Guide to Microbial Source Tracking: an Adaptive Framework Approach to Identify Sources of Fecal Pollution in Surface Waters

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ACRONYMS

Beach Action Value – BAV

Below Detection Limit – BDL

Colony Forming Units – CFU

Deoxyribonucleic Acid – DNA

Detectable but Not Quantifiable – DNQ

Environmental Protection Agency – EPA

Fecal Indicator Bacteria – FIB

Florida Department of Environmental Protection – FDEP

Gene Copies - GC

Human Polyomaviruses - HPyVs

Microbial Source Tracking – MST

Milliliters – mL

Most Probable Number – MPN

Pepper Mild Mottle Virus – PMMOV

Quantitative Polymerase Chain Reaction – qPCR

Recreational Water Quality Criteria – RWQC

Total Maximum Daily Load – TMDL

United States Environmental Protection Agency – USEPA

United States Geological Survey – USGS

TERMINOLOGY

cH8 – The H8 gene detected in culturable E. coli

H8 real time PCR – The H8 gene detected via real time PCR

CHAPTER 1: OVERVIEW

1.1. Impacts of Fecal Pollution on Microbiological Water Quality

Clean surface water and healthy aquatic ecosystems provide crucial benefits to society and other living organisms. Material benefits to society include drinking water and irrigation of food crops. Non-material benefits include recreational use, cultural value, and biodiversity while ecosystem services associated with healthy aquatic ecosystems include water filtration and geophysical hazard mitigation (Lynch et al., 2023; Wilson and Carpenter, 1999). Aquatic ecosystems face anthropogenic pressures that threaten to undermine their beneficial uses. One pressure that has notable effects is fecal pollution, which impacts surface waters globally (McLellan et al., 2024; Wear et al., 2021). Fecal pollution originating from many sources (e.g., sewage, stormwater runoff, agricultural waste, and wildlife deposition) introduces numerous microbiological and chemical contaminants that have the potential to injure human health, disrupt aquatic ecosystems, and cause economic loss (Dudgeon et al., 2006; Reid et al., 2019; Wear et al., 2021).

How humans interact with waterbodies can be defined in terms of primary and secondary contact. Primary contact is when there is immersion of the body or head and the potential for ingestion, such as during activities like swimming, surfing, or tubing (USEPA, 2012). For secondary contact, the recreational activities involve less potential ingestion of the water, but the water may instead splash onto the recreator, such as during boating or fishing (USEPA, 2024). The recreational water quality criteria set by the Environmental Protection Agency (EPA) is based on risk from primary contact (USEPA, 2012).

Fecal pathogens in contaminated surface waters can cause acute gastrointestinal illness, as well as infections of skin and conjunctiva (Colford et al., 2007; Dorevitch et al., 2012), or even more serious diseases such as meningitis (McGill et al., 2018; Petersen et al., 2023) or Guillain-Barre syndrome (Poropatich et al., 2010) in individuals exposed via primary contact (swimming) or secondary contact (e.g. boating, fishing). Swimming and fishing are activities of greatest interest in waterborne disease risk assessment due to their popularity (DeFlorio-Barker et al., 2018; USEPA, 2012; USEPA, 2024). An estimated 78% of adults participated in swimming or floating in 2008, with increased participation projected until 2060, while fishing attracted an estimated 31% of adults in the same period (Cordell, 2012), accounting for an estimated 4 billion surface water recreation events annually in the US (DeFlorio-Barker et al., 2018). Swimming and fishing

each result in an estimated 15 cases of acute gastrointestinal illness per 1000 recreators, translating into an estimated 50 million recreators affected by enteric (GI) illnesses per year with another 10 million affected by non-enteric illnesses contracted primarily through swimming (DeFlorio-Barker et al., 2018).

Sewage pollution is prevalent globally (McLellan et al., 2024), jeopardizing the health of aquatic organisms and benefits provided by healthy coastal and freshwater ecosystems, including coastal protection, food, jobs, biodiversity, recreation, and water filtration (Lynch et al., 2023; Wear et al., 2021). Coral reefs, oyster reefs, and salt marshes are valuable coastal ecosystems that have suffered considerable global decline and are demonstrably threatened by sewage pollution (Grabowski et al., 2012; Wear and Thurber, 2015; Wear et al., 2021). For example, increased nutrient loads and zoonotic pathogens in sewage have been associated with disease among coral species, reduced hard coral cover, fewer reef fish, and increased macroalgae in coral reefs (Hernández-Delgado et al., 2008; Redding et al., 2013; Reopanichkul et al., 2009; Sutherland et al., 2010). In freshwater ecosystems, biodiversity loss has been attributed in part to anthropogenic pollution including urban runoff and sewage (Dudgeon et al., 2006; Lynch et al., 2023; Reid et al., 2019). Impaired or reduced ecosystem function results in reduced material, non-material, and regulating benefits that, combined with human health costs, may represent significant economic loss and provide support for improved pollution mitigation efforts (Grabowski et al., 2012; Hernández-Delgado et al., 2008; Lynch et al., 2023; Redding et al., 2013; Reopanichkul et al., 2009).

