

Fecal Coliform Testing and DNA-Based Microbial Source Tracking in the Three Bays Estuarine System

Summary Report for the 1999 and 2000 Field Season

Three Bays Preservation, Inc.
Post Office Box 215
Osterville, MA 02655-0215

www.3bays.org



Spring 2001

TABLE OF CONTENTS

	Page #
Introduction	3
Sampling and Testing	
Results	4
Fecal Coliform Testing Results – Year 1999	
Fecal Coliform Testing Results – Year 2000	
DNA Microbial Source Tracking – Year 2000	
Summary	7
Figures	
Figure 1 – Fecal Coliform Data – Years 1999 and 2000	5
Figure 2 – Results of DNA-Based Microbial Source	6
Tracking – Year 2000	
Appendices	
Appendix A – Technical Approach and Methods for	10
Microbial Source Tracking	
Appendix B – Laboratory Procedures	12

**Summary of Fecal Coliform Testing and DNA-Based Microbial Source
Tracking
In the Three Bays Estuary for the Years 1999 and 2000**

INTRODUCTION

In addition to the measurement of nitrogen in the Three Bays system, our 1999 Water Quality Monitoring Program included sampling for fecal coliforms. Numerous human pathogens are spread by fecal contamination of water. These pathogens can be a risk to human health even at very low concentrations. Monitoring the level of indicator organisms, such as fecal coliforms, in water is used to assess the potential for the presence of organisms. The internationally accepted limit for bathing and other human water contact activities is 200 organisms per 100 ml (about 3.5 ounces). In the case of waters from which shellfish may be harvested for human consumption, the limit is 14 per 100 ml. The level of fecal coliforms in water is called the "coliform count". It is generally agreed that human fecal contamination is significantly more threatening to human health than contamination from animals.

Our 1999 sampling program revealed a number of alarmingly high summer and early fall coliform counts in the Marstons Mills River, Warren's Cove and Prince's Cove. In the past, high coliform counts measured by regulatory agencies in these same waters have been attributed to waterfowl and other wildlife. However, the pattern and timing of the 1999 samples indicated a significant potential for human-produced fecal contamination.

Until recently there has been no way to differentiate between human and animal fecal contamination measured in water samples. Knowing which source is the problem enables water quality management efforts to be more effective by directing source control efforts, especially those involving human sources, to where the problem is. Potential human sources include storm runoff, failing septic systems, and improperly operated boat toilet systems.

Early in the Year 2000, Three Bays Preservation learned that the University of Washington Department of Environmental Health had developed analytical procedures using DNA typing to determine the source of the coliform bacteria by identifying the genetic fingerprints of the contributing organisms. These procedures have been successfully used in a wide variety of applications to demonstrate their validity. In the Year 2000 Three Bays' monitoring program, this approach was used to determine the sources of the fecal pollution in the Marstons Mills River and northern areas of the estuary.

This document has been prepared to summarize the results from that part of the monitoring program that is focused on microbial contamination. For brevity and ease of understanding, the information is presented in a bulleted format.

Sampling and Testing

- Approximately 100 water quality samples were tested for fecal coliforms in 1999 and more than 200 in 2000.
- Of the fecal coliform tests performed in the year 2000, more than 70 of the water quality samples were subjected to DNA fingerprinting to identify the species from which the coliforms originated. Most of these tests were performed on samples taken in the northern waters of the estuary from Middle Pond to North Bay because of the higher coliform levels observed there in 1999.
- To determine the DNA patterns of those animals living in the area, approximately 110 stool samples were collected by a number of volunteers and shipped to the DNA testing laboratory. Human DNA characteristics were derived from a number of wastewater samples taken at the Hyannis Water Pollution Control Facility.

RESULTS

Fecal Coliform Testing Results-Year 1999

Our 1999 sampling program revealed a number of alarmingly high summer and early fall (July to October) coliform counts in the Marstons Mills River, Warren's Cove and Prince's Cove, as follows:

- Of 8 samples taken in Prince's Cove, 3 were above 200 the highest being 410.
- Of 13 samples taken in the Marstons Mills River, 7 were above 200. Three of these were over 500.
- Of 4 samples taken in Warren's Cove, all were over 200 and 2 were over 1000. Although not above 200, counts of 100 and 130 in North Bay were also of concern.
- Results from West Bay, Cotuit Bay, Eel, and Seapuit Rivers were generally good, averaging below 15 with very infrequent values of 30 or 40.

