IDEXX Summary 6Q

Topic:	Comparison of Colilert/Quanti-Tray with membrane filtration in natural waters
Title:	A long-term study comparing membrane filtration with Colilert defined substrates in detecting fecal coliforms and *Escherichia coli* in natural waters
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Report Highlights:

- 10 different environmental waters were analyzed over a time period of 36 months. Colilert/Quanti-Tray compared equally with Virginia's standard membrane filter technique (m-FC broth) with no significant differences found between the two testing methods.
- Colilert DST presents a laboratory protocol that is simpler to manage, quicker to process, and easier to quantify results than MF
- Membrane filter tests are labor and materials intensive and require a high degree of technical skill to obtain, interpret, and confirm results.
- Colilert's performance during warm and cold seasons and its remarkable precision over the range of stream qualities sampled offer a major benefit to testing laboratories, especially when compared to a standard MF protocol.

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by
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A long-term study comparing membrane filtration with Colilert® defined substrates in detecting fecal coliforms and *Escherichia coli* in natural waters

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Abstract

Assessment methods for determining the presence and number of fecal bacteria and *Escherichia coli* (*E. coli*) in waters, foodstuffs, sewage effluent, and soils have evolved from multiple tube fermentations (MTF's) to membrane filtrations (MF's) to, most recently, defined substrate technologies (DST's). Mounting evidence indicates Colilert DST (IDEXX, Westbrook, ME) to be a versatile assessment technique for detecting and enumerating *E. coli* over a range of applications. This study compared the performance of Colilert DST with a confirmed standard MF technique using m-FC broth (Millipore, Bedford, MA) in assessing *E. coli* in ten different environmental water samples obtained monthly over a 3-year period from the upper Appomattox River, VA. For the duration of the study, *E. coli* counts measured by Colilert DST were positively correlated (Pearson's correlation coefficient = 0.956; slope = 0.979; $p < 0.0001$) with *E. coli* counts measured by confirmed MF procedures. The results of a two-factor ANOVA revealed that Colilert DST counts compared equally to confirmed MF counts by year ($p = 0.974$), by stream sampled ($p = 1.0$), and by season ($p = 0.696$). *E. coli* counts were significantly lower during cold season months (Dec/Jan/Feb) than during warm season months (Jun/Jul/Aug) for each year contributing to marked variation in sample quality. Counts obtained by Colilert DST compared equally to those obtained by MF across all samples and dates for the three years. Colilert DST presents a laboratory protocol that is simpler to manage, quicker to process, and easier to quantify results than MF. These factors, plus the enhanced precision and versatility of Colilert DST over the span of this three-year study attests to its suitability for testing ambient surface waters.

Keywords: Defined substrates; Membrane filtration; Fecal coliform; *E. coli*

1. Introduction

Correlations between enteric diseases and contaminated water supplies in the 1850s (Snow, 1855) and the subsequent discovery of microbial disease agents in the 1880s led to the exploration of procedures to test for bacterial presence in water. Theodore Escherich (1885) suggested the use of *Bacillus coli* (later *Escherichia coli*) as a suitable indicator for fecal contamination since it was found in high densities in feces and was associated with the typhoid bacillus. In the early 20th Century, microbiologists (Eijkman, 1904; Leiter, 1929) learned to detect *E. coli* by observing gas production in glucose broths incubated at elevated temperatures. This test, called multiple tube fermentation (MTF) used serially diluted test samples in lactose broth. MTF became known as the Standard Test for Water Analysis and was a universally applied test for fecal pollution of water.

Goetz and Tsuneishi (1951) proposed and developed a membrane filtration (MF) procedure that was found to be more accurate than MTF and offered a considerable savings in time, labor, and cost. A variety of defined media were formulated and tested for use with MF (Clark et al., 1951; McCarthy et al., 1961; Geldreich et al., 1965; Rose et al., 1975; Levin et al., 1975; Presswood and Strong, 1978; Dufour et al., 1981; Messer and Dufour, 1998; USEPA, 2000) to isolate, enumerate, and identify indicator bacteria for water quality assessment. MF continues to be useful in monitoring drinking, natural, and wastewater samples and is standard practice in many public health laboratories in the United States (APHA, 1998).

