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Detection of *Stx*2 Gene of *Escherichia coli* and Elevated Levels of Fecal Bacteria in the Cattle Farming Regions of Lake Oconee

Dave S. Bachoon*, Munis M. Lukman, Christopher D. Burt
Department of Biological and Environmental Sciences, Georgia College and State University, Milledgeville, USA
Email: *dave.bachoon@gcsu.edu

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ABSTRACT

The presence of Total coliform, *Eschericha coli* and enterococci were enumerated in the cattle farming areas of the Oconee Watershed using colilert™ and enterolert™ IDEXX plates, respectively. Microbial Source Tracking (MST) using *Bacteroidales* molecular markers for ruminant (RuBac) and human (HuBac) specific bacterial groups were used to determine the source of the fecal pollution in the watershed. In the cattle farming regions of the watershed higher levels of fecal bacteria were detected compared to the levels of fecal bacteria at the forested and residential sites. MST indicated that the cattle farming regions (except DC2) of the lake was impacted by fecal pollution from a ruminant source such as cattle. In addition, qPCR for the *tuf* gene of *E. coli* and the *stx*2 gene that is commonly found in enterohemorragic *E. coli* O157:H7 were used to evaluate the presence of these bacteria in the study area. *E. coli* O157:H7 (*stx*2 gene) was detected only in the beef cattle regions of the watershed. The presences of *E. coli* and *stx*2 gene in the Oconee Watershed represent a potential public health risk because Lake Oconee and its tributaries are used for recreational activities as well as crop irrigation.

Keywords: Fecal Bacteria; Oconee Watershed; qPCR; *Eschericha coli*; *Tuf* Gene; Bacteroidale

1. Introduction

The significance of Shiga-toxin producing *Escherichia coli* (STEC) as a public health concern was recognized in 1982 during an investigation into an outbreak of hemorrhagic colitis in the United States. This outbreak was caused by consumption of poorly cooked ground beef in a fast-food restaurant chain in the western US From 1982 to 2002, a total of 350 *E. coli* O157:H7 outbreaks were reported in 49 US states, from which 325 outbreaks included a number of deaths [1]. Although the Shiga-toxin gene is found in all enterohemorrhagic *E. coli* (STEC), serotype O157:H7 alone is responsible for more than 73,000 cases of disease per year in the US, and it has been implicated in 250 deaths [1,2]; Cattle and other ruminants are natural reservoirs of *E. coli* O157:H7 that can introduce pathogenic *E. coli* into the environment through fecal shedding [3-6]. Estimates of occurrence vary, but it appears that both dairy herds and beef feedlots can have animals carrying STEC including *E. coli* O157:H7 [6,7].

Recent studies have demonstrated that the Lake Oconee Watershed is impacted by fecal pollution from urban development and cattle farming operations [8]. *Burt et al.* 2011 used Microbial Source Tracking (MST) markers to trace the source of *E. coli* in the Lake Oconee Watershed to bovine and human sources, and determined that the highest concentrations of *E. coli* in surface water were found in the cattle farming regions of the lake. Consequently, there is a high probability that Lake Oconee could be contaminated with STEC including *E. coli* O157:H7, and this could represent a serious public health risk when water from the lake is used for recreation or crop irrigation. Microbiological studies have found that waterborne transmission of STEC such as *E. coli* O157:H7 can occur through swimming, crop irrigation or from drinking contaminated water [7,9]. Moreover, the infectious dose of *E. coli* O157:H7 in humans is quite low [7]. Unfortunately, previous studies have not assessed the presence of STEC or *E. coli* O157:H7 in the Oconee Watershed.