Fecal Coliform Testing Results-Year 2000

Tidal Stations (Prince's Cove to Nantucket Sound)

- Average counts for the South (inner) end of Prince's Cove were about the same in 2000 as they were in 1999. However, the maximum value in 2000 was considerably higher than in 1999 (420 vs. 260—both in August).
- Average and maximum counts for Prince's Cove North (about 100 yards offshore from Prince Cove Marina) were about twice as high in 2000 than in 1999. Four tests were over 200 and 2 over 800, with a maximum of 950.
- The results from Warren's Cove were again alarmingly high in 2000 as in 1999. The average count was 600 with a maximum of more than 2600.
- Coliform counts in the northwest end of North Bay were considerably improved in the year 2000, showing about a 50 percent reduction in both average (25 vs. 50) and maximum (70 vs. 130) coliform values.
- Based on the great difference between the Warrens Cove results and those from the northwest end of North Bay, significant coliform die-off must be occurring in the channel between these two sampling locations.
- All other stations (West Bay, Cotuit Bay, Eel and Seapuit Rivers) were equally good in 2000 as in 1999.

Marstons Mill River Stations (Mystic Lake to Mill Pond)

- Mystic Lake and Middle Pond were slightly higher in 2000 than in 1999.
- The three stations in the river proper between Middle Pond and Mill Pond (at the lower end of the Herring Run, at River Road, and at the site of the Old Mill) were 50 to 75 percent lower in 2000 than in 1999. Perhaps this was due to greater rainfall in 2000.

A graphical presentation of all fecal coliform counts for the Years 1999 and 2000 for all of the sampling stations in the Three Bays Estuary is presented in Figure 1.

DNA Microbial Source Tracking – Year 2000

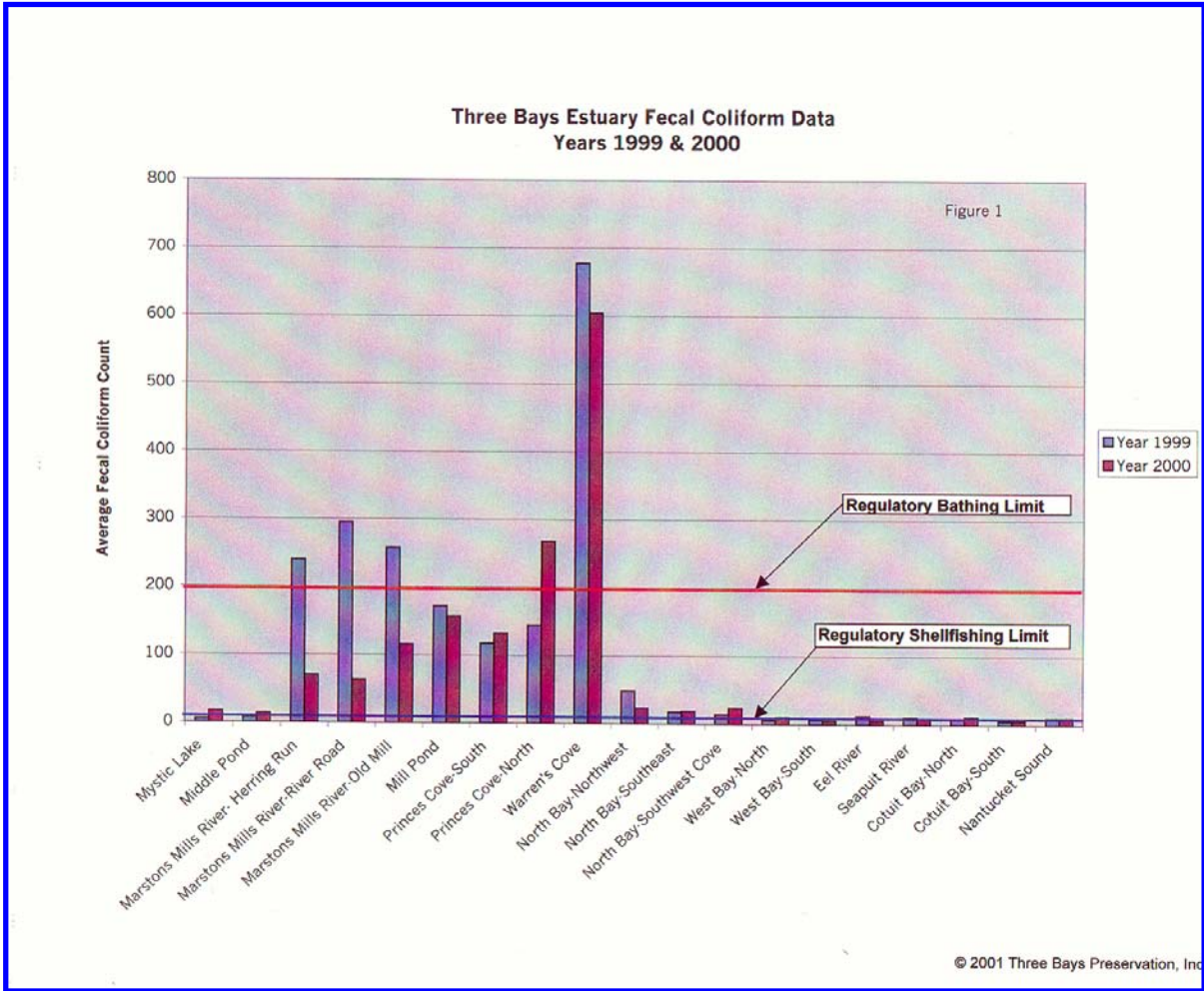
- DNA-based examinations of fecal coliform colonies revealed a wide variety of animal sources including humans, a variety of birds especially geese and gulls, and a wide variety of four-legged animals ranging from dogs, cats and horses to foxes, rodents, deer and raccoons.
- Two of the sampling stations, Prince's Cove North (near the marina) and the station at the opening to Warren's Cove, exhibited extremely high percentages of human fecal coliforms (44 percent and 53 percent, respectively). This is clear confirmation of our position that the high counts are due to humans and not birds.

