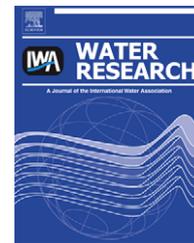


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# Seasonal variation in accurate identification of *Escherichia coli* within a constructed wetland receiving tertiary-treated municipal effluent

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## ABSTRACT

As the reuse of municipal wastewater escalates worldwide as a means to extend increasingly limited water supplies, accurate monitoring of water quality parameters, including *Escherichia coli* (*E. coli*), increases in importance. Chromogenic media are often used for detection of *E. coli* in environmental samples, but the presence of unique levels of organic and inorganic compounds alters reclaimed water chemistry, potentially hindering *E. coli* detection using enzyme-based chromogenic technology. Over seven months, we monitored *E. coli* levels using m-Coli Blue 24<sup>®</sup> broth in a constructed wetland filled with tertiary-treated municipal effluent. No *E. coli* were isolated in the wetland source waters, but *E. coli*, total coliforms, and heterotrophic bacteria increased dramatically within the wetland on all sampling dates, most probably due to fecal inputs from resident wildlife populations. Confirmatory testing of isolates presumptive for *E. coli* revealed a 41% rate of false-positive identification using m-Coli Blue 24<sup>®</sup> broth over seven months. Seasonal differences were evident, as false-positive rates averaged 35% in summer, but rose sharply to 75% in the late fall and winter. Corrected *E. coli* levels were significantly correlated with electrical conductivity, indicating that water chemistry may be controlling bacterial survival within the wetland. This is the first study to report that accuracy of chromogenic media for microbial enumeration in reclaimed water may show strong seasonal differences, and highlights the importance of validation of microbiological results from chromogenic media for accurate analysis of reclaimed water quality.

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## 1. Introduction

There is an increasing trend worldwide to require more efficient use of water resources, both in urban and rural environments. Toward this end, a large number of communities are utilizing reclaimed water, which has the advantage of being a constant and reliable water source. In the United States alone, an estimated 1.7 billion gallons (6.4 million m<sup>3</sup>) per day of wastewater is reused, and reclaimed water use on

a volume basis is growing at an estimated 15 percent per year (US EPA, 2004) demonstrating a growing acceptance of reclaimed wastewater as a clean, safe product.

With the growth in utilization of recycled water for municipal irrigation and other uses that result in human contact with the water product, accurate monitoring of water quality parameters increases in importance. Microbiological guidelines for reclaimed water reuse for irrigation are based on numbers of total coliforms, fecal coliforms and/or *Escherichia coli* (*E. coli*)

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(US EPA, 2004), and chromogenic media are often used for detection of these specific microbes or microbial groups in environmental samples. Though molecular analyses, including polymerase chain reaction (PCR), have been shown to be more sensitive and specific for detection of target microorganisms (Fratamico and Bagi, 2001), chromogenic media allow a better readability of the analysis results, are easier to use for water utility workers, and have the advantage of providing response in a shorter time compared with other methods (Edberg and Kontinick, 1986; Sartory and Howard, 1992).

Since 2002, the United States Environmental Protection Agency (US EPA) has approved 10 enzyme-based total coliform and *E. coli* detection tests for examination of drinking water (Olstadt et al., 2007). These tests are based on the detection of the enzymes  $\beta$ -D galactosidase and  $\beta$ -D glucuronidase which are associated with total coliforms and *E. coli*, respectively. One of these commercially available assays, m-Coli Blue 24<sup>®</sup> broth (Hach Company, Loveland, CO, USA), contains the metabolic dye 2,3,5-triphenyltetrazolium chloride that turns coliform colonies dark red, aiding in observations of total coliform growth. BCIF (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide), a chromogenic enzymatic substrate, is added to the broth for simultaneous detection of *E. coli*. *E. coli* produce  $\beta$ -D glucuronidase, which cleaves the BCIF substrate, releasing a blue chromogen, 5-bromo-4-chloro-3-indolyl, and colonies appear deep purple-blue. Quantification is achieved by counting the appropriately colored colonies.

