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Jenifer Turco Ph.D.
Valdosta State University, jturco@valdosta.edu

James A. Nienow
Valdosta State University, jnienow@valdosta.edu

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ENUMERATION AND IDENTIFICATION OF SELECTED BACTERIA IN WATER SAMPLES FROM THE ALAPAHOOCHEE RIVER SYSTEM

Jenifer Turco* and James A. Nienow
Department of Biology
Valdosta State University
1500 N. Patterson St.
Valdosta, GA 31698-0015
* Corresponding author: jturco@valdosta.edu

ABSTRACT

Escherichia coli and enterococci were enumerated in water collected bi-weekly for one year at ten stations in the Alapahoochee River system. The concentrations of E. coli were usually below a standard of 576 colony forming units (cfu) per 100 ml, except after heavy rain. At five stations the concentrations of enterococci were generally above a standard of 151 cfu/100 ml. Concentrations of both groups showed a direct but weak correlation with pH. For quality control, 772 colonies typical of E. coli and 773 colonies typical of enterococcus were tested by the appropriate verification procedures. Sixty-six (8.5%) of the former, and 57 (7.4%) of the latter, appeared to be false positives. Further study of 51 apparent false positive E. coli isolates showed that only 15.7% did not contain E. coli, indicating an actual false positive rate of about 1.3%. Further study of 18 false positive enterococcus isolates showed that 16.7% were Enterococcus species and 83.3% were Streptococcus species. Thus the actual false positive rate was estimated to be 6.2%.

Keywords: Escherichia coli, enterococci, bacteria, fecal coliforms, indicator, water quality, verification, false positive, pH, Alapahoochee River, Georgia

INTRODUCTION

The Alapahoochee River, a major tributary of the Alapaha River in southern Georgia, is formed from the confluence of Mud Creek and Grand Bay Creek on the border of Echols and Lowndes counties. Parts of this system have been listed as impaired by Georgia’s Environmental Protection Division (1, 2). As part of a larger project aimed at mitigating aquatic pollution in the watershed, the water quality of the Alapahoochee system was evaluated during a one year period beginning in May 2005 (3). The basic physicochemical properties of the water as well as biological and chemical indicators of water pollution were studied (3). Here we report on some aspects of the bacteriological portion of the study.
Currently the State of Georgia classifies its surface waters according to their designated use as follows: drinking water supplies, recreation, fishing, wild river, scenic river, and coastal fishing (4, 5). Most rivers and streams, including those in the Alapahoochee watershed, are designated as fishing. According to State guidelines, water quality is monitored using fecal coliform concentrations as the standard, with fishing streams listed as impaired if the geometric mean of four water samples in a thirty day period exceeds 200 colony forming units (cfu) per 100 ml between May and October or 1,000 cfu/100 ml between November and April; or if a single water sample exceeds 4,000 cfu/100 ml between November and April (4, 5).

In a 1976 publication, the United States Environmental Protection Agency (EPA) recommended using fecal coliforms (which can grow at 44.5° C; produce acid and gas during lactose fermentation; and include Escherichia coli) as indicators of fecal contamination, and suggested that a geometric mean of 200 cfu/100 ml not be exceeded in fresh water that is used for swimming (6). The EPA later estimated that this geometric mean concentration of fecal coliforms in fresh water would be associated with an illness rate of approximately 8 per 1,000 swimmers (7). In the mid-1980s, however, the EPA published documents indicating that the concentrations of E. coli and enterococci in fresh water at bathing beaches, but not the concentrations of fecal coliforms, were directly correlated with the occurrence of gastrointestinal illness in swimmers (7, 8). Therefore the EPA has recommended E. coli and/or enterococci (rather than fecal coliforms) as indicator bacteria for evaluating the quality of fresh water (7, 9).