The economic impact of fecal pollution is multifaceted. Most directly, medical expenses and lost productivity associated with waterborne illness cost an estimated $2.9 billion annually in 2007 USD (DeFlorio-Barker et al., 2018). Revenue from water-based recreation could also be affected, impacting local and regional economies. Recreation at Florida State Parks, which often contain water-based recreational opportunities, drew nearly 30 million visitors, with economic impacts of over $2.5 billion in fiscal year 2018-2019 (Borisova et al., 2020). Anglers alone spent $5 billion in a single year throughout Florida, more than in any other state (Borisova et al., 2020). Beach closures due to fecal pollution may have a negative impact on this sector of the economy, but non-health related costs of fecal pollution are understudied (Parsons et al., 2009; Rabinovici et al., 2004). Other forms of pollution or natural disturbance resulted in significant economic loss in tourism revenue, commercial and recreational fishing, boat sales, and more

(Beier et al., 2017; Guo et al., 2017; The Balmoral Group, 2020). Remediation programs for water bodies that consistently fail to meet regulatory standards for pollutants represent another form of economic loss associated with fecal pollution. These water bodies may be placed on the 303(d) impaired list and subject to total maximum daily load programs that cost $26,000 to $500,000 in 2000 USD per water body (USEPA, 2001). The term "impaired" refers to a water body that does not meet a water quality standard, such as the Recreational Water Quality Criteria (RWQC) set by the EPA (USEPA, 2012), which are based on fecal indicator bacteria levels.

1.2 Detecting & Quantifying Fecal Pollution

Regulatory standards designed to protect human health from fecal pollution rely on monitoring surface waters for fecal indicator bacteria (FIB). A report by Environment America found that 55% of beaches nationally experienced at least one day where FIB exceeded the USEPA Beach Action Value (BAV) in 2022 (Rumpler and Dutzik, 2023). Of the regional beaches evaluated, Gulf Coast beaches ranked worst with 84% of beaches exceeding the BAV criterion. Nationally, 5,090 beaches (79% of beaches) are considered "program beaches" that notify swimmers of unsafe conditions (USEPA, 2024). Of these beaches, 30% experienced either an advisory or closing in 2023 due to pollution. FIB are monitored at only 70% of program beaches, and more than half of closures associated with USEPA program beaches result from a combination of unknown sources of pollution (41%) and stormwater (20%; USEPA, 2024).

FIB, such as *Escherichia coli* and enterococci, are commensal, enteric organisms shed in the feces of most animals that serve as proxies for fecal pathogens (USEPA, 2012). Several epidemiology studies noted correlations between surface water FIB concentrations and illness, particularly in areas impacted by wastewater and urban runoff, leading to the development of water quality standards based on the density of FIB for fresh and marine water (Colford et al., 2007; USEPA, 2012; Wade et al., 2003). Measuring FIB offers a distinctly practical advantage: they are readily detectable using a few accessible, standardized methods (USEPA, 2009; USEPA, 2014). In contrast, measuring all possible fecal pathogens requires numerous assays and specialized processing methods to capture the high diversity and low concentrations present in sewage alone (Korajkic et al., 2018). The simplicity of monitoring one or two FIB to protect human health is offset by the limited information provided by these widely abundant organisms. Particularly limiting is the inability to distinguish between FIB of different sources (e.g. from

sewage, livestock, or wild animals), which pose varying risks to human health and require individualized intervention strategies (Harwood et al., 2014; Soller et al., 2010; Soller et al., 2014). This limitation is compounded by the fact that FIB can also reside in environmental reservoirs such as aquatic vegetation, sands, and sediment (Badgley et al., 2011; Ishii et al., 2007). FIB found in these matrices and FIB resuspended by disturbance to the water column may represent a past pollution event or signal environmental or naturalized strains (Devane et al., 2020; Korajkic et al., 2019). Naturalized strains refer to intestinal bacterial populations that survive and grow outside of their normal habitat, such as FIB in water and sediment (Badgley et al., 2010; Whitman et al., 2014). Naturalized strains are frequently found in sediments or aquatic vegetation but can be resuspended in water by fast water flow, precipitation, or recreational activities, inflating the FIB concentration in the water above regulatory thresholds (Badgley et al., 2011; Staley et al., 2013).

Sources of fecal pollution may be instead assessed by microbial source tracking (MST), thereby improving risk assessment and pollution mitigation efforts (Harwood et al., 2014). MST is an approach to source identification that utilizes tools such as qPCR to detect unique DNA sequences in host-associated microorganisms, termed MST markers (Harwood et al., 2014). The presence of MST markers in surface water provides evidence of fecal pollution by the associated host. However, relationships between FIB, MST markers, and pathogens are not always direct due to differences in relative abundance of the microbial groups in the feces of different hosts, and survival characteristics of the microorganisms in aquatic environments (Korajkic et al., 2018; Korajkic et al., 2019; Zimmer-Faust et al., 2017). MST marker DNA also persists through wastewater treatment, requiring additional testing to discern between treated effluent and sewage (Chern et al., 2022; Lobos et al., 2024; Srinivasan et al., 2011).