Despite their increasing use, the use of chromogenic media for detection of total coliforms and *E. coli* in environmental samples can present challenges. Potential problems include the presence of interferents such as humic acids, highly variable and complex sample matrices, and the presence of other confounding dominant native bacterial species (Noble and Weisberg, 2005; Pitkänen et al., 2007; Olstadt et al., 2007). The unique chemistry of reclaimed waters due to the presence of organic and inorganic compounds, including residual pharmaceuticals (Ternes et al., 2002; Drewes, 2004), endocrine disruptors (Lintelmann et al., 2003), nutrients (Magesan et al., 2000) and sodium and other salts (Bond, 1998), can potentially alter the accuracy of chromogenic media. In addition, reagent age has been shown to have a fundamental impact on the potential for obtaining false-positive reactions (Landre et al., 1998). Furthermore, it has been reported that some non-coliform bacteria produce  $\beta$ -D galactosidase (Palmer et al., 1993; Davies et al., 1995; Alonso et al., 1996), and while  $\beta$ -D glucuronidase production has been reported in 94–96% of *E. coli* strains (Manafi, 2000), it has also been found in species other than *E. coli* (Hartman, 1989; Geissler et al., 2000). Although these findings do not diminish the utility of chromogenic media, they do highlight the importance of confirmatory work necessary for accurate reporting of microbiological results.

The growth in use of recycled water worldwide increases the need for investigations of how the unique chemical characteristics of reclaimed water affect enzyme-based detection of *E. coli*. This study reports on *E. coli* detection using m-Coli Blue 24<sup>®</sup> broth in water samples collected over seven months in a constructed wetland receiving tertiary-treated reclaimed water. A suite of biological and chemical analyses were performed on all water samples, both to determine the accuracy of m-Coli Blue 24<sup>®</sup> in quantification of *E. coli* over several

seasons, and to identify water parameters that may alter broth performance.

## 2. Materials and methods

### 2.1. Study location and sample collection

Samples were collected at the Hayfield Tres Rios Demonstration Wetland (TRDW) at the 91st Avenue wastewater treatment plant located in Phoenix, Arizona, USA (33°23'28" N; 112°14'32" W; elevation 297 m). This site is a 1.2 ha constructed wetland filled with treated municipal effluent water that has undergone denitrification prior to release (average inflow 1890 m<sup>3</sup> d<sup>-1</sup>) (Williams and Adamsen, 2008). United States EPA guidelines suggest that reclaimed water used for wetland augmentation undergo secondary treatment prior to release (US EPA, 2004); tertiary-treated TRDW waters exceed this standard. A three-dimensional sampling array allows water sampling from 24 locations, including the wetland inlet and outlet, as well as zones at various depths throughout the wetland, including areas close to the sediment surface. Since its construction in 1995, this wetland has become a haven for local wildlife, a year-round home for a variety of waterfowl, and a temporary residence for migratory avian species (Tres Rios Wetlands, 2008).

Water sampling for microbiological and chemical analyses was performed every 3–4 weeks from July 2005 to February 2006 (total of 10 sampling dates). On sampling dates, a pump was used to pull water from the sampling sites into sterile 50 mL Whirl-pak<sup>®</sup> sample bags with 10 mg sodium thiosulfate (to suppress residual chlorine activity) (Nasco International, Modesto, CA, USA). Following collection of the Whirl-pak<sup>®</sup> sample, additional water from each location was collected in 1.0 L amber glass bottles and immediately analyzed for dissolved oxygen (DO) and temperature using a sensION 156 Portable Conductivity Meter (Hach Company). Water subsamples were also assayed on-site for pH (Corning pH meter with symphony probe, Corning B.V., The Netherlands) and electrical conductivity (EC) (Orion Research Model 101 Conductivity Meter, Thermo Scientific Orion, Waltham, MA, USA). The remainder of the water sample and the Whirl-pak<sup>®</sup> bags were placed on ice and transported to the laboratory, whereupon water subsamples were analyzed for nitrogen (NH<sub>3</sub>) using an Alpkem Automatic Analyzer (O-I Analytical, College Station, TX, USA) and a modified Kjeldahl nitrogen (N) procedure (Standard Methods, 1998).

### 2.2. Microbiological analyses

All water samples were processed for coliform/*E. coli* and heterotrophic plate counts within 6 h of collection. In the laboratory, samples (1.0 and 10.0 mL) were added to sterile 1X phosphate buffered saline (PBS) to a total volume of 25 mL that was then filtered through a sterile gridded membrane (0.45  $\mu$ m pore size) to capture bacteria (Whatman Pl., Kent, UK). The filter was then placed onto an absorbent pad in a 60 mm Petri dish containing 2.0 mL of m-Coli Blue 24<sup>®</sup> broth media. All dilutions were assayed in duplicate, and control plates with filtered PBS only were prepared periodically as well as before and after all samples were filtered to verify that

no cross-contamination occurred between samples. Inverted plates were then incubated at 35 °C for 24 h. Following incubation, colonies on the gridded filter were counted; maroon/red colonies were presumptive for coliforms, and dark blue colonies were presumptive for *E. coli*. After counting, a total of 40 colonies per sampling date (20 presumptive for *E. coli* and 20 presumptive for coliforms) were collected and archived for confirmatory analyses, described below.