In 1986, the EPA published specific criteria based on concentrations of E. coli and enterococci for evaluating fresh water that is used for swimming or other activities in which body immersion is considered likely; water used in these ways is referred to as primary contact recreational water (7, 9). These 1986 criteria were developed to protect swimmers from developing gastrointestinal illness as a result of ingesting contaminated water (7, 9). For primary contact recreational fresh water, the EPA has suggested using E. coli and/or enterococcus criteria corresponding to an illness rate of no more than 14 per 1,000 swimmers, and has recommended using criteria corresponding to an illness rate of 8 per 1,000 swimmers for fresh water that is heavily used for swimming (9). Two types of bacterial criteria are generally recommended for evaluating water quality: (i) the geometric mean of the concentrations of the indicator bacteria in several water samples and (ii) a single water sample maximum allowable concentration (SSM) of the indicator bacteria (7, 9). The EPA proposed different SSM criteria depending on the frequency of primary contact recreation. For infrequently used water, a SSM criterion based on the 95% confidence interval for the appropriate geometric mean can be applied; but for heavily used water, the confidence interval is reduced to 75% (7, 9).

Water used for activities in which body immersion is unlikely (e.g., fishing) is considered secondary contact recreational water. Although the EPA has not published bacterial standards specific for secondary contact recreational
water, one of its publications notes that “...states must adopt primary contact recreation wherever attainable for all surface waters within their jurisdiction...” (9). The EPA has also suggested possible approaches for evaluating the quality of secondary contact recreational water when the bacterial criteria for primary contact recreation cannot be met. Such approaches include using a criterion that is five times that used for primary contact recreational waters (9). Several issues have been raised concerning the EPA’s 1986 bacterial criteria for evaluating water quality (10). For example, many States have questioned whether these criteria can be appropriately applied to all waters in the United States (10).

In the present study, the concentrations of E. coli and enterococcus bacteria in aseptically collected water samples were determined using membrane filtration methods approved by the EPA (11, 12). According to the published 1986 EPA criteria (7, 9), the concentrations of both groups of bacteria indicate some degree of fecal contamination at each station, with higher levels of contamination in the Mud Creek–Alapahoochee River segment of the system. However, because the source of the contamination was not readily apparent we looked for intrinsic properties of the water which might be related to the increased concentrations in this segment. In addition, as part of our quality assurance/quality control procedures, 772 positive colonies in the E. coli method and 773 positive colonies in the enterococcus method were tested using the appropriate verification procedures (11, 12). Because the percentages of false positive isolates were higher than expected, we wanted to determine if the standard verification procedures were adequate for our water samples. Therefore, we used commercial identification systems to identify some of the apparent false positive isolates.

**MATERIALS AND METHODS**

**Collection of water samples and measurement of pH.**

Water samples were collected bi-weekly at four locations in Grand Bay Creek (GB1, GB2, GB3, and GB4), three locations in Mud Creek (MC1, MC2, and MC3), and three locations in the Alapahoochee River (AR1, AR2, and AR3) beginning on May 20, 2005 (Figure 1; see reference 3). GB1 and MC1 are located near the headwaters of Grand Bay Creek and Mud Creek, respectively. MC2 is located on the downstream edge of the Azalea City Industrial Park in Valdosta. MC3 is downstream of the Mud Creek Water Pollution Control Plant, which has a design capacity of 3.22 million gallons per day, and the confluence of Knights Creek with Mud Creek. The other stations are located in rural Lowndes, Lanier, and Echols counties. The protocol for collecting the water samples has been described previously (3). In brief, ~800 ml samples were collected aseptically in sterile polypropylene bottles away from the banks of the stream. Samples were kept on ice until processing; filtration of the samples in the laboratory was completed within 6 hours of the collection time. Beginning on September 23, 2005, water pH was measured in the field using a battery-operated Checker pH meter.
(Hanna Instruments, Woonsocket, RI). The last date for water collection and pH measurement was May 22, 2006.

**Figure 1.** The Alapahoochee watershed. GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River. Source: TIGER/United States Census Bureau.