1.3 The Adaptive Framework Approach for Identifying Fecal Pollution Sources

This document was developed to guide managers, regulatory agencies, and researchers in developing a practical, cost-effective strategy to identify sources of fecal pollution in a water body. Examples and interpretation are provided throughout the document.

The *adaptive framework approach* (Figures 1 and 2) described herein is designed to help formulate appropriate questions and develop an investigative strategy that is responsive to

findings during the study (e.g. adding and removing sites and specific analyses if needed). Key elements of this approach (Figure 1) include:

- Identifying objective(s) and developing overarching question(s)
- Gathering existing information about the watersheds and potential pollution sources
- Developing specific question(s) that address the overarching question(s)
- Identifying methods to answer question(s)
- Planning and implementing a sampling strategy
- Analyzing data
- Revisiting and refining question(s), methods, and sampling strategy as needed
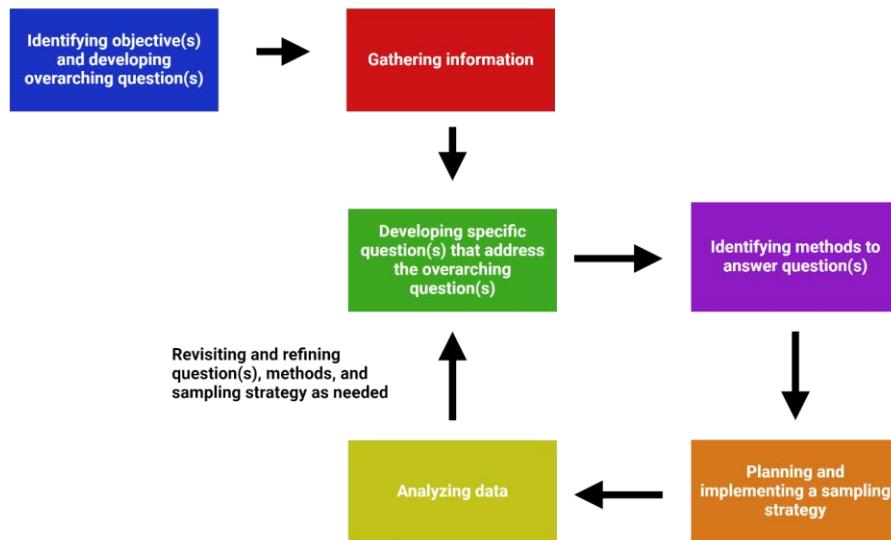
Figure 1. Key elements of the adaptive framework approach to identifying sources of fecal pollution. Each element is explored in detail in the guide document. Created in https://BioRender.com
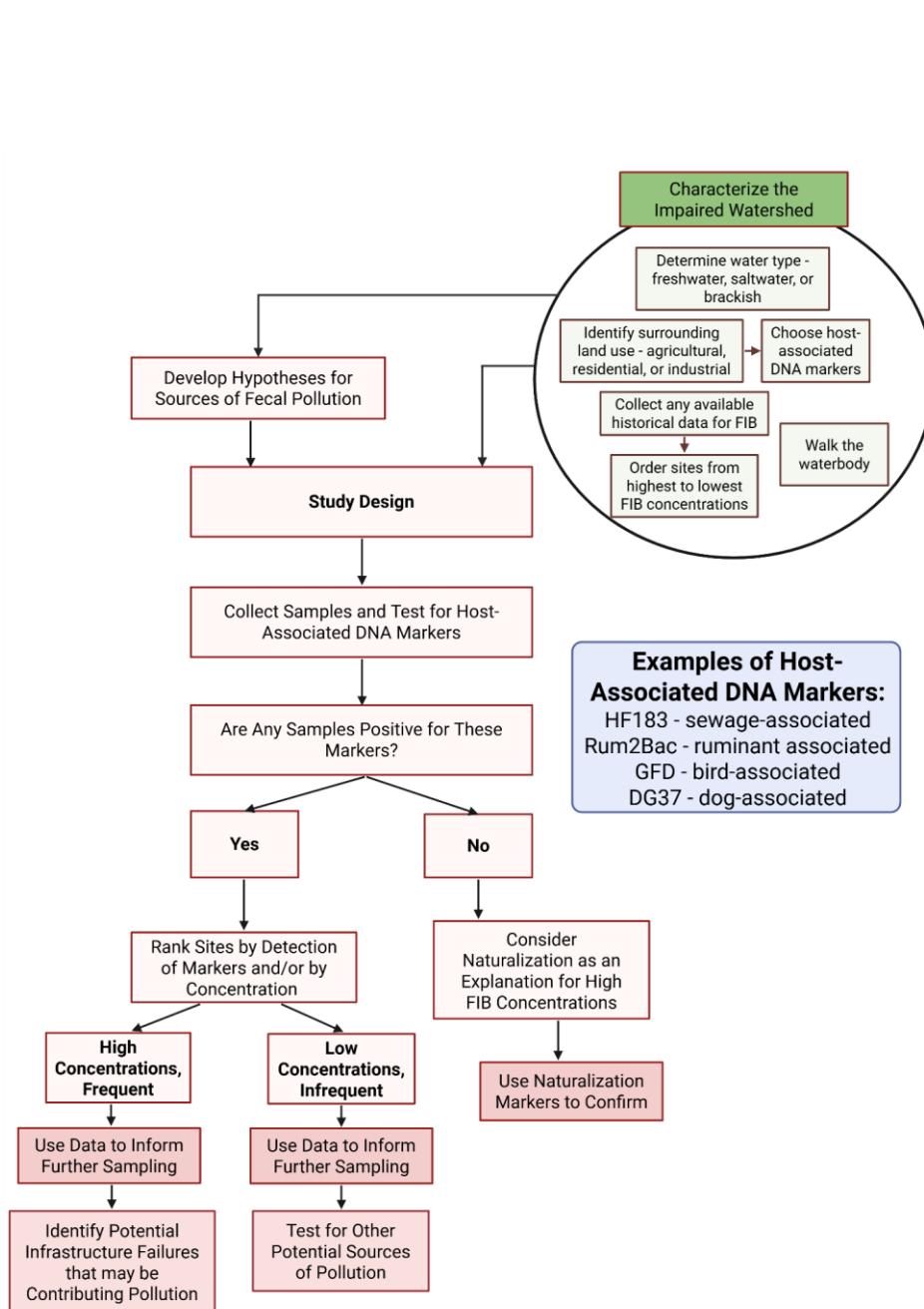
Figure 2. Breaking down the adaptive framework approach into bite-sized questions/answers via a decision tree. Created in https://BioRender.com