Heterotrophic bacteria were cultured using standard spread heterotrophic plate count (HPC) methods (Standard Methods, 1998). A total of 100 µL of each water sample was spread onto R2A non-selective media (Difco, Franklin Lakes, NJ, USA) and incubated for 5 days at 25 °C before counting. All HPC were performed in triplicate.

Confirmatory analysis on colonies presumptive for *E. coli* was performed using EPA Modified Method 1103.1 (US EPA, 2000). This verification procedure consists of four cultivation-dependent confirmatory tests. Generally, *E. coli* isolates would be expected to show negative results for cytochrome oxidase and citrate utilization, and positive results for both gas and indole production. Additional testing on all isolates presumptive for *E. coli* and coliforms was performed by PCR, using primers specific for the *sfmD* gene encoding a putative outer membrane export protein common to all known *E. coli* (Blattner et al., 1997; Kacliková et al., 2005). PCR reactions contained 300 nM of each primer, 12.5 µL Go Taq Green Master Mix (Promega Corp., Madison, WI, USA), 3 µL of the target isolate after regrowth in tryptic soy broth, and sterile water to a total volume of 25 µL. Reactions were performed on an Eppendorf Epigradient Master Cyclor (Eppendorf AG, Hamburg, Germany), using a program consisting of the initial denaturation at 94 °C for 15 min, 30 cycles with a denaturation at 94 °C for 15 s and annealing at 60 °C for 60 s, and a final extension at 72 °C for 8 min. Amplification product was detected by electrophoresis in a 2% agarose gel, stained with SYBR™ Safe DNA Gel Stain (Invitrogen Corp., Carlsbad, CA, USA) and visualized by transillumination by UV light. Presence of a DNA fragment of 106 bp indicated positivity for *E. coli*.

### 2.3. Statistical analyses

Comparisons of *E. coli* abundance and environmental variables within the same sampling date were performed using analysis

of variance (ANOVA). The effects of water quality variables (biological and chemical) on performance of m-Coli Blue® 24 broth (correct identification of *E. coli*) over sampling dates were determined using general linear models with results of confirmatory testing entered into the models as binary (positive = 0, negative = 1) variables. Data collected at the wetland inflow was omitted from all statistical analyses, as the *E. coli* levels in inflow waters were the result of water treatment efficacy only and were not affected by conditions within the wetland. pH data were transformed to hydrogen ion concentrations prior to statistical analysis. All statistical analyses were performed using Minitab Release 14.20 Statistical Software (Minitab Inc., State College, PA, USA). A significance level of  $p = 0.05$  was used for all statistical tests, and values reported are the mean (average) ± standard error of the mean.

## 3. Results and discussion

### 3.1. Water quality and environmental variables

Results of water chemistry parameters monitored at the TRDW over the seven-month sampling period are shown in Table 1. Water EC, though substantially higher than found in unpolluted natural waters (Wood and Dykes, 2002), agreed closely with values reported for recycled wastewater (Al-Nakshabandi et al., 1997; Bonachela et al., 2007). Ammonia (NH<sub>3</sub>) levels in the inflow were far lower than those found in reclaimed waters in Italy (8.6 and 35.3 mg L<sup>-1</sup>) (Aiello et al., 2007; Bonachela et al., 2007, respectively), but this lower N may reflect the treatment plant denitrification process undergone by Tres Rios waters prior to release to the environment. Matamoros et al. (2007) reported NH<sub>3</sub> concentrations averaging 24.1 mg L<sup>-1</sup> in UV/chlorine-disinfected reclaimed waters from several treatment plants in Spain, but these levels fell to 3.6 mg L<sup>-1</sup> from a plant with denitrification treatment. The TRDW water pH was within the range reported for reclaimed municipal wastewater in the southwestern United States (City of Tucson, 2007).