Bacterial pollution indicators chosen for fresh waters are predominantly comprised of the coliform bacteria—a group of small, facultative, Gram-negative bacilli (Family

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Enterobacteriaceae), which produce acid and gas from lactose within 48 h of incubation at 35 °C. In time, the use of 'coliforms' narrowed to 'fecal coliforms' and then to the more restrictive *E. coli* (and enterococci) norm currently approved for fresh water analysis by the U.S. Environmental Protection Agency (1986).

In the late 1980s another water assessment tool, defined substrate technology (DST), became available (Edberg et al., 1988). DST simultaneously detects total coliform bacteria and *E. coli* by enzymatic hydrolysis of specific substrates. The method screens for bacteria using selective inhibitors and elevated incubation temperatures to assess enzymatic activity.

One DST medium, Colilert® (Idexx Laboratories, Westbrook, ME), utilizes two substrates: *o*-nitrophenyl-β-D-galactopyranoside (ONPG), which screens for β-D-galactosidase, an enzyme found in lactose-fermenting bacteria and in some coliform bacteria, and 4-methylumbelliferyl-β-D-glucuronide (MUG), which screens for β-D-glucuronidase, an enzyme found in several bacterial species, but predominantly in *E. coli* (Tryland and Fiksdal, 1998). Colilert has recently been certified by the USEPA as a viable method for bacterial assessment of surface waters (USEPA, 2003).

Mounting evidence favors the use of Colilert over other assessment methods for testing freshwater (Edberg et al., 1991; Shadix and Rice, 1991; Cowburn et al., 1994; Fiksdal et al., 1994; Fricker and Fricker, 1996; Eckner, 1998; Grasso et al., 2000; Pisciotta et al., 2002; Yakub et al., 2002; Niemela et al., 2003), drinking water (Edberg et al., 1988; McFeters et al., 1992; Cowburn et al., 1994; Fricker et al., 1997; Niemela et al., 2003), wastewater effluent (Elmund et al., 1999; Kramer and Liu, 2002; Eccles et al., 2004), contaminated soils (Muirhead et al., 2004), and foodstuffs (Venkateswaran et al., 1996; Hara-Kudo et al., 2001) for these bacteria. MF and Colilert methods are commonly compared and discussed in water quality literature, but more comparisons are needed (Francy and Darner, 2000) to confirm the validity of Colilert.

This study compares the Colilert DST method with a standard MF method (using m-FC broth) used for testing Virginia surface waters for quantifying fecal coliforms and *E. coli* in natural freshwater samples over a 36-month period. This paper reports one of the first long-term comparison studies between Colilert and MF methods for ambient waters. The data were obtained from monthly bacterial assays of water samples from 10 locations, exhibiting a wide range of bacterial counts, along the upper Appomattox River and its tributaries in central Virginia. The results provide critical information confirming the accuracy of the Colilert method, its comparability to a standard assay, and its applicability for use.

2. Materials and methods

2.1. Sampling locations

On the third Tuesday of each month over a 36-month period (Jan 2001–Dec 2003), water samples were collected from 10 different locations ($n=396$) in the upper Appomattox River watershed in central Virginia, within Buckingham, Cumberland, Nottoway, and Prince Edward counties. All sites are located within a 25-mile radius of the town of Farmville, Virginia. Sample locations with GPS coordinates are presented in Table 1. For all sampling locations, the predominant land use of the sub-watersheds is agricultural (beef, dairy, poultry, row crops, and hay fields) interspersed with mixed hardwood forest and low housing density. Seven sampling sites were chosen from a list of stream sites revealing a history of non-compliance with Virginia state standards for fecal coliforms for contact waters (VA DEQ, 1998). Three additional sites, all in the Sayler's Creek watershed, were chosen for general interest.