CHAPTER 2: DEVELOPING AN OBJECTIVE-DRIVEN INVESTIGATIVE STRATEGY

Microbial source tracking studies are often designed to identify the source(s) of FIB in one or more water bodies, particularly in watersheds where impairment status has been determined (https://www.epa.gov/tmdl). This is a broad goal that requires further refinement to provide interpretable data and optimize resource use. Question(s) based on project goals and specific objective(s) must be developed to provide clear, purposeful answers.

2.1 Identifying overarching question(s)

Perhaps the most important aspect of any investigative process is development of a concrete, clear question, or series of questions. An ideal question is one that can be answered clearly using available tools and technology while supporting priorities (e.g., swimmer, angler, shellfish, and/or ecosystem health, total maximum daily load (TMDL) programs). The nature, scope, and outcomes of a study all depend on the specificity and appropriateness of questions posed. For example, if a Gulf Coast beach frequently exceeds the level of FIB set by state regulations (BAV) several questions may arise that require different approaches and target smaller or larger geographical areas. Some sample questions are below.

1. A generalized local-level question could be: *Which sources of contamination contribute to the frequency of BAV exceedance?* Knowledge of the surrounding area and historical observations, discussed in the following section, will help develop this question. If the beach is located in an urban area with impervious surface and storm drain outfalls, the question may be refined to: (A) *Does stormwater contribute to BAV exceedance?* (B) *Does sewage contribute to FIB levels in stormwater?*

2. An interest at the watershed-level could yield a question such as: *Do nearby water bodies contribute to BAV exceedance at this Gulf Coast beach?* One or more contributing water bodies may then be prioritized for further investigation.

3. A wider, regional question may be: (A) *Is sewage pollution an important source of FIB at Gulf Coast beaches?* (B) *Are livestock operations in the region an important source of FIB at Gulf Coast beaches?*

Many of these questions are simply starting points and further questions may arise throughout the course of the study. For more example questions, see Table 1.

Table 1. Questions about water quality can be about very local concerns, watershed-wide, or be on a wider geographic scale.

Highly local	Watershed-wide	Wider geographic questions
Why is the Perfectville Beach contaminated with FIB so often?	How widespread is the fecal pollution in water bodies in the watershed? Which areas are more or less contaminated, and what is/are source(s)?	What is the dominant cause of exceedance of RWQC at Florida Gulf of Mexico beaches? Does it differ by season?
Does the polluted water in the Perfectville Marsh affect water quality in Perfectville Lake?	FIB levels are high in most parts of the water body, and some areas that look pretty natural have no apparent sources. What's going on?	Which Gulf of Mexico state's beaches experience most frequent exceedance of RWQC? What is/are important sources?

2.2 Gathering information and recording observations about the water body

Many factors may influence FIB concentrations. Gathering information about the water body and nearby areas in the watershed will help refine the question and determine the scope of the study. Using a survey, such as the ones provided by the EPA (marine-routine-sanitary-survey-2023.pdf (epa.gov) and freshwater-routine-sanitary-survey-2023.pdf (epa.gov)), can aid in gathering this information.

2.2.1 Characterize surrounding land-use to identify possible contributing sources

Surrounding land use can impact the magnitude and source(s) of FIB in a water body. Urban, residential, and industrial land use may introduce human, pet, and wildlife (e.g., birds, deer) waste to a water body (Table 2). Sources may be directly deposited in surface water or conveyed

via stormwater, particularly in areas with large amounts of impervious surface cover. Agricultural land use may introduce livestock and wildlife waste. A meeting of stakeholders, e.g. representatives from regulatory agencies, nonprofit groups, citizen-scientists, is typically useful for capitalizing on knowledge about possible pollution sources. While all potential sources of FIB should be noted, not all sources pose equal risk to human health. Prioritizing the focus to high-risk sources in areas with multiple potential sources will improve resource allocation if priorities and question(s) of the study can still be addressed.