The limited variability in biological or chemical indicators between the water column and sediment surface (Table 1) indicates vertically well-mixed waters within the TRDW. The majority of the chemical and physical water properties was

**Table 1 – Measured biological and chemical properties of waters in the Tres Rios Wetland over seven months of sampling**

|                  | Coliforms<br>(CFU 100 mL <sup>-1</sup> )       | <i>E. coli</i><br>(CFU 100 mL <sup>-1</sup> ) | HPC<br>(CFU 100 mL <sup>-1</sup> )             | DO<br>(%)  | pH        | Temp<br>(°C) | EC<br>(µS cm <sup>-1</sup> ) | NH <sub>3</sub><br>(mg L <sup>-1</sup> ) |
|------------------|--|---|--|------------|-----------|--------------|------------------------------|--|
| Wetland inlet    | 220 (84)                                       | 0 (0)   | 7.1 × 10 <sup>4</sup> (2.3 × 10 <sup>4</sup> ) | 28.1 (1.0) | 7.0 (0.1) | 28.1 (1.8)   | 1777 (31)                    | 0.84 (0.16)                              |
| Water column     | 1.3 × 10 <sup>4</sup> (1.3 × 10 <sup>3</sup> ) | 848 (106)                                     | 4.2 × 10 <sup>6</sup> (3.5 × 10 <sup>5</sup> ) | 48.2 (3.4) | 7.2 (0.1) | 23.2 (0.9)   | 1761 (11)                    | 0.94 (0.06)                              |
| Sediment surface | 1.3 × 10 <sup>4</sup> (1.4 × 10 <sup>3</sup> ) | 808 (141)                                     | 4.1 × 10 <sup>6</sup> (4.4 × 10 <sup>5</sup> ) | 43.8 (3.1) | 7.2 (0.1) | 22.8 (1.0)   | 1781 (13)                    | 1.12 (0.12)                              |
| Wetland outlet   | 1.8 × 10 <sup>4</sup> (4.7 × 10 <sup>3</sup> ) | 580 (194)                                     | 4.7 × 10 <sup>6</sup> (1.1 × 10 <sup>6</sup> ) | 73.3 (9.6) | 7.6 (0.1) | 23.8 (3.0)   | 1748 (40)                    | 0.63 (0.11)                              |

Coliforms and *E. coli* were quantified using m-Coli Blue 24® broth, data shown represent gross bacterial numbers before confirmation. Numbers represent average of all samplings, with standard error of the mean in parentheses.

also stable horizontally (across the wetland) within sampling dates, with the exception of the water DO content, which more than doubled from the wetland inlet to the outlet on all dates (Table 1, column 4). This phenomenon was undoubtedly due to the presence of large algal mats throughout the wetland and the time and conditions of sample collection (always in mid-morning during sunny days). Under strong sunlight, photosynthetic activity of the algae would increase DO concentrations as water flowed through the wetland (Odum, 1956; Edwards and Owens, 1962). Algae also likely contributed to the rise in water pH from the inlet to the outlet of the wetland (Table 1, column 5), as pH increases resulting from hydrogen ion uptake during photosynthesis have long been confirmed in aquatic systems (Jørgensen et al., 1983; Axelsson, 1988).

Seasonal differences were evident in water quality and environmental variables (Table 2), as average water temperature fell and EC rose from the beginning to the end of the sampling period (Table 2, columns 6 and 7). Increased EC was observed only in water samples collected throughout the wetland and at the wetland outlet, while the inlet samples showed no seasonal changes in EC, indicating that precipitation inputs to the wetland from summer monsoon rainfall likely contributed to the sample dilution (lowered EC) found in early samplings. While precipitation totals for the entire sampling period were quite low (74 mm), the majority of rain fell in July and August (70 mm), while no precipitation was recorded in November through February (AZMET, 2007).

### 3.2. Indicator bacteria: relationships to water variables

No *E. coli* were isolated from the reclaimed water in the wetland inflow during the monitoring study. However, total coliforms and *E. coli* (using m-Coli Blue<sup>®</sup> 24 broth) and heterotrophic bacteria all increased dramatically from the wetland inlet to the outlet on all sampling dates (Table 1, columns 1–3). The TRDW provides seasonal and year-round habitat for a variety of waterfowl and other migratory birds, mammals, and amphibians (Tres Rios Wetlands, 2008), all of which have been shown to harbor *E. coli* and other coliform bacteria in their guts (Carr et al., 1976; Duellman and Trueb, 1994; Adeslyun, 1999; Hubálek, 2004). The relatively warm temperatures (Table 2, column 6) and the high nutrient status of the wetland waters (data not shown) might provide habitat for survival of enteric bacteria. Heterotrophic bacteria were present in the inlet waters (Table 1, column 3) but levels were two orders of magnitude higher within the wetland, providing further evidence for

the existence of exogenous bacterial sources within the wetland and/or proliferation of heterotrophs in the warm, nutrient-rich TRDW waters.