**Enumeration, verification, and additional testing of E. coli.**

Concentrations of *E. coli* were determined according to previously described procedures (3, 11). Briefly, three different sample volumes (selected at half-log intervals from 0.1, 0.3, 1.0, 3.0, 30.0, and 100.0 ml) were filtered in duplicate for each sample site. Each volume was filtered through a gridded membrane (pore size, 0.45 m), and the membrane was placed onto modified mTEC agar and incubated at 35°C for two hours. Then each plate was sealed in a plastic Whirl-pak bag and incubated in a water bath at 44.5°C for 22 hours. The magenta-colored colonies were counted with the aid of a dissecting microscope, and the number of colony forming units (cfu) in 100 ml of water was calculated. Modified mTEC agar contains sodium...
lauryl sulfate and sodium deoxycholate to inhibit gram positive bacteria. Gram negative bacteria that are \(\beta\)-D-glucuronidase positive (including most \(E.\ coli\) bacteria) form magenta-colored colonies on this medium due to the presence of 5-bromo-6-chloro-3-indolyl-\(\beta\)-D-glucuronide, which is broken down by \(\beta\)-D-glucuronidase to yield a colored compound (11, 13). The concentrations of \(E.\ coli\) in the water were compared to the EPA’s 1986 bacterial criteria for evaluating fresh water (7, 9). For the geometric means, values corresponding to illness rates of 8 per 1,000 and 14 per 1,000 swimmers were used. For the SSM criteria, values corresponding to the 95% confidence intervals for the respective geometric means were used (7, 9).

For each sampling day, three isolated magenta colonies from each site were used for verification. Each colony was used to inoculate a nutrient agar slant and a tube of trypticase soy broth. After incubation of these cultures overnight at 35°C, a portion of the slant culture was used for the cytochrome oxidase test. The broth culture was used to inoculate the following: (i) a tube of EC broth, which was incubated at 44.5°C for 24 hours and (ii) a Simmons citrate agar slant and a tube of tryptone broth (for the indole test), which were both incubated at 35°C for 48 hours. A colony was verified as \(E.\ coli\) if the bacteria were cytochrome oxidase negative, produced gas in EC broth when incubated as described, did not utilize citrate, and were indole positive.

Fifty-one apparent false-positive \(E.\ coli\) isolates were studied in more detail. Each isolate was streaked for isolation onto MacConkey agar and incubated at 35°C for one to two days. Isolated colonies were then subcultured onto a MacConkey agar plate and a nutrient agar slant, and identified using the Enterotube II system (BD Diagnostics, Sparks, MD), according to the manufacturer’s specifications.

**Enumeration, verification, and additional testing of enterococci.**

Concentrations of enterococci were determined according to previously described procedures (3, 12). The filtration procedure itself was similar to that described in the preceding section for monitoring \(E.\ coli\). In the enterococcus procedure, however, each membrane was placed onto a plate of mL agar and incubated at 41°C for 24 hours. Colonies with blue halos were counted with the aid of a dissecting microscope and the number of cfu in 100 ml of water was calculated. mL agar contains nalidixic acid, sodium azide, triphenyl tetrazolium chloride, and cycloheximide to inhibit the growth of other microorganisms (12, 14, 15). Bacteria that are \(\beta\)-glucosidase positive (including most enterococcus bacteria) form colonies with a blue halo on mL agar due to the presence of indoxyl-\(\beta\)-D glucoside, which is broken down to yield a colored compound (12, 14). The concentrations of enterococci in the water were compared to the EPA’s 1986 bacterial criteria for evaluating fresh water (7, 9), as described for \(E.\ coli\).

For each sampling day, three isolated colonies with blue halos from each site were used for verification. Each colony was used to inoculate a brain heart infusion agar slant and a tube of brain heart infusion broth. After incubation
of these cultures overnight at 35°C, the broth culture was used to inoculate the following: (i) a tube of brain heart infusion broth, which was incubated at 45°C for 48 hours, (ii) a tube of brain heart infusion broth plus 6.5% NaCl, which was incubated at 35°C for 48 hours, and (iii) a plate or slant of bile esculin agar, which was incubated at 35°C for 48 hours. The original brain heart infusion agar slant culture was gram stained after incubation for 24-48 hours. A colony was verified as enterococcus if it contained gram positive cocci, grew in brain heart infusion broth at 45°C, grew in brain heart infusion broth plus 6.5% NaCl at 35°C, and hydrolyzed esculin as evidenced by formation of a black precipitate on bile esculin agar.