Traditionally, enterohemorragic *E. coli* strains were detected from water samples using time consuming culture based enrichment methods [10,11]. Today, quantitative polymerase chain reaction (qPCR) methods have emerged as another option to rapidly detect the presence of STEC in environmental samples without the need for

*Corresponding author.

isolating and culturing the bacterium [7,12]. Quantitative PCR assays for the *tuf* gene of shigella-like *E. coli* strains has been used to quantify *E. coli* in environmental samples [13]. While the shiga-like toxin genes (*stx*1 and *stx*) that are phage encoded and is present in enterohemorrhagic *E. coli* strains were used to indicate the presence of shiga-toxin producing *E. coli* strains [12]. These qPCR assays provide rapid and cost effective means of detecting and quantifying the presence of *E. coli* and the potential of shiga-toxin producing *E. coli* strains in the Oconee Watershed.

For this study, our aims were to investigate the parameters of surface waters in the Oconee Watershed that are indicative of the presence of STEC, and use MST approach with *Bacteoridales* markers for human (HuBac) and cattle (RuBac) specific bacterial groups to track a potential source of STEC in the Lake Oconee watershed [14-16]. In addition, physiochemical parameters and the level of fecal indicator bacteria at each site were determined as an index of water quality.

2. Material and Methods

2.1. Study Sites

Cattle fecal contamination in the Upper Oconee River Basin was established using three main tributaries of Lake Oconee: the Oconee River, Apalachee River, and Sugar Creek (**Figure 1**). The Oconee River Basin covers 5326 square miles of Central Georgia, and falls within the Level 3 Piedmont and Southeastern Plains Ecoregion. The Upper Oconee Basin is made up of the Oconee River, Apalachee River, Indian Creek, and Murder Creek sub-

