods.

Only one utility who had agreed to participate in this portion of the study reported positive coliform samples in their distribution system monitoring (Pinellas County Utilities, PCU). Follow-up samples were collected by PCU and submitted to ASI on several occasions, but none of these samples, 100 mL or 2 L, were positive for coliforms (data not shown). Due to the extremely limited sample size, no conclusions were drawn regarding the use of increased volume samples after total coliform positive results.

TESTING TWO PROCEDURES IN DIFFERENT LABORATORIES: ROUND ROBIN TESTING

Two separate 2 L samples were sent to each of nine laboratories, and the target concentration of organisms present was *Enterobacter aerogenes* (5 CFU/2 L) and *E. coli* (5 CFU/2 L). Subsequent testing showed the mean concentrations to be 5.67 CFU/2 L for *E. coli* and 4.67/2 L for *Enterobacter aerogenes.* The laboratories were not asked to confirm their results. In addition three separate deionized water samples were spiked with target organisms and sent as a concentrate to be added to the participating laboratory's own tap water. Three further samples prepared in 100 mL of Williston deionized water were also sent. All laboratories correctly reported coliforms to be present in both samples, although one laboratory did not find coliforms in one sample using the Coliscan® method. For *E. coli*, two laboratories reported negative results with Colilert-18®. The results are shown in Table 3.23.

Table 3.23
Round Robin results for 2 L samples seeded with low concentrations of *Enterobacter aerogenes* and *E.coli* both at approximately 5 cfu/2L

Laboratory	Sample	Colilert 18		Coliscan®		In-house enumeration	
		TC	EC	TC	EC	TC	EC
1	1	P	P	7	8	4.7	6.7
1	2	P	P	3	2	1.9	7.3
2	1	P	A	7	12	4.7	6.7
2	2	P	P	1	6	1.9	7.3
3	1	P	P	4	8	4.7	6.7
3	2	P	P	2	12	1.9	7.3
4	1	P	P	3	2	4.7	6.7
4	2	P	A	1	6	1.9	7.3
5	1	P	P	5	4	4.7	6.7
5	2	P	P	3	10	1.9	7.3
6	1	P	P	4	8	4.7	6.7
6	2	P	P	1	14	1.9	7.3
7	1	P	P	5	6	4.7	6.7
7	2	P	P	3	7	1.9	7.3
8	1	P	P	3	6	4.7	6.7
8	2	P	P	3	7	1.9	7.3
9	1	P	P	4	6	4.7	6.7
9	2	P	P	0	2	1.9	7.3

TC = *Enterobacter aerogenes*
EC = *E. coli*

The results from the 2 L samples were largely in agreement from laboratory to laboratory, but the numbers of samples are too small to draw further conclusions.

The summary data from the remaining samples for Colilert-18® are shown in Table 3.24.

Table 3.24
Summary data from the Round Robin comparison of methods for Colilert-18® for three samples prepared in Williston deionized water and three prepared in the participating laboratory's tap water.

	Number of organisms added		Reported range		Reported mean	
	Total coliform	*E. coli*	Total coliform	*E. coli*	Total coliform	*E. coli*
Williston water	46	28	34-53	22-38	43.1	27.8
Williston water	23	13	14-29	10-21	22.9	13.2
Williston water	23	14	18-38	9-25	25.1	14.5
Utility water	48	25	25-56	15-34	42.4	22.8
Utility water	19	10	12-25	5-15	18.2	10.3
Utility water	20	13	14-25	8-19	19.6	12.7

The results obtained at the various participating laboratories (which were all certified for performing analysis for the TCR) were very similar to those obtained at the by the laboratory that prepared the samples with no major differences seen. This was expected because all participating laboratories were familiar with the use of Colilert® or Colilert-18®.

Comparative data for the Coliscan® method are shown in Table 3.25.

Table 3.25
Summary data from the Round Robin comparison of methods for Coliscan® for three samples prepared in Williston deionized water and three prepared in the participating laboratory's tap water.

