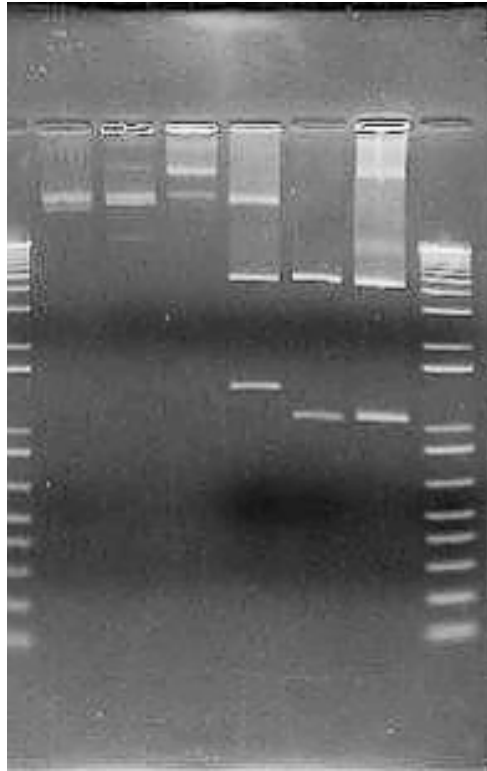


**Identification of Bacteria Sources
in Green Hill Pond
Using Polymerase Chain Reaction (PCR)**



DNA fingerprints: the result of PCR and gel electrophoresis.
(Photo credit: <http://csmres.jmu.edu/Bioweb/Bio480/Fall01/Tues5/results.htm>).

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Table of Contents

LIST OF TABLES.....	3
TABLE OF FIGURES.....	4
ABSTRACT.....	5
1.0 INTRODUCTION.....	6
1.1 STUDY AREA.....	6
2.0 METHODS.....	6
2.1 STUDY PLAN.....	7
2.2 SCAT SAMPLING.....	8
2.3 DRY WEATHER.....	8
2.4 WET WEATHER.....	9
3.0 RESULTS.....	11
3.1 DRY WEATHER FECAL COLIFORM CONCENTRATIONS.....	11
3.2 WET WEATHER FECAL COLIFORM CONCENTRATIONS.....	11
3.3 KNOWN SOURCE SAMPLES.....	12
3.4 BACTERIA SOURCE IDENTIFICATIONS.....	12
<i>Allen's Cove</i>	13
<i>Factory Brook</i>	13
<i>Teal Brook</i>	14
<i>Teal and Factory Brook Cove</i>	15
<i>Unnamed Brook 2</i>	15
<i>Gooseberry Cove</i>	16
<i>Unnamed Brook 1</i>	16
<i>Data overview</i>	17
3.5 EVALUATION OF DATA QUALITY.....	19
<i>Fecal coliform</i>	19
<i>Bacterial source identifications</i>	19
<i>Representativeness</i>	20
4.0 DISCUSSION.....	23
Conclusions.....	25
5.0 REFERENCES.....	28

LIST OF TABLES

Table 1. List of sources in the source library (Chikarmane, 2003).....	7
Table 2. Dates, numbers and types of sampling conducted in Green Hill Pond.....	7
Table 3. Dates and numbers of collection for different animal scat types.	8
Table 4. Dry weather sampling dates and locations.	8
Table 5. Wet weather sampling dates and locations.	10
Table 6. Dry weather geometric mean fecal coliform concentrations.	11
Table 7. Wet weather mean and geometric mean fecal coliform concentrations.	11
Table 8. Mean and geometric mean fecal coliform concentrations per 100g of animal scat.	12
Table 9. Identification of isolates and similarity index (SI) for Allen’s Cove locations.	13
Table 10. Identification of isolates and similarity index (SI) for Factory Brook locations.	14
Table 11. Identification of isolates and similarity index (SI) for Teal Brook locations.....	14
Table 12. Identification of isolates and similarity index (SI) for Teal-Factory Cove.	15
Table 13. Identification of isolates and similarity index (SI) for Unnamed Brook 2 stations	16
Table 14. Identification of isolates and similarity index (SI) for Gooseberry Cove stations.....	16
Table 15. Identification of isolates and similarity index (SI) for Unnamed Brook 1 stations.	17
Table 16. Positively identified isolates at each station with a Similarity Index of 0.7 or greater.....	17
Table 17. Positively identified isolates at each station with a Similarity Index of 0.8 or greater.....	18
Table 18. Results summarized by location and source species (SI of 0.7 or greater).....	18
Table 19. Percent of samples containing a particular species by location (SI of 0.7 or greater).	19
Table 20. Confidence intervals for the replicate data collected in Green Hill Pond.....	19
Table 21. Percent agreement for bacteria source identification results.....	20
Table 22. Summary of other bacterial source tracking projects.....	22

TABLE OF FIGURES

Figure 1. Dry weather sampling stations including scheduled, eliminated and new stations.9
Figure 2. Wet weather sampling stations including scheduled, eliminated and new stations.....10
Figure 3. Bacteria sources identified at all Green Hill Pond stations (SI of 0.7 or greater).....23
Figure 4. Percent contribution of sources to Green Hill Pond areas (SI of 0.7 or greater).24
Figure 5. Most prominent species found in Green Hill Pond.....26

ABSTRACT

A bacteria source tracking study was performed in Green Hill Pond and its tributaries during the fall of 2002. This study utilized Polymerase Chain Reaction (PCR), to identify bacteria sources in Green Hill Pond, the first time this technique has been used in Rhode Island to track sources of bacteria in surface waters. This technique assumes that bacteria in the intestines of different species of animals are relatively unique. Bacteria were collected from feces of animal species present in the watershed to create a known source library. The DNA from bacteria in the surface water samples was then compared to the DNA in the library to determine the closest match.

For the Green Hill Pond bacteria source tracking project, 70 samples were collected from known sources. Species included but were not limited to Canada goose, deer, dog, mute swan, otter, rabbit and raccoon. Samples from 9 septic systems in the area, including several that were failing were also collected. A total of 58 surface water samples were collected in the areas of Allen's Cove, Gooseberry Cove, Factory Brook and Teal Brook.

From each water sample, up to 5 bacterial isolates were analyzed using PCR. The result of PCR for an individual bacterial isolate is a distinct banding pattern. Banding patterns of bacteria from unknown sources in the water samples were compared to the library of known sources. This comparison was done using a cluster analysis method, which determines the closest match in the source library. The match between known and unknown is calculated from the number of matching bands and the result is expressed as a similarity index (SI). For this study, we determined that an SI of 0.7 was sufficient for identification.

A total of 251 isolates were analyzed for this project. Thirty-one isolates had an SI less than 0.7 and therefore were considered unknown (12%). Wildlife accounted for 38% of the isolates, followed by birds (31%), unknown (12%), humans (11%) and dogs (8%). Overall, wildlife and waterfowl accounted for the majority of the isolates analyzed, and animals accounted for 87% of the positively identified isolates. The dominant source species were generally consistent within each area and differed between areas. Bacteria from wildlife were more prominent in the isolates analyzed from Factory and Teal Brooks and in Factory Teal Cove. Human bacteria also appeared regularly in this area. Birds and dogs were more prominent in the isolates taken from samples in Allen's Cove, Gooseberry Cove and Unnamed Brook 2. The contribution of bacteria from humans was highest in Unnamed Brook 2 and merits a further investigation of this area. Verification of these results using another bacteria source tracking method is recommended to validate the results of this study.

1.0 INTRODUCTION

Elevated bacterial concentrations in Green Hill Pond are responsible for shellfish area closures in the pond since 1994. The purpose of this Bacterial Source Tracking project was to test a molecular technique that can determine the sources of bacteria in the coves and tributaries in Green Hill Pond. The technique works by comparing the DNA fingerprint of unidentified bacteria in Green Hill Pond samples to the DNA fingerprint of bacteria from known, identifiable sources.

1.1 Study area

This project focused on several areas in Green Hill Pond that do not meet water quality standards. In these areas an optical brightening study was conducted to determine if the sources were related to septic systems. The results found no evidence of contributions from septic systems. Also, bracketing of suspected sources did not provide any definitive source information. The areas of focus included the Teal and Factory Brook watersheds, Unnamed Brook 2, Gooseberry Cove (the cove that Unnamed Brook 2 flows into) and Allen's Cove. These areas have discrete, manageable watersheds and the small size of these areas facilitated collecting reliable known and unknown sources.

2.0 METHODS

This project included collecting identifiable scat samples and surface waters in Green Hill Pond and its tributaries. Scat samples were collected to establish known sources from the study area and to contribute to a DNA library of known sources. RIDEM Fish and Wildlife personnel assisted in searching for and identifying the source of these samples in the field. The criteria for identification consisted of location, consistency, size and shape of the scat. Species included but were not limited to domestic dog, raccoon, rabbit, deer, otter, mute swan, and Canada goose. Solid samples were collected in sterile whirlpak bags. Water samples were collected in sterile BAL Laboratory Nalgene bottles.