Monthly differences in bacterial levels were evident, as both total coliform levels and *E. coli* increased significantly from the summer (July and August) to winter (January and February) samplings (Fig. 1; Table 2). Variability in *E. coli* counts from m-Coli Blue 24<sup>®</sup> broth on each sampling date was correlated to total coliform number ( $r^2 = 0.776$ ;  $p = 0.009$ ) but not to heterotrophic bacteria ( $r^2 = 0.218$ ;  $p = 0.244$ ). Temperature and EC accounted for significant portions of variability in *E. coli* (temperature:  $r^2 = 0.767$ ;  $p = 0.010$ ; and EC:  $r^2 = 0.699$ ;  $p = 0.019$ ). An inverse relationship between *E. coli* and temperature was observed (Fig. 1); this result was surprising, given that *E. coli* survival is promoted by warmer, rather than cooler, temperatures in some systems (Anderson et al., 1983; Chandran and Hatha, 2005). However, recent work has revealed that decreases in water temperature may promote increased survival among *E. coli* exposed to environmental stressors, possibly resulting from temperature-induced changes in fluidity of cell membranes (Sampson et al., 2006; Cebrián et al., 2008), providing an explanatory mechanism for the observed increases in *E. coli* in Tres Rios waters during the cooler months.

### 3.3. Confirmatory testing with culture-dependent methods

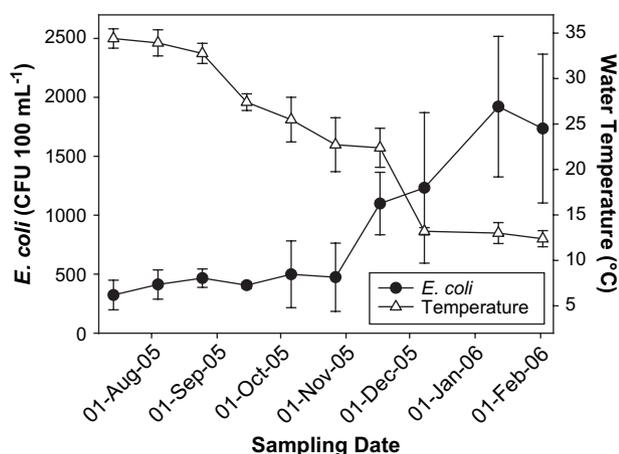
On each sampling date, 20 colonies presumptive for *E. coli* were removed from the m-Coli Blue 24<sup>®</sup> plates and archived for confirmatory analyses. Confirmatory testing using the four-step EPA Modified Method 1103.1 revealed that 55% (110 of 200 colonies) of collected isolates showed results presumptive for typical *E. coli* (Table 3, row 1), indicating a false-positive rate of 45% using the broth medium. Six additional patterns of substrate use were also observed during the confirmatory testing (Table 3). These utilization patterns alone do not provide sufficient information for presumptive identification of the bacteria, though the second most commonly observed pattern (Table 3, row 2) is common to *Enterobacter aerogenes* and *Enterobacter cloacae* (Health Canada, 2002), both of which have been reported as sources of false positives in studies utilizing m-Coli Blue 24<sup>®</sup> broth as a growth medium (Grant, 1997).

Collection date was the only variable significantly ( $r^2 = 0.69$ ;  $p < 0.001$ ) related to the rates of false-positive identification of *E. coli* over the sampling period, a result undoubtedly related to the increasing rates of false positives