Eighteen apparent false positive enterococcus isolates were evaluated in additional tests. Each isolate was streaked for isolation onto brain heart infusion agar and incubated for one to two days at 37°C. Isolated colonies were subcultured onto brain heart infusion agar slants, gram stained, tested for catalase, inoculated onto mEI agar, and tested again for ability to grow in brain heart infusion broth at 45°C and ability to grow at 35°C in brain heart infusion broth plus 6.5% NaCl. Each isolate was then identified using the Biolog MicroLog System, release 4.2 (Biolog, Inc., Hayward, CA), as specified by the manufacturer. Briefly, each isolate was grown overnight on Biolog Universal Growth agar plus 5% sheep blood at 37°C in a humidified atmosphere that contained 6.5% carbon dioxide. Then a suspension of the isolate was prepared in the appropriate inoculating fluid and adjusted for turbidity as recommended by the manufacturer. One hundred fifty microliters was inoculated into each well of a GP2 MicroPlate, and the plate was incubated at 37°C in a humidified atmosphere that contained 6.5% carbon dioxide. The plates were read manually after 4-6 hours of incubation and again after 16-24 hours of incubation.

RESULTS AND DISCUSSION

Enumeration of *E. coli* and enterococci.

On most sampling days, the concentrations of *E. coli* in the water samples were below or close to 576 cfu/100 ml (Figure 2). This concentration represents the 95% confidence limit for a geometric mean associated with an illness rate of 8 per 1,000 swimmers; the 95% confidence limit can be applied to fresh water that is seldom used for swimming (7, 9). Two prominent exceptions occurred during the study period, on June 3, 2005, and on April 9, 2006, when the concentrations of *E. coli* exceeded 1,000 cfu/100 ml at all stations, with the exception of station MC1 on April 9, 2006. On June 3, 2005, the concentrations of *E. coli* were ≥ 4,000 cfu/100 ml in the water from all stations. In addition, on October 7, 2005, the concentration of *E. coli* exceeded 2,000 cfu/100 ml in water from station MC2. Heavy rain occurred within the 24 hour period prior to sample collection on each of these days, and may explain these higher concentrations of *E. coli*. Since the 95% confidence limit for a geometric mean associated with an illness rate of 14 per 1,000 swimmers (7, 9) is 2,507 cfu/100 ml, clearly all stations exceeded this
value at least once. This concentration of 2,507 cfu/100 ml represents the highest concentration suggested by the EPA for primary contact recreational fresh water that is seldom used for swimming (7, 9).

Figure 2. *Escherichia coli* concentrations in water collected from various stations during the study period. GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River.

The geometric means of the concentrations of *E. coli* in water from station MC1 and all stations in Grand Bay Creek over the entire study period were less than 126 cfu/100 ml (Table I), which corresponds to an illness rate of 8 per 1,000 swimmers (7, 9). In contrast, the geometric means of the concentrations of *E. coli* in water from stations MC2, MC3, and the Alapahoochee River stations exceeded this standard, but were less than 548 cfu/100 ml (Table I), which corresponds to an illness rate of 14 per 1,000 swimmers (7, 9). Thus, when one considers the *E. coli* criteria corresponding to an illness rate of 14 per 1,000 swimmers in fresh water that is seldom used for primary contact recreation (as suggested by the EPA), all stations exceeded the single sample maximum allowable concentration, but no station exceeded the geometric mean criterion. Because the thermotolerant *E. coli* bacteria form a subset of the fecal coliform group, it is likely that the geometric means for the concentrations of fecal coliforms would have been higher than the geometric means for the observed concentrations of *E. coli* in this study. Between May and October, the geometric means for the concentrations of *E. coli* were above 200 cfu/100 ml for water from stations MC2, MC3, and all of the Alapahoochee River stations (data not
shown); these data suggest that the water at these stations would have been evaluated as impaired according to the fecal coliform standard used by the State of Georgia. Our findings are consistent with those of the Environmental Protection Division of the Department of Natural Resources of the State of Georgia, which has determined that portions of Mud Creek and the Alapahoochee River are impaired (1, 2).

