A comparison of ten USEPA approved total coliform/*E. coli* tests

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ABSTRACT

Since 2002, the United States Environmental Protection Agency (USEPA) has approved ten enzyme-based total coliform and *E. coli* detection tests for examination of drinking water. These tests include: Colilert®, Colilert-18®, Colisure®, m-Coli Blue 24®, Readycult® Coliforms 100, Chromocult®, Coliscan®, E * Colite®, Colitag™ and MI Agar. The utility of the enzyme based test systems is based on both the ability of the test to detect the target organisms at low levels and the ability of the test system to suppress the growth of non-target organisms that might result in false positive results. Differences in the ability of some of these methods to detect total coliform and *E. coli*, as well as suppress *Aeromonas spp*., a common cause of "false positive" results, have been observed. As a result, this study was undertaken to elucidate the strengths and weaknesses of each method. Water samples were collected from three geographically and chemically diverse groundwaters in Wisconsin. One-hundred milliliter aliquots were individually spiked with both low concentrations (one to ten organisms) and high concentrations (fifty to one-hundred) of each of five different total coliform organisms (*Serratia, Citrobacter, Enterobacter, E. coli, & Klebsiella*). These spiked samples were used to test the capability of ten enzyme-based test systems to both detect and enumerate the spiked organisms. In addition, 100 ml samples were independently spiked with two different strains of *Aeromonas spp*. at six different levels, to assess the ability of each enzyme-based test to suppress *Aeromonas spp*. Analysis of the data indicated that wide variability exists among USEPA approved tests to detect and quantify total coliforms, as well as suppress *Aeromonas spp*.

Key words | *Aeromonas spp.*, enzyme-based test, *E. coli*, groundwater, total coliform

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INTRODUCTION

Protection of groundwater from microbial contamination is an important public health priority (Kramer *et al.* 1996; Blackburn *et al.* 2004). Recent epidemiological studies clearly show that gastrointestinal disease due to ingestion of drinking water is occurring at significant levels in the United States (Craun *et al.* 1997; Hancock *et al.* 1998; Blackburn *et al.* 2004) and Canada (Payment 1997). Furthermore, the United States Centers for Disease Control reported in their last 10 year summary of waterborne disease outbreaks that over 70% of the documented outbreaks occurring in the U.S. were associated with contaminated well water (Craun *et al.* 1997). These facts underscore the need for sensitive, reliable laboratory methods to identify microbial contamination in groundwater that might pose a potential risk of illness.

Over the past ten years, enzyme-based methodologies which simultaneously detect both total coliforms and *E. coli*, have become widely accepted as the standard for microbiological analysis of water. These tests are based on the detection of the enzymes ß-D galactosidase and ß-D glucuronidase which are uniquely associated with total coliforms and *E. coli*, respectively. Enzyme-based coliform

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and *E. coli* methods must include enhancements in order to work effectively in a variety of water matrices. For example, buffers, salts and micro-nutrients are added to enhance enzyme expression. These additives are particularly important in tests that permit enumeration, where the enzyme production from a single organism must be detected. Another important ingredient might be an antibiotic (i.e. Cefsulodin) added to suppress the activity of non-coliforms while leaving the coliforms unaffected. For example, *Aeromonas*, a non-coliform, is known to produce small amounts of ß-D galactosidase. A concentration of 1000 (cfu/100 ml) unsuppressed *Aeromonas* in a 100 ml water sample could trigger a false positive result (Kluender *et al.* 1997; Landre *et al.* 1998).

During the 1990s, three USEPA approved enzyme based methods (Colilert®, Colilert-18®, Colisure®) were thoroughly tested, characterized and subsequently became widely used for testing Wisconsin groundwaters (*Standard Methods 9223B 1998*; US EPA Federal Register 1989*a*, *b*; US EPA Federal Register 1992; US EPA Federal Register 1994). Recently, seven new enzyme-based products have been approved by the USEPA. The seven more recently approved tests are m-Coli Blue 24®, E ∗ Colite® (US EPA Federal Register 1999), Readycult® Coliforms 100, Chromocult® (US EPA Federal Register 2002), Coliscan® (USEPA Laboratory Certification Manual 2005), Colitag™ (US EPA Federal Register 2004) and MI Agar (US EPA Federal Register 1998). Fricker *et al.* 2003 have reported specific problems with some of the newly approved products, however, no comprehensive studies detailing the side-by-side performance of all these new tests have been published (Fricker *et al.* 2003). Preliminary work performed at the Wisconsin State Laboratory of Hygiene suggested differences in the efficacy of some of the newly approved enzyme substrate methods. These preliminary findings led to the project described in this report. The objectives of the project were threefold; 1) to determine the capabilities of all of the USEPA approved enzyme substrate methods to detect the presence or absence of total coliform and *E. coli* in three chemically diverse Wisconsin groundwaters, 2) to determine the ability of each product to accurately detect and quantify the number of total coliforms and *E. coli* in these groundwaters, 3) to determine each product's ability to suppress various concentrations of two Wisconsin environmental strains of *Aeromonas spp*., which may represent a non-coliform and heterotrophic bacteria that are likely to occur as a false positive interference (Faber *et al.* 1997; Kluender *et al.* 1997; Landre *et al.* 1998).

METHODS

The ten enzyme-based tests evaluated during this project were all USEPA approved methods for drinking water analysis (USEPA Lab. Certification Manual 2005). Although there are many similarities in the approved methodologies, seven of the methods have distinctive characteristics and features. Each method is identified and described below.

1) Colilert® (IDEXX, Westbrook, Maine, WP200): Colilert® uses ONPG (ortho-nitrophenyl-ß-D-galactopyranoside) and MUG (4-methyl-umbelliferyl-ß-D-glucuronide) as substrates to simultaneously detect total coliform and *E. coli*. Total coliforms produce the enzyme ß-D galactosidase which will cleave the colorless ONPG substrate releasing a yellow chromogen easily detected by the unaided eye. Additionally, *E. coli* produces ß-D glucuronidase which cleaves the MUG substrate releasing 4-methyl umbelliferone, a fluorogen which fluoresces with a distinct blue glow when observed under a long wave (365–366 nm) ultraviolet light. Colilert® results are determined after 24–28 hours of incubation at 35°C. Colilert® was approved in a presence/absence format, but quantification is available using a most probable number multi well card available from the manufacturer (US EPA Federal Register 1989*a*, *b*; US EPA Federal Register 1992).