**Table 2 – Bi-monthly biological and chemical properties of waters in the Tres Rios Wetland**

|          | Coliforms<br>(CFU 100 mL <sup>-1</sup> )       | <i>E. coli</i><br>(CFU 100 mL <sup>-1</sup> ) | HPC<br>(CFU 100 mL <sup>-1</sup> )             | DO<br>(%)   | pH        | Temp<br>(°C) | EC<br>(μS cm <sup>-1</sup> ) | NH <sub>3</sub><br>(mg L <sup>-1</sup> ) |
|----------|--|---|--|-------------|-----------|--------------|------------------------------|--|
| July–Aug | 1.0 × 10 <sup>4</sup> (1.6 × 10 <sup>3</sup> ) | 370 (90)                                      | 5.8 × 10 <sup>6</sup> (6.8 × 10 <sup>5</sup> ) | 46.6 (15.1) | 7.1 (0.1) | 33.8 (1.6)   | 1707 (9)                     | 1.49 (0.18)                              |
| Sep–Oct  | 1.0 × 10 <sup>4</sup> (1.5 × 10 <sup>3</sup> ) | 459 (98)                                      | 3.2 × 10 <sup>6</sup> (2.9 × 10 <sup>5</sup> ) | 43.3 (11.3) | 7.3 (0.1) | 25.0 (3.1)   | 1759 (13)                    | 1.02 (0.11)                              |
| Nov–Dec  | 1.7 × 10 <sup>4</sup> (1.7 × 10 <sup>3</sup> ) | 1185 (145)                                    | 4.5 × 10 <sup>6</sup> (4.6 × 10 <sup>5</sup> ) | 51.8 (26.7) | 7.2 (0.1) | 16.9 (2.9)   | 1864 (15)                    | 0.70 (0.05)                              |
| Jan–Feb  | 1.8 × 10 <sup>4</sup> (4.0 × 10 <sup>3</sup> ) | 1798 (402)                                    | 4.0 × 10 <sup>6</sup> (9.0 × 10 <sup>5</sup> ) | 43.8 (9.8)  | 7.3 (0.2) | 13.7 (3.0)   | 1895 (40)                    | 1.17 (0.26)                              |

Coliforms and *E. coli* were quantified using m-Coli Blue 24<sup>®</sup> broth, data shown represent gross bacterial numbers before confirmation. Numbers represent average of all samplings, with standard error of the mean in parentheses.



**Fig. 1 – Average E. coli and water temperature across the Tres Rios Wetland over seven-month sampling period. Data points represent average values from 23 sampling locations (excluding the wetland inlet), while error bars represent the standard error of the mean.**

over successive sampling dates. In July and August and in September and October, the rates of false-positive identification of *E. coli* on m-Coli Blue 24<sup>®</sup> broth plates averaged 28% (17 of 60 colonies) and 45% (18 of 40 colonies), respectively. During this time period, none of the measured environmental, biological, or chemical variables showed significant relationships with false-positive identification. On later sampling dates, correct identification of *E. coli* on m-Coli Blue 24<sup>®</sup> broth fell sharply, and rates of false positives averaged 68% (41 of 60 colonies) in November and December and 85% (34 of 40 colonies) in January and February. During the later samplings, false-positive identification of *E. coli* was significantly correlated to the HPC ( $r^2 = 0.22$ ;  $p = 0.043$ ).

The high rate of false-positive identification of *E. coli* (45%) using the chromogenic medium was not wholly unexpected. The target enzyme,  $\beta$ -D glucuronidase, is present in 94–96% of *E. coli* strains (Manafi, 2000), but has also been reported in *Citrobacter* spp. (Gauthier et al., 1991; Geissler et al., 2000) and *Enterobacter* spp. (Geissler et al., 2000), as well as *Salmonella*, *Shigella*, and *Yersinia* spp. (Hartman, 1989), and *Klebsiella*

and *Serratia* spp. (Alonso et al., 1996). The reason for the production of  $\beta$ -D glucuronidase by these organisms is not known, but it has been suggested that the reaction may be plasmid-mediated (Brenner et al., 1993).

**3.4. Confirmatory testing by PCR**

All isolates presumptive for *E. coli* and coliforms (total of 400 colonies) were subjected to PCR reactions oriented to the *sfmD* gene encoding a putative outer membrane export protein of all *E. coli* (Blattner et al., 1997; Kacliková et al., 2005). All of the 110 isolates confirmed to be *E. coli* by the EPA Method (above) also showed the presence of PCR product of the expected size (106 bp) (Fig. 2). In addition, eight isolates that were not identified as *E. coli* using the EPA Method also showed PCR product of 106 bp, reducing the rate of false-positive identification using m-Coli Blue 24<sup>®</sup> broth over the seven-month sampling period to 41%. All of the isolates confirmed by PCR but not by culturing had shown results in three of the four confirmatory tests that were consistent with *E. coli*, but two had shown no indole production (Table 3, row 7), and six showed no gas production (Table 3, row 5). The US Centers for Disease Control reports *E. coli* to be 98% positive for indole, but that rare, indole-negative strains do exist (Manual of Clinical Microbiology, 2003). The lack of observed gas production could have resulted from leaks in the syringes designed to capture the gas produced in the laboratory incubations, but alternatively, these six strains could have been anaerogenic (non-gas producing) *E. coli*. Anaerogenic strains are relatively rare among *E. coli* (comprising <10% of known strains) (Edwards and Ewing, 1986), but have been shown to be culturable using membrane filtration and m-Coli Blue 24<sup>®</sup> broth (Grant, 1997).