All samples were brought to BAL Laboratory for fecal coliform analysis and preparation on petri dishes for transport to Cape Cod Community College. Fecal coliform enumeration was performed on solid and water samples. Analysis of the solid samples began with the addition of a buffer solution to bring the total volume to 100 ml. After analysis by BAL laboratories the samples were used to inoculate petri plates containing selection medium that only allows for growth of *E. coli*. The petri plates were then sent via US Mail to Dr. Hemant Chikarmane at Cape Cod Community College. Dr. Chikarmane was responsible for conducting the PCR analysis and performing comparisons of bacteria to determine the closest match.

Polymerase Chain Reaction or PCR is a molecular laboratory technique used to amplify certain portions of the DNA strand. The purpose to using this technique is to create a genetic fingerprint that is reproducible and comparable to the fingerprints of other individuals. The method works by using primers to mark the segments of DNA that will be amplified. The primers are short sequences of DNA that attach at the beginning and end of a specified DNA sequence. The primers form a template for the sequence that is to be copied many times over. The key to this method is a molecule (specifically a polymerase) derived from the hot spring bacterium *Thermus aquaticus* (Taq polymerase for short). Taq polymerase is used to extend the primers during the 72⁰C portion of the reaction to create and recreate the selected segment. PCR involves using a series of thirty heating and cooling reactions to perform the amplification. The DNA is unzipped during the heating reaction (94-96⁰C) and then cooled (56-64⁰C) during which the primers anneal to the DNA molecule. The mixture is then heated to 72⁰C during which Taq polymerase extends the primers

(fills in the molecule after the primers). When this series of events is repeated thirty times the result is about one billion copies of the desired DNA segment. The resulting mixture of segments is separated using gel electrophoresis creating a “fingerprint” made up of a specific reproducible pattern of bands.

The banding pattern is then compared to the patterns in the library of known sources using cluster analysis to determine the source of the bacteria. The more bands two individuals share increases the likelihood that they are related. The library used for this analysis included the known sources that were collected specifically for this project in addition to sources collected by Dr. Chikarmane in Cape Cod. The source library includes 447 different isolates from 22 different species including 48 human isolates. A complete listing of sources in the source library is presented in Table 1 (Chikarmane, 2003).

Table 1. List of sources in the source library (Chikarmane, 2003).

Source	Number of isolates	Source	Number of isolates
Human	55	Deer	29
Rat	30	Cow	23
Mouse	36	Horse	18
Raccoon	29	Alpaca	3
Otter	21	Sheep	12
Chipmunk	11	Goat	4
Dog	32	Pig	6
Cat	25	Goose	32
Hamster	8	Gull	36
Rabbit	19	Swan	18
		Total	447

2.1 Study plan

Sampling was conducted on eight dates. Five dry weather surveys were conducted during the months of September and October 2002. Both scat samples and water samples were collected during these dry weather surveys. The majority of the septic system sampling was conducted on September 25, 2002. The various sampling surveys and numbers of samples collected are presented in Table 2.

Table 2. Dates, numbers and types of sampling conducted in Green Hill Pond.

Date	Scat sampling	Dry weather sampling	Wet weather sampling	Septic system sampling
9/12/02	23	3		
9/15/02			4	
9/16/02			9	
9/19/02	8	8		1
9/25/02				8
10/3/02	11	6		
10/8/02	12	8		
10/16/02			14	
10/22/02	7	6		
Totals	61	31	27	9

Sampling during wet weather was conducted during two storm events. The first event occurred late on September 15, 2002. NOAA's hourly rain data website (http://precip.fsl.noaa.gov/hourly_precip.html) reported that Westerly and Newport received 0.9 inches of rain on September 15th. DEM staff sampled tributaries that evening, and in-pond samples were collected the following morning. The second wet weather event was sampled on October 16, 2002 when 1.02 inches of rain was recorded in Westerly and Newport (http://precip.fsl.noaa.gov/hourly_precip.html).

2.2 Scat sampling

Scat sampling was conducted on five separate occasions in the Green Hill Pond area. Scat was collected from eleven different animal species. A number of scat samples from each species were collected on different dates, totaling 61 scat samples. The various animal scat types found including 5 different birds and 6 different mammals are presented in Table 3. Septic system samples were collected during two days. The septic system bacteria samples are assumed to be representative of human bacteria.

Table 3. Dates and numbers of collection for different animal scat types.

Animal species	9/12/02	9/19/02	9/25/02	10/3/02	10/8/02	10/22/02	Total
Canada goose	3	2			2	2	9
Cormorant	2						2
Deer	4			4	1		9
Dog	1	2		1	4	3	11
Horse				1			1
Mallard duck	2				3		5
Mute swan	3	2			1		6
Otter	1	2					3
Rabbit	4			2			6
Raccoon	2			3			5
Gull	1				1	2	4
Septic		1	8				9
Total	23	9	8	11	12	7	70

2.3 Dry weather

Dry weather sampling stations and dates are presented in Table 4. The locations of the stations are presented in Figure 1. Tributary and in-pond sampling was conducted during dry weather on five dates. One of the original dry weather stations and two of the wet weather stations were not sampled due to drier than normal conditions. As a result, the study focus was shifted from Unnamed Brook 1 to Factory Brook.

Table 4. Dry weather sampling dates and locations.

Date	9/12/02	9/19/02	10/3/02	10/8/02	10/22/02
AC1	X	X	X	X	X
UN2- UP					
UN2-D	X	X	X	X	X
TB-UP		X	X	X	X
TB-D		X	X	X	X
FB-UP		X	X	X	X
FB-D		X	X	X	X
AC1-Bottom		X			
UN1- Cove	X				

Scheduled station locations X – stations actually sampled
Shaded boxes denote additional sampling stations

DEM's original intention was to sample Unnamed Stream 2 during dry weather and both unnamed streams during wet weather. We were able to identify a location to sample Unnamed stream 2 where it entered the pond, however upstream of that point was a small wooded wetland complex with no well defined channel. This wetland drains into the pond through a corrugated pipe at the head of the Cove behind Gooseberry Island. DEM could find no specific location to sample upstream in Unnamed Brook 2.

Unnamed Brook 1 was not flowing during dry weather or during the first wet weather event at either of the upstream or downstream locations. A sample was taken during the second wet weather event at the upstream location, but no downstream location was found flowing at any time. It is most likely that the stream crossed India Point Road, flowed into a wetland area, then infiltrating into the ground and not entering the pond. In the cove where Unnamed Brook 1 discharges a single sample was collected.

Figure 1. Dry weather sampling stations including scheduled, eliminated and new stations.



2.4 Wet weather

Wet weather sampling stations and dates are presented in Table 5. Locations of the wet weather sampling stations are presented in Figure 2. Some modifications were also made to the wet weather sampling plan. During the first wet weather event the pipes discharging into Allen's Cove were not sampled. Ultimately one pipe was sampled during wet weather. Alternatively, one station was added along Factory Brook and another station was added in the cove to which Factory and Teal Brooks discharge.

Table 5. Wet weather sampling dates and locations.

Date	9/15/02	9/16/02	10/16/02
AC1	X	X	X
AC2		X	X
DP02			
DP06			X
DP07			
UN2-UP			
UN2-D		X	X
UN2 Cove		X	X
UN1-UP			X
UN1-D			
UN1 - Cove			
TB-UP	X	X	X
TB-D		X	X
FB-D	X	X	X
FB-UP	X	X	X
TBFB-Cove	X	X	X

Scheduled station locations X - stations actually sampled
 Shaded boxes denote additional sampling locations

Figure 2. Wet weather sampling stations including scheduled, eliminated and new stations.



3.0 RESULTS

3.1 Dry weather fecal coliform concentrations

Dry weather sampling was conducted on the five dates presented in Table 4. Dry weather geometric mean fecal coliform concentrations are presented in Table 6. A single sample was collected at three of the stations. For sample AC1 Bottom, the bottom of Allen Cove was disturbed and a sample was taken to see whether disturbing the sediments would affect fecal coliform concentrations and the resulting source species identification. At station UN1 Cove, a single sample was collected on the first sampling run because Unnamed Brook 1 had not outlet to the pond at that time. At station Gooseberry Cove a sample was collected next to the spit of sand where swans congregate to see if there was any influence on the concentrations in that cove.

Dry weather fecal coliform concentrations were high in Factory and Teal Brooks and Unnamed Brook 2. In Factory Brook the highest concentrations were at the downstream location. In Teal Brook the higher concentration was located at the upstream station. The station with the highest dry weather concentration was UN2D, which had a geometric mean concentration of 1,573 fc/ 100 ml.

Table 6. Dry weather geometric mean fecal coliform concentrations.