2) Colilert-18® (IDEXX, Westbrook, Maine, WP200-18): Colilert-18® is very similar to Colilert® however additional nutrients are added which allow the test to be read following 18 hours, rather than 24 hours of incubation (US EPA Federal Register 1992).

3) Colisure® (IDEXX, Westbrook, Maine, WCLS200): Colisure® uses CPRG (chlorophenol red ß-D-galactopyranoside) and MUG (4-methyl-umbelliferyl-ß-D-glucuronide) as substrates to simultaneously detect total coliform and *E. coli*. Total coliforms produce the enzyme ß-D galactosidase which cleaves the yellow CPRG substrate releasing a magenta red chromogen easily detected by the unaided eye. Similarly, *E. coli* produces ß-D glucuronidase

which cleaves the MUG substrate releasing 4-methyl umbelliferone, a fluorogen which emits distinct blue fluorescence when observed under a long wave (365–366 nm) ultraviolet light. Colisure® results are determined after 24–48 hours of incubation. Colisure® is approved as a presence/absence method; however, quantification is available using a most probable number multi well card available from the manufacturer (US EPA Federal Register 1994; US EPA Federal Register 1999).

4) m-Coli Blue 24® (Hach/Millipore Billerica, MA, MOOPMCB2): m-Coli Blue 24® is a membrane filter method capable of quantifying total coliform and *E. coli* after a 24 hour incubation at 35°C. The water sample is filtered through a membrane and placed onto an absorbent pad containing the m-Coli Blue 24® broth media. Total coliform detection with m-Coli Blue 24® relies on antibiotic inhibitors to suppress the growth of non-coliforms while using a metabolic dye TTC (2, 3, 5 triphenyltetrazolium chloride), that turns the colonies red, aiding in observation of colony growth. BCIG (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide), a chromogenic enzymatic substrate, is added for simultaneous detection of *E. coli*. *E. coli* produces ß-D glucuronidase which cleaves the BCIG substrate releasing the blue chromogen, 5-bromo-4-chloro-3 indolyl. Total coliform colonies appear red and *E. coli* colonies appear a deep purple-blue color. Quantification is achieved by counting the appropriately-colored colonies. This product was approved by USEPA as a presence/absence test for total coliform and *E. coli* in drinking water (US EPA Federal Register 1999).

5) Readycult® Coliforms 100 (EMD Chemicals Inc., Gibbstown NJ, 1.01295.0001): Readycult® Coliforms 100 is available in a presence/absence format for the detection of total coliforms or *E. coli* after 24 hour incubation at 35°C. This product uses X-GAL (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and MUG (4-methyl umbelliferyl ß-D glucuronide) to simultaneously detect total coliforms and *E. coli*. Galactosidase produced by total coliforms cleaves the colorless X-GAL substrate releasing a blue-green chromogenic color change. *E. coli* produces ß-D glucuronidase which cleaves the MUG substrate releasing 4-methyl umbelliferone, a distinct blue fluorogen observable under a long wave (365–366 nm) ultraviolet light. Readycult® also contains an additive, IPTG (isopropyl-ß-D-galactoside), a ß-D-galactosidase inducer to aid in total coliform detection. Additionally, this product includes the amino acid tryptophan in its formulation which permits a quick and easy detection of indole production for verification of *E. coli* presence (US EPA Federal Register 2002).

6) Chromocult® (EMD/Merck Laboratories, Gibbstown, NJ, unavailable in US): Chromocult® requires a 24 hour incubation at 35°C. Chromocult® uses ß-D-galactosidase and cleaves the Salmon-GAL substrate and causes a salmon to red color of the coliform colonies. *E. coli* cleaves both Salmon-GAL and X-glucuronide, so that positive colonies take on a dark-blue to violet color (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide) to detect total coliforms and *E. coli*. Similar to Readycult® and manufactured by EMD, the breakdown of the Salmon-GAL by the galactosidase produced by total coliform will form red colored colonies and cleaving of the X-GLUC substrate by the glucuronidase produced by *E. coli* results in a blue-violet colored colony due to the mixture of the red and blue chromogens. Quantification is achieved by counting the appropriately-colored colonies. It should be noted that this product, as marketed in Europe, contains the antibiotic Cefsulodin to suppress the growth of non-coliform bacteria. However, the addition of Cefsulodin has not been approved by the US EPA for use in the US and was not evaluated in this study. In addition, Chromocult® is presently not being distributed in the United States (US EPA Federal Register 2002).

7) Coliscan® (Micrology Laboratories L.L.C.Goshen, IN, 250MF): Coliscan® is a 24 hour broth based membrane filtration method which uses the substrate RedGal® (6-Chloro-3-Indolyl-ß-D-galactoside) for the detection of total coliforms and X-Gluc (5-Bromo-4-Chloro-3-Indolyl-ß-D-glucuronide) for the simultaneous detection of *E. coli*. Galactosidase produced by total coliforms cleaves the colorless RedGal® substrate resulting in the formation of pink/red colonies. Glucuronidase produced by *E. coli* cleaves the X-Gluc substrate resulting in blue/violet colony coloration due to the combination of the red and blue chromogens. Quantification is achieved by counting the appropriately-colored colonies. Confirmation of *E. coli* maybe performed with the addition of Kovacs Reagent to *E. coli*-like colonies (USEPA Laboratory Certification Manual 2005).