	Number of organisms added		Reported range		Reported mean	
	Total coliform*	*E. coli*	Total coliform*	*E. coli*	Total coliform*	*E. coli*
Williston water	39	27	22-39	15-35	33.7	23.7
Williston water	21	9	10-27	7-14	17.7	10.0
Williston water	20	12	10-29	5-18	18.2	10.8
Utility water	23	12	17-28	12-15	18.3	9.1
Utility water	17	9	1-23	1-13	12.8	6.8
Utility water	16	8	2-26	0-14	13.1	6.1

*Note: The values for "total coliform" were calculated by the addition of the values for *E. coli* and *E. aerogenes*

The results generated by the different laboratories showed some variation and one laboratory failed to detect *E. aerogenes* in one sample and a different laboratory failed to detect *E. coli* in a sample. Overall, the results generated with Coliscan® were lower than those seen with Colilert-18®. The total mean recovery of total coliforms was 28.7 per sample for Colilert-18® and 19.0 for Coliscan®. For *E. coli* the corresponding means were 16.9 and 11.1 organisms per sample for Colilert-18® and Coliscan® respectively. When data for coliforms were analyzed using statistical procedures contained in ISO 17994, the expanded uncertainty was 16.0 with values for X_L and X_H being 30.5 and 62.4 respectively, showing that significantly more coliforms were recovered with Colilert-18® than with Coliscan®. Similar analysis for *E. coli* gave an expanded uncertainty of 18.4 and values for X_L and X_H of 35.9 and 72.7, showing that Colilert-18 ®recovered significantly more *E. coli*.

INVESTIGATION INTO THE BACTERICIDAL EFFECTS OF TAP WATER

The initial results obtained by testing the survival of four strains of coliform organisms are shown in Table 3.26.

Table 3.26.
Log inactivation of coliform organisms after one hour contact time in different waters. The initial concentration of organisms in each sample was typically between 100 and 400 cfu/mL.

Water source	*E.coli*	*K.terrigena*	*H.alvei*	*E.aerogenes*
Site 1 treated 1	0.4	0	0.8	0.2
Site 1 treated 2	0.7	**1.5**	0.7	ND
Site 1 treated 3	0.7	**1.0**	0.7	ND
Site 1 treated 4	**1.3**	**1.8**	**1.2**	**1.4**
Site 1 treated 5	0.4	0.5	ND	ND
Site 1 treated 6	0.1	0.4	ND	ND
Site 2 treated 1	**1.8**	0.7	**2.4**	0.7
Site 2 treated 2	**1.6**	**1.5**	ND	ND
Site 2 treated 3	**1.4**	**1.2**	1.5	0.9
Site 1 treated 7	0	0.1	ND	ND
Site 1 treated 8	0	0	ND	ND
Carbon filtered Site 1 treated 1	0	0	0	0
Carbon filtered Site 1 treated 2	0	0	0	0
Site 1 raw 1	0.2	0.3	0	0.1
Site 1 raw 2	0	0.1	0	0
Site 1 raw 3	0	0	ND	ND
Site 2 raw	0	0	ND	ND
Site 3 treated	0	0	0	0
Groundwater 1	0.1	0	ND	ND
Groundwater 2	0.1	0	ND	ND
Groundwater 3	0	0	ND	ND
Groundwater 4	0	0	ND	ND
Site 4 treated	0	0	ND	ND
Site 5 treated	0	0	ND	ND
Site 6 treated	0	0	ND	ND
Site 7 treated	0	0	ND	ND
Site 8 treated	0	0.1	ND	ND
Site 9 treated	0	0.1	ND	ND
Site 10 treated	0	0.1	ND	ND
Site 11 treated	0	0.1	ND	ND

ND = not done
Note: Site 1 was sampled on multiple dates; each line in the table represents a different date
Bold values indicate log reduction > 1

Treated water from "Site 2" showed a bactericidal effect on each occasion that it was tested whereas treated water from "Site 1" varied in its bactericidal effect. None of the other waters tested showed significant bactericidal activity and filtration of treated Site 1 water through a carbon filter removed the bactericidal effect. The raw water used at Site 1 appeared to have no significant bactericidal effect and thus it was hypothesized that the effect was due to a water treatment chemical. Comparison of the treatment regimens at the water treatment plants at Site 1

and Site 2, which use the same source water, indicated that the effect may be due to the use of Superfloc C572, a coagulation aid. This was therefore investigated by preparing dilutions of the polymer in deionized water and exposing bacteria for different time periods. Mean results of five sets of experiments are shown in Table 3.27.