Station	Geometric mean fecal coliform concentration fc/100ml	Number of samples
AC1	15	5
AC1 Bottom	50	1
FBD	341	4
FBUP	113	4
TBD	75	4
TBUP	141	5
UN1 Cove	65	1
UN2D	1,573	5
Gooseberry Cove	290	1

3.2 Wet weather fecal coliform concentrations

Wet weather sampling occurred during two storms, and consisted of one round of sampling during each storm. The wet weather fecal coliform data in Table 7 is presented in the form of means and geometric means because only 1 or 2 samples were collected at many of the stations. Highest concentrations were found in Teal, Factory and the Unnamed Brooks.

Table 7. Wet weather mean and geometric mean fecal coliform concentrations.

Station	Mean fecal coliform concentration fc/100 ml	Geometric mean fecal coliform concentration fc/100 ml	Count
AC1	89	25	3
AC2	90	85	2
DP06	7850	7,765	2
FB-D	13,833	7,958	3
FB-UP	6600	4,893	3
TB-D	1080	566	2
TBFB-Cove	3583	1,105	3
TB-UP	8567	8,052	3
UN1-UP	11000	11,000	1
UN2- Cove	1588	290	3
UN2-D	11050	4,806	2

3.3 Known source samples

Known source samples, including identified animal scat samples, were collected during every dry weather sampling run on five dates in the fall of 2002 (see Table 1). Some scat samples were difficult to find fresh. The cormorant, horse and gull samples were all fairly old, and the cormorant guano had to be scraped off a rock. Fecal coliform concentrations of these scat samples were analyzed, and the mean and geometric mean concentrations are presented in Table 8. Most of the other scat samples were fairly fresh, however, and the resulting fecal coliform concentrations reflect the freshness.

Fecal coliform concentrations were highest in the scat from mammals, ranging from the tens of millions (dog) to billions (deer and otter) of fecal coliform per 100 grams of scat (wet weight). The rabbit scats had low concentrations that might reflect the apparent age of the scat at the time of collection. Waterfowl had lower concentrations, ranging from thousands (duck) to millions (Canada goose).

Table 8. Mean and geometric mean fecal coliform concentrations per 100g of animal scat.

Animal species	Mean Fecal coliform concentration fc/100 g wet wt	Geometric mean Fecal coliform concentration fc/100 g wet wt.	Count
Canada goose	1.42×10^{10}	5.29×10^6	9
Cormorant	1	1	2
Deer	8.4×10^9	2.26×10^9	8
Dog	8.62×10^9	2.9×10^7	10
Duck	8.64×10^4	6.3×10^3	4
Horse	20	20	1
Otter	3.21×10^9	1.72×10^9	3
Rabbit	1.8×10^9	1.41×10^3	6
Raccoon	3.43×10^{10}	4.88×10^8	5
Gull	10^3	23	4
Mute swan	4.61×10^6	2.13×10^4	6

3.4 Bacteria source identifications

The PCR fingerprint results from each isolate analyzed were compared to the known source library using a cluster analysis. Each isolate analyzed using PCR has a specific band pattern associated with it which forms the DNA fingerprint of that isolate. The number of specific bands in part depends upon the number of primers used as described in the Section 2.0 above.

The results are in the form of a percent similarity known here as the Similarity Index (SI). The Similarity Index describes how closely related the banding pattern of each isolate is to the banding pattern of a specific animal type in the source library. A percent similarity of 70% (0.7) or greater indicates a likely match. The reliability of the result increases with an increasing percent similarity. In general the similarity index values ranged from 0.7 to 0.9 with very few isolates having a similarity index lower than 0.7. Any isolate with a similarity index less than 0.7 was omitted from any final data analysis. Of the 251 analyzed isolates, 31 were omitted for having a low similarity index, leaving 220 isolates.

Allen's Cove

The results for all Allen's Cove locations are presented in Table 9. Forty-nine isolates were analyzed from samples taken during wet and dry weather in Allen's Cove. Nine of the isolates had an SI less than 0.7. Of the remaining isolates, the majority (26 isolates, or 65%) matched with birds. Wet weather sources were primarily bird (15 isolates, 60%) and dog (5 isolates, 20%). The second and third most prevalent sources overall were dogs (7 isolates, 18%) and raccoons (3 isolates, 8%). A single isolate identified as human was found in a dry weather sample taken from the center of Allen's Cove.

Table 9. Identification of isolates and similarity index (SI) for Allen's Cove locations.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
Station AC1 (Center of Cove)						
9/12/02	42	Deer (0.7)				
9/16/02 wet	3	Bird (0.5)*	Bird (0.8)	Bird (0.7)		
9/16/02 wet rep	240	Dog (0.8)	Bird (0.7)	Bird (0.6)*		
9/19/02	60	Raccoon (0.6)*	Bird (0.9)	Bird (0.9)	Bird (0.7)	Bird (0.8)
10/3/02	2	Human (0.7)	Bird (0.7)	Bird (0.8)		
10/8/02	9	Dog (0.7)	Bird (0.9)	Dog (0.8)	Bird (0.8)	
10/16/02 wet	23	Bird (0.6)*	Bird (0.8)	Dog (0.8)	Bird (0.6)*	Dog (0.8)
10/22/02	<1					
Station AC2 (NW Corner of cove, wet weather only)						
9/16/02 wet	60	Cow (0.6)*	Dog (0.8)	Raccoon (0.7)	Bird (0.8)	Bird (0.8)
10/16/02 wet	120	Otter? (0.6)*	Bird (0.7)	Bird (0.8)	Bird (0.8)	Dog (0.8)
Miscellaneous Allen's Cove samples						
AC1 Bottom 9/19/02 (disturbed sediments)	50	Bird (0.7)	Bird (0.8)	Bird (0.7)	Bird (0.6)*	Bird (0.6)*
DP06 10/16/02 (wet)	9,000	Deer (0.7)	Deer (0.8)	Bird (0.8)	Bird (0.8)	Raccoon (0.7)
DP06 rep 10/16/02 (wet)	6,700	Bird (0.7)	Bird (0.8)	Raccoon (0.8)	Bird (0.8)	Bird (0.8)

* denotes samples excluded from further analysis

Factory Brook

Results for the two Factory Brook stations are presented in Table 10. A total of 64 isolates were analyzed from two sampling locations in Factory Brook. Eleven of the analyzed isolates had an SI less than 0.7. Of the 53 remaining isolates, two primary bacteria sources emerged in Factory Brook. Twenty-five isolates were identified as birds or rabbits, comprising 47% of the total isolates analyzed in Factory Brook. Deer and rodents were also prevalent as sources in Factory Brook with 10 positively identified isolates (32% of the total).

Human also emerged as a source at the upstream station in Factory Brook with 6 isolates or about 11% of the total. Other sources in the area included raccoon (3 isolates, 6%), and dog (1 isolate, 2%). Birds and rabbits remained the predominant sources in wet weather runoff. Wet weather sources included birds as the highest (6 isolates, 24%), rabbits as second highest (5 isolates, 16%), raccoons as third highest (3 isolates, 12%). A single human isolate was detected during wet weather (4%).

Table 10. Identification of isolates and similarity index (SI) for Factory Brook locations.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
FBD (Factory Brook and Teal Drive)						
9/15/02 wet	28,000	Bird (0.7)	Bird (0.8)			
9/16/02 wet	12,000	Bird (0.7)	Bird (0.8)	Rabbit (0.9)		
9/19/02	500	Rabbit (0.7)	Rabbit (0.8)	Rabbit (0.7)	Rat (0.8)	
10/3/02	190	Deer (0.8)	Bird (0.6)*	Rabbit (0.8)	Dog (0.9)	Cow? (0.6)*
10/8/02	530	Deer (0.7)	Rat (0.8)	Bird (0.8)	Rabbit (0.8)	Rabbit (0.8)
10/16/02 wet	1,500	Bird (0.6)*	Rabbit (0.7)	Rabbit (0.7)	Bird (0.8)	Bird (0.8)
10/22/02	270	Deer (0.7)	Deer (0.8)	Rat (0.8)	Rabbit (0.7)	Bird (0.6)*
FBUP (Factory Brook and Matunuck Schoolhouse Road)						
9/16/02 wet	14,000	Deer (0.7)	Rat (0.7)	Rodent (0.6)*	Raccoon (0.8)	Rabbit? (0.6)*
9/19/02	560	Bird (0.6)*	Rabbit (0.7)	Bird (0.7)	Human (0.8)	Human (0.9)
10/3/02	110	Human (0.7)	Rat (0.8)	Deer (0.8)	Rat (0.7)	Deer (0.8)
10/8/02	91	Rodent (0.6)*	Bird (0.8)	Bird (0.8)	Human (0.8)	Rabbit (0.7)
10/16/02 wet rep	2700	Rat (0.8)	Rabbit (0.7)	Raccoon (0.8)	Raccoon (0.8)	Bird (0.6)
10/16/02 wet	3100	Bird (0.55)*	Bird (0.8)	Deer (0.8)	Deer (0.8)	Human (0.8)
10/22/02	29	Rodent (0.6)*	Human (0.9)	Bird (0.8)	Rabbit (0.8)	Rabbit (0.8)

*denotes samples excluded from further analysis

Teal Brook

Results for the two Teal Brook stations are presented in Table 11. Fourteen water samples were collected at two locations in Teal Brook, and a total of 72 isolates were analyzed from these samples. Of those isolates 4 had an SI less than 0.7. Of the remaining 68 isolates, the most prevalent source in Teal Brook was rabbit with 18 isolates (26%). The second and third highest sources were raccoon (14 isolates, 20%) and bird (9 isolates, 13%). Human sources were ranked fourth in Teal Brook with 7 identified isolates (10%).