ArcGIS can be a useful tool in determining land use and impervious surface cover around sites of interest. If you are not already familiar with how to do this using ArcGIS, there are multiple online tutorials to use to familiarize yourself, such as this one (https://www.youtube.com/watch?v=gHEE7nke82U). Using ArcGIS, you can investigate the map of the area surrounding your waterbody of interest and calculate the percentage of land use and land cover.

2.2.2 Locate infrastructure maps to identify possible contributions

Aged or failing sewer and septic infrastructure present a high-risk source of fecal pollution. Obtain GIS maps of sewer and stormwater infrastructure to identify potential points-of-failure near the water body. Lift stations, manholes, and sewer lines near or intersecting the water body should be noted. Septic tanks near water bodies may also contribute to FIB loads. Note the proximity and density of septic tanks near water bodies.

2.2.3 Characterize physical and natural aspects of the water body and watershed to identify other possible contributing factors

FIB from past pollution events and/or environmental strains may be present in environmental habitats such as soil, aquatic vegetation, and sediment. Each of these environmental reservoirs, under permissive conditions, may contribute FIB to surface waters. Observe stream flow, which can increase adsorption and entrainment of FIB to sediment particulates in slow-flow conditions and generate turbulence that causes resuspension of sediment-associated FIB during fast-flow conditions. Warm, subtropical waters, nutrient-laden sediments, the presence of aquatic vegetation, and shaded conditions from tree cover may shelter FIB from otherwise unfavorable conditions and contribute to FIB load. Make note if the water body is tidally influenced, as that

will impact potential sampling times. If sampling at marine or tidally-influenced sites, it will be necessary to only sample on outgoing tides to prevent any backwash from further downstream.

Examine the structure of the water body for tributaries and potential sources of runoff (e.g., storm drain outfalls, drainage canals or ditches). Branch points of streams can perform like nodes in a network to differentiate contaminated tributaries from cleaner ones. Sampling sites along streams with contributing tributaries should be strategically situated to discern between tributaries that may/may not contribute to fecal pollution.

2.2.4 Obtain historical FIB data to perform spatial and temporal analysis of the water body

Locate historical FIB data compiled by monitoring agencies for the water body of interest. Sources of updated, publicly available FIB data in Florida include a) Water Atlas (https://wateratlas.usf.edu/), b) Impaired Waters Rule database (http://publicfiles.dep.state.fl.us/DEAR/IWR/), and c) Watershed Information Network (https://prodenv.dep.state.fl.us/DearWin/public/wavesSearchFilter?calledBy=menu). Spatial and temporal analysis is best performed on datasets with multiple sites per water body that were monitored over several years, preferably at least five years.

Once adequate data (5-10 years prior if possible) have been obtained, organize data by site, year, and organism (e.g., *E. coli*, enterococci). Note cases where land use has changed over the 5-10 year time period and explore changes in FIB levels that correspond with changing land use. Check for uniformity in units (e.g., most probable number (MPN)/100 mL or colony forming units (CFU)/100 mL). The USEPA standardized methods for FIB (Method 1600 and 1603) and RWQC report data in CFU/100 mL, making this the ideal unit if available data are reported this way. Next, perform a $\log_{10}$ transformation to reduce variability that can exist in datasets. After data are organized and transformed, calculate geometric means to minimize the effect of outliers in the data. Display geomean FIB concentrations spatially (sites depicted) and temporally (months and/or years depicted). Determine whether FIB concentrations vary by site, month, and/or year.

Spatial differences: Sites with higher concentrations of FIB make good starting points for a study. FIB concentrations at this site could be influenced by pollution sources in the surrounding/upstream areas or physical and natural aspects of the waterbody. Refer to land use

and infrastructure maps to identify possible sources and areas of highest priority. Sites with low FIB concentrations can provide a useful negative control site.

Temporal differences: Several biotic and abiotic factors can influence FIB patterns over time. Migratory birds could increase FIB in a seasonal manner and, as natural sources of FIB, may be candidates for natural source exclusion from costly TMDL programs (Nguyen et al., 2018). Precipitation and season are abiotic factors that can influence FIB concentrations (Gonzalez et al., 2021). Precipitation and FIB concentrations are frequently correlated; one study of man-made lakes in Florida observed a greater overall impact of rainfall compared to land use on FIB concentrations (Staley et al., 2013). Another study of a Florida river observed reduced FIB concentrations and greater evidence of wildlife sources in a drought year compared to a year of average rainfall (Shehane et al., 2005). Precipitation values can be collected from a number of sources, including USGS gauges (https://dashboard.waterdata.usgs.gov/app/nwd/en/) or Weather Underground (https://www.wunderground.com/), as long as the reporting station is within close proximity to the study area.