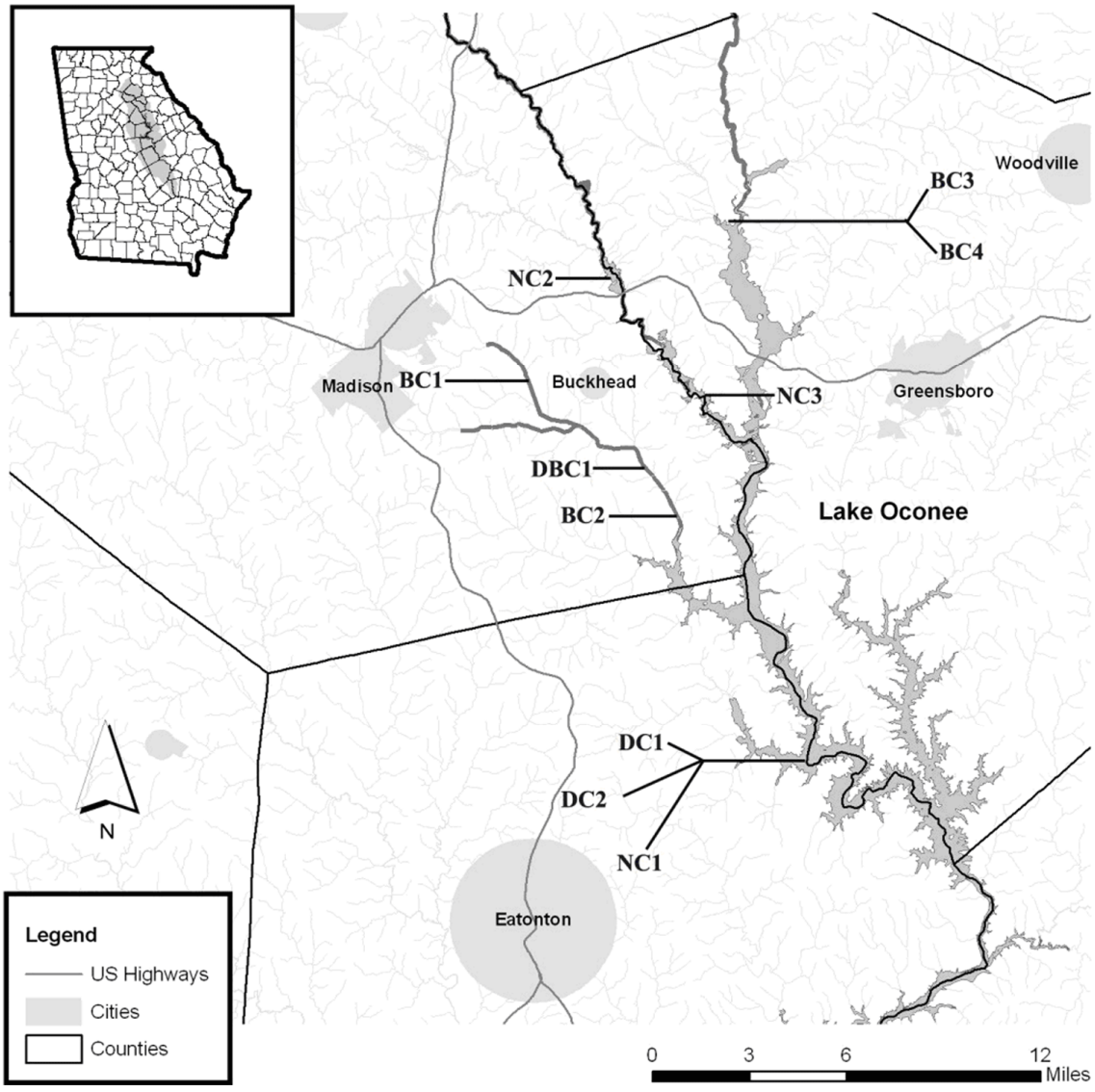

Figure 1. Map of Upper Oconee watershed with sampling sites labeled. DC = Presence of dairy cattle; BC = Presence of beef cattle; DBC = Presence of both beef and dairy cattle; NC2, NC3 = Forested; NC1 = Modern suburban.

watersheds which meet at Lake Sinclair. The Oconee River flows south from Athens and joins Greenbriar Creek just east of Lake Oconee in Greene County. Beef cattle farms at Dyar Pasture (BC3 and BC4) are on the Oconee River. BC3 allows cattle direct access to the river while BC4 has riparian buffers and fencing prohibiting cattle from entering surface waters. The Apalachee River watershed forms the western boundary of National Forest lands in Greene County, GA. US 278/GA 12 intersects this watershed in north Greene County (NC2) while Swords Boat Ramp (NC3) is located at the southern end of the river. No cattle are present at these sampling sites. Sugar Creek supplies the central portion of Lake Oconee, and is intersected by Seven Island Rd (BC1) in Morgan County. At site BC1 there is a grazing heard of 25 - 50 beef cattle, and the cattle are restricted from the creek using riparian buffers with fencing. Cattle activity has broken the fencing at BC1 allowing cattle to enter the creek. Mt Zion Rd (BC2) in Putnam County also intersects Sugar Creek, and was used as a sampling site due to its close proximity to a beef cattle operation. Three sampling sites, DC1, DC2, and NC1 were on the shoreline of Lake Oconee; DC1 is characterized as a site with fencing completely excluding cattle from the lake while DC2 is 100 m downwind from DC1, and is influenced by a creek that receives runoff from the cattle operation flowing through riparian area before it enters the lake. NC1 is characterized as being a modern suburban site with no cattle activity.

2.2. Sample Collection and Physical Parameters

Water samples were collected in April 2010, from the top 25 cm of surface waters and stored on ice for <24 hrs before processing. Physical parameters were measured on site during each sampling event. All physical parameters (water temperature (˚C), total dissolved solids, turbidity (NTU), pH, and dissolved oxygen (mg/L)) were measured based on manufacturer's protocol using a Horiba U-52G meter (Kyota, Japan).

2.3. Biological Parameters

The geometric means of enterococci, total coliform, and *E. coli* were determined at each site. The IDEXX Quanti-Tray Sealer 2X (Westbrook, ME, USA) was used for enumeration of total coliform, *E. coli*, and enterococci. The reagents Colilert-18 and Enterolert were used following the manufacturer's protocol for the quantification of each respective bacterium and bacterial numbers were based on the table provided by the manufacturer.