Table 11. Identification of isolates and similarity index (SI) for Teal Brook locations.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
TBD (Teal Brook and Teal Drive)						
9/16/02 wet	2,000	Bird (0.6)*	Raccoon (0.7)	Raccoon (0.8)		
9/19/02	90	Rabbit (0.7)	Rabbit (0.8)	Bird (0.7)	Rat (0.8)	Rat (0.8)
10/3/02	140	Deer (0.8)	Rabbit (0.8)	Human (0.7)	Raccoon (0.8)	Raccoon (0.8)
10/8/02	32	Rabbit (0.6)*	Raccoon (0.7)	Rabbit (0.8)	Rat (0.7)	Bird (0.8)
10/16/02 wet	160	Raccoon (0.7)	Rabbit (0.8)	Rabbit (0.8)	Rabbit (0.8)	
10/22/02	80	Bird (0.7)	Rabbit (0.8)	Raccoon (0.8)	Raccoon (0.8)	Rabbit (0.8)
TBUP (Teal Brook and Matunuck Schoolhouse Road)						
9/16/02 wet rep	12,000	Raccoon (0.8)	Raccoon (0.8)	Rabbit (0.7)		
9/16/02 wet	5000	Bird (0.7)	Bird (0.8)	Rat (0.8)	Rabbit (0.8)	Bird (0.8)
9/19/02	280	Rat (0.7)	Rabbit (0.8)	Rat (0.8)	Rabbit (0.8)	Rabbit (0.8)
10/3/02	250	Raccoon (0.7)	Rat (0.8)	Rat (0.8)	Human (0.7)	Human (0.8)
10/8/02	140	Human (0.8)	Human (0.8)	Rabbit (0.7)	Raccoon (0.8)	Rabbit (0.8)
10/8/02 rep	200	Rabbit (0.6)*	Bird (0.7)	Bird (0.7)	Rabbit (0.7)	Rabbit (0.8)
10/16/02 wet	8,700	Deer? (0.6)*	Human (0.7)	Human (0.9)	Raccoon (0.8)	
10/22/02	40	Deer (0.7)	Raccoon (0.8)	Bird (0.8)		

*denotes samples excluded from further analysis

Other isolates identified in Teal Brook included rodent (8 isolates, 12%) and deer (3 isolates, 5%). Raccoons were most prevalent in wet weather with 6 isolates out of 19 (32%), followed by rabbit (5 isolates, 26%) and bird (5 isolates, 26%). Other sources during wet weather included human (2 isolates, 10%), rat (1 isolate, 5%), and deer (1 isolate, 5%).

Teal and Factory Brook Cove

Results for Teal and Factory Brook Cove are presented in Table 12. The cove near the mouths of Teal and Factory Brook flow was sampled three times only during wet weather. Twelve isolates were analyzed from these samples and two of those isolates had an SI less than 0.7. Of the remaining 10 isolates, rabbits were the most prevalent source with 5 isolates (55%). The second most common source in this cove during wet weather was human with 2 isolates (20%). Raccoons had 2 isolates (20%), and a single bird isolate was found (10%) during wet weather. The Teal and Factory Brook Cove results are consistent with upstream stations in that rabbits were the predominant species identified as contributing to these areas.

Table 12. Identification of isolates and similarity index (SI) for Teal-Factory Cove.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
Teal Brook – Factory Brook Cove TBF (Cove in Green Hill Pond into which Teal and Factory Brooks flow)						
9/16/02 wet	10,000	Rodent (0.6)*	Rodent (0.6)*			
9/16/02 rep wet	300	Bird (0.8)	Raccoon (0.8)	Rabbit (0.8)	Human (0.7)	Rabbit (0.8)
10/16/02 wet	450	Rabbit (0.8)	Raccoon (0.7)	Human (0.9)	Rabbit (0.8)	Rabbit (0.8)

*denotes samples excluded from further analysis

Unnamed Brook 2

Results for the Unnamed Brook 2 stations are presented in Table 13. Thirty isolates were analyzed from samples taken at the mouth of Unnamed Brook 2 and additional 14 isolates were analyzed at a station in the cove near the mouth of Unnamed Brook 2 during wet weather. Three of the isolates had an SI less than 0.7. Of the remaining 41 isolates the most common source was bird (17 isolates, 42%). Humans were the second most prevalent source in Unnamed Brook 2 with the highest number of human isolates (13 isolates, 32%). Raccoons (5 isolates, 12%) and dogs (5 isolates, 12%) followed in abundance, and a single rabbit isolate was identified (2%).

In wet weather the most common source was human with 9 isolates (39%). Second and third most common sources in wet weather were bird (7 isolates, 30%) and dogs (5 isolates, 22%). The relative abundance of human bacteria is unexpected since few houses exist in this area. It appears that high fecal coliform concentrations are associated with multiple human isolates in a sample. This may indicate a failing septic system in the area. This finding should be investigated further.

Table 13. Identification of isolates and similarity index (SI) for Unnamed Brook 2 stations

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
Unnamed Brook UN2D (just before Unnamed Brook 2 enters Green Hill Pond)						
9/12/02	560	Bird (0.8)	Bird (0.7)	Bird (0.8)	Raccoon (0.8)	
9/16/02 wet	21,000	Dog (0.75)	Dog (0.8)	Human (0.8)	Human (0.8)	Human (0.8)
9/19/02	2,300	Raccoon (0.6)*	Rabbit (0.7)	Human (0.8)	Bird (0.7)	Bird (0.8)
10/3/02	2,700	Bird (0.8)	Bird (0.8)	Bird (0.7)	Raccoon (0.7)	Raccoon (0.8)
10/8/02	9,900	Raccoon (0.6)*	Human (0.8)	Human (0.8)	Bird (0.7)	Bird (0.8)
10/16/02 wet	1,000	Raccoon (0.7)	Dog (0.8)	Bird (0.7)	Raccoon (0.7)	Bird (0.8)
10/22/02	280	Human (0.9)				
UN2 Cove						
UN2 Cove 9/16/02 wet	4,600	Dog? (0.6)*	Dog (0.8)	Dog (0.8)	Human (0.8)	
UN2 Cove 10/16/02 wet	120	Human (0.7)	Bird (0.7)	Bird (0.8)	Bird (0.8)	Human (0.8)
UN2 Cove wet 10/16/02 rep	1,100	Bird (0.7)	Human (0.8)	Human (0.8)	Human (0.8)	Bird (0.8)

*denotes samples excluded from further analysis

Gooseberry Cove

Results for the two Gooseberry Cove stations are presented in Table 14. Gooseberry Cove is located just outside the Unnamed Brook 2 area. Two samples were taken during dry weather, one in the center of the cove and the second near "Swan Point". Five isolates from each sample were analyzed, and one isolate from each sample had an SI less than 0.7. Three of the isolates in the center of the cove came from dogs, and the other isolate came from birds. All of the isolates from the sample near 'Swan Point' came from birds.

Table 14. Identification of isolates and similarity index (SI) for Gooseberry Cove stations.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
Gooseberry Cove						
Gooseberry Cove 10/8/02	290	Bird (0.5)*	Bird (0.8)	Dog (0.8)	Dog (0.8)	Dog (0.8)
Gooseberry Island (at Swan Point) 9/19/02	3,900	Bird (0.8)	Bird (0.8)	Bird (0.8)	Bird (0.7)	Bird (0.6)*

*denotes samples excluded from further analysis

Unnamed Brook 1

Results for the two Unnamed Brook stations are presented in Table 15. Due to unusual drought conditions in 2002 Unnamed Brook 1 was not flowing. A single dry weather sample was taken in the cove near where Unnamed Brook 1 normally enters Green Hill Pond. Three of the five isolates were identified as bird; the remaining two were from dogs. A single wet weather sample was collected in a wooded area where Unnamed Brook 1 normally flows. The water in this stream was primarily coming from the roadway and the sources were the same as the cove with 3 bird isolates and 2 dog isolates.

Table 15. Identification of isolates and similarity index (SI) for Unnamed Brook 1 stations.