Table 1
Water sampling sites within the upper Appomattox River watershed, Virginia

Site	Location (lat./long.)	VA county
Angola Creek 17 (ANG17)	37.355N/−78.555W	Cumberland
Appomattox River 1 (APP1)	37.388N/−78.619W	Prince Edward/ Buckingham
Appomattox River 2 (APP2)	37.307N/−78.388W	Prince Edward/ Cumberland
Buffalo Creek 15 (BUF15)	37.268N/−78.482W	Prince Edward
Green Creek 16 (GRE16)	37.328N/−78.305W	Cumberland
Little Saylors Creek 5 (SAY5)	37.316N/−78.263W	Prince Edward
Little Saylors Creek 6 (SAY6)	37.289N/−78.273W	Prince Edward
Big Saylors Creek 7 (SAY7)	37.308N/−78.228W	Prince Edward
Big Saylors Creek 8 (SAY8)	37.288N/−78.221W	Nottoway
Vaughans Creek 14 (VAU14)	37.351N/−78.560W	Prince Edward

Sampling locations are located near agricultural pastures to varying degrees within the piedmont region of southcentral Virginia.

2.2. Sampling protocol

Water samples were collected according to standard guidelines (APHA, 1998), with at least one field duplicate sample obtained each month at a randomly chosen site. All samples were taken on the designated sampling dates, collected in sterile 18 oz. Whirl-Pak® bags (Nasco, Fort Atkinson, WI), and stored on ice for transport to the laboratory. All samples were processed by MF and Colilert DST within 6 h of collection with separate aliquots aseptically transferred from the same sample container for comparison ($n=792$). Additionally, one sample duplicate was randomly selected each month within each test category.

2.3. Membrane filtration

Fecal coliforms were assayed using a 1% sample dilution (1 ml test sample: 99 ml sterile phosphate buffered dilution water) processed according to APHA (1998). Membrane filters ((0.45 um pore size) Millipore, Bedford, MA) were transferred to 50 mm petri plates containing 2 ml of m-FC broth (Millipore) and incubated for 24 ± 2 h at $44.5°\pm0.2°$ C (APHA, 1998). Two sample blanks were processed during the MF to insure quality of dilution water each month. Each blue fecal coliform colony was confirmed for fermentation of lactose (EC broth, BDL, Sparks, MD), IMViC response (broth/agar media - DIFCO Labs, Detroit, MI) and lack of oxidase (BBL Dryslide, BDL, Sparks, MD) activity. Confirmation of *E. coli* from other fecal coliform

bacteria is enhanced by IMViC testing accompanied by the elevated (44.5 °C) incubation temperature (Leclerc et al., 2001), otherwise referred to as the 'IMVeC' test (Mossel, 1982). Those isolates not confirming as *E. coli* were not further identified and were not used in the tabulation of the bacterial count. MF results were recorded as number of fecal coliform colony-forming units (CFUs) per 100 ml test sample for all comparisons. The selectivity of the elevated incubation temperature reduced interference effects due to background bacterial presence on the m-FC medium. After the 44.5 ° C incubation, few (<10) background (non-fecal coliform) colonies developed on any MF plate. Since many streams in this study contained high numbers of fecal coliform bacteria, the appropriate sample dilution for MF processing was 1%, which limited the sensitivity threshold of the test for sample counts to <100 CFU/100 ml for plates with no colony. All fecal coliform counts of '<100' were ascribed values of 50 CFU/100 ml for data analyses.

2.4. Colilert DST

Total coliforms and *E. coli* were assayed using the Colilert Quanti-tray 2000 technique using a 25% sample dilution (25 ml test sample: 75 ml sterile phosphate buffered dilution water), processed according to manufacturer's instructions, and incubated in the same chamber and conditions as the MF plates. Two sample blanks were processed during each monthly DST to insure quality of dilution water. After incubating, wells showing chromogenicity (yellow) and fluorescing under UV (365 nm) illumination were counted positive for *E. coli*. All enumerations were performed using a most probable number (MPN)-based system with a quantification range between <1 and 9 676 CFU per 100 ml when using a 25 ml sample dilution. Aliquots of 100 randomly selected wells showing both chromogenic and fluorogenic responses were aseptically transferred using sterile, disposable 3 cc medical syringes for confirmation of *E. coli* as described for presumptive fecal coliforms in the MF procedure. Since all initial confirmations were positive for *E. coli*, additional confirmations were reduced to quarterly intervals. Reports indicate identity confirmation is not required when using Colilert on freshwater samples (Fricker et al., 1997; Niemela et al., 2003). Each lot of Colilert media was tested with Quanti-Cult® (Chrisope Technologies, Lake Charles, LA) cultures to check media response. Colilert test results were recorded as number of total coliform bacteria (not used in this comparison) and as number of *E. coli* (CFUs/100 ml) for all comparisons.