2.4. DNA Extraction from Environmental Samples

DNA was extracted from cattle and sewage fecal samples following the procedure of the MoBio Ultraclean™ Soil DNA Kit. Cattle fecal samples were collected from dairy and beef cattle farms. Filters of water samples were processed with the MoBio Ultraclean™ Soil DNA Kit (Carlsbad, CA) using a modification of the "Alternative Protocol" given by the manufacturer [17,18]. In brief, this involved separating the bead solution from the beads and placing it in a 15-mL centrifuge tube containing the filter. Solutions S1 and IRS were placed in the tube and vortexed vigorously for 15 min. The solution was removed from the centrifuge tube and placed in the bead tube. From this point on the manufacturer's protocol was followed. Extracted DNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE).

2.5. qPCR Setup and Controls

Lyophilized *E. coli* O157:H7 genomic DNA from the European Commission: Institute for Reference Materials and Measurements IRMM-449 and *E. coli* strain B genomic DNA Sigma® D4889 were used as positive controls in *tuf* gene qPCR assays. Only *E. coli* O157:H7 IRMM-449 was used as a positive control for *stx* gene assay. In both instances, *Bifidobacterium adolescentis* genomic DNA ATCC® number 15703D™ was used as a negative control. Extracted DNA from cow manure and sewage were used as positive environmental controls for source tracking of ruminant and human *Bacteroides* respectively.

Following extraction of environmental samples, DNA was amplified using the Bio-Rad CFX96 (Hercules, CA). Samples were run using optimized qPCR assays [12,13, 15,16]. Each qPCR contained a 25 µL volume with 12.5 µL of iQ™ SYBR® Green 2 X Supermix, 9.5 µL deionized H_2O, 250 nM of primer (**Table 1**), and 1 µL (~10 ng) of template DNA.

Total *E. coli* detection using the *tuf* gene as a target was performed under the following conditions: initial denaturing at 95˚C for 5 min; 40 cycles of 95˚C for 30 s, 60˚C for 30 s, and 72˚C for 30 s; a final elongation step at 72˚C was omitted in each protocol as it has been deemed unnecessary. The *stx* gene primers (**Table 1**) were used for the detection of STEC using the following conditions: initial denaturing at 95˚C for 5 min; 45 cycles of 95˚C for 15 s, 54˚C for 10 s, and 72˚C for 15 s. Serial dilutions of *E. coli* O157:H7 DNA was used to generate all standard curves from 2.97×10^6 gene copies to 2.97×10^1 gene copies. Environmental samples were assessed for possible PCR inhibition by amending with Bifidobacteria DNA as described by *Bachoon et al.* 010. Changes of less than two C_T value were observed, which indicates that the extracted DNA did not contain impurities that significantly inhibited the PCR (Bachoon et al. 2010). The detection limit for STEC was 1.50×10^2 gene copies per 100 mL of water sample.

2.6. Microbial Source Tracking

The source of the fecal contaminant was tracked using ruminant (RuBac) and human *Bacteroides* (HuBac) specific primers (**Table 1**). The SYBR Green qPCR conditions for detection of ruminant *Bacteroidales* were 95˚C for 10 min; 40 cycles of 95˚C for 15 s and 60˚C for 1 min. Conditions for HuBac were: 95˚C for 10 min; 40 cycles of 95˚C for 30 s and 60˚C for 45 s. Primers for HuBac and RuBac were tested for cross-reaction to insure specificity for targets. Distinctive T_m curves for HuBac and RuBac PCR assays are shown in **Figure 2**.