Date	Fecal coliform concentration fc/100 ml	Source species and Similarity index (SI)				
		Isolate #1	Isolate #2	Isolate #3	Isolate #4	Isolate #5
Unnamed Brook 1 stations						
UN1 Cove 9/12/02	65	Bird (0.7)	Dog (0.8)	Bird (0.8)	Bird (0.8)	Dog (0.8)
UN1UP 10/16/02 wet weather	11,000	Bird (0.7)	Bird (0.8)	Dog (0.8)	Dog (0.8)	Bird (0.7)

*denotes samples excluded from further analysis

Data overview

A summary of the number of positively identified isolates with a Similarity Index of 0.7 or greater for each station is presented in Table 16. These results indicate that birds were the single largest contributor of fecal coliform to the areas sampled, comprising about 30% of the total. The second highest contributors of fecal coliform were rabbits with about 15.5% of the total. Humans were the third largest contributor of fecal coliform with about 11% of the total. The remaining isolates were raccoon (11%), dog (8%), rat/rodent (6%), and deer (6%). For each sample collected, between one and five isolates were analyzed. The number of isolates analyzed per sample depended upon the number of individual bacteria colonies on the petri dishes prepared and provided by BAL Laboratories. Five isolates were analyzed for most samples.

Table 16. Positively identified isolates at each station with a Similarity Index of 0.7 or greater.

Station	Bird	Dog	Raccoon	Human	Rabbit	Rat/ Rodent	Deer	Unidentified	Total
AC1	15	5	0	1	0	0	1	7	29
AC2	5	2	1	0	0	0	0	2	10
DP06	6	0	2	0	0	0	2	0	10
FBD	7	1	0	0	10	3	4	4	29
FBUP	5	0	3	6	5	4	5	7	35
GB Cove	1	3	0	0	0	0	0	1	5
Gooseberry I	4	0	0	0	0	0	0	1	5
TBD	3	0	8	1	9	3	1	2	27
TBFB	1	0	2	2	5	0	0	2	12
TBUP	6	0	6	5	9	5	1	2	34
UN1 Cove	3	2	0	0	0	0	0	0	5
UN1 UP	3	2	0	0	0	0	0	0	5
UN2 Cove	5	2	0	6	0	0	0	1	14
UN2D	13	3	5	7	1	0	0	2	31
Total	77	20	27	28	39	15	14	31	251
Total Percent	30.7%	8.0%	10.8%	11.2%	15.5%	6.0%	5.6%	12.4%	100%

Several studies have suggested that an SI of 0.8 or greater would provide more confidence in the results. This was suggested by Oscar Pancorbo (pers. comm.) and also described in EPA's Wastewater Technology Fact Sheet on Bacterial Source Tracking (2002). The results, using samples with a similarity index of 0.8 or greater are presented in Table 17. There were 99 isolates with a similarity index of 0.7 or less, leaving 152 isolates with a similarity index of 0.8 or greater. While the total number of isolates changed with this stricter interpretation of the data interestingly, the ranking of the sources did not change and only the individual percent contributions of birds and rabbits decreased significantly. Also, the total percent contribution from wildlife decreased from 39% for an SI of 0.7 or greater to 25% for an SI of 0.8 or greater.

Table 17. Positively identified isolates at each station with a Similarity Index of 0.8 or greater.

Station	Bird	Dog	Raccoon	Human	Rabbit	Rat/ Rodent	Deer	Unidentified	Total
AC1	9	4	0	0	0	0	0	16	29
AC2	4	2	0	0	0	0	0	4	10
DP06	5	0	1	0	0	0	1	3	10
FBD	5	1	0	0	5	3	2	11	29
FBUP	4	0	3	5	2	2	4	15	35
GB Cove	1	3	0	0	0	0	0	1	5
Gooseberry I	3	0	0	0	0	0	0	2	5
TBD	1	0	5	0	8	2	1	10	27
TBFB	1	0	1	1	5	0	0	4	12
TBUP	3	0	5	4	6	4	0	13	35
UN1 Cove	2	2	0	0	0	0	0	1	5
UN1 UP	1	2	0	0	0	0	0	2	5
UN2 Cove	3	2	0	5	0	0	0	4	14
UN2D	7	2	2	7	0	0	0	12	30
Total	49	18	17	22	26	11	8	98	251
Total Percent	19.5%	7.2%	6.8%	8.8%	10.4%	4.4%	3.6%	39.4%	100%

To further summarize the data, samples were separated into four areas, which include Allen's Cove, Teal Brook, Factory Brook and Unnamed Brook 2. The isolates were additionally divided into five categories, which include bird, dog, human, wildlife and unknown. The wildlife category includes raccoon, rabbit, rat, rodent and deer. The results of this summary are presented in Table 18. Birds were the primary source of isolates in Allen's Cove. In Factory and Teal Brooks wildlife dominated the isolate sources. The highest contributor of isolates in Unnamed Brook 2 was birds, however, in this area humans also figured prominently. The number of human isolates found in Unnamed Brook 2 was equal to the total number of human isolates identified in all other areas.

Table 18. Results summarized by location and source species (SI of 0.7 or greater).

Location	Bird	Dog	Human	Wildlife	Unidentified	Total
Allen's Cove	26	7	1	6	9	49
Factory Brook	12	1	6	34	11	64
Teal Brook	9	0	6	42	4	61
Teal Factory Cove	1	0	2	7	2	12
Unnamed Brook 2	18	5	13	6	3	45
Gooseberry Cove	5	3	0	0	2	10
Unnamed Brook 1	6	4	0	0	0	10
Totals	77	20	28	95	31	251

The percentage of samples containing an isolate from a given species was calculated to evaluate the relative consistency of individual source species over time in the different areas of Green Hill Pond. The results are presented in Table 19. Samples containing isolates from birds in wet and dry weather were common in all areas. Wildlife isolates were also common in wet and dry weather samples in Teal and Factory Brooks. Samples containing bacteria from dogs were more common during wet weather in Allen's Cove and Unnamed Brook 2. Samples containing bacterial isolates from humans were most prevalent in Unnamed Brook 2. In Factory and Teal Brooks Human bacteria was present in about a third of wet and dry weather samples.

Table 19. Percent of samples containing a particular species by location (SI of 0.7 or greater).

Location (number of samples/ isolates)	Bird		Dog		Human		Wildlife	
	Total percent	% wet and dry samples	Total percent	% wet and dry samples	Total percent	% wet and dry samples	Total percent	% wet and dry samples
Allen's Cove (12/41)	92 %	100% w	42%	57 % w	8 %	0 % w	33 %	43 % w
		80 % d		20 % d		20 % d		20 % d
Teal Brook (14/58)	42 %	20 % w	0%	0 % w	29 %	20 % w	100 %	100% w
		56 % d		0 % d		33 % d		100% d
Factory Brook (14/53)	57 %	60 % w	7%	11 % w	29 %	20 % w	93 %	100%w
		56 % d		0 % d		33 % d		89 % d
Unnamed 2 (10/41)	70 %	60 % w	30%	60 % w	70 %	80 % w	40 %	20 % w
		80 % d		0 % d		60 % d		60 % d

3.5 Evaluation of data quality

Fecal coliform

Four replicate samples were collected to assess data quality during the study period. These four samples represent six percent of the total number of unknown samples taken. BAL laboratories also analyzed a laboratory split sample for station AC1, sampled on October 16, 2002 and at AC2 on September 16, 2002. The precision of the mTEC membrane filtration technique for fecal coliforms is 35% at the 95% confidence interval (Rippey et al., 1987). Replicates collected in this study were compared to the confidence interval criteria mentioned above using a 95% confidence interval of 35% above and below the mean of the two values to assess data precision. The results are presented in Table 20. All of the replicates fell within their respective confidence interval except for UN2 Cove, indicating that the data are of adequate quality.

Table 20. Confidence intervals for the replicate data collected in Green Hill Pond.

Station	Initial fecal coliform concentration fc/100 ml	Replicate fecal coliform concentration fc/100 ml	Mean fecal coliform concentration fc/100 ml	95% Confidence interval fc/100 ml	Does the data fall within the confidence interval?
TBUP	140	200	170	111 – 230	Yes
FBUP	2700	3100	2900	1885 – 3925	Yes
DP06	9000	6700	7850	5103 to 10598	Yes
UN2 Cove	44	120	82	53 – 111	No
AC2	60	80	70	46 – 95	Yes
AC1	23	27	25	16 – 34	Yes

Bacterial source identifications

The same four replicates described above were used to assess the consistency of the bacterial source identification results. Those same samples were also analyzed using PCR. The agreement between each original sample and its replicate sample are compared in Table 21. The animal sources that the two samples had in common were determined and a percent agreement was calculated by dividing the number of isolates in common by the total number of isolates. This ratio ranged between 30% to 100%. The average percent agreement was 65%. The results may reflect the level of precision inherent in the PCR analysis. The agreement may also indicate that fecal coliform concentrations at some stations reflect the influences of many animal species and the consistency between replicate samples would be improved by increasing the numbers of isolates analyzed per sample.

Table 21. Percent agreement for bacteria source identification results.