- As the samples taken at Prince Cove South contained only 20 percent human coliforms compared to 44 percent at the north end, it is likely that the reason for the high coliform counts at the north end is due to human wastes from moored or docked boats, homes, and/or the marina.
- Results from the north end of Warren's Cove are of relatively low human origin (17 percent) yet the south end is 53 percent. As there are but 1 or 2 boats moored in the Cove large enough to have their own toilets, it appears that the extremely high coliform counts observed at the south end must originate from one or more of the septic systems that border the cove, and less significantly from the avian component (42 percent).
- Relatively high human percentages of about 30 percent were identified at the southeast end of North Bay and at the River Road sampling station on the Marstons Mills River.
- The human component was present in all other stations sampled and ranged from 10 to 20 percent.

The results of the DNA testing program are presented in Figure 2.

SUMMARY

- From a fecal coliform and human component standpoint, the most highly contaminated stations in the estuary are the Prince's Cove North (marina area) and Warrens Cove (south end) stations. The numerical data for these locations in both 1999 and 2000 are significantly above regulatory public health limits. Summertime body contact activities in these locations should be avoided.
- The high coliform counts in both locations coupled with the high human component are urgent matters that need to be approached immediately by health officials and by home and boat owners in the immediate area to identify and remediate the human sources.
- Three Bays Preservation staff has presented the data, conclusions, and recommendations summarized in this Report to the Town's Coastal Health Resource Coordinator, Public Health Division. The Town plans to conduct a detailed Sanitary Survey of the entire Three Bays shoreline in the summer of 2000. Weekly coliform sampling adjacent to the Princes Cove boat-launching ramp is also planned.
- Three Bays Preservation will continue to perform coliform testing in North Bay, Prince's Cove, Warren's Cove, and the Marstons Mills River, along with appropriate DNA analyses. We will also assist the Town with immediate notification of high coliform results, as well as with the implementation of the new No-Discharge regulations for the Three Bays.



APPENDICES

APPENDIX A

TECHNICAL APPROACH AND METHODS for MICROBIAL SOURCE TRACKING

INTRODUCTION

Numerous human pathogens are spread by fecal contamination of water. Examples are *Vibrio cholera*, *Salmonella typhi*, *Giardia lamblia*, *Cryptosporidium parvum* and Hepatitis A. These pathogens can be a risk to human health even at very low concentrations. Due to difficulties in the detection, identification, and enumeration of specific human pathogens in environmental and food samples, the concept of indicator organisms and related methodologies were developed and implemented in the late 1800's. Indicator organisms are used to assess the potential for the presence of pathogens. These organisms must be prevalent in feces, found in higher concentrations than pathogens, be more resistant to disinfectants (more persistent in the environment), and easy to quantify. The group of bacteria referred to as fecal coliforms meet these criteria. A formal definition of this group is that they are facultatively anaerobic bacilli that ferment lactose with the production of gas within 48 hours at a temperature of 44.5 deg C. A prevalent and well-studied member of this group is the species *Escherichia coli* (E. Coli).

The concept of indicator organisms is the principal component of regulatory microbiology. The major limitation of this concept is that it is an oversimplification of the complex dynamics of microbial ecology, physiology, and genetics. It is true that often the presence of indicators can be associated with fecal contamination. However, it is also true that in many instances there may be little or no correlation between the presence of indicator organisms and the presence of fecal contamination and human pathogens.