2.5. Statistical analyses

E. coli counts as measured using the Colilert method and the MF method were compared using an independent-samples *t*-test (across all years and all streams sampled). A Pearson's product moment correlation analysis was used to examine the relationship between *E. coli* counts measured with the Colilert method and *E. coli* counts measured using the MF method. *E. coli* counts were also compared using three 2-factor analysis of variance (ANOVA) tests. The first ANOVA compared type of test (Colilert vs. MF) and year of data collection (2001, 2002, and 2003) as independent variables. A second ANOVA compared type of test and stream sampled as independent variables (10 streams total, see Table 1); the third ANOVA used type of test and season as independent variables.

Seasonal analyses were conducted due to temperature variations during warm and cold seasons in the region where samples were obtained. The piedmont region of Virginia has a seasonal climate (average temp for Dec/Jan/Feb is 10.4°/9.4°/11.1 ° C, respectively; for Jun/Jul/Aug it is 29.8°/31.5°/30.7 ° C) with an average difference of 21 ° C between cold and warm months (Southeast Regional Climate Center, 2004; Van der Leeden, 1998). Therefore, bacterial counts obtained by MF and Colilert DST for 'cold months' (Dec., Jan., and Feb.) and 'warm months' (June, July, and Aug.) were used to examine the performance of both techniques with respect to seasonal temperature differences. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL).

3. Results

3.1. Bacterial confirmations

Most (98.7%; 3 074/3 114) of the fecal coliform colonies isolated on m-FC broth from MF revealed positive confirmations for *E. coli*. The identities of the unconfirmed isolates were not obtained. Previous studies have found other enterics (i.e. *Klebsiella*, *Enterobacter*, *Citrobacter*, and others) in confirmatory tests of MF results (Chao et al., 2003; Eccles et al., 2004). All 100 of the Colilert Quanti-Tray wells chosen for bacterial confirmation (revealing positive chromogenic and fluorogenic reactions) tested positive for the presence of *E. coli*.

3.2. Independent samples t-test and Pearson's correlation analysis

Results of an independent-samples t-test indicated no significant difference ($p=0.401$) between number of *E. coli* measured using Colilert (mean ± SE: 522.99 ± 77.86) and MF method (635.82 ± 79.82) over the course of the study. Furthermore, Pearson's correlation analysis demonstrated a significant positive relationship (correlation coefficient = 0.956; $p<0.0001$) between *E. coli* counts obtained from Colilert and MF tests (Fig. 1) over the range of bacterial counts.

3.3. Two-factor analysis of variance

Results of a two-factor ANOVA on *E. coli* counts by type of test (Colilert and MF) and year of data collection (2001, 2002, and 2003) indicated no significant main effect for type of test ($p=0.305$) and no significant interaction between type of test and year of data collection ($p=0.974$; Fig. 2). There was a significant main effect for sampling year ($p<0.001$) and post-hoc analysis (Tukey multiple comparisons) indicated a significant decrease in average *E. coli* counts from 2001 to 2002 (with no significant difference between counts for 2002

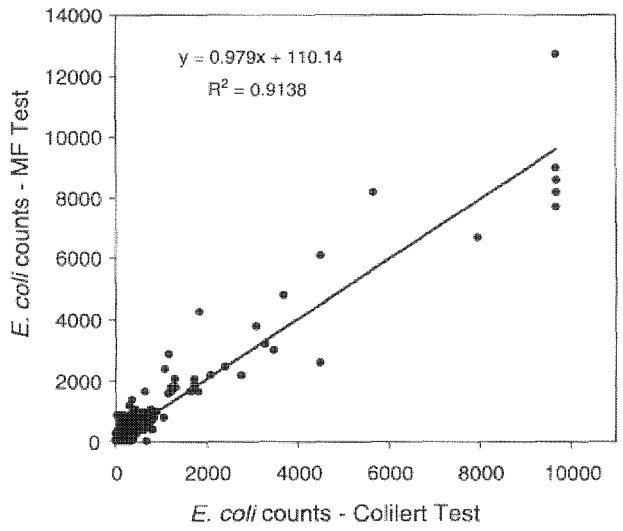

Fig. 1. Significant positive relationship (Pearson's correlation coefficient = 0.956; $p<0.0001$) between *E. coli* counts as measured using the Colilert (x-axis) and MF (y-axis) methods.