Table 1 | Chemical characteristics of the sampling sites

	Site 1		Site 2		Site 3	
pH	8.1	8.4	7.4	7.4	6.44	6.26
Alkalinity (mg/l)	331.53	331.17	100	101.1	10.22	9.87
Hardness (mg/l)	3.36	3.97	100.2	98.15	12.5	11.11
Soluble Iron (mg/l)	0.002	0.004	0.15	0.39	0.19	0.07
Conductivity (uS/cm)	898	891	202.1	201.4	117	106.7

8) E ∗ Colite® (Charm Sciences Inc., Lawrence, MA, ECO-100): E ∗ Colite® uses a dehydrated enzyme substrate media to determine presence or absence of total coliforms and *E. coli* within 48 hours at 35°C. The substrates used for detection of total coliforms and *E. coli* include X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and MUG (4-methyl umbelliferyl ß-D-glucoside). Galactosidase produced by total coliforms cleaves the colorless X-Gal substrate causing a blue-green chromogenic color change. *E. coli* produces ß-D glucuronidase which cleaves the MUG substrate releasing 4-methyl umbelliferone, a distinct blue fluorogen which is observable under a long wave ultraviolet light (365–366 nm). Confirmation of *E. coli* presence may be performed with the addition of Kovak's Reagent (US EPA Federal Register 1999).

9) MI Agar (S & S Biosciences, Fisher, B14985): MI Agar is a media used in conjunction with membrane filtration. The substrates involved with this method are MUGal (4-methy-lumbelliferyl-ß-D-galactopyranoside) for detection of total coliform and IBDG (Indoxyl-ß-D-glucuronide) for detection of *E. coli*. The galactosidase cleaves the MUGal substrate releasing a fluorogen that causes total coliform colonies to fluoresce under long wave UV light (365–366 nm). Glucuronidase produced by *E. coli* cleaves the colorless IBDG substrate releasing a blue chromogen resulting in blue colonies in visible light. Quantification is achieved by counting the appropriately-colored colonies (Brenner *et al.* 1993; US EPA Federal Register 1998).

10) Colitag™ (CPI International, Santa Rosa, CA, 4600-0012): Colitag™ is available in a presence/absence format for the detection of total coliforms or *E. coli* after 22–26 hours of incubation at 35°C. Colitag™ uses ONPG (ortho-nitrophenyl-ß-D-galactopyranoside) and MUG (4-methyl-umbelliferyl-ß-D-glucuronide) to simultaneously detect total coliforms and *E. coli*. Total coliforms produce ß-D galactosidase which will cleave the colorless ONPG substrate releasing a yellow chromogen easily detected by the unaided eye. *E. coli* produces ß-D glucuronidase which cleaves the MUG substrate releasing 4-methyl umbelliferone, a distinct blue fluorogen observable under a long wave

Table 2 | Spike level specifications

	Enterobacter		*Klebsiella*		*E. coli*		*Citrobacter*		*Serratia*	
Spike Level	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH
Range (cfu)	9–13	83–131	6–14	48–66	5–11	67–118	6–18	72–83	10–18	101–194
Average (cfu)	11	107	10	57	8	84	11	77	15	145
Standard Dev.	2.1	24.0	4.0	9.0	3.1	29.2	6.4	5.5	4.6	46.7
Relative STD	19%	22%	40%	16%	38%	35%	58%	7%	31%	32%

Table 3 | Spike level specifications for *Aeromonas spp*. strain #1 and strain #2

Aeromonas spp. strain #1						
Range	1–8	2–96	26–1000	320–11000	6100–110000	350000–1500000
Average	5	52	2200	5500	58000	770000
Standard Deviation	4	47	490	5300	52000	635000
Relative STD	72%	91%	22%	96%	90%	82%
Aeromonas spp. strain #2						
Range	4–8	28–116	760–4000	4600–14000	4100–150000	310000–2200000
Average	6	65	2000	8300	73000	940000
Standard Deviation	2	46	1800	5000	67000	1090000
Relative STD	35%	70%	90%	60%	92%	116%

Table 4 | Presence/absence data results from Site 1 water samples spiked with (1–10) and (50–100) organisms

	Site 1				**P = Present**		**A = Absent**			
	Citrobacter spp.		***Enterobacter spp.***		***E. coli***		***Klebsiella spp.***		***Serratia spp.***	
Product	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**
Colilert	P	P	P	P	P	P	P	P	P	P
Colilert-18	P	P	P	P	P	P	**A**	P	P	P
Colisure-24	P	P	P	P	P	P	P	P	**A**	**A**
Colisure-48	P	P	P	P	P	P	P	P	P	P
Coliscan w/CF	P	P	P	P	P	P	P	P	P	P
Coliscan	P	P	P	P	P	P	P	P	P	P
MI Agar	P	P	P	P	P	P	P	P	P	P
mColiBlue 24	P	P	P	P	P	P	P	P	P	P
Chromocult	P	P	P	P	P	P	P	P	P	P
Readycult	P	P	P	P	P	P	P	P	**A**	**A**
E * Colite-28	**A**	P	P	P	P	P	P	P	**A**	**A**
E * Colite-48					P	P				
Colitag	P	P	P	P	P	P	P	P	P	P

ultraviolet light (365–366 nm). Colitag™ also contains a low pH buffered medium, TMAO (Trimethylamine-N-oxide) which is metabolized into trimethylamine. Trimethylamine, a basic compound, neutralizes low pH which aids in resuscitation of chlorine-stressed organisms (US EPA Federal Register 2004).

Sampling sites

Three sampling sites were chosen to include geographically, geologically and chemically diverse groundwaters. The chemistry of each groundwater was determined using standard methods analyses for alkalinity, pH, hardness, conductivity and soluble iron (*Standard Methods* 1998). Samples were tested from two sampling events and the results are summarized in Table 1. Site 1 is from a source with high hardness that was softened using ion exchange resin. Site 1 is characterized by a high pH, alkalinity and conductivity and a low amount of soluble iron and a low hardness due to softening. Site 2 was moderately hard water with a neutral pH, and moderate alkalinity and conductivity. Site 3 had a low hardness, pH, alkalinity, and conductivity. There was good correlation in the water chemistry values for each individual site each time chemical analysis was performed.