Table 3.27
Counts of *E. coli* after exposure to Superfloc C572 at different concentrations and times of exposure

Matrix	T = 0 min	T = 30 min	T = 2 hours	T = 6 hours
DI water	293	262	232	250
DI water	296	268	230	244
DI water	260	258	234	218
DI water	268	230	230	236
DI water	270	254	254	258
Mean	**277**	**254**	**245**	**241**
0.1 mg/L polymer	169	83	8	1
0.1 mg/L polymer	176	62	10	1
0.1 mg/L polymer	196	68	13	0
0.1 mg/L polymer	159	63	13	0
0.1 mg/L polymer	194	59	14	1
Mean	**179**	**67**	**12**	**1**
1.0 mg/L polymer	203	196	120	70
1.0 mg/L polymer	202	202	119	62
1.0 mg/L polymer	185	203	108	64
1.0 mg/L polymer	200	194	123	62
1.0 mg/L polymer	181	181	148	55
Mean	**194**	**195**	**124**	**63**
5.0 mg/L polymer	183	156	129	92
5.0 mg/L polymer	156	140	120	71
5.0 mg/L polymer	166	157	147	77
5.0 mg/L polymer	165	132	118	81
5.0 mg/L polymer	173	169	157	86
Mean	**169**	**151**	**134**	**81**

The results indicate that as the concentration of the polymer increases the amount of microbial inactivation decreases which is counter intuitive. Nonetheless this trend has been seen with every set of experiments conducted with Superfloc C572. It is not clear if this apparent bactericidal effect is in some way related to the coagulating properties associated with this polymer. All experiments were conducted in deionized water and this may have had an impact on the results obtained. However, attempts were made to determine if coagulation of bacteria was occurring during the contact period but no indication that this occurred was found. Clearly, such results require more in depth research and clarification but the resources were not available for this to be done as part of this study. However, it is very clear from these results that some degree of inactivation occurs that increases with time but decreases with increasing concentration. At the higher concentrations of polymer that were used, there was approximately

50% reduction in bacterial counts over a six hour time period. However, at the lowest concentration tested (0.1 mg/L) the bacterial concentration decreased by two orders of magnitude within the same time period.

CHAPTER 4
SUMMARY AND CONCLUSIONS

This study focused on the comparison of microbiological methods approved for use in the United States and the United Kingdom. The initial part of the work was to undertake an extensive literature review to consider previous studies that had sought to determine the performance of microbiological methods used for the examination of water. Thereafter, an extensive experimental phase was undertaken in an effort to determine the relative performance of the methods with both pure cultures and naturally contaminated waters. Two of these methods (one membrane filtration method and one most probable number, presence/absence method) were then selected for further study at two locations within the US. The methods, Coliscan® and Colilert-18®, were selected based upon their performance in the experimental work and after discussions with the Project Advisory Committee. The methods were used to compare performance with both 100 mL and 2 L volumes of water. Finally, the two methods were compared in a "round robin" comparison at nine laboratories in the US. It was not the intent of this study to develop a "league table" of method performance and indeed this would not be possible since the various approaches led to different performance criteria. However, data was generated to enable interested parties to determine the strengths and weaknesses of the various methods.

Based on the overall data set, it is clear that methods that utilize the activity of the enzyme β-D-galactosidase for the detection of total coliforms recover more coliforms than do methods that utilize fermentation of lactose. This is not because of an inherent greater sensitivity of the procedures for individual strains of coliforms, but rather because of the ability to detect organisms that slowly ferment lactose. Conventional lactose-based methods do not always detect lactose fermentation in the 18-24 hours of incubation that is typically used. Whilst the enzyme-based methods detect a broader diversity of coliform organisms, the same basic genera are generally detected (Fricker and Eldred, 2009). The argument put forward by some workers that the coliforms that do not rapidly ferment lactose are of no significance to public health is incorrect. For example some strains of *E. coli* do not ferment lactose within 24 hours although they do give a positive β-D-galactosidase reaction within the same time period. The difference between two strains of a single species of total coliform that do and do not ferment lactose rapidly is merely the presence (or absence) of a single enzyme, lactose permease. The presence of this enzyme does not indicate any particular hygienic significance

