ANTIBIOTIC RESISTANCE ANALYSIS OF FECAL COLIFORMS TO DETERMINE FECAL POLLUTION SOURCES IN A MIXED-USE WATERSHED

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(Received 13 November 2001; accepted 1 July 2002)

Abstract. Antibiotic resistance analysis was performed on fecal coliform (FC) bacteria from a mixed-use watershed to determine the source, human or nonhuman, of fecal coliform contamination. The study consisted of discriminant analysis of antibiotic resistance patterns generated by exposure to four concentrations of six antibiotics (ampicillin, gentamicin sulfate, kanamycin, spectinomycin dihydrochloride, streptomycin sulfate, and tetracycline hydrochloride). A reference database was constructed from 1125 fecal coliform isolates from the following sources: humans, domestic animals (cats and dogs), agricultural animals (chickens, cattle, and horses), and wild animals. Based on similar antibiotic resistance patterns, cat and dog isolates were grouped as domestic animals and horse and cattle isolates were grouped as livestock. The resulting average rate of correct classification (ARCC) for human and nonhuman isolates was 94%. A total of 800 FC isolates taken from the watershed during either a dry event or a wet event were classified according to source. Human sources contribute a majority (>50%) of the baseflow FC isolates found in the watershed in urbanized areas. Chicken and livestock sources are responsible for the majority of the baseflow FC isolates found in the rural reaches of the watershed. Stormwater introduces FC isolates from domestic (~16%) and wild (~21%) sources throughout the watershed and varying amounts (up to 60%) from chicken and livestock sources. These results suggest that antibiotic resistance patterns of FC may be used to determine sources of fecal contamination and aid in the direction of water quality improvement.

Keywords: antibiotic resistance, fecal pollution, watershed

1. Introduction

The Environmental Protection Agency National Watershed Database 305(b) report (Environmental Protection Agency, 1999) ranks fecal coliform (FC) bacteria as the most widespread pollution problem in the nation's rivers and streams. Nonpoint sources of fecal contamination that contribute to pollution are frequently difficult to identify. The health risk to humans is greater from human than from animal sources (Sinton *et al.*, 1993). The ability to differentiate between human sources (failing septic systems and leaking sewer lines) and nonhuman sources (domestic animals, agricultural animals, and wild animals) could aid in water quality maintenance, public health assessment, and environmental pollution management.

Although FC are routinely monitored in many public waters, tests that measure the presence/absence or concentration of FC provide no indication of the source

Environmental Monitoring and Assessment **85:** 87–98, 2003.

of contamination. Other methods have been developed to address the origin of fecal contamination. The ratio of FC to streptococci was believed to be indicative of the source, with a high ratio (>4) indicating animal sources and a low ratio (<0.7) indicating human sources (Feachem, 1975), but the method has proven unreliable and is no longer in use (American Public Health Association, 1992). The species-specificity of certain bacteriophages (bacterial viruses) (Rusin *et al.*, 1992) and fecal streptococci (FS) (Devriese *et al.*, 1993) has been utilized in source determination, although with only limited success. Molecular methods, such as fatty acid profiling (Simmons, 1994), DNA fingerprinting (Devriese *et al.*, 1993), random amplified polymorphic DNA analysis (Berg, 1994), and 16S ribosomal DNA markers (Bernhard and Field, 2000) have shown promise, but they can be excessively laborious, costly, or complicated for routine use.

The approach that has shown the most potential is based upon the classification of bacteria by antibiotic resistance patterns. Initial studies developed the multiple antibiotic resistance (MAR) index by testing bacterial isolates for growth inhibition in the presence of a number of single concentration antibiotics (Krumperman, 1983). Antibiotic use in humans and animals can result in the occurrence of antibiotic-resistant bacteria and the patterns of antibiotic resistance can be specific to the bacterial host (Holmberg *et al.*, 1984). The MAR index has been improved by increasing the range of antibiotic concentrations tested and by using discriminant analysis (DA) to categorize the antibiotic resistance patterns with a high degree of certainty. The improved method, antibiotic resistance analysis (ARA), has been applied to fecal pollution source-tracking with success. ARA studies using fecal streptococci have differentiated fecal pollution from human sources (sewage) and animal sources (turkey, chicken, dairy cattle, beef cattle, wild) from geographically distinct sites with correct classification rates of $\geq$95% (Wiggins *et al.*, 1999; Sinton *et al.*, 1993). ARA using fecal streptococci was recently used to successfully determine human and non-human fecal pollution sources in a geographically contiguous watershed with a correct classification rate of $\geq$95% (Hagedorn *et al.*, 1999). This study is the first attempt to apply ARA using FC to determine the source of fecal pollution in a watershed.