Sampling and processing

Each site was sampled on two occasions, once for the total coliform and *E. coli* detection and quantification experiments and once for the *Aeromonas spp.* suppression experiments, for a total of six sampling events. Each sample consisted of 60 liters of water collected in three, 20 liter

Table 5 | Presence/absence data results from Site 2 water samples spiked with (1–10) and (50–100) organisms

	Site 2				**P = Present**		**A = Absent**			
	Citrobacter spp.		***Enterobacter spp.***		***E. coli***		***Klebsiella spp.***		***Serratia spp.***	
Product	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**
Colilert	P	P	P	P	P	P	P	P	P	P
Colilert-18	P	P	P	P	P	P	P	P	P	P
Colisure-24	P	P	P	P	P	P	P	P	**A**	P
Colisure-48	P	P	P	P	P	P	P	P	P	P
Coliscan w/CF	P	P	P	P	P	P	P	P	P	P
Coliscan	P	P	P	P	P	P	P	P	P	P
MI Agar	P	P	P	P	P	P	P	P	P	P
mColiBlue 24	P	P	P	P	P	P	P	P	P	P
Chromocult	P	P	P	P	P	P	P	P	P	P
Readycult	P	P	P	P	P	P	P	P	**A**	**A**
E ∗ Colite-28	P	P	P	P	P	P	P	P	**A**	**A**
E ∗ Colite-48					P	P				
Colitag	P	P	P	P	P	P	P	P	P	P

Cubitainers™, and transported to the laboratory within 24 hours of collection.

Samples used for the detection and quantification of total coliforms and *E. coli* were prepared by dispensing a 100 milliliter aliquot from the Cubitainers™ while mixing with magnetic stir bars. For each of the three sites, five different total coliform organisms were spiked at two different concentrations and tested in triplicate for each of the ten methods including a variation of Coliscan® and one additional incubation period for Colisure® and E ∗ Colite®. Complete analysis for the total coliform/*E. coli* detection and quantification objective resulted in 1098 samples. Samples (100 ml) were also prepared from each of the three sites for use in the *Aeromonas* spp. suppression experiments. Each sample was spiked with two strains of *Aeromonas spp*. which were prepared at six different ten fold dilutions (with a range of 1×10^1 to 1×10^6 cfu/100 ml) and tested in triplicate for each of the ten methods including a variation of Coliscan® analysis and one additional incubation period for Colisure® for a total of 1296 samples.

The total coliform (*E. coli, Klebsiella spp., Enterobacter spp., Citrobacter spp., and Serratia spp*.) and *Aeromonas spp*. organisms used for this study were isolated from actual drinking water samples analyzed at the Wisconsin State Laboratory of Hygiene. These single passage environmental organisms were isolated, identified using API 20E and frozen at −80°C prior to use in this study. The cultures were prepared for spiking the day before the spiking procedure by thawing at room temperature, inoculating onto nutrient agar slants and incubating for 24 hours at 35°C. The next day, the isolates were harvested from the slant into a 99 milliliter blank of phosphate buffered dilution water (USEPA Method 1605 2001). Serial dilutions were then

Table 6 | Presence/absence data results from Site 3 water samples spiked with (1–10) and (50–100) organisms

	Site 3				**P = Present**		**A = Absent**			
	Citrobacter spp.		***Enterobacter spp.***		***E. coli***		***Klebsiella spp.***		***Serratia spp.***	
Product	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**	**<10**	**50–100**
Colilert	P	P	P	P	P	P	P	P	P	P
Colilert-18	P	P	P	P	P	P	P	P	P	P
Colisure-24	**A**	**A**	**A**	P ∗	P ∗	P ∗	P ∗	P ∗	P ∗	P ∗
Colisure-48	P ∗	P ∗	P ∗	P ∗	P ∗	P ∗	P ∗	P ∗	P	P
Coliscan w/CF	P	P	P	P	P	P	P	P	P	P
Coliscan	P	P	P	P	P	P	P	P	P	P
MI Agar	P	P	P	P	P	P	P	P	P	P
mColiBlue 24	**A**	**A**	**A**	P ∗	**A**	**A**	**A**	**A**	P	P
Chromocult	P	P	P	P	P	P	P	P	P	P
Readycult	P	P	P	P	P	P	P	P	**A**	**A**
E ∗ Colite-28	P	P	P	P	P	P	P	P	**A**	**A**
E ∗ Colite-48					P	P				
Colitag	P	P	P	P	P	P	P	P	P	P

performed to create bacterial suspensions containing low levels (1–10) and high levels (50–100) of organisms per ml which were subsequently added as one ml aliquots to the 100 ml water samples for use in the experiments (USEPA Method 1605 2001; *Standard Methods* 1998). The actual spike concentrations of the prepared dilutions were determined using a Heterotrophic Plate Count test (*Standard Methods* 1998). Since the Colisure® and E ∗ Colite® test systems allow the test to be read out at either 24/28 (total coliform) and 48 hours (*E. coli*), results were determined and recorded at each time. Tables 2 and 3 represent Heterotrophic Plate Count statistical data regarding bacterial concentrations dispensed during each spiking procedure.

For the total coliform and *E. coli* detection portion of the experiment, triplicate pairs of individual water samples were spiked with one ml aliquots of the spike material from each strain of bacteria at levels that resulted in 1–10 and 50–100 bacteria in the 100 ml test vials.

Table 7 | The percent failure rate of each enzyme-based test to detect the presence or absence of total coliform bacteria and *E. coli*

USEPA Approved Product	Failure Rate
Colilert®	0%
Colilert-18®	3.3%
Colisure®24 hours	20%
Colisure®-48 hours	0%
Coliscan® w/CF	0%
Coliscan®	0%
MI Agar	0%
mColiBlue 24®	23%
Chromocult®	0%
Readycult®	20%
E ∗ Colite®-28 hours	20%
E ∗ Colite®-48hours	0% (for *E.coli* only)
Colitag™	0%

For the *Aeromonas spp*. suppression procedure, two strains of *Aeromonas spp*. were obtained through the same culture protocol as described above. One-hundred ml samples spiked with 10^1, 10^2, 10^3, 10^4, 10^5 and $10^6$of each *Aeromonas spp*. strain were prepared. This spiking protocol was repeated on three separate days for each of the three sampling sites. On one occasion, as seen in Table 9 for *Aeromonas spp*. strain #1, the spike level was one log lower for each spike level due to spiking error.