There is much disagreement amongst microbiologists on the best experimental procedures to be used for the comparison of microbiological methods. Whilst most agree that naturally contaminated drinking water samples would be ideal, the extremely low incidence of positive samples means that their use is impractical for developing enough data to statistically show differences in performance. In recent years, some workers have advocated the use of pure cultures to compare the performance of methods. In our study the use of very low numbers of cells derived from pure cultures did lead to some useful experimental data. However, this approach only examined two aspects of the overall performance of the methods. The use of pure cultures allows a researcher to determine if the various methods are able to support the growth of specific strains of organisms and give appropriate reactions to allow their identification as coliforms or *E. coli*. The results obtained may indicate that some methods are more likely to recover a wider range of target organisms but these results are based solely on the selection of

strains used. In order to be clear about the relative sensitivities of different methods, a large number of pure cultures would be required and the experimental design would need to provide quantitative data in order to determine inherent sensitivity. Despite these caveats, the work reported here with pure cultures did indicate that some methods were inherently less sensitive than others. However, the results are based on the number of target organisms used. Substantial differences were demonstrated between data sets generated using mean counts of 1.5 and 3.0 CFU/100 mL. This reflects an inherent difference in sensitivity between methods with the particular strains of organisms used. It may not reflect the sensitivity of the methods with a broader range of organisms. Nonetheless the results with pure cultures of both *E. coli* and *Enterobacter aerogenes* did suggest that ColiSure® and m-ColiBlue24® were significantly less sensitive than other methods. These results were supported to some extent by other experimental procedures.

The inherent sensitivity of a method is a product of many factors, but one of the most important is the type of chemicals used to inhibit the growth of non-target organisms. The inhibitors used (including elevated temperature of incubation) determine the extent to which non-target organisms can grow. Substantial growth of non-target organism can have many detrimental effects, including:

1. Overgrowth that out competes the target organisms
2. Production of bacteriocins preventing the growth of target organisms
3. Growth that masks the reaction used to detect target organisms
4. Growth that produces false positive results.

The optimum choice of inhibitors is therefore of paramount importance in the design of a culture method. In addition to the factors listed above, some inhibitors may inhibit the growth of target organisms, particularly those that are sub-lethally injured, for example by exposure to sub-optimal levels of chlorine.

Experiments were performed to compare the performance of each medium at recovering both total coliforms and *E. coli* from raw water that had been diluted to give a countable range of target organisms. There were apparent differences in the performance of the methods, but very few were of statistical significance. Additionally there was no consistent trend for most methods in their performance in recovering total coliforms and *E. coli.* During the work with raw source waters it became very clear that the typical target range of countable bacteria used for comparison studies was not suitable. ISO 17994 suggests that the best range of colony forming units of the target organism to use for comparison studies is 20-50. At these levels some less inhibitory media can be overgrown even when the target organisms are total coliforms. When the target is *E. coli* the number of target organisms needs to be in the range 1-10 CFU/ 100 mL or the plates can easily be overgrown by non-target organisms. This is particularly true of membrane filtration methods that are designed to detect total coliforms and *E. coli* simultaneously by incubation of samples at 35-37°C. The use of this lower target range means that, in general, many more samples are required in order to detect a statistically significant difference between two procedures. Furthermore, it was determined that the two procedures for dealing with zero values in the ISO 17994 protocol can lead to different outcomes when this lower range of target organisms is used as there are more zero values encountered.

With disinfected samples, there were more statistically significant results with total coliforms with most methods outperforming the reference method (tergitol TTC) However, with *E. coli* only two methods (Coliscan® and Colilert-18®) detected significantly more target organisms than the reference method. These two methods were chosen for further work using

large volume samples in Florida and California. Presence/absence methods were also compared with disinfected waters and no significant difference in the number of samples found to contain coliforms could be found between Colilert®, Colilert-18®, ReadyCult® and ColiSure®. However, for *E. coli*, both Colilert® and Colilert-18® detected significantly more target organisms than did ReadyCult®. There was no significant difference between ReadyCult® and Colisure®. These experiments were conducted with a limited range of matrices and there is no information on the effect of matrix on recovery and consequently these results should be treated as preliminary and laboratories should do further testing if they consider changing their procedures.

Where specificity is a concern, it was clear that the procedures that are based upon β-D-glucuronidase should be used. These were more specific *for E. coli* than the methods based upon lactose fermentation. For total coliforms, methods that utilize β-D-galactosidase reactions tended to be more specific, but the differences were less clear. False negative results varied considerably between the methods with lactose-based methods having more false negative coliform results due largely to the presence of non-lactose fermenting organisms.