2. Methods

Antibiotic resistance patterns can be geographically specific. Therefore, a local FC ARA reference database was generated from FC isolates from known sources in the Big Creek watershed. Multiple (3) samples were taken from each known source and the samples were composited prior to analysis. Isolates were collected from the following known sources: sewage influent (Big Creek Water Reclamation Plant), 250 isolates; dogs and cats (Atlanta Humane Society), 125 isolates, each; cattle (cattle pond on the upper reach of Foe Killer Creek at Charlotte Drive and Rucker Road in Alpharetta, GA), 125 isolates; horses (Wills Park Equestrian

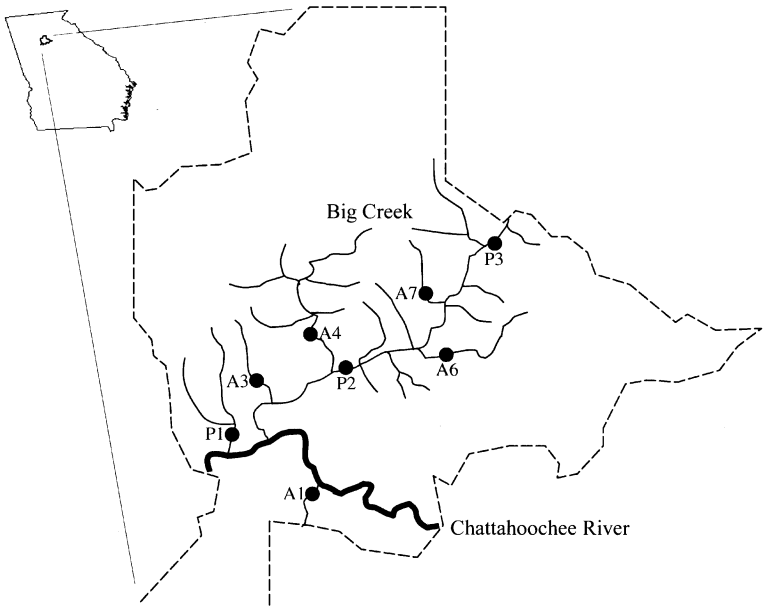

Figure 1. The study area showing the location of Big Creek, the Chattahoochee River, and sampling sites (solid circles). The Chattahoochee River flows from east to west in the study region.

Center in Alpharetta, GA), 125 isolates; chicken (chicken farm near Cumming, GA), 125 isolates; and wild animals (spring-fed creek in an uninhabited valley in the Chattahoochee National Forest near Dawson, GA), 250 isolates. Samples were collected from existing water quality monitoring sites in the Big Creek watershed for unknown or mixed source FC. Samples were collected during a dry event (<0.1 inch of rain in the preceding 72 hr) on 20 April, 2000 and again during a wet event (>0.1 inch of rain within 24 hr preceded by a dry event) on 4 June, 2000. The sites sampled were P1, P2, P3, A1, A3, A4, A6, and A7 (Figure 1). A total of 50 isolates were collected from each sample. These sites were chosen because they offer the greatest coverage of the watershed and represent the major individual tributaries to Big Creek. Sites P3 and A1 are not on Big Creek, but are adjacent to the watershed and these sites are included in the study for comparison. After collection, all samples were placed on ice in coolers and processed within 6 hr.

FC were isolated using Standard Methods 9222D (Feachem, 1975), the same method used in routine regulatory FC analyses, for data consistency with ongoing monitoring efforts. Sewage and fecal samples were suspended and serial diluted in FC Buffer (3×10^{-4} M H_2KPO_3, 2×10^{-3} M $MgCl_2 \cdot 6H_2O$, pH 7.2±0.2). After isolation, individual blue-pigmented FC colonies were transferred to 96-microwell plates containing 0.2 mL of mFC broth (Difco, Detroit, MI; pH 7.4, prepared to manufacturers specifications and used within 72 hr, stored at 4 °C) and incubated

at 44.5±0.2 °C for 48 hr. Wells that exhibited growth and formed a dark blue color after incubation were considered to contain FC cultures.