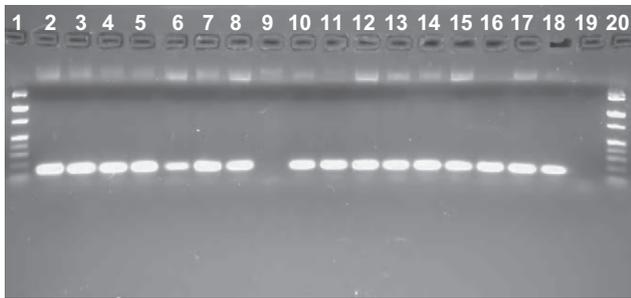
The 200 isolates presumptive for coliforms were also analyzed by PCR. Of these, eight isolates showed a PCR product of 106 bp, indicating a rate of false negatives of 4%. False negative reactions on m-Coli Blue 24<sup>®</sup> broth may be caused by *E. coli* (including most *E. coli* O157 strains) that do not express the  $\beta$ -D glucuronidase enzyme (Martins et al., 1993).

When the *E. coli* levels determined using m-Coli Blue 24<sup>®</sup> broth were corrected for the false-positive rates on each sampling date, the strong seasonal trends observed in the uncorrected data were no longer evident, with only limited

**Table 3 – Results of four-step culture-dependent confirmatory testing for E. coli (US EPA, 2000)**

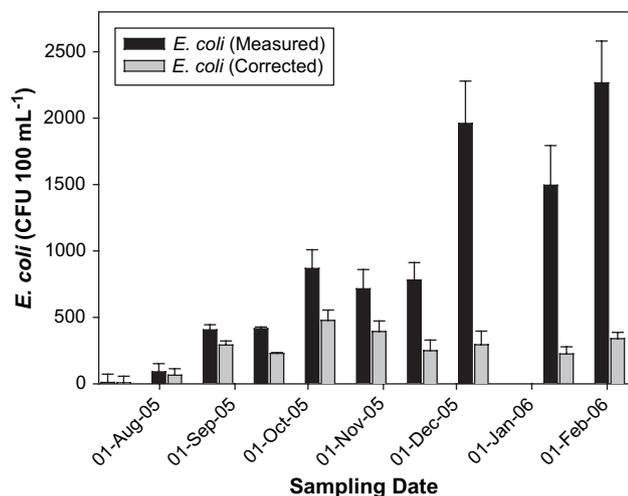
| Cytochrome oxidase | Gas production | Citrate utilization | Indole production | Isolates         |                  |
|--------------------|----------------|---------------------|-------------------|------------------|------------------|
|                    |                |                     |                   | Number           | Percent of total |
| -                  | +              | -                   | +                 | 110 <sup>a</sup> | 55.0             |
| +                  | -              | +                   | -                 | 27               | 13.5             |
| -                  | -              | +                   | -                 | 26               | 13.0             |
| -                  | +              | +                   | +                 | 19               | 9.5              |
| -                  | -              | -                   | +                 | 12               | 6.0              |
| +                  | +              | -                   | +                 | 4                | 2.0              |
| -                  | +              | -                   | -                 | 2                | 1.0              |
| Totals             |                |                     |                   | 200              | 100.0            |

Symbols refer to positive (+) and negative (-) test results.  
 a Isolates show expected results for typical *E. coli*.



**Fig. 2 – Results of confirmatory PCR of selected *E. coli* isolates from Tres Rios Wetland. Lanes 1 and 20: 50 bp standard molecular weight ladder; Lane 2: positive control strain (ATCC #25922); Lanes 3–8, 10–18: positive *E. coli* isolates showing PCR-generated fragment of 106 bp; Lane 9: negative *E. coli* strain; Lane 19: no template (water only) control.**

month-to-month variability (Fig. 3). Corrected data revealed that water leaving the wetland had the highest *E. coli* levels in mid- to late-autumn, a period when the wetlands offer a haven for migrating waterfowl (Tres Rios Wetlands, 2008). Unlike the uncorrected data, *E. coli* counts in the TRDW were no longer correlated with total coliforms ( $r^2 = 0.076$ ;  $p = 0.372$ ) or with temperature ( $r^2 = 0.078$ ;  $p = 0.358$ ), but EC still accounted for small, but significant portions of variability in *E. coli* levels ( $r^2 = 0.298$ ;  $p < 0.001$ ). EC is a complex phenomenon, affected not only by the concentrations of total dissolved ions in water but also by their mobility and valence (Standard Methods, 1998) and thus, identifying mechanisms for the *E. coli*/EC correlation would be beyond the limits of the collected data. However, several studies report that bacterial survival in water is enhanced by ion addition at very low concentrations (<25 ppt), while cell death increases measurably at higher salt concentrations