Station	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Percent agreement
DP06	Deer	Deer	Bird	Bird	Raccoon	80%
DP06 rep	Bird	Bird	Raccoon	Bird	Bird	
FBUP	Rat	Rabbit	Raccoon	Raccoon	Bird	30%
FBUP rep	Bird	Bird	Deer	Deer	Human	
TBUP	Human	Human	Rabbit	Raccoon	Rabbit	50%
TBUP rep	Rabbit	Bird	Bird	Rabbit	Rabbit	
UN2 Cove	Human	Bird	Bird	Bird	Human	100%
UN2 Cove rep	Bird	Human	Human	Human	Bird	
Mean percent agreement						65%

Representativeness

The sampling of tributaries and the pond resulted in the collection of four to five dry weather and two to three wet weather water samples for each location. Typically five isolates were collected from each water sample collected. With the samples aggregated by animal groups for the four areas in Table 19, the characterization of source species is based on the analysis of between 43 and 58 isolates in 10 to 14 water samples in each area.

The use of molecular methods to identify source species is rather new. Protocols have not been well established for determining the appropriate number of samples and isolates, as well as the amount of sampling needed to determine the confidence that could be assigned to results such as those stated in this report. To provide some insight on the representativeness of the results, we surveyed methodologies used in recent studies of a similar nature and interviewed researchers active in the field. A comparison between descriptions of this and similar studies is presented in Table 22. The parameters used to assess confidence in a study were considered to be the size and complexity of the study area, the number of isolates per water sample, the number of isolates per station or area, and the number of samples collected per station.

As shown in the table, other studies have typically analyzed a greater number of isolates per water sample than the present study. For instance, 20 isolates per sample were analyzed for the Charles River and about 10 isolates per sample were analyzed for two estuaries in Maine. A larger number of isolates per sample is desirable from the standpoint that the diversity of the sources would more accurately emerge. When the number of isolates is increased in a sample with bacteria from a number of host species, the results would be expected to better reflect that diversity. It is possible that by using a lower number of isolates, the results could be biased by the small sample set, giving an inaccurate percentage of isolates attributable to a particular host species.

DEM contacted Dr. Oscar Pancorbo, Director of the Wall Experimental Station in Lawrence, MA, who is active in the Bacterial Source Tracking field and was one of the principal investigators of the Charles River study. As Table 22 shows, the Charles River study was relatively intensive from the standpoint of the number of samples per station and the number of isolates per sample. Dr. Pancorbo pointed out that the DEM study of Green Hill pond did have a relatively high number of water samples representing each station or area, and hence a relatively high number of bacterial isolates upon which to base its findings for each area. Dr. Pancorbo also pointed out that using a smaller number of isolates for each sample is counterbalanced by the greater number of samples collected and analyzed from each area. To some extent, the representativeness of using a greater number of

samples (hence isolates) for each station compensates for the use of fewer isolates selected from each 100 ml sample. The characterization provided above in section 3 is therefore believed to be comparable to that provided in other studies.

It may also be important to consider the scale of the watershed in which these studies are taking place. In many cases the proportions of number of samples collected and isolates analyzed are very different than in the Green Hill Pond Study. For instance in Maine, two watersheds were sampled. The Webahannet watershed is 25 square miles in size and the Little River watershed is 48 square miles. A total of 300 isolates were collected from each watershed. In New York, 489 total isolates were collected from four watersheds totaling 2395 square miles. The Green Hill Pond watershed is 6 square miles and 250 isolates were analyzed. The Four Mile Run study in Virginia is similar with a watershed area of 19.7 square miles and 439 isolates analyzed. The Green Hill Pond study was focused in a relatively small, discrete area, so the source-unknown relationship is expected to be more direct.

Another strength of the present study is the amount of site-specific information provided by the scat surveys. Peter Hartel, a professor at the University of Georgia has investigated temporal and spatial variability in isolate ribotypes and believes that variability is affected by both time and geography (Hartel et al., 2003). In one study, Hartel compared ribotypes from farm animals in two locations in Georgia and one location in Idaho. Hartel found that ribotypes could distinguish between animal species but ribotypes from different geographic areas varied within species too much for libraries from different areas to be effective (Hartel et al. 2002). In another study Hartel, focused on the diets of penned and wild deer and determined that diet affected the diversity of ribotypes in these animals (Hartel, Summer and Segars, 2003). Hartel's work suggests that many factors affect the ribotypes present in a given species in a given area at a given time. The best way to limit the variability of the library is to collect as many known samples as possible in the same area and at the same time as the study.

As Table 22 shows, 70 reference samples were collected from the immediate survey area in Green Hill Pond. This number compares favorably with the numbers used elsewhere. For example the study in Maine was conducted during the winter, and researchers found it difficult to obtain viable isolates from the 10 known samples they collected (C. Dalton, pers. comm.). In the New York study, 57 known samples were collected. In the Four Mile Run study 94 known samples were collected. In the Green Hill Pond study 70 known source samples were collected which was approximately the same as the number of water samples collected (58).

DEM believes that the sources identified during this study provide an adequate representation of the bacteria sources present in the Green Hill Pond area during the study period. It is possible, however, that other sources present in the watershed were not identified during this study. The possible reasons for a non-identification of a source could be that not enough isolates were analyzed from each sample, or that 2002 was a drought year affecting possible groundwater inputs to the pond. Because this was a discrete study that took place during two months in 2002, it is also possible that the bacteria analyzed may not be representative of the bacteria sources to the pond during the entire year.

Many researchers across the country are working to perfect microbial source tracking techniques. For Green Hill Pond, additional information would be desirable. A follow-up study would help to check the repeatability of DEM's findings.

Table 22. Summary of other bacterial source tracking projects.

Project location	Method used	Watershed area mi ²	Isolates per sample / total isolates	Number of samples / stations	Number of source samples
Green Hill Pond	RAPD-PCR	6	5 / 250	58 / 4 areas	70
Charles River ¹	REP-PCR (Pancorbo)	308	20 / 800	120 / 19	Unknown
Webhannet Estuary ²	Ribotyping (Steve Jones)	25	10 / 300	20 / 20 (not all used for BST analysis)	10
Little River Estuary ²		48			
New Croton Reservoir ³	Ribotyping (M. Samadpour)	360	Up to 5 / 489 total isolates	234 / 4 (outflow of each reservoir)	57
Delaware Reservoirs ³		569			
Catskill Reservoirs ³		1012			
Cannonsville Reservoirs ³		454			
South Carolina ⁴	Multiple Antibiotic Resistance	19	unknown / 198	Unknown / 23	123 (isolates from sewage)
Four Mile Run, Virginia ⁵	Pulsed Field Gel Electrophoresis	19.7	~8-10 / 439	55 / 31	94
Source Molecular Corporation ⁶	Ribotyping	Depends on project	5-8	Depends on project	Provide source sample
Microbio Services ⁷	Pulsed Field Gel Electrophoresis, Ribotyping	Depends on project	5-8	Depends on project	Provide source sample

¹Oscar Pancorbo, Director Wall Experimental Station, Lawrence, MA. Personal communication.

²Cayce Dalton, Maine Sea Grant. Personal Communication. www.umseagrant-mst.org

³Water quality monitoring in source water areas for New York City: And integrative watershed approach. A report on the first year of monitoring. www.strodcenter.org

⁴Kelsey, R. H., G. I. Scott, D. E. Porter, B. Thompson and L. Webster. 2003. Using Multiple antibiotic resistance and land use characteristics to determine sources of fecal coliform bacterial pollution. Environmental Monitoring and Assessment. 81:337-348.

⁵Simmons, G. E., Jr., D. F. Wayne, S. Herbein, S. Myers, E. Walker. 2002. Estimating nonpoint source fecal coliform sources using DNA profile analysis. In (T. Younos, ed.) Advances in Water Monitoring Research, pp. 143-168. Water Resources Publications, LLC. Denver, CO.