3. Results

3.1. Physiochemical Parameters

A total of 10 sites representing three major tributaries of Lake Oconee, as well as sites on the lake itself, were sampled for one month (n = 50). Seven sites were located at cattle farms and three sites were from regions of the lake without cattle farms (**Figure 1**, **Table 2**). The physiochemical parameters DO, pH and temperature were similar at all the study sites, and turbidity was higher at the cattle farming sites (~55 NTU) compared to the cattle free regions (~36 NTU) of the watershed (**Table 2**). These parameters are typical for surface waters in the Lake Oconee region [8,19]. Surface water from dairy and beef cattle farming areas exhibited similar physical parameters throughout the course of the study. Dissolved oxygen averaged 10.40 mg/L, pH averaged 7.02 with values ranging from 6.56 to 7.74, and turbidity displayed an average of 37.81 NTU. Sites DC1 and DBC1 displayed higher turbidity levels throughout sampling, 55.20 ± 17.17 NTU and 53.12 ± 31.84 NTU respectively. Temperatures for all sites ranged from 10.45˚C to 22.71˚C, with an average temperature of 16.79˚C (**Table 2**).

3.2. Fecal Indicator Bacterial Enumeration

Enterococci, total coliform, and *E. coli* were enumerated, in duplicate, five times in April and their geometric

Table 1. Target gene primer sets for the detection of *E. coli, stx*2 and source tracking of ruminant and human fecal bacteria.

Target	Primer	Sequence (5'-3')	Annealing Temp. (˚C)	Product Size (bp)	Reference
tuf	TEco1553	TGGGAAGCGAAAATCCTG	60	258	Maheux *et al.* 2009
	TEco1754	CAGTACAGGTAGACTTCTG			
*stx*2	JMS2F	CGACCCCTCTTGAACATA	54	108	Jothikumar & Griffiths 2002
	JMS2R	GATAGACATCAAGCCCTCGT			
Ruminant *Bacteroidales*	BacR_f	GCGTATCCAACCTTCCCG	60	100	Reischer *et al.* 2006
	BacR_r	CATCCCCATCCGTTACCG			
Human	HuBac566f	GGGTTTAAAGGGAGCGTAGG	60	116	Layton *et al.* 2006
Bacteroidales	HuBac692r	CTACACCACGAATTCCGCCT			

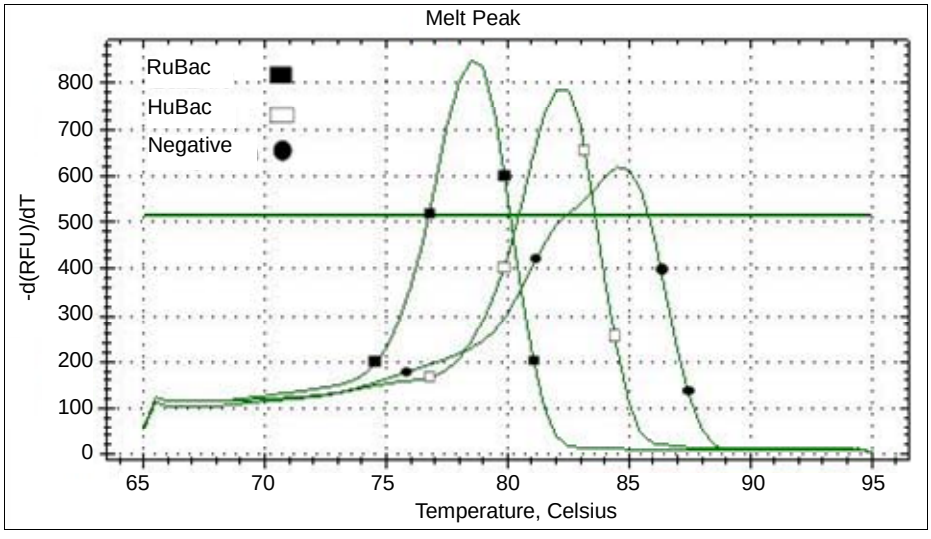

Figure 2. T_m curve profiles of SYBR Green qPCR assay. T_m was 78.1˚C for RuBac and 82.5˚C for HuBac. A no template negative control peaked at 85˚C.