**Fig. 3 – Measured and corrected *E. coli* levels in the outflow of the Tres Rios Wetland over seven-month sampling period. Data represent the average of duplicate assays run on outflow samples on each sampling date, and error bars represent the standard error of the mean.**

(Kaspar and Tamplin, 1993; Heller et al., 1998). Therefore, it is not wholly unexpected that *E. coli* levels and EC in TRDW waters would show significant correlations.

### 3.5. Factors affecting accuracy of m-Coli Blue 24<sup>®</sup> broth

Although the accuracy of m-Coli Blue<sup>®</sup> 24 broth in identification of *E. coli* has been shown to be high (94%) in spiked samples of drinking water (Bernasconi et al., 2006), the performance of this chromogenic medium with environmental samples has been less stellar. In a study designed to contrast the performance of 10 enzyme-based sampling methods in detecting *E. coli* in natural waters, the percent failure rate was highest (23%) in m-Coli Blue 24<sup>®</sup> broth (Olstadt et al., 2007). Olstadt et al. (2007) reported that the performance of m-Coli Blue 24<sup>®</sup> was most problematic in groundwater samples with low pH and low alkalinity, and speculated that accurate water testing might require a media formulation with greater buffering capacity. In addition, the authors felt that high levels of background heterotrophic bacteria ( $4.2 \times 10^6$  CFU 100 mL<sup>-1</sup>) may have contributed to the poor performance of the medium (Olstadt et al., 2007). Pitkänen et al. (2007) also speculated that background heterotrophic growth hindered the performance of different chromogenic media.

Although the HPCs were significantly correlated with false positives on the m-Coli Blue 24<sup>®</sup> plates only during later sampling dates, HPCs in the Tres Rios water in July through October were not significantly different from those measured in November through February (Table 2, column 3). Nonetheless, over the seven-month sampling period, HPC levels in the Tres Rios waters agreed closely with those observed to be problematic by Olstadt et al. (2007), indicating that the high background flora could have hindered the performance of m-Coli Blue 24<sup>®</sup> broth through the length of the present study. It is plausible that seasonal changes in water temperature or chemistry may have promoted the growth of a  $\beta$ -D-glucuronidase-producing bacterial species other than *E. coli*, increasing the false-positive rate in winter months.

This is the first study in which a strong seasonal shift in false-positive rates has been observed while using a chromogenic medium to quantify *E. coli*. Additional investigations, with increased measurement of water quality parameters, are needed to identify the seasonal changes in reclaimed water chemistry and/or biology that affect detection of *E. coli* when using enzyme-based technology. Because the observed monthly and seasonal trends in *E. coli* levels changed radically upon correction following confirmatory work, this study presents a strong example of the need for confirmatory analysis of microbiological data arising from chromogenic media. Barring confirmation, bacterial analyses of reclaimed water based on selective isolation and culturing techniques should be interpreted with caution.

## 4. Conclusions

Many water microbiology testing methods have been developed for use on relatively clean samples, but testing of reclaimed water samples may present challenges due to the presence of potential interferents to specific methodological

approaches. In the TRDW, measurements over seven months using m-Coli Blue 24<sup>®</sup> broth revealed increasing levels of *E. coli* over time, with highest levels in mid-winter (January). When data were corrected for the false positives, *E. coli* levels fell sharply, with the highest numbers in mid-autumn when the wetland is in high usage by migratory waterfowl. This work, showing radically different results following confirmatory analysis of initial microbiological findings, highlights the extreme importance of validation of data from chromogenic media for accurate analysis of reclaimed water quality. Rates of *E. coli* mis-identification using m-Coli Blue 24<sup>®</sup> broth rose steadily through the sampling period. Furthermore, the corrected *E. coli* numbers were significantly correlated to water EC. Together, these findings suggest that seasonal shifts in the as yet unidentified chemical quality of the reclaimed water entering the TRDW exert some control over *E. coli* survival and proliferation.

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