**Table I.** Water pH, geometric means of bacterial concentrations and calculated corresponding illness rates during the study period for all stations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Station&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GB1</td>
</tr>
<tr>
<td>E. coli Geometric mean&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85</td>
</tr>
<tr>
<td>Illness rate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.4</td>
</tr>
<tr>
<td>Enterococci Geometric mean&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73</td>
</tr>
<tr>
<td>Illness rate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.2</td>
</tr>
<tr>
<td>pH Standard mean</td>
<td>4.4</td>
</tr>
<tr>
<td>Minimum</td>
<td>3.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>5.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River.

<sup>b</sup>Geometric means are given as cfu/100 ml.

<sup>c</sup>The corresponding illness rates per 1,000 swimmers were calculated as previously described (7, 9).

The concentrations of enterococci in the water samples exhibited some different trends, as well as some similar trends, when compared with the concentrations of *E. coli* (Figure 3). For example, at sites MC2 and MC3 of Mud Creek, and at all three sites in the Alapahoochee River, the concentrations of enterococci were generally higher than the concentrations of *E. coli*, and well above 151 cfu/100 ml, the 95% confidence level for the geometric mean corresponding to an illness rate of 8 per 1,000 swimmers (7, 9). Furthermore, the concentrations of enterococci at these stations sometimes exceeded the standard of 656 cfu/100 ml, the 95% confidence level for the geometric mean corresponding to an illness rate of 14 per 1,000 swimmers. This concentration is the highest suggested by the EPA for primary contact

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recreational fresh water that is seldom used for swimming (7, 9). Although the concentrations of enterococci were usually lower in water from the remaining stations, water from all stations exceeded the standard of 656 cfu/100 ml at least once (Figure 3).

![Graph](image)

**Figure 3.** Enterococcus concentrations in water collected from various stations during the study period. GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River.

As was the case with *E. coli*, some of the highest concentrations of enterococci were detected in water collected within 24 hours after heavy rain. However, increases in the concentrations of enterococci did not always parallel the observed rainfall-associated increases in the concentrations of *E. coli* (Figures 2 and 3). Water from all sites on Mud Creek and the Alapahoochee River had elevated concentrations of enterococci above 4,000 cfu/100 ml on June 3, 2005; and water from the remaining sites, with the exception of GB1, had concentrations of enterococci above 1,000 cfu/100 ml on this date. On April 9, 2006, another rain day, water from stations MC2, MC3, and all Alapahoochee River stations, had increased concentrations of enterococci above 2,500 cfu/100 ml. Of the remaining stations, only stations GB1, GB2, and GB4 had enterococcus concentrations above 151 cfu/100 ml; the concentrations at these stations ranged from 173-264 cfu/100 ml. On the third rain day, October 7, 2005, water from station GB4 had a concentration of 255 cfu/100 ml; water from stations GB1 and MC1 had concentrations of enterococci above 656 cfu/100 ml (which were higher...
than normal); and water from stations MC2, MC3, and all Alapahoochee River stations had concentrations of enterococci above 1,500 cfu/100 ml. The remaining stations (GB2 and GB3) had concentrations of enterococci less than 151 cfu/100 ml. These data suggest that rainfall influences the concentrations of enterococci and E. coli differently (3). In addition, water from stations AR1 and AR2 had elevated concentrations of enterococci, more than 1,500 cfu/100 ml, on October 21, 2005; and water from stations MC3 and AR3 had concentrations of 734 and 701 cfu/100 ml, respectively; the reason for these increases is unknown. From late November to January, the concentrations of enterococci in the water from stations GB1 and GB2 exceeded 1,000 cfu/100 ml at least twice. These increases during the winter months appeared to correlate with an increase in the percentage of enterococcus isolates that failed to meet the criteria for verification, and may reflect changes in the types of bacteria present during this time. Thirteen (43.3%) of the 30 isolates from December 16, 2005 failed to meet the criteria for verification as enterococci; and 49 (86.0%) of the 57 total apparent false positive enterococcus isolates for the entire year were obtained from the end of November to the beginning of March (3).