Cultures (10) from each source were confirmed as FC by verifying gas production after incubation for 3 hr at 35.0±0.5 °C, then 21±2 hr at 44.5±0.2 °C in A-1 broth (per 1 L ddH_2O; tryptone, 20.0 g; lactose, 5.0 g; sodium chloride, NaCl, 5.0 g; salicin, 0.5 g; polyethylene glycol *p*-isooctylphenyl ether (Triton X-100), 1.0 mL). A reference strain, *E. coli* No. 11775 (American Type Culture Collection, Rockville, MD), was included as a positive control.

FC cultures were tested for resistance to six antibiotics (Sigma-Aldrich, St. Louis, MO) at the following final concentrations: ampicillin (12.5, 25, 50, and 100 μg mL^{-1}), gentamicin sulfate (6.25, 12.5, 25, and 50 μg mL^{-1}), kanamycin (12.5, 25, 50, and 100 μg mL^{-1}), spectinomycin dihydrochloride (2.5, 5, 10, and 20 μg mL^{-1}), streptomycin sulfate (12.5, 25, 50, and 100 μg mL^{-1}), and tetracycline hydrochloride (1.25, 2.5, 5, and 10 μg mL^{-1}). Sterile antibiotic solutions were added to tempered (44.5 °C) mFC agar (Difco), mixed, poured into petri dishes, and used immediately upon setting. Each set of petri dishes consisted of one plate of each concentration of each antibiotic and one control plate without antibiotics. Cultures were transferred from 96-microwell plates to petri dishes with a sterilized 50-pin replicator fashioned from an Immunology TSP (Nalge Nunc International Corp., Naperville, IL) and incubated at 44.5±0.2 °C for 24 hr. Isolates were recorded as resistant to an antibiotic if a dark blue colony was observed after incubation. Cultures that failed to develop colonies on mFC agar without antibiotics were not used for further analysis. Duplicates of approximately 5% of the known source isolates were performed to check for consistency.

DA was performed on the data for the ability of each isolate to grow in the presence of each concentration of each antibiotic. DA is a multivariate technique used to (1) separate distinct sets of observations into predefined classes and (2) categorize new observations into these classes. The DA process first generated discrimination rules according to the known source isolate sets, then compared each unknown isolate to the known source isolate sets and classified each isolate into one of the known sources. Each analysis generated a classification table that was used to determine the average rate of correct classification (ARCC) (Wiggins, 1996). The analysis was performed with SYSSTAT Version 9.0 (SPSS, Inc., Chicago, Il) using the linear DA option, which assumes that all groups share a common covariance matrix and that the measurement variables are multivariate normally distributed. Prior probabilities for each group were set to equal.

3. Results

The maximum antibiotic resistances of isolates from known sources were used to develop discrimination rules for the Big Creek watershed (Table I). Sewage isolates were largely resistant to spectinomycin and, to lessor degrees, ampicillin,

TABLE I

Maximum antibiotic resistance of fecal coliforms from known sources

Drug	Isolates from each source (% of total[a])						
	Cat	Cattle	Chicken	Dog	Horse	Sewage	Wild
(μg mL^{-1})	(n = 125)	(n = 125)	(n = 125)	(n = 125)	(n = 125)	(n = 250)	(n = 250)
Ampicillin							
5	0	0	0	0	0	0	0
10	0	0	0	2	0	6	0
20	0	72	4	1	72	16	0
40	0	28	96	0	28	28	0
Gentamycin							
6	0	48	28	2	48	2	0
12.5	0	0	0	0	0	0	0
25	0	0	0	0	0	1	1
50	2	0	0	0	0	0	1
Kanamycin							
12.5	0	42	5	0	58	2	0
25	0	17	8	0	7	1	0
50	0	0	14	0	0	4	0
100	1	3	74	0	3	14	0
Spectinomycin							
5	0	0	0	0	0	0	0
10	2	90	0	0	90	1	0
20	97	0	0	86	0	82	1
40	2	0	0	14	0	16	0
Streptomycin							
10	1	0	0	2	0	2	1
20	1	0	8	2	0	19	1
40	1	97	29	1	97	4	1
80	1	3	63	1	3	2	0
Tetracycline							
5	97	63	0	94	30	28	62
10	1	14	26	0	16	2	1
20	0	6	7	1	39	2	0
40	0	18	54	4	15	29	0

[a] n, total number of isolates from each source.