As the samples were spiked, a heterotrophic plate count was performed within 30 minutes for each of the prepared suspensions to determine the actual spike concentration. An unspiked "blank" of the sample water was tested using each enzyme-based product as well as Ampicillin-Dextrin agar with vancomycin (USEPA Method 1605 2001) to determine any background total coliform or *Aeromonas spp*. in the unspiked water which could adversely influence the results of the spiked samples. The results from "blank" analysis prior to sample spiking of each of the three water matrices indicated that there were no positive samples for background target organisms (total coliforms, *E. coli* and *Aeromonas*). Each lot of the enzyme-based product used for this project was tested using a positive control (*E. coli*), a negative control (*Aeromonas spp.*) and a sterility check. After completion of the spiking procedure, the samples were processed using each of the test methods, following the protocols provided by either journal articles or the manufacturers (Brenner *et al.* 1993; Charm Sciences, Inc. 2004; CPI International 2004; Edberg *et al.* 1991; EMD Chemicals, Inc. 2004; Fricker *et al.* 1997; Fricker *et al.* 2003; Geissler *et al.* 2000; Hach Company 2004; IDEXX Laboratories, Inc. 2004; Manafi & Rosmann 1999; McFeters *et al.* 1995; Micrology Laboratories 2004; USEPA Federal Register 1989*a*, *b*, 1992, 1994, 1998, 1999, 2002, 2004; USEPA Method 1604 2002).

RESULTS

Federal regulations regarding the occurrence of total coliforms and *E. coli* in drinking water are based on the presence or absence of the organisms rather than the numbers of organisms detected. Consequently, any test method must be capable of reliably producing this presence/absence result. Tables 4, 5 and 6 show the ability

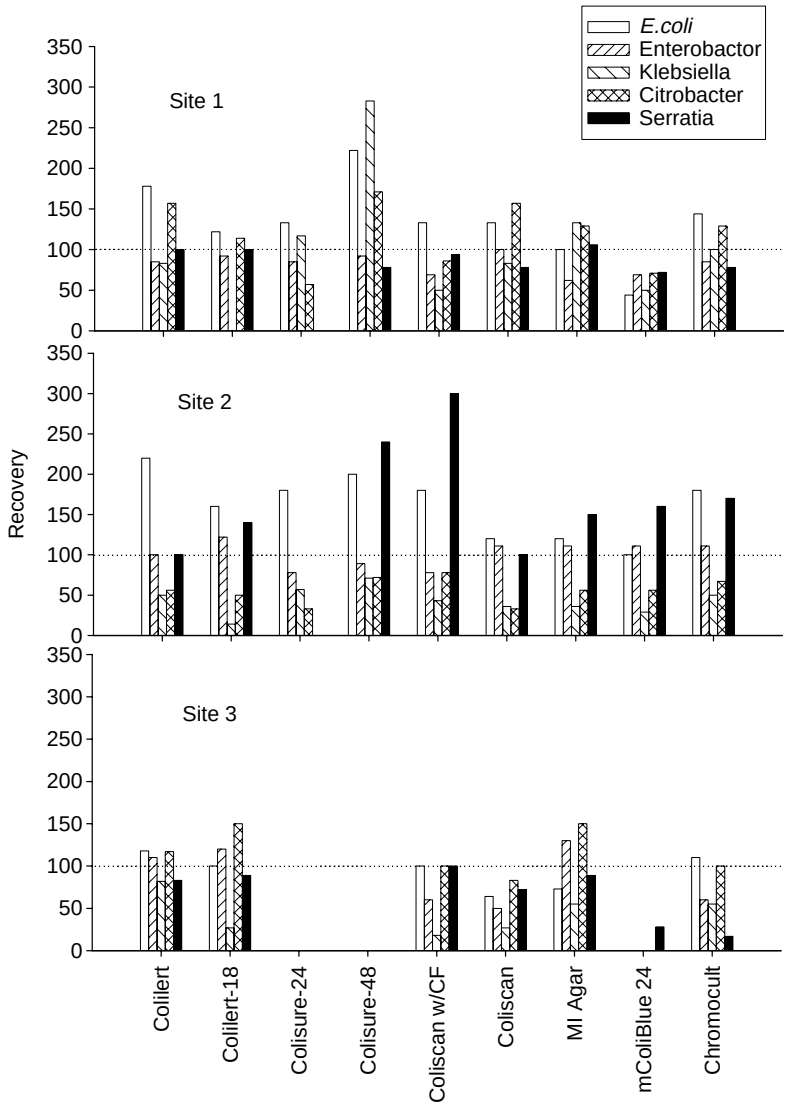

Figure 1 | Percent recovery data for water samples from each sampling site spiked with 1–10 bacteria of each total coliform genus.

of each of the evaluated methods to detect total coliforms and *E. coli* in a presence/absence format. In most cases, the triplicate analyses performed on each sample to increase the robustness of the data set had identical results between the replicates. However, there were exceptions when using the low and high level concentrations of *Klebsiella, Enterobacter, E. coli, Citrobacter and Serratia* where the triplicate analysis yielded two "present" results and one "absent" or two "absent" and one "present" result. These were treated as a present result in the tables. Triplicate analysis was only treated as "absent" if all three replicates were negative. These phenomena occurred only for Colisure® incubated for 24 and 48 hours as well as m-Coli Blue 24® analysis of Site 3 water and are noted with an asterisk on Table 6. The expected result for these first objective trials was "coliform present" and, for the *E. coli* spike, "*E coli* present" for all samples. Surprisingly, this was not the case in 27 of the 366 tests which exhibited a 7.3% false negative rate.