and 2003; see Fig. 2). Similarly, results of a two-factor ANOVA on *E. coli* counts by type of test and stream sampled also demonstrated no significant main effect ($p=0.301$) for type of test and no significant interaction ($p=1.0$) between type of test and stream sampled (Fig. 3). A significant main effect for stream ($p<0.001$) indicated that mean *E. coli* counts were different across streams (see Fig. 3). *E. coli* counts obtained by MF and Colilert methods for 'cold months' (Dec., Jan., and Feb.) and "warm months" (June, July, and Aug.) were also compared using a two-factor ANOVA. Again, there was no significant main effect for type of test ($p=0.696$) and no significant interaction ($p=0.741$), suggesting that *E. coli* counts obtained using Colilert and MF methods were not different within warm and cold seasons (see Fig. 4).

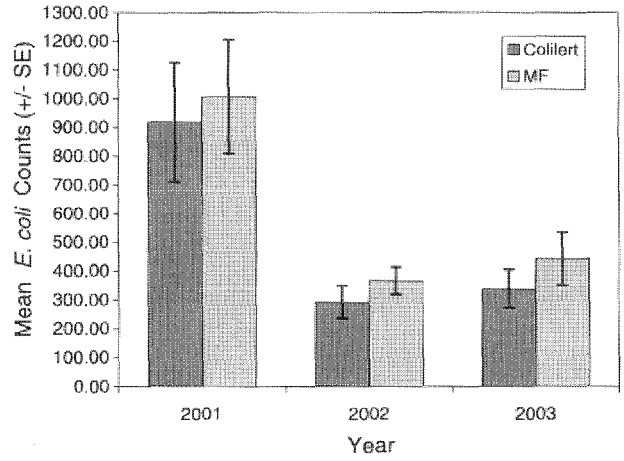

Fig. 2. Mean *E. coli* counts (±SE) per year of data collection with separate bars for Colilert and MF counts. No significant main effect for type of test and no interaction between type of test and year of data collection as indicated by two-factor ANOVA ($p>0.05$) suggest that the two methods were not different regardless of sampling year. A significant main effect for year followed by Tukey multiple comparisons indicated that *E. coli* counts were significantly higher for the first sampling year compared to 2002 and 2003 counts.

A significant main effect for season ($p=0.048$) indicated that *E. coli* counts were significantly lower during colder months than during warmer months (see Fig. 4).

4. Discussion

Virginia's 50,527 free-flowing stream miles have approximately 11,384 miles (22.5%) that are assessed by VA DEQ for at least one designated use parameter (2004). Of those 6938 miles were determined impaired, and 442 out of 494 watersheds contained at least one impaired waterway (VA DEQ, 2004). The leading cause of impairment for designated uses in Virginia rivers and streams is violation of bacterial standards (VA DEQ, 2004). As freshwater outlets bring high amounts of fecal bacteria (fecal coliforms and enterococci) to coastal areas (Noble et al., 2000), the impact becomes magnified.

Fecal contamination of surface waters is considered by many to be a serious health threat in the state of Virginia, and many laboratories are involved in efforts to monitor the problem. Labs certified for fecal coliform testing in Virginia use a standardized MF technique for presumptive analysis (VA DEQ personal communication). While most labs certified for water quality assessment in Virginia are committed to using the MF test protocol, interest is growing in the Colilert method and comparison studies under a wide range of conditions are being conducted (VA DEQ, personal communication).

This study indicates that the ability to measure *E. coli* using Colilert compares favorably to the Virginia-state standard MF for measuring fecal coliforms in natural waters, as long as all parameters of collection, preservation, and testing are constant. Over the 36 months of this study, Colilert DST produced comparable results to MF for measures of bacteria within sampling years, during cold or warm seasons, and within streams exhibiting high (e.g. ANG 17, APP 2, SAY 5, and SAY 6) or low (e.g. SAY 7 and SAY 8) bacterial numbers. At each sampling location, bacterial counts obtained by DST compared favorably with MF with no significant differences found between testing methods.