TABLE II

Classification of fecal coliforms from cat, cattle, chicken, dog, horse, sewage, and wild sources

Source	No. (%) of isolates classified as:							
	Cat	Cattle	Chicken	Dog	Horse	Sewage	Wild	ARCC
Cat	(96)	(1)	(0)	(1)	(0)	(0)	(2)	(96)
Cattle	(0)	(74)	(2)	(0)	(23)	(0)	(0)	74
Chicken	(0)	(1)	(94)	(0)	(6)	(0)	(0)	94
Dog	(83)	(1)	(0)	(15)	(0)	(1)	(0)	15
Horse	(0)	(46)	(3)	(0)	(51)	(0)	(0)	51
Sewage	(33)	(1)	(1)	(11)	(2)	(50)	(2)	50
Wild	(0)	(2)	(0)	(0)	(0)	(0)	(98)	98
Total								70

streptomycin, and tetracycline. Dog and cat isolates were nearly identical in that both groups demonstrated resistance to high concentrations of spectinomycin and low concentrations of tetracycline. Horse and cattle isolates demonstrated similar resistances to spectinomycin and streptomycin. Chicken isolates were highly resistant to ampicillin, kanamycin, streptomycin, and tetracycline. Wild isolates demonstrated resistance only to low concentrations of tetracycline.

Discriminant analysis of antibiotic resistance patterns of FC from cat, cattle, chicken, dog, horse, sewage, and wild sources generated an ARCC of 70% (Table II). Although this is significantly greater than random classification ($1.0 \div 7$ classes = 14% ARCC), the target ARCC for this project is $\geq 90\%$. The results indicate poor discrimination between the cat and dog isolates, the horse and cattle isolates, and the sewage and cat isolates. Only 15% of the dog isolates were accurately classified and 83% were misclassified into the cat class. Almost half of the horse isolates (46%) were misclassified into the cattle class and 23% of the cattle isolates were classified into the horse class. Half of the sewage isolates (50%) were accurately classified, with most of the misclassified cases falling into the cat (33%) and dog (11%) classes. The antibiotics spectinomycin, streptomycin, kanamycin and ampicillin were the most important measurement variables. The antibiotics gentamicin and tetracycline were relatively unimportant for classification. Based on the results of Table II, the original cat and dog isolates were combined into one domestic group, and the original cattle and horse isolates were combined into one livestock group.

Discriminant analysis of antibiotic resistance patterns of FC from chicken, domestic, livestock, sewage, and wild sources generated an ARCC of 87% (Table III). The results indicate a high degree of classification accuracy for domestic, livestock, poultry and wild groups (96–98%). However, only 51% of the sewage group were

TABLE III

Classification of fecal coliforms from chicken, domestic, livestock, sewage, and wild sources[a]

Source	No. (%) of isolates classified as:					
	Chicken	Domestic	Livestock	Sewage	Wild	ARCC
Chicken	(96)	(0)	(4)	(0)	(0)	96
Domestic	(0)	(98)	(1)	(0)	(1)	98
Livestock	(3)	(0)	(97)	(0)	(0)	97
Sewage	(1)	(43)	(3)	(51)	(2)	51
Wild	(0)	(0)	(2)	(0)	(98)	98
Total						87

[a] Cat and dog sources were grouped to form the domestic classification. Cattle and horse sources were grouped to form the livestock classification.

TABLE IV

Classification of fecal coliforms from sewage and animal sources[a]

Source	No. (%) of isolates classified as:		
	Sewage	Animal	ARCC
Sewage	(76)	(24)	76
Animal	(29)	(71)	71
Total			72

[a] Chicken, domestic, livestock, and wild sources were grouped to form the animal classification.

accurately classified due, primarily, to misclassification (43%) into the domestic class. Again, the antibiotics spectinomycin, streptomycin, kanamycin and ampicillin were the most important measurement variables. The antibiotics gentamicin and tetracycline were relatively unimportant for classification. Based on Table III, the original measurement variables were combined into two groups: sewage and animal, a combination of domestic, livestock, poultry and wild isolates.

Discriminant analysis of antibiotic resistance patterns of FC from sewage and animal sources generated an ARCC of 72% (Table IV). The results indicate an unacceptable degree of misclassification between the sewage and animal classes (24–29%). Returning to Table III, the original measurement variables were combined into four groups: chicken, sewage/domestic, livestock, and wild isolates.