For Site 1, (Table 4) the test methods were capable of detecting total coliforms and *E. coli* with four exceptions. E ∗ Colite® incubated for 28 hours, Colisure® read at 24

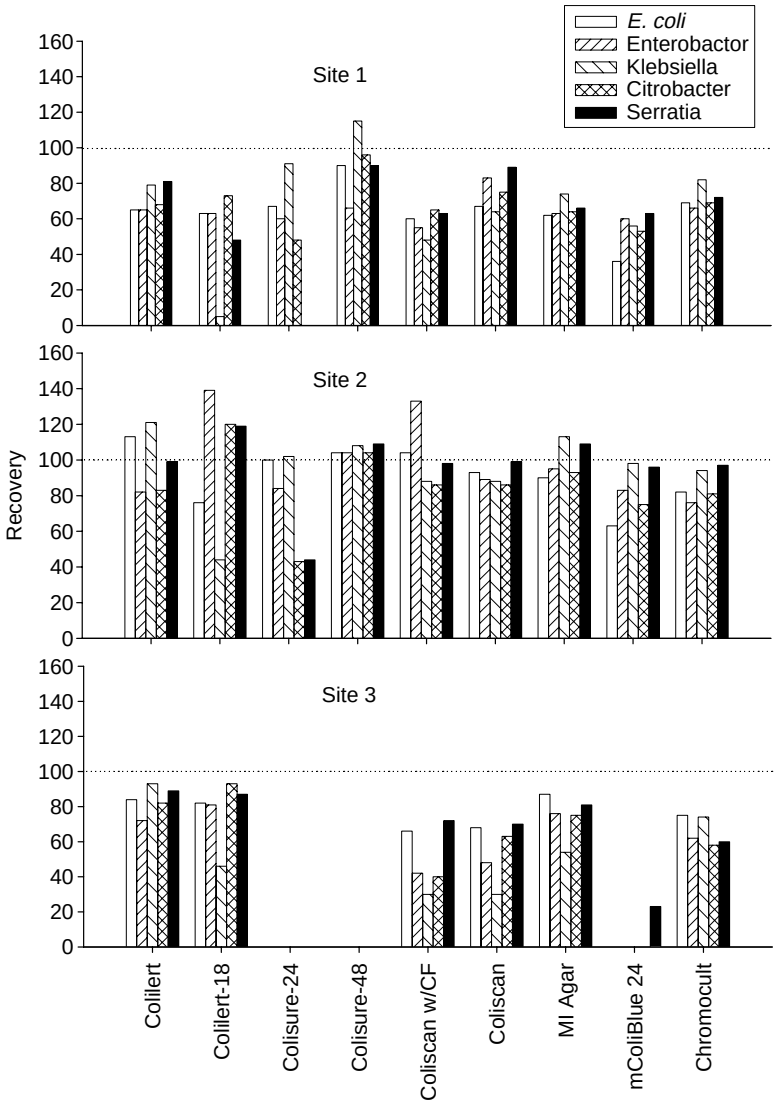

Figure 2 | Percent recovery data for water samples from each sampling site spiked with 50–100 bacteria of each total coliform genus.

hours and Readycult® were unable to detect *Serratia spp.* at both spike levels of <10 and 50–100 cells. In addition, the E * Colite® product also failed to detect the <10 *Citrobacter spp.* spike whereas Colilert-18® failed to detect *Klebsiella* spp. at <10 cells. In water samples from Site 2 (Table 5), Colisure® read at 24 hours once again failed to detect *Serratia* spp. at the <10 spike level, but not at 48 hours. E * Colite® and Readycult® at both of the spike levels in Site 2 failed to detect *Serratia* spp.. Detection variability was least acceptable at Site 3 (Table 6). For example, Colisure® read at 24 hours failed to detect *Enterobacter spp.* at the <10 concentration and *Citrobacter spp.* at both <10 and 50–100 cells. mColiBlue24® was incapable of detecting *E. coli*, *Klebsiella spp.* and *Citrobacter spp.* at both spike levels and *Enterobacter spp.* at <10 cells. Readycult® and E * Colite® did not detect *Serratia spp.* at both spike levels. A summary of the failure rates for each test is presented in Table 7.

Colilert®, Colisure® read at 48 hours, Coliscan®, MI agar, Chromocult®, and Colitag® performed as expected and were all capable of detecting the presence of total coliforms and *E. coli* in all samples tested. The most alarming of these results is the inability of m-ColiBlue 24® to detect *E. coli* at Site 3. The failures of the various methods appear to be both organism and sample matrix dependant.

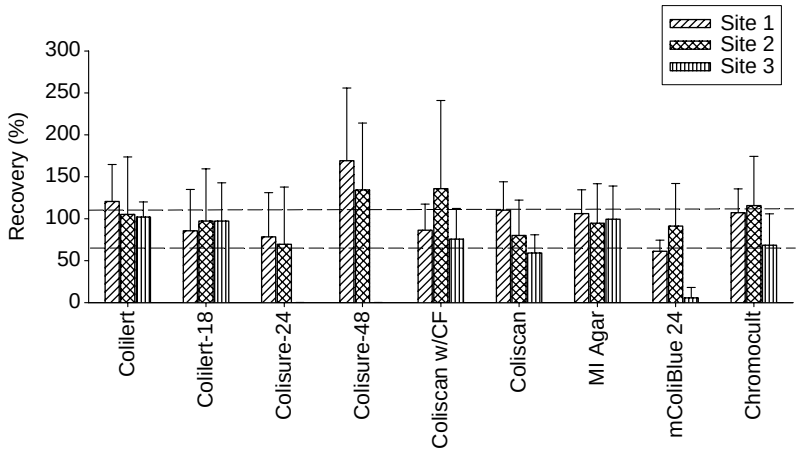

Figure 3 | Average percent recovery plots for all samples spiked with 1–10 organisms including error bars representing one standard deviation from the mean.

Enumeration methods

In addition to the ability to simply detect the presence/absence of total coliforms and *E. coli*, Colilert®, Colilert-18®, Colisure®, Coliscan®, MI Agar, mColiBlue 24® and Chromocult® also have the ability to enumerate organisms. In the enumeration portion of the experiments, analyses were once again performed in triplicate. The triplicate test results were arithmetically averaged and compared to the heterotrophic plate count results as a recovery percentage. Preliminary observations of the data showed that there was an apparent difference in enumeration capabilities of the tests based on the sample matrix. Consequently, Figures 1 and 2 depict the percent recovery of each of the spike organisms for each enumeration capable method stratified by sample site (matrix). Figure 1 demonstrates the results low level spike (1–10 organisms) and Figure 2 displays the high level spike (50–100 organisms). The stratified graphs allow facile comparisons of recoveries for all of the methods and each of the organisms across all three sample types.