2.2.5 Walk the water body to inspect areas of priority and surrounding areas for further evidence of sources or contributing factors

After gathering information virtually by identifying locations with high FIB concentrations and potential sources of pollution, plan a field reconnaissance. Record observations while progressing through the water body, starting at the most downstream site and ending at the most upstream site. This should be performed in one day, as a preliminary sampling event. Collect samples for culturable FIB at areas of historically high FIB levels or suspicious areas (e.g., suspected illicit connection, runoff from cattle ranch, sanitary sewage overflow). Record GPS coordinates at each collection site. Potential sources identified during reconnaissance should be noted, including storm drain outfalls, sewer infrastructure (manholes, pump stations), and homeless encampments. Document animal sightings (wild and domestic), dog parks, on-site packaging facilities, and farms (see Table 2). Note any homes near the water body that may have septic systems. Record width and depth of the water body as well as surrounding characteristics such as tree cover and vegetation.

Table 2. Sources of fecal pollution frequently associated with various land use categories

Land Use Category	Associated Source(s)
Urban	Sewage[a], dogs[b], birds
Residential	Sewage, dogs, birds
Industrial	Sewage, birds
Agricultural	Sewage, cattle & other livestock, birds, deer, and wildlife
Natural	Birds, deer, and other wildlife

[a] The broad category "sewage" includes human feces.

[b] Although dog feces are a potential source of fecal contamination in water bodies, the Harwood Lab in Tampa, FL rarely detects this source using marker DG37.

2.3 Generate specific question(s)

Once the water body has been characterized and sufficient historic data analyzed, the team can begin to develop specific questions that should be addressed by the study. Answers to these specific questions help address the overarching project question, and therefore, objective. One example overarching, broad question for a project directed at exploring BAV exceedances at a tidally-influenced beach is: *what factors contribute to E. coli and enterococci levels along the beach?* This question could provide a starting point for many related, specific questions. One direction of interest may be addressing whether connected water bodies or structures contribute to FIB concentrations at the beach. In the case of this hypothetical beach, perhaps two storm drain outfalls are present along the length of the beach with a river between. Sampling at each storm drain outfall and slightly upstream of the confluence of the river and beach during low tide may reveal high FIB concentrations in outgoing water in one or more (or none) of these contributing areas. Another specific question may be whether FIB concentrations are influenced by seasonal precipitation. Sampling during wet and dry seasons may reveal the influence of weather patterns. Study objectives aimed at source identification should aim to answer specific questions about the waterbody and contributing fecal sources which can be answered by

assessing MST marker detection frequency and concentration, supported by FIB data and information on surrounding land use.

2.4 Finding a partner in MST

Governmental agencies, academic researchers, and commercial laboratories may provide MST services, generally for a fee, or in collaboration on a grant or contract. A list of agency and university scientists who may be able to help identify a MST partner in various Gulf of Mexico states is shown below (Table 3). A web search of "source tracking commercial lab" will identify commercial laboratories that perform MST.

Table 3. Potential microbial source tracking partners or those who can assist in finding a research partner.

Laboratory Name	Contact Information	Website
Florida DEP Laboratory	Biology Program, Anita Nash	Florida DEP Laboratory | Florida Department of Environmental Protection
US Environmental Protection Agency	National Exposure Research Laboratory; Asja Korajkic	US EPA Microbial Source Tracking
USF Environmental Microbiology Lab	Valerie "Jody" Harwood	Dr. Valerie J. Harwood Microbial Source Tracking Lab at USF Tampa (theharwoodlab.wixsite.com)

CHAPTER 3: METHODS FOR DETECTING FECAL POLLUTION & IDENTIFYING SOURCES

3.1 Fecal indicator bacteria

For over a century, the conventional approach for estimating the presence of pathogens in surface waters has involved the measurement of FIB such as fecal coliforms, *E. coli* and enterococci. FIB should be cultured throughout the duration of any microbial source tracking study for several purposes: (1) they represent the regulatory rule (USEPA, 2012), and serve as a comparison against water quality criteria and other studies; (2) they are a measure of viable fecal microorganisms, in contrast to methods that measure only DNA, and (3) to provide a basis of comparison against which the level of MST marker genes can be compared.

Investigators should determine which FIB is appropriate for the water bodies of interest during the early planning stages of a water quality study. The USEPA recommends assessing water quality by culturing *E. coli* or enterococci for freshwater and enterococci for estuarine and marine sites (USEPA, 2012). It can be informative to culture *E. coli* and enterococci, particularly if MST methods that rely on cultured FIB are used (see section 3.2 below). Follow USEPA Method 1600 and 1603 for selectively culturing enterococci and *E. coli* in water (USEPA, 2009; USEPA, 2014), respectively for the most reproducible and defensible results. Alternatively, the EPA Colilert method (Method 9223 B-2004 Colilert®) is also approved for *E. coli* in surface waters (USEPA, 2004). A less expensive method for culturing *E. coli* that provides results comparable to the EPA methods has been recently published (Calarco et al., 2024).