The examination of water samples larger than 100 mL offers the potential for greater sensitivity. The research reported here demonstrated clearly that examination of 2 L samples detected coliform organisms much more frequently than examination of 100 mL irrespective of whether Colilert-18® or Coliscan® was used as the method. Interestingly, Colilert-18® detected coliforms in twice as many samples as did Coliscan® when 100 mL samples were examined, whereas the reverse trend was seen with 2 L samples, where Coliscan® significantly outperformed Colilert-18®. The examination of larger volume samples is clearly of benefit when looking for total coliforms, although the increase seen was site specific and probably reflects the concentration of organisms present. However, the choice of medium for these larger samples requires more investigation.

The same pattern of results was not seen in round robin testing when comparing the two methods for their ability to recover coliforms from 2 L samples although Coliscan® did detect *E. coli* in slightly more samples than did Colilert-18®. However, again with 100 mL samples, the results submitted by the participating laboratories clearly showed the greater sensitivity of Colilert-18® over Coliscan® for these samples.

Work was undertaken to investigate the observation that the tap water at one laboratory was bactericidal even after residual disinfectant was removed. It was found that the inhibitory effect was due to a polymer used at the treatment plant (Magnifloc 572C). The inhibitory effect was seen at low concentrations 0.1 – 0.5 mg/L. The effect was less marked at higher concentrations.

FUTURE RESEARCH

This research has demonstrated the superiority of methods based upon the enzymes β-D-galactosidase and β-D-glucuronidase for detecting total coliforms in water both in terms of specificity and sensitivity. It was not the intent of this research to create a “league table” of methods but this is an area of research that many laboratories would welcome. Such research would need to be performed on a multi-laboratory basis to ensure that a wide variety of strains of bacteria and types of matrix were examined. Perhaps this should be done in concert with the

regulatory purposes of the methods, since labs are required to be accredited or certified for use of the methods to report data under drinking water regulations?

It is clearly time that the regulations for wastewater, biosolids and recreational waters be reviewed in view of the findings of this study. It would be more appropriate if regulations covering these matrices also used *E. coli* rather than "fecal coliforms". While this process has begun, further work is required to determine the potential effects of using *E. coli* as a regulated parameter. Furthermore, the methodologies to be used for these matrices are not clearly defined.

There is also a need for more work to be undertaken on the benefits of large volume sampling for coliforms. A previously published Research Foundation report "Strategies for Managing Total Coliform and *E. coli* in Distribution Systems" also found benefits to using larger volume samples. Two areas should be considered. Firstly, the methodology needs to be examined in greater detail than was possible here. Methods in current use are designed for 100 mL samples and may not perform well with larger volumes with the increase in competing flora that would be present. Secondly, the correlation between results seen with large volume samples and other water quality parameters needs to be examined. This work should cover not only water quality data but also processes such as disinfection procedures. The ability of large volume samples to detect ingress due to mains breaks or low pressure events should be investigated as well as the effects of disinfectant residual and biofilm growth.

The unexpected finding that a chemical used as a coagulation aid in water treatment processes had a bactericidal effect requires further investigation. The presence of the chemical could lead to misleading bacteriological results. The research should cover other coagulation aids to determine if this is a widespread effect. Further, a neutralizer for such compounds needs to be found to prevent it having an effect after samples have been taken and before they are examined.

CHAPTER 5
RECOMMENDATIONS TO UTILITIES

Microbiological monitoring of drinking water supplies has two components, regulatory monitoring and "operational" monitoring. Whilst the former is mandated by law and is designed to demonstrate that a water system is supplying drinking water that is safe, the latter form of monitoring is often the most critical. However, for both purposes the methodology used should be sensitive, specific and accurate with minimal time for a confirmed result. For smaller systems in particular, the method used should also be simple and inexpensive to perform, requiring minimal microbiological expertise (however in the US, these would all be contracted out to full labs so it might not be an issue except for expense). This study has provided data that indicate that methods based upon detection of activity of the two enzymes β-D-galactosidase and β-D-glucuronidase tend to be more specific, sensitive and accurate than the more traditional methods based upon lactose fermentation. In particular, those methods with the highest degree of specificity can be used without a confirmation step, leading to a much faster confirmed result. This improved confidence in the result allows utilities to respond more rapidly to microbiological threats and also only to respond to genuine microbiological results. A sample that indicates the presence of *E. coli*, based upon positive β-D-galactosidase and β-D-glucuronidase reactions, is very likely to contain *E. coli*. Whereas finding a lactose fermenting colony on mFC or MLSB medium may be the result of the presence of an environmental thermotolerant coliform. It is this greater degree of confidence in the initial result that is the most compelling reason to use the newer, enzyme-based methods.