The most obvious observation is the inability of Colisure® and mColiBlue 24® to significantly recover any level of coliforms spiked into the Site 3 water. This result was so striking that it was suspected that a testing error might have been involved. It was decided to re-run this portion of the analysis in order to rule out this possibility. The re-test resulted in verification of the initial results.

It also becomes apparent that individual test methods vary in their ability to recover specific coliform organisms. For example Colilert-18® does a poor job in recovery of *Klebsiella*. Another example is that Colisure®, when read at 24 hours does a poor job recovering *Serratia*. The other

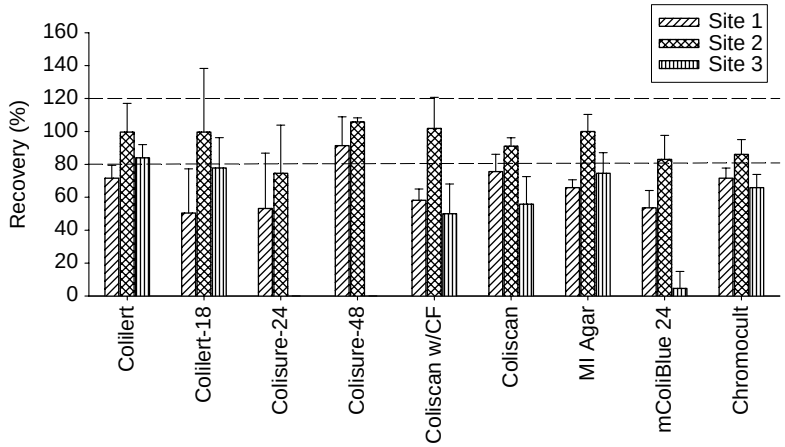

Figure 4 | Average percent recovery plots for all samples spiked with 50–100 organisms including error bars representing one standard deviation from the mean.

Table 8 | Product ability to suppress two different strains of *Aeromonas spp.* at multiple levels of contamination spiked into water collected from Site 1

	Site 1											
Organism	***Aeromonas spp.* strain #1**						***Aeromonas spp.* strain #2**					
Spike amount	10^1	10^2	10^3	10^4	10^5	10^6	10^1	10^2	10^3	10^4	10^5	10^6
Product												
Colilert	−	−	−	−	−	−	−	−	−	−	−	−
Colilert-18	−	−	−	−	−	−	−	−	−	−	−	−
Colisure-24	−	−	−	−	+	−	−	−	−	−	−	−
Colisure-48	−	−	−	−	+	−	−	−	−	−	−	−
Readycult	−	−	−	−	−	−	−	−	−	−	−	−
E ∗ Colite-28	−	−	−	−	−	−	−	−	−	+	−	+
Colitag	−	−	−	−	−	−	−	−	−	−	−	−
Coliscan w/CF	−	−	−	−	−	−	−	−	−	−	−	−
Coliscan	−	−	−	−	−	−	−	−	−	−	−	−
MI Agar	−	−	−	−	−	−	−	−	+	+	+	+
mColiBlue 24	−	−	−	−	−	−	−	−	−	+	+	+
Chromocult	+	+	+	+	+	+	+	+	+	+	+	+

valuable information gathered from these graphs is the obvious effect of sample matrix on the ability of individual test systems to recover the spiked organisms.

With each site representing different water quality characteristics, the figures (Figures 3 and 4) for each test spiked with 1–10 (low) and 50–100 (high) organisms demonstrate significant differences among the methods ability to recover the spiked organisms at a 100% level. The figures represent the mean recovery for all total coliform organisms for each test at each site. The error bars represent one standard deviation from the mean. The most significant information derived from these figures is the fact that tests with error bars that overlap 100% have expected recoveries that are not statistically different from 100% but tests with error bars that cover a wide range are expected to have results that have wide variations. Clearly, all tests had higher variability for the low spikes, which is a result of the larger uncertainty in the spike determination for the low level spikes. Since the Safe Drinking Water Act states that only one organism detected is considered an unsafe sample and a follow-up sample is necessary, this information could be useful for decisions regarding which enzymatic test to use for determining the efficacy of water treatment (LeChevallier *et al.* 1996; McMath *et al.* 1999; Williams & Braun-Howland 2003).

Perhaps the most important aspect of a product's performance lies in its ability to perform on actual world samples where large numbers of non-target organisms can interfere with the test results. The *Aeromonas* spp. suppression experiment was aimed at testing this aspect of product performance. The expected result is that a product will suppress the growth and galactosidase production of the non coliform organisms even when the non coliform organism level is in the 10^5 range. The data from the

Table 9 | Product ability to suppress two different strains of *Aeromonas spp.* at multiple levels of contamination spiked into water collected from Site 2

	Site 2											
Organism	***Aeromonas spp.* strain #1**						***Aeromonas spp.* strain #2**					
Spike amount	10^0	10^1	10^2	10^3	10^4	10^5	10^1	10^2	10^3	10^4	10^5	10^6
Product												
Colilert	−	−	−	−	−	−	−	−	−	−	−	+
Colilert-18	−	−	−	−	−	−	−	−	−	−	−	−
Colisure-24	−	−	−	−	−	−	−	−	−	−	−	−
Colisure-48	+	+	−	−	−	+	−	+	−	−	−	−
Readycult	−	−	−	−	−	−	−	−	−	−	−	−
E ∗ Colite-28	−	−	−	−	−	−	−	+	+	+	+	+
Colitag	−	−	−	−	−	−	−	−	−	+	+	−
Coliscan w/CF	−	−	−	−	−	−	−	+	+	+	+	+
Coliscan	−	−	−	−	+	+	−	−	−	+	+	+
MI Agar	−	−	−	−	−	−	+	+	+	+	+	+
mColiBlue 24	−	−	−	−	−	−	+	+	+	+	+	+
Chromocult	+	+	+	+	+	+	+	+	+	+	+	+

Aeromonas spp. suppression analysis are presented in Tables 8, 9 and 10, once again stratified by sampling site. The data indicates that there was a wide diversity in the ability of the various products to suppress *Aeromonas spp.* Generally, Colilert®, Colilert-18®, Colitag®, Colisure® and Readycult® all displayed acceptable suppression capability. The remaining products (E ∗ Colite®, Coliscan®, MI, m-Coli Blue 24® and Chromocult®) were unable to suppress *Aeromonas spp.* at various contamination levels. This was particularly true with *Aeromonas spp.* strain #2. The Chromocult® product could not suppress *Aeromonas spp.* even at the minimum spike level with either of the strains. MI Agar did not suppress # 2 at Site 1 and 2 but was successful in suppressing *Aeromonas spp.* strain #2 at Site 3. Once again, as in the recovery experiments, there was a marked difference observed between the various sample matrices.