Table 2. Measurements of dissolved oxygen (DO) concentration (mg/L), pH, turbidity (NTU), temperature (˚C), and total dissolved solids (mg/L) at each site. All values displayed are means ± standard deviation.

Site	DO	pH	NTU	Temp	TDS
DC1 (Dairy)	9.11 ± 0.46	7.08 ± 0.22	55.20 ± 17.17	19.24 ± 1.85	0.06 ± 0.010
DC2 (Dairy)	9.30 ± 1.03	7.01 ± 0.24	37.10 ± 12.29	19.32 ± 1.22	0.04 ± 0.005
DBC1 (Dairy/Beef)	10.14 ± 0.71	6.95 ± 0.08	53.12 ± 31.84	16.23 ± 2.87	0.03 ± 0.003
BC1 (Beef)*	10.55 ± 1.67	7.05 ± 0.30	26.16 ± 11.29	18.97 ± 2.13	0.03 ± 0.008
BC2 (Beef)	11.48 ± 1.17	7.11 ± 0.44	29.24 ± 11.29	14.26 ± 2.93	0.05 ± 0.004
BC3 (Beef)*	10.86 ± 1.53	6.91 ± 0.42	36.90 ± 22.78	14.31 ± 3.46	0.05 ± 0.007
BC4 (Beef)	10.82 ± 1.29	6.97 ± 0.39	28.28 ± 23.05	15.22 ± 2.48	0.05 ± 0.004
NC1 (Forested)	10.92 ± 2.16	7.11 ± 0.28	34.46 ± 9.42	17.56 ± 5.82	0.04 ± 0.003
NC2 (Forested	10.55 ± 0.62	7.09 ± 0.21	41.16 ± 14.10	16.32 ± 2.01	0.05 ± 0.003
NC3 (Suburban)	10.31 ± 1.88	6.97 ± 0.25	36.52 ± 7.74	16.48 ± 1.79	0.04 ± 0.006

*Sites where cattle had direct access to surface water.

means were taken to dampen effects of a single sampling event. Cattle free sites exhibited the lowest levels of fecal contamination in all instances. NC3 displayed the lowest levels of *E. coli* and enteroccoci (45.8 MPN/100mL and 11.9 MPN/100mL respectively), while the modern suburban site at NC1 displayed the lowest coliform numbers (588.2 MPN/100mL). Highest levels of coliform bacteria were found at DC1 (4208.2 MPN/100mL), which was a site with fencing only. However, cattle farms with direct access (BC3) and riparian buffered sites with fencing (DBC1) had similar numbers of fecal coliform (2878.1 MPN/100mL and 2685.6 MPN/100mL). DBC1 also displayed the second highest totals of enterococci and *E. coli* (261.7 MPN/100mL and 713.2 MPN/100mL). The highest levels of both enterococci and *E. coli* (382.2 MPN/100mL and 1152.2 MPN/100mL) were measured at another site (BC1) where cattle had direct access to the water.

3.3. qPCR Assessment of *E. coli* and *Stx*

Slurries of fecal samples from beef cattle farms and the dairy cattle farm were assayed for the presence of *E. coli and* STEC, and indicated that *E. coli* and *stx* gene were present in both types of fecal samples (data not shown). The greatest amount of *E. coli* 8.13×10^3 gene copies per 100 mL were detected at BC1 (7.68×10^3), BC2 (1.93×10^3) and BC 6.1×10^3 (**Table 3**). *Stx* was detected only at beef cattle sites (BC1 to BC4). Forested and urban areas of the watershed did not have detectable levels of *stx* (**Table 3**).