Another important decision is which environmental matrices are relevant to the study. The RWQC focuses on bacterial populations in water, but other matrices such as sediment, sand, and aquatic vegetation can contribute to FIB levels in surface waters. Sediment and aquatic vegetation often harbor higher concentrations of FIB than water (Badgley et al., 2010; Byappanhalli et al., 2012; Coulliette & Noble, 2008; Nguyen et al., 2018). Appendices B and C describe the process of culturing FIB from sediment and aquatic vegetation. Water bodies with inconsistent sources evidenced by MST, an abundance of silty sediment, shaded conditions from tree cover, decaying vegetation and/or abundant aquatic vegetation are good candidates for FIB culture from matrices in addition to water. FIB concentrations in sand at bathing beaches may

also be of interest. These matrices of FIB could be explored as sources of naturalized FIB populations.

3.2 Microbial source tracking

MST can be used to identify source(s) of fecal pollution in a water body, determine the magnitude of fecal pollution, locate point source(s), and improve human health risk assessment, but must be interpreted with appropriate knowledge of the watershed and recognition of method limitations (see Sections 2.2 and 3.2.2). Reliance solely on nucleic acid-based MST methods may overestimate untreated sewage contamination and human health risk, particularly in areas with recycled water use, as MST marker DNA persists through wastewater treatment (Chern et al., 2022; Lobos et al., 2024; Srinivasan et al., 2011). Methods such as testing for the H8 gene in culturable *E. coli* (Lobos et al., 2024; Senkbeil et al., 2019) can assist in discriminating between sewage contamination vs. recycled water. The most complete understanding of sources of contamination in a water body typically is achieved by analyzing relationships among FIB, multiple MST markers, and environmental parameters, which may offer valuable insight into likely sources of FIB and/or seasonal relationships (Brandt et al., 2025; Gonzalez-Fernandez et al., 2021; Goshu et al., 2021; Nguyen et al., 2018).

After gathering background information (Chapter 2.2) and generating specific questions (Chapter 2.3), MST practitioners must determine which host sources are most likely to contribute to fecal load and/or present high human health risk in the water bodies to be studied. Project objectives should inform selection of source(s) investigated by MST; however, it is important to understand that effective MST markers do not exist for all possible animal hosts. Table 4 contains a list of MST markers that the Harwood Lab has found useful in Florida and other geographic regions, or that have substantial support in the literature. Known cross-reactivity, or detection of a given MST marker in non-target hosts, is also shown in Table 4. Knowledge of animal populations in the study area is necessary to make informed decisions about selection of MST markers. For example, in an area with an abundant deer population, it is prudent to back up the use of HF183 with a secondary human/sewage marker (Boehm et al., 2013; Nguyen et al., 2018).

Another important consideration in study design if whether the water body(ies) may be impacted by recycled water or other disinfected discharges, since HF183 is often abundant in treated wastewater (Lobos et al., 2024).The H8 gene in culturable *E. coli* helps discriminate between sewage contamination and treated wastewater discharges (e.g., recycled water) inputs to surface waters, as it does not survive the wastewater treatment process (Senkbeil et al., 2019), unlike HF183 or other DNA markers. H8 is present in ~17% of *E. coli* isolates in sewage (Lobos et al., 2024), so prevalence of the marker in a water body with diluted sewage influence can be an issue. Similar markers with higher prevalences in sewage, such as *hycj*M (Deng et al., 2015), are being explored for use in detected culturable, sewage-associated *E. coli* in the Harwood Lab.

Qualitative risk assessment based on source(s) identified by MST marker genes can be valuable for prioritizing areas for further testing or remediation, but quantitative microbial risk assessment (QMRA) provides more actionable predictions about the probability of illness from exposure to the water. Co-occurrence of MST marker genes and fecal-borne pathogens has enabled researchers to ascertain risk of illness when certain concentrations of MST marker genes are present, termed risk-based thresholds (Ahmed et al., 2018; Boehm et al., 2015; McLellan et al., 2018; Schoen & Ashbolt, 2010; Symonds et al., 2016). In sewage-impacted waters the presence of one sewage-associated marker, HF183, at 525 gene copies/100 mL of water is predicted in one study to result in gastrointestinal illness equivalent to RWQC FIB criteria of 32 illnesses per 1000 recreators (Boehm and Soller, 2020). In contrast, another study found that the HF183 risk-based threshold was 26,600 gene copies (GC) per 100 mL for scenarios involving fresh sewage (Ahmed et al., 2024). In the EPA GOM study, our QMRA model included measurements of adenovirus and norovirus in sewage which resulted in a HF183 risk-based threshold of 31,600 GC/100 mL (Brandt & Harwood, 2025, https://tampabay.wateratlas.usf.edu/microbial-source-tracking/). Although risk-based thresholds require multiple assumptions and are typically based on risk from one source, e.g. sewage, they can provide a benchmark and basis for prioritization of effort among sites or water bodies.

Table 4. Select bacterial and viral markers: targets, reported specificity and sensitivity, source for assay, and well-documented cross-reactivity (the detection of a marker in a non-target host species). N/A indicates that there is no published evidence of cross-reactivity. This list is not exhaustive and instead reflects the markers previously used by the authors of this document. Markers with an asterisk are patented by the EPA and require permission or a license to use.