DISCUSSION

Enzyme based methodologies have become widely accepted as the industry standard for water microbiological testing (*Standard Methods 9223B* 1998). The USEPA has approved ten of these methods for use in testing drinking water (US EPA Federal Register 1989*a*, *b*; US EPA Federal Register 1992; US EPA Federal Register 1994; US EPA Federal Register 1999; US EPA Federal Register 2002; US EPA Federal Register 2004; USEPA Laboratory Certification Manual 2005), irregardless of the fact there is a paucity of side-by-side comparison data available that labs can use in choosing a product for purchase. The data produced in this study suggests that there are significant differences between the ten USEPA approved methods both in the ability to detect total coliforms and *E. coli* and in their ability to suppress false positive results from the non coliform

Table 10 | Product ability to suppress two different strains of *Aeromonas spp.* at multiple levels of contamination spiked into water collected from Site 3

	Site 3											
Organism	***Aeromonas spp.* strain #1**						***Aeromonas spp.* strain #2**					
Spike amount	10^1	10^2	10^3	10^4	10^5	10^6	10^1	10^2	10^3	10^4	10^5	10^6
Product												
Colilert	−	−	−	−	−	−	−	−	−	−	−	−
Colilert-18	−	−	−	−	−	−	−	−	−	−	−	−
Colisure-24	−	−	−	−	−	−	−	−	−	−	−	−
Colisure-48	−	−	−	−	−	−	−	−	−	−	−	−
Readycult	−	−	−	−	−	−	−	−	−	−	−	−
E ∗ Colite-28	−	−	−	−	−	−	−	−	−	−	+	+
Colitag	−	−	−	−	−	−	−	−	−	−	−	−
Coliscan w/CF	+	+	+	+	+	+	−	+	+	+	+	+
Coliscan	+	+	+	+	+	+	+	+	+	+	+	+
MI Agar	−	−	−	−	+	+	−	−	−	−	−	−
mColiBlue 24	−	−	−	−	−	−	−	−	−	−	−	−
Chromocult	+	+	+	+	+	+	+	+	+	+	+	+

organisms, such as, *Aeromonas spp*. Furthermore, this study demonstrates performance differences attributable to sample matrix differences.

Some of the methods evaluated were unable to detect certain species of total coliform from all of the groundwater matrices examined. The most significant of these findings was the inability of mColiBlue 24® to detect *E. coli* even in high concentrations in the Site 3 matrix. The Site 3 groundwater characterized by a high level of background heterotrophic bacteria, low pH and low alkalinity (Table 1) was the most problematic. Although the interaction of these parameters with test performance is not entirely understood, one might speculate that low pH and low alkalinity level samples such as the Site 3 water may require a media formulation with greater buffering capacity. The data suggests the possibility that the Colisure® and mColiBlue24® may not provide enough acid-neutralizing capacity to provide accurate results whereas the other products were capable of maintaining their integrity and efficacy in the water samples exhibiting these characteristics. Another possible explanation would be associated with the high level of background bacterial contamination. The background heterotrophic plate count (HPC) for Site 3 groundwater was 418 cfu/ml, whereas the background counts for Site 1 and Site 2 were 16 cfu/ml and 3 cfu/ml, respectively. This increased level of heterotrophic bacteria may have influenced the ability of mColiBlue24® and Colisure® products to provide accurate results, whereas the other products were less sensitive to background bacteria.

The presence of high levels of *Aeromonas spp.* in water samples, which may have a low level of galactosidase production, can lead to false positive results if the organisms are not adequately suppressed by the media additives. In this study, major differences between products and their ability to suppress *Aeromonas spp*. were observed. Tables 8,

9 and 10 indicated differences in product abilities to suppress *Aeromonas spp.* between sites and between *Aeromonas spp.* strains. There was no apparent pattern to each product's inability to suppress *Aeromonas spp.* With the exception of Colilert-18® and Readycult®, all methods at some point in this study were unable to suppress *Aeromonas spp.* In some instances, a product would be unable to suppress *Aeromonas spp.* at a lower spike level yet completely suppress *Aeromonas spp.* seeded at a greater spike level. This anomaly could not be explained. However, if this anomaly is ignored, Colilert® and Colisure® could be added to the list of media that demonstrated sufficient suppression in all three groundwater sample matrices.

Variability for different enzyme-based products to suppress different strains of *Aeromonas spp.* was observed. For example, the strain #1 of *Aeromonas spp.* seeded in Site 1 and Site 2 water was completely suppressed by MI agar however, MI agar failed to suppress *Aeromonas spp.* strain # 2. Conversely, MI agar failed to suppress *Aeromonas spp.* strain #1 and completely suppressed # 2 in the presence of groundwater collected from Site 3.

Product inconsistencies were also observed. For example, in Table 9 Colisure® read at 48 hours was unable to suppress *Aeromonas spp.* strain #1 at lower concentrations but able to suppress *Aeromonas spp.* strain #1 at higher concentrations.

CONCLUSIONS

Further research with enzyme-based methods is needed to increase the amount of data to better understand the implications of these results. Additional investigation regarding how chemical characteristics and concentration of background heterotrophic bacteria may affect detection of total coliforms and *E. coli* when using enzyme-based technology is also needed. Future research will focus more on which methods surpass other methods in their ability to accurately detect low levels of chlorine-stressed total coliform and *E. coli* as well as in suppression of *Aeromonas spp.*, as well as other non-coliform bacteria that may interfere with proper operation of the enzyme-based product. The study does stress the need for careful side-by-side evaluation of any method used in all sample matrices prior to being used for the analysis of drinking water samples (and source water) where the results will be used for making public health decisions.

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