TABLE V

Classification of fecal coliforms from chicken, sewage/domestic, livestock, and wild sources

Source	No. (%) of isolates classified as:				
	Chicken	Livestock	Sewage/ domestic	Wild	ARCC
Chicken	(94)	(6)	(0)	(0)	94
Sewage/Domestic	(0)	(3)	(96)	(1)	96
Livestock	(3)	(97)	(0)	(0)	97
Wild	(0)	(2)	(0)	(98)	98
Total					96

TABLE VI

Classification of fecal coliforms from human and nonhuman sources[a]

Source	No. (%) of isolates classified as:		
	Human	Nonhuman	ARCC
Human	(96)	(4)	96
Nonhuman	(6)	(94)	94
Total			94

[a] Members of the sewage group that were classified as domestic were removed and the remaining members were reclassified as human. Chicken, domestic, livestock, and wild sources were grouped to form the nonhuman classification.

Discriminant analysis of antibiotic resistance patterns of FC from chicken, livestock, sewage/domestic, and wild sources generated an ARCC of 96% (Table V). The results indicate a high degree of classification accuracy for chicken, livestock, sewage/domestic and wild classes (94–98%). The antibiotic spectinomycin is by far the most important measurement variable, followed by streptomycin and kanamycin. The antibiotics ampicillin, gentamicin, and tetracycline were relatively unimportant for classification. Members of the sewage isolates that were misclassified as domestic in Table III (121 observations) were removed and the remaining isolates were reclassified as human (Appendix). Based on this reclassification, the original measurement variables were combined into two groups: human and nonhuman, a combination of chicken, domestic, livestock, and wild isolates.

Discriminant analysis of antibiotic resistance patterns of FC from human and nonhuman sources generated an ARCC of 94% (Table VI). The antibiotics ampicillin, streptomycin, and spectinomycin were the most important measurement variables. The antibiotics gentamicin, kanamycin and tetracycline were relatively unimportant for classification. Based on the results of Table VI, the target ARCC of $\geq$90% was achieved. The unknown isolates were then classified according to the same classification rules as in Table VI and nonhuman isolates were further classified as in Table III.

Discriminant analysis was performed on a total of 400 unknown isolates from 8 sites in the Big Creek watershed (Table VI). Isolates classified as human predominated (>50%) at site P1 during both wet and dry sampling events and at site A3 during the dry sampling event. The remaining isolates from P1 and A3 were mostly livestock (12–14%), except during the wet sampling event, when domestic and wild contributions became significant (16 and 20–22%, respectively). Site P2 had a significant number of human (44%) or livestock (44%) isolates during the dry event. The human contributions were largely replaced, however, by domestic (16%) and wild (20%) sources during the rain event. Sites P3 and A1 had lower numbers of isolates that classified as human (14 and 22%, respectively) and nearly identical numbers of livestock isolates (40–42 and 36–38%) during both wet and dry sampling events. The contributions from chicken isolates to P3 (42%) and A1 (32%) during the dry sampling event were reduced by the presence of domestic (16%) and wild (20%) isolates during the wet event. Sites A4 and A6 were nearly identical in that livestock predominated (90–92%) over low numbers of human isolates ($\leq$8%) during dry events, but nonhuman isolates became much more evenly distributed over the four classes, chicken (36–46%), domestic (16%), livestock (10–18%), and wild (20%), during the wet event. Site A7 had a low number of isolates that classified as human (6%) and nearly equal contributions from chickens (48%) and livestock (46%) during the dry event, although an increase in livestock (60%) and contributions from domestic (16%) and wild (20%) replaced the chicken group during the wet event.