⁶Source Molecular Corporation, Miami, FL. Commercial service. www.sourcemolecular.com

⁷Microbioservices, Gainesville, FL. Commercial service. www.microbioservices.com

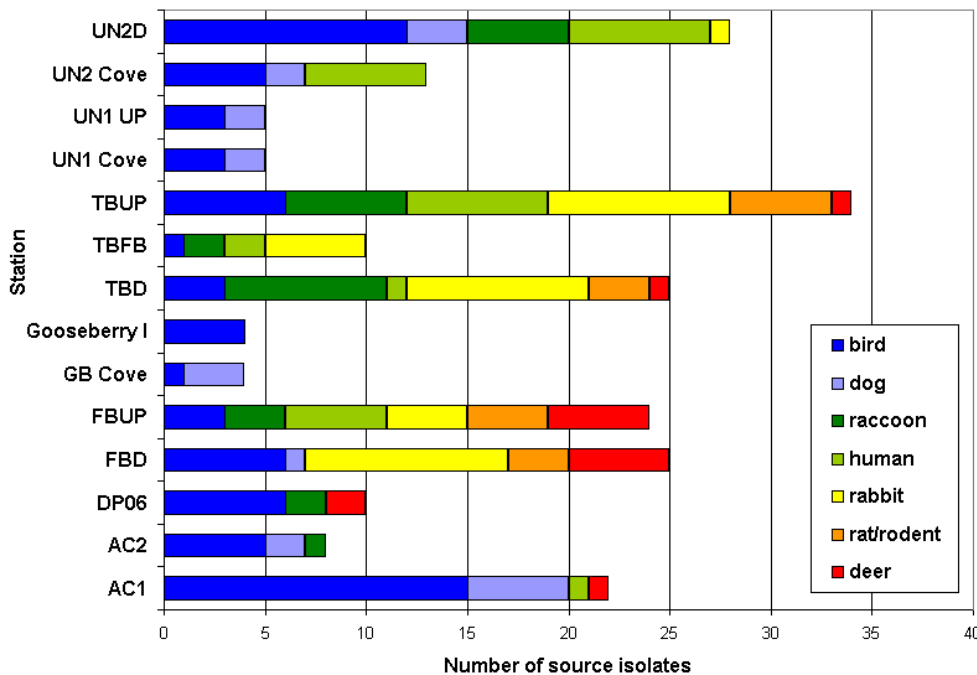
4.0 DISCUSSION

Of the 220 isolates analyzed with a similarity index of 0.7 or greater from the Green Hill Pond watershed 87% were from waterfowl, wildlife, or pets. Waterfowl and wildlife comprised 80% of the total number of isolates identified. These categories specifically included birds, raccoon, otter, rabbit, rat, rodent, and deer. Bacteria from humans represented about 11% of the total isolates analyzed and domestic pets (dogs) accounted for about 8% of the isolates analyzed.

Several other recent studies found similar results to those found here. A study in Tampa Bay, Florida using Multiple Antibiotic Resistance analysis (MAR) found that 73.6% of analyzed *E. coli* isolates were identified as non human (Rose, et al., 2001). In Virginia’s Four Mile Run the majority (83%) of *E. coli* isolates were determined, using Pulsed Field Gel Electrophoresis to be nonhuman as well (Simmons et al.). In the Four Mile Run study human isolates were found in only localized areas. One third of the isolates identified in Four Mile Run were of bird origin. In Murrells Inlet, South Carolina, another MAR study found that 97% of surface waters contained bacteria from non-human sources (Kelsey et al, 2003).

In the Green Hill Pond study, the source species varied at different stations in the pond. The breakdown by species identified at each station is presented in Figure 3. The percent contributions of these sources in the sample areas of Green Hill Pond are presented in Figure 4. For example, rabbits, rats and other rodents were found primarily at the lower Factory and Teal Brook stations. The wooded wetlands and open grassy areas in these watersheds provide ideal habitat for these types of animals. DEM staff also noted that rabbit and deer scats were more abundant in those areas. This representation is simplified

Figure 3. Bacteria sources identified at all Green Hill Pond stations (SI of 0.7 or greater).

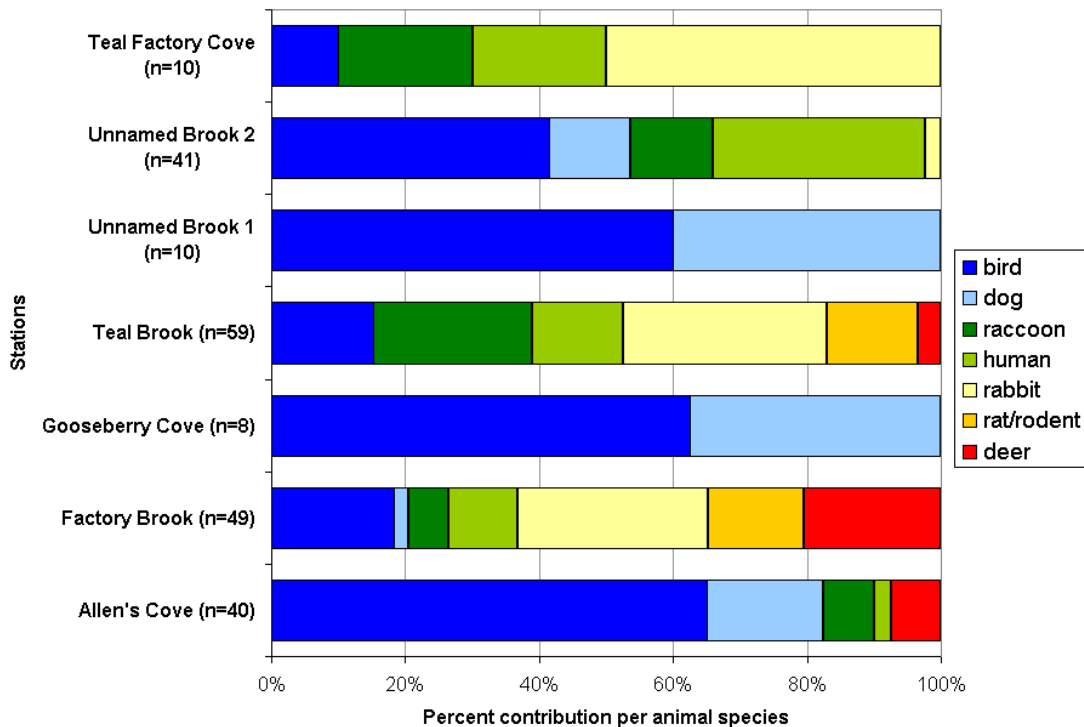


and shown spatially in Figure 5, where deer, rats and rodents are grouped into the wildlife species category.

Humans factored significantly in samples taken at the Factory, Teal, and Unnamed Brook 2 stations. Seven of the ten samples (70%) collected in the Unnamed Brook 2 area contained human bacteria (Table 18); about a third of the isolates were identified as human. The human contribution in Unnamed Brook 2 is interesting because the majority of its watershed is wetland or wooded wetland and contains few houses. This area should be investigated further. Human bacteria were significant at the upstream station in Factory Brook, where five of seven samples contained human bacteria. This result is also interesting because like Unnamed Brook 2, the watershed is wetland and wooded wetland and contains few houses. It is certainly possible that one or more houses in each area has a failing septic system. At the time of sampling, one known failing septic system was located along Teal Brook adjacent to station TBUP, where three of seven samples contained human bacteria. The significant human signal in water samples at TBUP are assumed to be due to that failing system. Plans are underway to replace this failing system with a new and innovative septic system.

Bacteria from birds were found at stations throughout the study area, but the highest contributions were seen in Allen’s Cove and in the Unnamed Brook 2 area (Figure 5). Large Canada goose and mute swan populations reside in Green Hill Pond, congregate in the coves and on lawns, and contribute to the fecal coliform levels in these areas. The wooded and wetland areas also provide habitat for a variety of smaller birds, which can also contribute bacteria to the pond. Bird feces can reach the pond in two ways. The first mechanism is direct deposition on surface waters or along the intertidal zone where feces are subsequently immersed on the following high tide. A second

Figure 4. Percent contribution of sources to Green Hill Pond areas (SI of 0.7 or greater).



pathway for bird droppings is through deposition on land, on vegetation in upland or wetland areas, or on impervious surfaces such as roofs and roads, where they are subsequently washed into surface waters via overland flows and carried to the pond.

Dog bacteria were found at all stations except those in Teal Brook. Numerous dogs reside in the area, as ample quantities of dog scat were available for sampling by DEM staff. Rabbits also contributed significantly to the bacteria in Factory and Teal Brooks. The lower Teal and Factory Brook subwatersheds had large areas of grass and shrubs that provide habitat for rabbits, rats, and other rodents. DEM staff also found the feces of deer, raccoons and rabbits in these areas. Deer figured most prominently in Factory Brook, which has the largest wooded area of all the areas sampled.

The majority of bacteria analyzed during this study in Green Hill Pond came from animal sources. Some of these sources can be addressed by public education and by mitigating stormwater runoff. Runoff from impervious sources is considered controllable and should be reduced to the maximum extent practicable. There are a number of things the general public can do to help. Dog owners should pick up dog waste whenever they walk their dogs. Educating the public to not feed birds can help reduce the nuisance waterfowl populations and thus, the contribution from birds. Bacteria from birds are controllable to some extent. They can be further reduced by creating buffer strips along waterfront properties to discourage Canada geese from congregating and grazing on lawns. The populations of geese and swans can be controlled through hunting. The swan population can also be reduced by adding eggs.