3.4. Microbial Source Tracking

Microbial Source Tracking using putative DNA markers for human (HuBac) and ruminants (RuBac) were used to identify the major source of fecal pollution in the study sites of the lake (**Table 4**, **Figure 2**). All dairy and beef cattle sites, except DC2, displayed the marker for ruminant contamination, while cattle free sites did not (**Figure 2**). The HuBac marker for human fecal pollution was detected at BC2, BC3, BC4, and DBC1 and two cattle free sites NC2 and suburban site NC3.

Table 3. Total gene copies of *E. coli* and *stx*2 per sampling site. Values displayed are means ± standard deviation. Percentages displayed are based on mean values.

Site	Total *E. coli* Gene Copies (10^3)	*stx*2 Gene Copies (10^3)
DC1	1.15 ± 2.33	0
DC2	3.15 ± 2.98	0
DBC1	4.05 ± 2.37	0
BC1	8.13 ± 9.13	0.61 ± 0.339
BC2	7.68 ± 1.04	1.93 ± 2.91
BC3	7.71 ± 1.48	6.10 ± 16.2
BC4	4.50 ± 2.44	2.10 ± 1.75
NC1	1.21 ± 2.21	0
NC2	1.71 ± 4.74	0
NC3	1.43 ± 0.975	0

4. Discussion

In 2007, the State of Georgia identified seventy-two stream segments in the Oconee River Basin as water quality limited due to elevated fecal coliform levels [20]. Research on the sources of fecal pollution in the Oconee River Basin is very limited, but poultry and cattle opera-

Table 4. Molecular Source Tracking of *Bacteroidales* putative markers of ruminant and human fecal bacteria detected by qPCR.

Site	Ruminant (RuBac)	Human (HuBac)
DC1	+	−
DC2	−	−
DBC1	+	+
BC1	+	−
BC2	+	+
BC3	+	+
BC4	+	+
NC1	−	−
NC2	−	+
NC3	−	+

Figure 2, shows SYBR Green assay detection peaks for RuBac and HuBac.

tions along with urban development have been suggested as major sources of fecal bacteria in the Oconee Watershed [8,19,21]. Whenever there is a high incident of cattle fecal contamination in an environment there will be an increase in the likelihood of detecting STEC including *E. coli* O157:H7 in that area because cattle are a natural reservoir of these pathogenic bacteria [6,22]. Therefore, in this study we focused our efforts on the detection of *stx* as a potential indicator of STEC in the cattle farming areas of the watershed.

Ongoing research in our lab monitors fecal pollution in the Oconee Watershed. Not surprising the highest levels of fecal indicator bacteria were detected in the cattle farming regions of the watershed. At the beef cattle site BC1, cattle have direct access to surface water which subsequently led to the highest geometric means of *E. coli* and enterococci (**Table 5**). This agrees with previous research that demonstrated that high level of fecal bacteria were present in the cattle this farming region of the Oconee Watershed [8,19]. Most of the areas where cattle were present, except for the cattle farming site BC3, exceeded the state regulatory standard for *E. coli* levels of 126 CFU/100mL in recreational waters [22]. In contrast, relatively low levels of fecal bacteria including *E. coli* were detected in the forested and urban areas of the watershed (**Table 3**). Low levels of fecal pollution are typical for forested and modern suburban regions of the Oconee Watershed [8,19].