The sensitivity of the Colilert method to low bacterial numbers is well documented (McFeters et al., 1992; Niemela et al., 2003), and the test is noted for its ability to recover stressed cells in a variety of testing applications (Covert et al., 1992; Eckner, 1998; Jiang et al., 2002; Eccles et al., 2004). Colilert shows a precision to (1 cell/100 ml due to the high sensitivity of MUG substrate to the presence of *E. coli* using the MPN estimate. Reports indicate β-D-glucuronidase is found in greater than 95% of *E. coli* strains (Berger, 1994), is expressed globally among these strains (Venkateswaran et al., 1996), and remains stable at 44.5 °C (Tryland and Fiksdal, 1998). Similarly, Colilert sensitivity was also noted in tests of potable water (Cowburn et al., 1994; Fricker et al., 1997; Niemela et al., 2003). In this study, the Colilert method correlated well with the MF method with samples exhibiting low (<300 bacteria/100 ml) *E. coli* counts (Figs. 1 and 3).

In freshwater samples with chronically high *E. coli* counts (>300 bacteria/100 ml), a 1% MF sample dilution affects

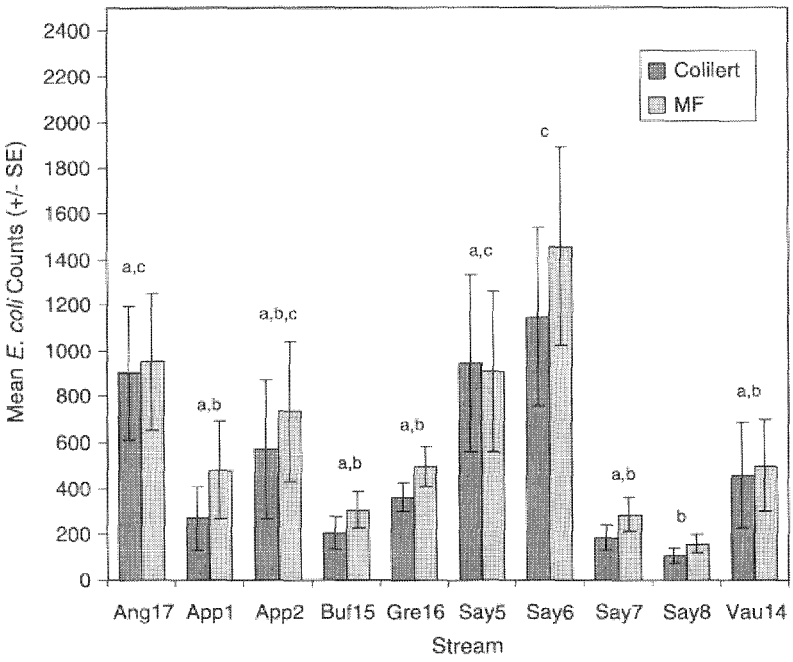

Fig. 3. Mean *E. coli* counts (±SE) per stream with counts from Colilert and MF methods shown separately. No significant main effect for type of test and no significant interaction (as demonstrated by two-factor ANOVA; $p > 0.05$) indicated that *E. coli* counts were not different using the two methods for any of the 10 streams sampled. A significant main effect ($p < 0.05$) for stream suggested differences in *E. coli* counts between streams sampled. Letters above error bars show significant differences among streams as indicated by Tukey multiple comparisons post-hoc analysis.

the degree of sensitivity of the final count to 1 CFU (reported as 100 cells/100 ml). In high-count samples, Colilert produced comparable results to MF with outstanding frequency as illustrated by the Pearson correlation (Fig. 1) and the ANOVA comparisons per individual stream (Fig. 3). Significantly higher *E. coli* counts were obtained during the warm months of the year (Fig. 4) and, during these months, the Colilert DST method compared equally to the MF method.

High-count samples may contain a mix of biologics in addition to fecal bacteria. Attempts to use DST substrates (i.e. ONPG and MUG) for rapid, nearly instantaneous assessments of coliform bacteria and *E. coli* have been compromised by non-coliform heterotrophs (Tryland and Fiksdal, 1998) and by plant and algal enzymes (Davies et al., 1994). Additionally, tests of tropical water sources using standard Colilert DST methods reveal interference reactions from non-coliform bacteria (Pisciotta et al., 2002; Chao et al., 2003). However, the majority of these reports include suggestions to ameliorate such interferences either by modifying the test protocol or by adding confirmatory tests for *E. coli*. Although the capacity of Colilert to accurately measure total coliforms and *E. coli* in tropical waters remains unclear, recent reports indicate that the Colilert DST method is useful in measuring *E. coli* in sewage effluent, sludge, and soils containing a high diversity of organisms and enzymes (Kramer and Liu, 2002; Yakub et al., 2002; Eccles et al., 2004; Muirhead et al., 2004) as well as in environmental freshwaters from temperate regions.