The choice of specific method is often a matter of personal preference within a laboratory but the factors of sensitivity, specificity, accuracy, simplicity and time to result are the most important factors to consider in choosing an appropriate method. Cost is also an issue, with the more recently developed, enzyme-based procedures costing more in materials than the traditional, lactose-based membrane filtration methods. However, there are many potential savings to be made when using a method that gives a more rapid and accurate result. The cost of re-sampling can be reduced with fewer false positives, and the ability to act quickly and decisively based on an accurate result can lead to considerable cost benefits. Furthermore, a reduction in "false alarms" would reduce the number of boil water advisories issued and the subsequent injuries associated with these. The enzyme-based methods generally require less time to prepare and afford better quality assurance; this in itself can be a substantial cost saving. There are also more tangible costs associated with the lactose-based methods including a dedicated incubator or waterbath set at 44°C for confirmations, additional media for confirmation steps and the associated quality assurance together with increased recording and reporting. The impact of the additional confirmatory steps is often overlooked when calculating the true cost of a procedure.

An argument that is sometimes put forward to support the use of traditional, membrane-based methods is that these methods allow the microbiologist to see background flora and hence have a better understanding of overall water quality. This argument is seriously flawed. All media for the detection of total coliforms, irrespective of their basic composition, contain compounds specifically designed to inhibit the growth of non-coliforms. That some media fail to inhibit a proportion of the non-coliform flora is a failing of the medium and not a benefit. Growth of background flora can inhibit the growth of coliforms or impair the ability to recognize

them. If information on the general microbiological nature of the water is required, then a method such as heterotrophic plate count should be used in conjunction with methods specifically designed to detect coliforms and *E. coli*.

During the performance of this study, there has been considerable interest in the authors producing a "competitive matrix" which would indicate the "best" method or methods for the examination of drinking water for the presence of total coliforms and *E. coli*. We have largely resisted this but in order to try and help utilities some points to be considered are listed below:

1. Methods based upon membrane filtration require more laboratory equipment and analyst skill
2. Methods based upon the fermentation of lactose for the identification of the presence of total coliforms give a high proportion of false negative results
3. Methods based upon fermentation of lactose (e.g. m-Endo) require substantially more analyst interpretation and the presence of "sheen" colonies is often difficult to detect
4. Methods based upon the fermentation of lactose for the detection of total coliforms and *E. coli* require confirmation tests and this extends the time to result to about 72 hours
5. Methods based upon detection of the expression of the enzymes β-D-galactosidase and β-D-glucuronidase give confirmed results in 18-28 hours (depending on the method)

Essentially, the simplest methods are those that are presence/absence tests and in general these give the fastest time to a confirmed result. They are almost all based upon the detection of the two enzymes β-D-galactosidase and β-D-glucuronidase (and hence are more accurate than lactose-based methods) and require the least interpretation. It is not the intent of this report to recommend a single product and reference to Table 2.1 may help those who wish to examine their choices further. Much has been discussed about the effect of different matrices on the performance of microbiological methods. There is no doubt that the matrix has some effect, although these effects have not been studied systematically. It is clear however, that with matrices with high counts of background (i.e. non target) organisms, MPN-based methods are the easiest to interpret and give the most accurate results.

Whilst there is no compelling reason to change the volume of water examined with microbiological tests used for regulatory purposes, the use of larger samples can be of considerable benefit when investigating the source of coliform organisms in water supply systems. In this study, the use of two liter (2 L) samples facilitated the detection of considerably more coliform-positive samples than did the use of 100 mL samples. In previous work, we have utilized larger volume sampling to determine the source of coliforms within a distribution system. A medium-sized system was experiencing a high rate of total coliform positives and these were thought to be all biofilm derived. The use of large volume samples showed that in fact, whilst there was considerable regrowth of coliforms within the distribution system, there were significant numbers of coliforms entering the distribution system from the treatment plant. These were seldom detected by 100 mL samples because the concentration of organisms was of the order of 1 CFU/L or less. Utilities should consider the use of large volume samples for investigative purposes. One area of concern here is that the methods currently available were all designed for 100 mL samples and their performance may be impaired when larger volumes of water are concentrated.