For the one-year period during which samples were collected, the geometric means for the concentrations of enterococci in water from all stations were above 33 cfu/100 ml (Table I), which corresponds to an illness rate of 8 per 1,000 swimmers (7, 9). However, water from station MC1 and all stations in Grand Bay Creek had geometric means lower than 144 cfu/100 ml (Table I), which corresponds to an illness rate of 14 per 1,000 swimmers (7, 9). In contrast, water from stations MC2 and MC3, as well as all stations in the Alapahoochee River, had geometric means that were 2.9- to 3.9-fold higher than the 144 cfu/100 ml standard; these values correspond to illness rates ranging from 18.4 to 19.5 per 1,000 swimmers (Table I). Interestingly, although the geometric means for the concentrations of enterococci and E. coli were similar for station MC1 (43 and 40 cfu/100 ml, respectively), the geometric means for the concentrations of enterococci at stations MC2 and MC3 were (respectively) 9.8-fold and 13.0-fold higher, compared with only 4.4-fold and 7.4-fold higher geometric means (respectively) for the concentrations of E. coli (Table I).

If one uses enterococcus concentrations corresponding to an illness rate of 14 per 1,000 swimmers as the criterion for impairment, as suggested by the EPA for fresh water that is seldom used for primary contact recreation (9), all stations exceeded the single sample maximum allowable concentration; and stations MC2, MC3, and all stations in the Alapahoochee River exceeded the geometric mean criterion as well. Thus the enterococcus studies suggest a higher level of fecal contamination in the Alapahoochee system than do the E. coli studies. However, the results with both types of bacteria indicate that the water from stations MC2, MC3, and all stations in the Alapahoochee River is lower in quality than the water from the remaining stations.
Verification and additional testing of *E. coli*.

Verification tests were performed on 772 colonies typical of *E. coli* (3 colonies per station for each sampling day), and 66 of these (8.5%) appeared to be false positives since they did not meet one or more of the four criteria for verification (cytochrome oxidase negative, gas production in EC broth, indole positive, citrate negative). Fifty-one of these apparent false positives, as well as two isolates that were verified as *E. coli* (controls), were studied further. As expected, the two control isolates yielded a single colony type on MacConkey agar, produced magenta colonies on mTEC agar, and were identified as *E. coli* using the Enterotube II system.

Sixteen (31.4%) of the 51 tested, apparent false positive isolates yielded a single colony type on MacConkey agar, produced magenta colonies on mTEC agar, and were identified as *E. coli* using the Enterotube II system. Each of these 16 had originally failed to meet only one of the criteria for verification: one isolate appeared to be cytochrome oxidase positive, 11 did not produce gas in EC broth, three were indole negative, and one appeared to be citrate positive. Retesting showed that the cytochrome oxidase positive isolate was actually negative for cytochrome oxidase, and that the citrate positive culture was actually citrate negative.

Twenty-seven (52.9%) of the 51 tested, apparent false positive isolates yielded at least two types of colonies on MacConkey agar. *E. coli* bacteria that produced magenta colonies on mTEC agar were identified from each of these 27 isolates using the Enterotube II system. Twenty of these 27 isolates (which had originally tested citrate positive) also contained another type of citrate positive bacteria that produced white colonies on mTEC agar. Six additional isolates contained another type of bacteria that produced magenta colonies on mTEC agar, and the remaining isolate contained two additional types of bacteria: one producing magenta colonies on mTEC agar and the other producing white colonies on mTEC agar. Bacteria that yielded white colonies on mTEC agar were identified as *Enterobacter* (three isolates), *Klebsiella* (three isolates), *Citrobacter* (two isolates), *Serratia* (one isolate), *Salmonella* (one isolate), or could not be identified with certainty using the Enterotube II system (11 isolates). Non-*E. coli* bacteria that produced magenta colonies on mTEC agar included *Enterobacter* (one isolate), *Salmonella* (one isolate), and bacteria that could not be identified with certainty using the Enterotube II system (five isolates).