4. Discussion

The purpose of this study was to determine the contributions of human and nonhuman sources to FC contamination in a mixed-use watershed. Based on the classifications in Table VII, human sources appear to contribute a substantial portion of the baseline (dry event) FC isolates found in Big Creek and near its confluence with the Chattahoochee River. Chicken and livestock sources are responsible for the majority of the baseline FC isolates found in the upstream reaches of the Big Creek watershed. Runoff from rainfall appears to dilute the baseline readings throughout the watershed while introducing chicken, domestic, and wild FC isolates. Although the proportions of human, chicken, and livestock FC isolates vary from site to site,

TABLE VII

Classification of fecal coliforms from unknown sources in and near the Big Creek watershed

Site	Sample	Fecal coliforms (CFU 100 mL^{-1})	Classification (%)				
			Human	Chicken	Domestic	Livestock	Wild
P1	Dry	100	88	0	0	12	0
	Wet	200	58	0	17	4	22
P2	Dry	90	44	12	0	44	0
	Wet	209	8	0	16	56	20
P3	Dry	50	16	42	0	42	0
	Wet	145	12	12	16	40	20
A1	Dry	80	26	32	0	38	4
	Wet	100	18	10	15	36	20
A3	Dry	500	86	0	0	14	0
	Wet	1100	8	2	16	54	20
A4	Dry	260	8	0	0	92	0
	Wet	600	8	46	15	10	21
A6	Dry	39	6	4	0	90	0
	Wet	46	8	36	16	18	22
A7	Dry	40	6	48	0	46	0
	Wet	30	4	0	16	60	20

the proportions of domestic (15–17%) and wild (20–22%) FC isolates are relatively constant and appear only during wet events.

ARA is heavily dependent upon the antibiotics chosen for analysis. The antibiotics tested in this study were sufficient to generate an ARCC of 94% between human and nonhuman sources. Ampicillin, spectinomycin, and streptomycin were the most important measurement variables for differentiating human and nonhuman FC. The addition of kanamycin as a measurement variable allows the differentiation of the nonhuman sources into chicken, domestic, livestock, and wild sources with an ARCC of >96%. Many other antibiotics are routinely administered to chickens, livestock, and humans, and some specialty antibiotics are administered only to specific groups. Although the ARCC generated in this study is quite acceptable, another antibiotic or set of antibiotics may more efficiently discriminate between the classes used. Further studies may determine the most effective antibiotic(s) for FC classification in this study area.

Several points must be considered during construction of the known source FC database. First, the known sources must be representative of the classification requirements. For example, the purpose of this study was to differentiate human FC

from nonhuman FC contamination. Consequently, the database was constructed from human and nonhuman sources. Second, the known source samples must be representative of the known source population. A database generally requires a few hundred isolates per known source to become sufficiently representative and accurate. This was true for the known source dog and cat isolates in this study because they were all very similar. However, the sewage influent isolates, expected to be primarily human, included mixed FC isolates that were classified as originating from dog or cat sources. Similar results have been seen before (Wiggins *et al.*, 1999) and the problem may be avoided by sampling septage or human volunteers. Finally, the current use of ARA for determining sources of fecal contamination relies on the development of known source databases that are 'snapshots' of the water systems. No long-term studies have been performed to ascertain the temporal variability or drift of antibiotic resistance patterns. Therefore, unknown FC isolates should be analyzed close in time to the construction of the known source FC database.

The known source FC database developed in this study can be used for determination of sources of FC isolates from other waters. However, the known source database should initially be tested with known source FC isolates from the new watershed system. If these isolates are classified with sufficient accuracy (>90%), then the database is representative of the watershed system. If the isolates are not accurately classified, there are two options for improving the database. The first option is to determine the most influential existing measurement variables and improve the classification accuracy by focusing primarily on those variables. The second option is to construct a new database from known source isolates specific to the watershed under analysis.

This is one of the first uses of ARA of FC in a natural watershed to determine sources of fecal contamination. Although ARA has been used in several recent studies to identify sources of fecal contamination, fecal streptococci were used as the test organisms rather than FC. The reasons for choosing FS are that, unlike FC, FS are recoverable from treated biowastes (Wiggins *et al.*, 1999) and FS may persist longer in the environment than FC (Environmental Protection Agency, 1999). For the purposes of this study, FC were the preferred test organism because FC monitoring is an integral part of the existing water sampling plan for the Big Creek watershed and an extensive historical database is already in existence. In addition, FC testing is the most widely-used form of bacteriological monitoring in public- and private-use waters and the techniques used in this study may be directly adapted to ongoing monitoring plans. As such, ARA of FC can provide a relatively quick, inexpensive, and accurate tool for purveyors of public health, environmental protection, and improved water quality to determine sources of fecal pollution in mixed-use watersheds.

Acknowledgements

This work was supported by the City of Atlanta, Georgia. I thank J. Rozza for critical discussion and K. Autrey and V. Pope for assistance with manuscript preparation.

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