The utility of the indicator concept is further limited by the lack of appropriate methodologies for tracking organisms associated with contamination to their potential sources. Sources of water pollution can be divided into two general groups, point and non-point sources. Point sources of pollution have defined discharge points such as pipes-- municipal and industrial wastewaters for example. Non-point sources of pollution do not have defined discharge points. Because of their diffuse nature, nonpoint sources are difficult to identify and control. Nonpoint sources of microbial pollution include wildlife, agricultural practices, on-site septic systems, commercial and recreational boating, aquaculture, and industrial practices. This impediment to the identification and control of sources of microbial pollution in water adversely affects the deci-

sion-making process of water quality and fisheries resources management.

Each year millions of dollars are spent on fecal and total coliform assays to determine the extent of bacterial pollution in water environments, and to satisfy increasingly rigid regulatory requirements concerning microbiological quality of water. Knowing the sources rather than just monitoring the level of pollution enables water quality management efforts to be more effective by directing source control measures where the greatest problem is. Although there are human pathogens associated with fecal pollution of animal origin, the risk to human health would presumably be greater if contamination is caused primarily by human sources (mainly due to presence of human viruses). For these reasons, there is a need for the Microbial Source Tracking method described below to be used along with conventional analysis to more fully understand and address a bacterial pollution problem.

Microbial Source Tracking

In response to limitations of conventional methods, the Microbial Source Tracking methodology was developed by Dr. Mansour Samadpour of the Department of Environmental Health at the University of Washington (Samadpour, 1990). MST can be summarized in two steps. The first step is the molecular characterization of strains of the study organism, in this case *E. coli*, by DNA fingerprinting, specifically referred to here as ribotyping. Secondly, ribotypes of *E. coli* strains isolated from potential sources are matched with the ribotypes of strains isolated from receiving water to determine the extent and distribution of each sources contribution to contamination.

The data resulting from an MST analysis can be used in: understanding the sources, distribution, and movement of microbial populations in the environment.

- conducting risk and exposure assessment studies of the potential human effects associated with microbial pollution
- design and implementation of source controls
- studying the effects of control measures
- environmental litigation

Definitions

An *isolate* is a pure culture of bacteria established from a source using sterile technique and appropriate growth media. The intent is that the culture originates from a single organism.

A *strain* is a classification of a group of organisms within a bacterial species based on relatedness resulting from clonal descent. A clone is defined as all the individuals (descendants) derived from a single individual (progenitor) by asexual reproduction (fission). The progenitor and descendants are genetically identical unless mutation occurs. A working definition of clone is: a group of bacterial cultures that have been isolated independently (from different sources, at different times, and in different places) and have so many genotypic and phenotypic characteristics in common that the most likely explanation for their relatedness is that they are of clonal origin.

A *ribotype* is a DNA pattern obtained from the DNA operon, or gene, that codes for ribosomal RNA (rRNA). This operon is highly conserved (not easily mutated) and can be used to distinguish between bacterial strains of the same species over many generations in the environment (Atlas et al., 1992) (Selander et al., 1987). Thus, within a population of a given bacterial species there may be numerous isolates belonging to a single strain that can be distinguished from other strains of the same species by a unique ribotype.

MST makes use of the ability to classify organisms based on their genetic fingerprints into groups of clonal descent, or strains, as described above. The second concept forming the basis for the source tracking theory is that of resident vs. transient strains (Hartl and Dykhuizen, 1984). A bacterial strain that has adapted to a particular environment, or host (e.g. animal intestinal tract), is capable of colonizing that environment and competing favorably with members of the indigenous flora. These are called resident strains. Resident strains are usually shed over a long period of time from their host, thus providing a characteristic signature of their source. A transient strain is a bacterial strain that is introduced into a new environment, or host (e.g. into an animal by ingestion), but cannot colonize and persist in that environment. If the host is sampled over time for a given species of bacteria, a few resident strains are consistently observed in the system while a larger number of transient strains are seen passing through.

Rationale

Given that bacterial population structure is clonal and if within each species different clones have adapted to specialized environments, then it should be possible to:

- study a collection of bacterial isolates from a contaminated site (e.g. receiving water) and from possible sources of contamination

- divide the isolates into groups of clonal origin
- match the isolates from the contaminated site to the sources
- identify the contributing sources.

This requires the selection of an appropriate methodology for interstrain differentiation of bacteria. The method of choice needs to be sensitive enough to allow for dividing the species of interest into groups of clonal origin, and the results should be reproducible. The method should also be easy enough to perform, and the results should allow for comparing a large number of bacterial isolates. Ribosomal RNA typing with the use of appropriate restriction enzymes is the method of choice in MST studies of fecal coliforms. In special circumstances such as source tracking studies of *E. coli* O157:H7 (an *E. coli* strain associated with several food-related outbreaks), it has been necessary to develop and use other methods of differentiation.