DNA was extracted from 0.2 um nucleopore filters of water samples and used for qPCR SYBR Green assays of *E. coli* and *stx* at each study site (**Table 3**). We assumed that the filters would recover bacteria cells and allow the majority of any free bacteriophage to pass through and be discarded. Using qPCR, the highest levels of *E. coli* were detected in the cattle farming regions of the watershed. This agrees with the IDEXX enumeration levels of *E. coli* in the watershed (**Table 5**) and studies that have recovered 10^2 to 10^8 CFU *E. coli* O157:H7 per gram in feces and fresh manure [23-25]. It has been documented that the presence of *E. coli* O157:H7 can be determined in a sample based on PCR detection of one of six virulence genes including Shiga toxin 2 [26,27]. However studies have indicated that the *stx* gene can be detected in free bacteriophage from sewage samples and therefore detection of the *stx* gene does not always confirm the presence of STEC in environmental samples [9]. Among the sites, only areas influenced by beef cattle showed presence of *stx* and therefore these sites would be a likely source of shiga-toxin producing *E. coli* strains. It was unexpected that *stx* was not detected at the dairy farming sites because *stx*2 was detected in fecal samples from both dairy and beef cattle farms used in this study (data not shown), and previous research has indicated STEC is shed from beef and dairy cattle [7,27]. However, it has been suggested that depending on their diet beef cattle may shed greater quantities of STEC compared to dairy cattle [3]. Overall qPCR detected approximately 50% less *E. coli* at the dairy farm sites compared to the beef cattle sites, and IDEXX enumeration of *E. coli* also indicated that there were lower levels of *E. coli* at dairy cattle sites (DC1 and DC2). In addition, qPCR indicated that a large proportion of total *E. coli* detected at the beef cattle site BC3 contained the *stx*2 gene and could be STEC strains. One reason for high levels of *stx* was attributed to access of the cattle at this site. The presence of *stx* is cause for concern because it suggest the presence of STEC in the lake which can be health risk to the public because these pathogens can survive in aquatic environments for up to a year [7,25].

Often it is important to determine the source of fecal pollution in an environment before the most appropriate mitigating steps can be developed to alleviate the prob-

Table 5. IDEXX plating geometric means (MPN/100mL) for month of April 2010.

Site	Enterococci	Coliform	*E. coli*
DC1	118.6	4208.2	317.8
DC2	172.3	1744.6	163.0
DBC1	261.7	2685.6	713.2
BC1	382.2	1789.8	1152.2
BC2	129.4	1860.9	451.2
BC3	41.6	2878.1	52.9
BC4	26.1	1893.0	104.2
NC1	10.8	588.2	91.6
NC2	39.1	1763.8	90.7
NC3	11.9	769.4	45.8

lem. Recently MST methods have emerged as a reliable approach to rapidly identify the source of fecal contamination in environmental samples based on the association of particular bacterial groups with specific host animals [14-16]. Although it was expected that a major source of fecal pollution in the cattle farming regions of the lake would be from cattle, MST with *Bacteroidales* markers for ruminant (RuBac) was used as a confirmatory test for the presence of cattle fecal pollution. A PCR SYBR Green assay for RuBac and HuBac was used to detect the presence of cattle and human fecal pollution in the water samples, respectively (**Figure 2**). The RuBac marker was detected at all the cattle farming sites except DC2. The HuBac marker was detected at four of the cattle farming sites and at two cattle free sites (**Table 4**). The detection of the RuBac marker at the cattle farming sites agrees with previous MST research that demonstrated that cattle are the major source of fecal pollution in this region of the Oconee watershed [8,19,28]. Even though we did not observe cross reaction between the HuBac primers and cattle fecal DNA in our controls, it was still possible that the detection of the HuBac marker in some of the cattle farming sites could be attributed to the tendency of this PCR assay to cross react with cattle fecal samples [15,29]. This reasoning is supported by the fact that there were no houses in close proximity to the cattle farms in the watershed. In addition recent MST research on fecal pollution in this area of the Oconee watershed has demonstrated that cattle are a major source of fecal pollution [19]. Therefore it appears that cattle fecal pollution was the likely source of *stx*2 gene and possible STEC in this region of the Oconee Watershed.

5. Conclusion

There were high levels of fecal indicator bacteria present in the cattle farming regions of the Oconee Watershed. Quantitative PCR was an effective means for detecting the presence of *stx* gene as a potential indicator of STEC in environmental samples and in this study the cattle farming regions were the only areas of the watershed where *stx*2 was detected. The presence of *stx*/STEC in the Oconee Watershed represents a potential public health risk because the water in this area is used for recreational and agricultural purposes.

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