None of the remaining eight (15.7%) of the 51 tested, apparent false positive isolates contained *E. coli*, as determined by the Enterotube II system. All of these eight isolates had initially failed two or more of the *E. coli* verification tests. Six of these were pure cultures that produced magenta colonies on mTEC agar; four were identified as *Salmonella* and the other two could not be identified using the Enterotube II system. The remaining two of these eight cultures that did not contain *E. coli* were mixed. One contained *Salmonella* that produced white colonies on mTEC agar and *Serratia* that produced magenta colonies on mTEC agar. The other contained *Salmonella*
that produced magenta colonies on mTEC agar and an unidentified bacterium that produced white colonies on mTEC agar.

When one considers all 51 tested, apparent false positive isolates, *E. coli* was found in 43 (84.3%) of them. Therefore, a reasonable estimate of the false positive rate in this study is 1.3% (15.7% of 8.5%). This value is consistent with previously published estimates (11).

Two factors caused initial overestimation of the percentage of apparent false positive *E. coli* isolates in this study. First, there were some difficulties associated with picking the colonies. For example, white, mucoid, spreading colonies sometimes grew on the plates and complicated the process of picking well-isolated colonies for verification. The detection of more than one type of bacteria in 29 (56.9%) of the 51 tested, apparent false positive isolates most likely reflects these difficulties. In future studies, streaking the picked colonies for isolation before conducting the verification tests would eliminate this problem, and permit a more accurate estimation of the percentage of false positives.

The second factor that contributed to initial overestimation of the percentage of apparent false positives was the verification procedure used. If one considers only the 22 tested, apparent false positive isolates that yielded pure cultures, 16 (72.7%) were identified as *E. coli* using the Enterotube II system. These 16 cultures had failed only one of the original criteria for verification. The remaining six pure cultures that were not identified as *E. coli* had each failed two or three of the verification criteria. It seems likely that a more accurate estimation of the percentage of false positives would result if one routinely identified those isolates that failed only one of the criteria for verification using a commercial system such as the Enterotube II. When one considers only these 22 isolates that yielded pure cultures, one can estimate the false positive rate in this study to be about 2.3% (27.3% of 8.5%). This rate is about twice as high as previously published estimates (11).

**Verification and additional testing of enterococci.**

Verification tests were performed on 773 colonies typical of enterococcus (3 colonies per station for each sampling day), and 57 (7.4%) appeared to be false positives since they did not meet one or more of the criteria for verification (gram positive cocci, growth at 35° in brain heart infusion broth plus 6.5% NaCl, hydrolysis of esculin, and growth in brain heart infusion broth at 45°C). All 57 isolates hydrolyzed esculin, and 56 of them contained only gram positive cocci. Fifty-five of these isolates failed to grow in brain heart infusion broth plus 6.5% NaCl, and three of these 55 also failed to grow in brain heart infusion broth at 45°C. There was one isolate that met all criteria for verification except growth at 45°C, and one additional isolate was a mixed culture. Eighteen of these apparent false positives, as well as two isolates that were verified as enterococci (controls), were studied further. As expected, the two control isolates were identified as *Enterococcus* species using the Biolog MicroLog System.
The 18 apparent false positive isolates chosen for study had initially met all verification criteria except for growth at 35°C in brain heart infusion broth plus 6.5% NaCl. Seventeen of these 18 isolates were cultured from water collected between late November and early March. As mentioned previously, it was during this period that 86% of the apparent false positive enterococcus isolates were obtained. All 18 isolates were pure cultures of gram positive cocci, produced blue colonies on mEL agar, and grew in brain heart infusion broth at 45°C. Surprisingly, three isolates grew in brain heart infusion broth plus 6.5% NaCl when they were re-tested; whereas the remaining 15 isolates did not. All isolates were catalase negative. Of the three isolates that grew in brain heart infusion broth plus 6.5% NaCl when they were retested, two were identified as *E. faecalis*; and the third isolate was *Enterococcus mundtii*. Why these three *Enterococcus* isolates failed to grow in brain heart infusion broth plus 6.5% NaCl when they were initially tested is not clear. The remaining fifteen isolates were members of the genus *Streptococcus*. *S. gallolyticus* was identified most frequently (seven isolates). This species has been isolated from the feces of various animals, including cows, horses, and pigs (16). Three isolates were *S. uberis*, a species that has been isolated from the feces of cows as well as various environmental sources (17). One isolate was *S. mutans/ratti* and the other four *Streptococcus* isolates could not be identified beyond the genus level. Thus 15 (83.3%) of the 18 isolates were truly false positives; therefore, the false positive rate for enterococcus in this study can be estimated as 6.2% (83.3% of 7.4%), a rate consistent with that reported by other researchers (12). In the present study, the standard verification procedure for enterococcus provided a more realistic estimate of the rate of false positives than did the standard verification procedure for *E. coli*.