APPENDIX B

LABORATORY PROCEDURES

Bacterial Culture and Isolation

The water samples were analyzed for fecal coliform enumeration by the Barnstable County laboratory according to the membrane filter method (APHA, 1992). After analysis, the plates were refrigerated and shipped overnight to the University of Washington lab. Morphologically appropriate colonies (round, blue, and flat) were chosen from these plates and streaked for isolation onto MacConkey media and incubated at 37°C for 24 hours. The fecal samples were similarly transported directly to the U.W. lab. They were swabbed heavily onto MacConkey media plates and incubated at 37°C for 24 hours. Characteristic colonies (round, purplish-red, typically flat) were chosen from these plates to be streaked for isolation, again on MacConkey media.

Isolated colonies that fermented lactose on MacConkey were then restreaked onto Trypticase Soy Agar (TSA). Biochemical analysis was done to positively identify *E. coli*. This was done by inoculating each isolate into a tryptophane broth and onto a sodium citrate slant and incubating at 37°C for 24 hours. Isolates that were able to produce indole from tryptophane and not able to utilize sodium citrate as a sole source of carbon were positively identified as *E. coli*. These isolates were then assigned an isolate number and cultured again on TSA to obtain enough cells for storage in LB-15% glycerol freezing media at -70°C and for genomic (chromosomal) DNA isolation.

DNA Isolation and Digestion

Confluent growth of each isolate was scraped with a sterile flat-headed toothpick from TSA plates and suspended in Tris-EDTA buffer. The suspension was mixed well by pipetting up and down. To lyse the cells sodium dodecyl sulfate (SDS) and proteinase K (Pharmacia, Piscataway, N.J.) were added. This was followed by phenol extraction to remove cellular material other than DNA. The preps were vortexed and then centrifuged for five minutes. The top aqueous layer containing DNA was removed and extracted with chloroform to further purify the DNA. DNA was precipitated out of solution by adding 2.5-3 times the prep volume of absolute ethanol. The DNA was spooled onto a glass capillary pipette, washed with absolute ethanol, dried, and resuspended in enough sterile distilled water to obtain a consistent DNA concentration among all preps.

Restriction endonuclease digestions of each DNA prep were done by using 10 units of appropriate

restriction enzymes (Boehringer Mannheim, GmbH, Germany) as instructed by the manufacturer and 4 μL of DNA. Each 20 μL digestion prep was incubated at 37°C overnight. The preps were then centrifuged and 3 μL of stop dye was added to arrest the digestion reaction and prepare for loading into gels for electrophoresis.

Gel Electrophoresis and DNA Probing

The fragments of DNA produced by the enzyme digestion were resolved by agarose gel electrophoresis. The DNA fragments were then transferred from the gel by blotting onto a Nitrans filter (Schleicher & Schuell, Keene, N.H.) in high salt solution (Maniatis et al., 1982) (Southern, 1975). These blots were baked at 80°C for one hour.

The blotted DNA was then hybridized with a radioactively labeled ribosomal RNA (rRNA) probe prepared using rRNA purified from *E. coli* (Stull et al., 1988) (Maniatis et al., 1982). The probe was labeled with [γ -32P] dCTP, using random primers and incubated at 37°C for 30 minutes. The double stranded DNA molecules are denatured into single strands during the blotting process. During the hybridization reaction, the single stranded probe joins to single stranded DNA that contain segments of the ribosomal RNA operon. Hybridization of the probe to the blotted DNA was done under stringent conditions.

After hybridization the blots were washed, dried, and then exposed to X-ray film (Kodak, Rochester, N.Y.) with an intensifying screen at -70°C. Two to three different time exposures were done to ensure all DNA bands that hybridized with the probe would be visible on film. The X-ray image of the DNA banding produced in this way for each isolate is termed an autoradiogram. The actual banding pattern is a ribotype.

Ribotyping and Analysis

The autoradiograms from one gel or blot are examined to identify those with identical banding patterns and the same ribotype, and therefore who belong to the same strain. Using an algorithm developed in the U.W. lab, the ribotypes were converted to an alphanumeric pattern.

The data for each isolate was entered into a computer database (using Microsoft Access 2.0). Isolates were sorted by ribotypes. Potential ribotype matches between isolates obtained from water and source samples, source samples of the same type, source samples of different types, and different water samples were confirmed by further inspection of the autoradiograms.