This study has demonstrated that a polymer added as a coagulation aid has bactericidal properties and its presence in treated water can lead to inaccurate microbiological results. Utilities that use such polymers should do their utmost to ensure that there is no residual polymer in the final water or that if this is unavoidable that they are able to demonstrate that it has no impact on microbiological data.

The majority of positive coliform samples encountered by water utilities contain very few target organisms and the work presented here has shown that at these low levels (e.g. 1 cfu/100 mL) there is a significant chance that the presence of these organisms will be missed. This is true for any of the methods used but clearly some methods are more sensitive than others. Nonetheless, no method is 100% accurate. Furthermore all methods can give rise to false positive results and care should be taken in interpreting "presumptive" results in the absence of confirmation. However, the data presented here demonstrate that there are clear differences between the accuracy of some methods and this should be borne in mind when selecting a method for routine use. The growth of non-target organisms occurs with all methods although the amount of background growth may differ between methods depending on the inhibitors used. A reduction in the growth of background flora is desirable but some inhibitory agents may also impact the growth of target organisms. Thus water utilities should carefully consider the methodology that they use.

While differences were seen between methods in this study and some methods were apparently significantly better than others, this may not be the case with all water matrices. Comparative trials such as the ones presented here can only give an indication of performance and each individual laboratory should confirm that the method they choose performs well with their matrices. Similarly, if a laboratory changes the methodology they use, care should be taken in the interpretation of results. It is clear from the data presented here that most methods that detect total coliforms on the basis of β-D-galactosidase will detect more coliforms than methods that rely upon lactose fermentation. If a laboratory chooses to change their methodology from a lactose-based method to one based upon β-D-galactosidase then they can expect to see more frequent positive coliform samples. This reflects the change in procedure and not any deterioration of water quality.

The use of large volume samples may be beneficial in detecting total coliforms but the use of large volume samples is not to be recommended for routine use. They may however, be useful during investigations of the source of coliforms and for "special" samples such as those taken after mains repairs or after mains breaks. However care should be taken when using large volume samples as false positive results are more likely to occur and positive results should always be confirmed.

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ABBREVIATIONS

%	Percent
°C	Degrees Centrigrade
ANOVA	Analysis of Variance
ASI	Analytical Services, Inc.
ATCC	American Type Culture Collection
BCIG	5-Bromo-4-chloro-3-indolyl -D-galactopyranoside
BGB	Brilliant Green Bile
BGLB	Brilliant Green Lactose Bile
CFU	Colony Forming Units
DEFRA	Department for Environment, Food and Rural Affairs
DI	Deionized (water)
DST	Defined substrate technology
DWI	Drinking Water Inspectorate
EPA	Environmental Protection Agency
h	Hour
HPC	Heterotrophic plate count
IBDG	Indoxyl-/3-D-glucuronide
IM-MF	Injury Mitigating Membrane Filtration
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISO	International Organization for Standardization
L	Liter
LT (broth)	Lauryl tryptose
LTB	Lauryl tryptose broth
LTLSB	Lactose-Tryptone-Lauryl Sulphate Broth
MF	Membrane Filtration
mFC	Membrane Fecal Coliform agar
mg	Milligram
MI (agar)	4-Methylumbelliferyl-B-D-galactopyranoside, Indoxyl-B-D-glucuronide agar
mL	milliliter
MLGA	Membrane Lactose Glucuronide Agar
MLSB	Membrane Lauryl Sulfate Broth
MPN	Most Probable Number
MTF	Multiple Tube Fermentation
MUG	4-methylumbelliferyl-β-D-glucuronide

NCTC	National Collection of Type Cultures
ONPG	Ortho-Nitrophenyl-β-galactoside
p	Probability
P/A	Presence / Absence
Salmon-gal	6-Chloro-3-indolyl-beta-D-galactopyranoside
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TTC	Lactose Tergitol with 2,3,5-Triphenyltetrazoliumchloride
UK	United Kingdom
цm	Micron
US	United States
USEPA	United States Environmental Protection Agency
x-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
X_H	Value of the relative difference at the approximate upper 95% confidence limit
X_L	Value of the relative difference at the approximate lower 95% confidence limit