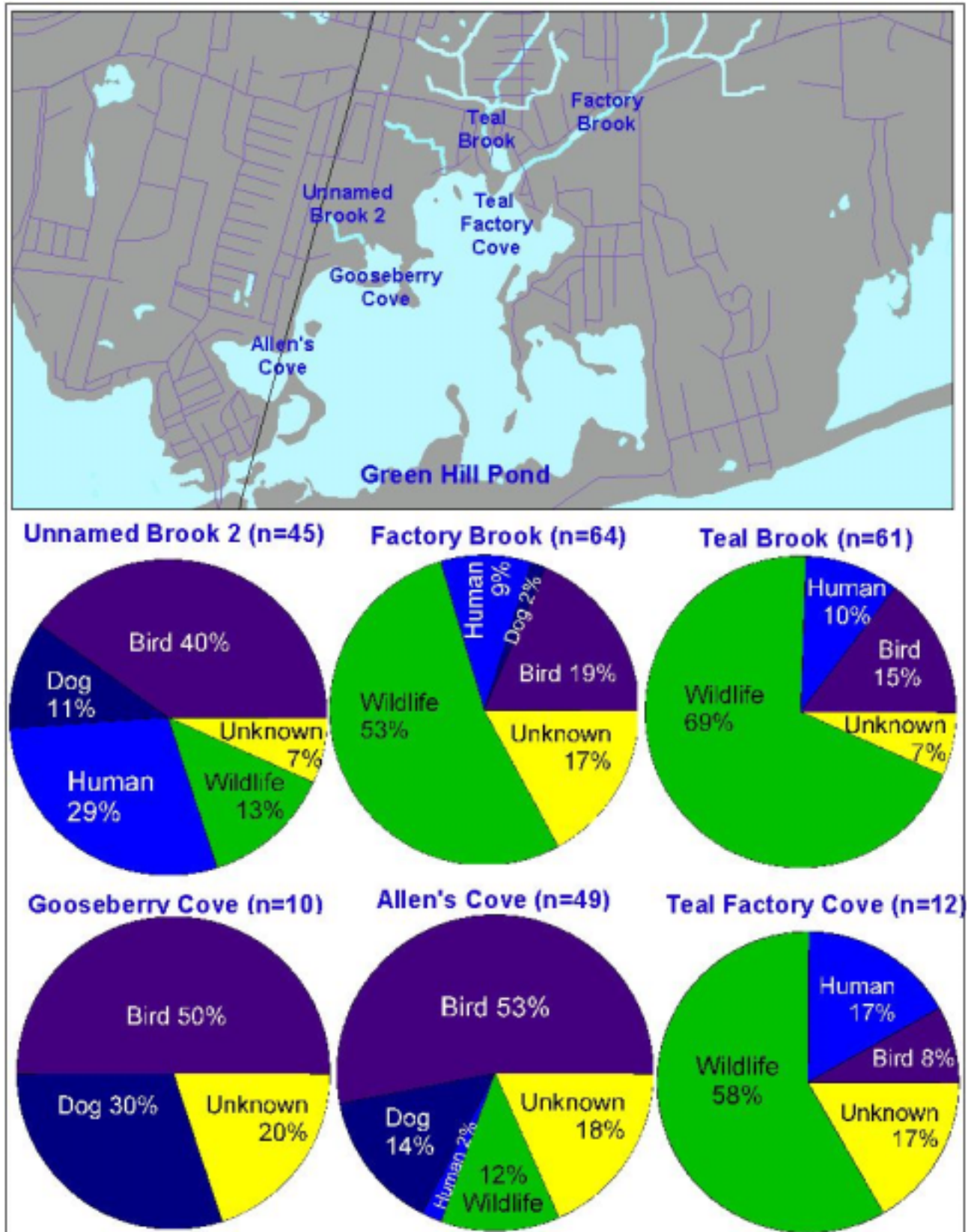
Human sources most likely enter the streams from failing septic systems. One stream in particular, Teal Brook, was sampled just downstream of a known ISDS failure, and the results indicate that human bacteria were present in the stream. Failures in Individual Septic Disposal Systems, or ISDSs can occur for several reasons. Some of the more common failures occur when the soils around the system become clogged due to overloading causing untreated sewage to surface. Problems similar to these have been documented in the Green Hill Pond area.

Human bacteria must be controlled. Several programs are already underway in the Green Hill Pond watershed that will address contributions of human bacteria entering the pond. There is an inspection program underway in South Kingstown where all the septic systems in the town will be inspected. This inspection program will help to identify poorly functioning or failing septic systems so that improvements can be made. The Environmental Protection Agency and the University of Rhode Island have a joint program installing new and innovative septic systems in the Green Hill Pond area and on Block Island. Many new systems have already been installed and several more are planned.

Conclusions

This study has shown that Polymerase Chain Reaction can be a useful tool in identifying sources of bacteria in small coastal areas. The results appear to be reasonable given the potential sources are relatively consistent within each area and relate well to land use in the area. For example, samples from streams (Factory and Teal Brooks) flowing through wooded wetlands populated by wildlife, were dominated by bacteria from wildlife. Human bacteria were found at a station a short distance

Figure 5. Most prominent species found in Green Hill Pond.



from a known and sampled failing septic system. In areas (Allen's and Gooseberry Coves) where Canada geese and mute swans congregate and were observed during the study, the majority of isolates were from birds.

More investigation is needed to elucidate the findings for some areas. Unnamed Brook 2, had the highest percentage of human bacteria, however the area has few homes. At the upstream station in Factory Brook human bacteria were found at a similar percentage as in Teal Brook, however no septic systems exist near the station.

This study compares favorably to other attempts at using molecular or phenotypic methods to determine the source of bacteria. Although fewer isolates were collected per sample relative to other studies, we believe a sufficient number of samples were collected and a similar or greater number of isolates were analyzed per station. One strength of the study was its spatially and temporally relevant source library collected during the study. Another strength is that the size and complexity of the study area was also relatively small when compared to those of other studies. We encourage a follow-up study in this area to help validate the findings of this study and confirm the sources of bacteria to Green Hill Pond.

DEM believes that the sources identified during this study provide an adequate representation of the bacteria sources present in the Green Hill Pond area during the study period. DEM recommends using BST techniques during non-drought conditions and at a time of high groundwater and/or high domestic water use (Study conducted Sept- Oct 2002) to further evaluate potential septic system influences on the pond. In the meantime, the study results provide sufficient justification for initiation and continuation of efforts to control bacteria sources to the pond. Though less prevalent than waterfowl and wildlife species, any indication of human borne bacteria is unacceptable. The efforts of South Kingstown and Charlestown to ensure the proper functioning of all septic systems through establishment of wastewater management districts should be continued. Additionally, the study results indicate that greater attention to pet waste clean up and management/control of nuisance waterfowl and wildlife populations is necessary to reduce bacteria concentrations in Green Hill Pond. A more thorough discussion of implementation measures addressing ways of reducing and eliminating sources will be available in the Green Hill Pond TMDL.

5.0 REFERENCES

- Environmental Protection Agency. 2002. Wastewater Technology Fact Sheet: Bacterial Source Tracking. Environmental Protection Agency: Office of Water. EPA 832-F-02-010. 11pp.
- Cayce Dalton, Maine Sea Grant. May 1, 2003. Personal Communication. www.umseagrant-mst.org
- Chikarmane, H. M. 2003. *E. coli* Source Identification Report of the Rhode Island Department of Environmental Management.
- Hartel, P. G., A. L. Funk, J. L. Hill, J. D. Summer, E. A. Frick, and M. B. Gregory. 2003. Variability of *Escherichia coli* ribotypes in a complex watershed during varied flow conditions. Water Resources (in press).
- Hartel, P. G., J. D. Summer, J. L. Hill, J. V. Collins, J. A. Entry and W. I. Segars. 2002. Geographic variability of *Escherichia coli* ribotypes from animals in Idaho and Georgia. Journal of Environmental Quality. 31:1273-1278.
- Hartel, P. G., J. D. Summer, and W. I. Segars. 2003. Deer diet affects ribotype diversity of *Escherichia coli* for bacterial source tracking. Water Research (in press)
- Kelsey, R. H., G. I. Scott, D. E. Porter, B. Thompson and L. Webster. 2003. Using Multiple antibiotic resistance and land use characteristics to determine sources of fecal coliform bacterial pollution. Environmental Monitoring and Assessment. 81:337-348.
- Pancorbo, Oscar. Division and Station Director, Wall Experiment Station, Massachusetts Department of Environmental Protection. April 23, 2003. Personal communication.
- Rippey, S. R., W. N. Adams and W. D. Watkins. 1987. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach. Journal Water Pollution Control. 59:8 795- 798.
- Rose, J. B. J. H. Paul, M. R. McLaughlin et al. 2001. Healthy Beaches: Tampa Bay: Microbiological monitoring of water quality conditions and public health impacts. Final Project Report.
- Simmons, G. E., Jr., D. F. Wayne, S. Herbein, S. Myers, E. Walker. 2002. Estimating nonpoint source fecal coliform sources using DNA profile analysis. In (T. Younos, ed.) Advances in Water Monitoring Research, pp. 143-168. Water Resources Publications, LLC. Denver, CO.
- Stroud Water Research Center. 2001. Water quality monitoring in the source water areas for New York City: An integrative watershed approach. A report on the first year of monitoring. Contribution No. 2001007. Submitted to NY Department of Environmental Conservation. www.strodcenter.org.u