**Relationship between concentrations of bacteria and water pH.**

Because we noticed that the concentrations of enterococci seemed to be lower in water from stations GB3, GB4, and MC1, we wondered if some intrinsic factor(s) in the water might account for the lower concentrations of enterococci at these stations. Measurements showed that water from these stations, as well as the other stations in Grand Bay Creek, generally had a lower pH than water from the remaining stations (Table I). Therefore, we examined the possible relationship between bacterial concentrations and the pH of the water by regressing bacterial counts on measured pH values. In general, there was a significant relationship between the concentrations of both enterococci and *E. coli* and the pH of the water; this relationship appears stronger when the data from samples collected within 24 hours after heavy rain are omitted (Figures 4 and 5). The regression equations for *E. coli* and enterococci are given by

\[
E. \text{ coli concentration} = -175 + 64.5 \times \text{pH}
\]

\[
\text{Enterococcus concentration} = -376 + 120.5 \times \text{pH}
\]

In both cases, the regression slopes are highly significant (p < 0.001), suggesting that the pH of the water or some factor correlated with pH, directly
influences the concentrations of these two groups of bacteria. If pH is indeed responsible, it is possible that circumneutral discharges from the Azalea City Industrial Park and the Mud Creek Water Pollution Control Plant, perhaps in concert with freshwater springs derived from the underlying limestone karst formations, increase the pH of this segment of the system (3). The increased pH could then enhance the survival, and perhaps growth, of *E. coli* and enterococci in the water, leading to higher concentrations of both groups downstream. Previous studies have demonstrated that the lower boundary for the growth of *E. coli* in culture is between pH 3.8 and 4.0 (18). Our own preliminary experiments have demonstrated an effect of pH on enterococci isolated from the Alapahoochee system. Survival of these enterococci in buffered water at pH values ≤ 5 was impaired during a five-day period, and their growth in brain heart infusion broth was inhibited at pH values ≤ 4 (19, unpublished data). These data are consistent with the results of other researchers who showed that the growth of *Enterococcus faecalis* strain AT1 is inhibited at a pH of 4.7, and that this organism is killed by incubation at a pH of 2.5 for three hours (20). Likewise, Nakajo et al. (21) found that the growth of *E. faecalis* JCM8728 is inhibited at pH values ≤ 4. The concentrations of bacteria discharged from the Mud Creek Water Pollution Control Plant, which had a geometric mean equal to 3.1 fecal coliforms per 100 ml during 2003 (2), are unlikely to be high enough to account for the concentrations of bacteria observed downstream of the treatment plant. It should be noted that the $R^2$ values for both pH regressions are low, 0.32 for *E. coli* and 0.17 for enterococci, indicating that pH is not the sole factor influencing the concentrations of bacteria.

![Figure 4](http://digitalcommons.gaacademy.org/gjs/vol65/iss2/8)  
*Figure 4*. Relationship between *Escherichia coli* concentrations and pH of the water in the absence of rain. The predicted values were calculated from the following regression equation: *E. coli* concentration = $-175 + 64.4 \times \text{pH}$. GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River.
Figure 5. Relationship between enterococcus concentrations and pH of the water in the absence of rain. The predicted values were calculated from the following regression equation: Enterococcus concentration = -367 + 120.5*pH. GB1-GB4, stations on Grand Bay Creek; MC1-MC3, stations on Mud Creek; AR1-AR3, stations on the Alapahoochee River.

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REFERENCES