Overall, measures of stream quality improved between the first and subsequent years of the study. Possible reasons for this improvement will not be discussed but could pertain to differences in precipitation, timing of sampling relative to

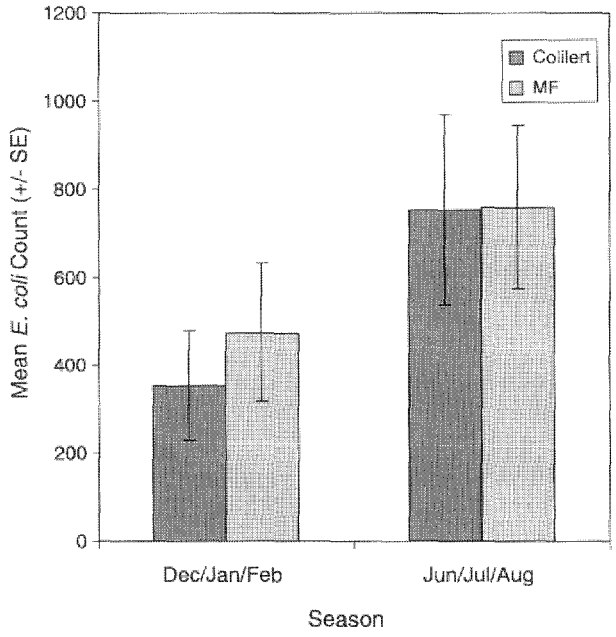

Fig. 4. Mean *E. coli* counts (±SE) per cold season (Dec., Jan., and Feb) and warm season (June, July, Aug.) with counts from Colilert and MF methods shown separately. No significant main effect for type of test and no significant interaction (as demonstrated by two-factor ANOVA; $p > 0.05$) indicated that *E. coli* counts were not different for Colilert and MF methods in cold or warm seasons. A significant main effect for season ($p < 0.05$) indicated greater *E. coli* counts during warm months.

precipitation events, reduction of agricultural activity, implementation of best management practices or combinations of these reasons. Preliminary data obtained prior to 2001 from these sampling locations compared with the higher counts obtained during that year.

5. Conclusions

Colilert DST proves to be a reliable testing method for the determination of *E. coli* when compared with a standard MF method for assessing surface waters of the upper Appomattox River watershed, Virginia. Throughout this long-term study, the Colilert DST method performed well over a range of conditions that included high and low bacterial counts and different sampling seasons. The documented sensitivity of the Colilert method under various stream quality conditions suggests that it is not only comparable to a standard MF method, but that it also exhibits a greater versatility than the MF method due to its ability to precisely quantify high and low bacterial counts.

MF techniques are used for a wide variety of bacterial assessments and have wide applications in bacterial testing protocols. For fecal coliform analyses, Colilert DST screens specifically for *E. coli* so no further confirmatory tests are needed resulting in fewer false positive outcomes. MF tests are labor and materials intensive and require a high degree of technical skill to obtain, interpret, and confirm results. In contrast, the Colilert system is pre-packaged, which removes set-up time and decreases the likelihood of a mistake during prepwork. Additionally, the Colilert system can be used by persons with minimal training and still provide reliable results. Therefore, compared to the MF method, the Colilert DST method overcomes many of the procedural difficulties of MF, allows labs to process samples more quickly, and results in a lower cost per sample.

Colilert DST proved to be a reliable assessment method throughout the entire period of this study. Its performance during warm and cold seasons and its remarkable precision over the range of stream qualities sampled offer a major benefit to the testing laboratory. Given the faster setup and processing time, reduced requirement for glassware and equipment, and lack of need for additional incubations and time for confirmatory testing, Colilert DST is an improved method for assessing *E. coli* contamination for fresh water stream samples.

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