Marker Name	Target	Sensitivity / Specificity	Known Cross-Reactivity	Source for Assay
HF183	Human/Sewage	94% [1] / 95%	Deer[5], dogs[6], pigs[7], cows[7], pigeons[7]	Green et al., 2014a
HumM2*	Human/Sewage	100% / 97%	Elk[8], deer[7], pigs[7], cows[7], pigeons[7]	Shanks et al., 2009
H8	Human/Sewage	100% / 92%	H8 gene in *K. pneumoniae*	Senkbeil et al., 2019
CPQ_056	Human/Sewage	100% / 93%	Gull, dogs	Stachler et al., 2017
HPyVs	Human/Sewage	100% / 100%	N/A	McQuaig et al., 2006
PMMoV	Human/Sewage	100%[2] / 85%	Chicken[2], seagull[2]	Zhang et al., 2006
DG37	Dog	85% / 100%	N/A	Green et al., 2014b
GFD	Bird	58% / 100%	N/A	Green at al., 2012
LA35	Poultry	80%[3] / 93%	N/A	Weidhaas et al., 2010
Rum2Bac	Ruminant	97% / 100%	N/A	Mieszkin et al. 2010
CowM3*	Cattle	100%[4] / 97%	Deer[6], Humans[9]	Shanks et al., 2010

1. Ahmed, Masters, & Toze, 2012
2. Rosario et al., 2009
3. Nayak, Weidhaas, & Harwood, 2015
4. Xue & Feng, 2019
5. Nguyen et al., 2018
6. Linke et al., 2021
7. Boehm et al., 2013
8. Stachler et al., 2017
9. Xue, 2016

3.2.1 Sample collection

Water must be collected in sterile containers, filtered onto a membrane, and processed for nucleic acid purification. USEPA Method 1696 (USEPA, 2019) presents recommendations for assessing the sewage-associated MST marker HF183 in surface waters. A simplified protocol for collecting and processing water to concentrate bacterial and DNA and/or RNA for other MST markers is presented in Appendix D and E, respectively. Microbial source tracking markers have also been identified in natural matrices (e.g., aquatic vegetation, sediment, soils, and sand) using protocols presented in Appendix F and G.

3.2.2 Selecting Bacterial and Viral Marker Genes for MST

Diversity of bacterial and viral pathogenic species, low prevalence in human populations, and low concentrations in feces generally preclude source identification by bacterial and viral pathogen markers. Instead, many widely-used MST markers are found in non-pathogenic fecal bacteria or fecal viruses associated with specific hosts (Table 4). Ideal bacterial marker genes are present at high concentrations in host feces, enabling ready detection when diluted in surface waters, and exhibit high specificity and sensitivity in the geographic region (Bernhard & Field, 2000; Harwood & Stoeckel, 2011; Harwood et al., 2014).

Viral pathogens are typically present in low concentrations in sewage, but several sewage-associated viral marker genes have proven useful in MST field studies (Table 4). Viral MST marker genes are thought to better reflect the fate and transport of viral pathogens compared to bacterial markers (Diston et al., 2015; Harwood et al., 2014). Sample processing for viral marker genes frequently requires one or more additional steps compared to bacterial markers, including sample acidification prior to processing, larger processing volumes, the use of dead-end hollow fiber ultrafiltration, and/or concentration by precipitation or other means (Gonzalez-Fernandez et al., 2021; Gonzalez-Fernandez et al., 2023; Korajkic et al., 2021; Symonds et al., 2014).

Studies of large or complex watersheds with many possible sources of fecal pollution may include a broad selection of MST markers, while projects with very focused objectives may employ a more selective approach to MST marker selection. For example, when project objectives dictate that many possible sources of fecal pollution are identified in a mixed-use water body (e.g., residential, natural, and agricultural land use patterns) MST marker selection

Commented [AL2]: I think we can talk about what to consider when selecting MST markers including the levels in non-target animals. Let me know what you think.

Commented [NB3R2]: From Dr. Harwood: Discuss in fourth paragraph

may include human/sewage-associated, livestock-associated, and wildlife-associated markers. Conversely, studies with limited resources predominantly concerned with human health risk will likely benefit from prioritizing human/sewage-associated MST markers. This is particularly true if the study is deployed in areas containing high densities of wastewater conveyance systems, stormwater outfalls, or onsite wastewater disposal (septic) systems.

Once possible contributing source(s) have been identified, MST markers should be selected. Multiple markers, both bacterial and viral, may be available for a given source (Table 4 for some examples), but some may not perform well in a given geographic region due to factors such as abundance in target hosts and presence in non-target hosts. HF183 is a useful sewage marker (Table 4), but the usefulness of HF183 can depend on the potential for confounding circumstances where it is applied. DNA can survive through wastewater treatment, leading to high concentrations of HF183 in areas where recycled water or other treated wastewater discharge is prevalent. Furthermore, HF183 may have poor specificity in areas with abundant deer populations. While the sensitivity is still high, the specificity is much lower.

Performance testing of markers is a critical first step in MST studies carried out in geographic regions where markers have not previously been validated. Reference materials such as sewage from applicable sources (e.g. centralized wastewater treatment facilities, septic systems) and feces of animals whose population density and proximity to water indicate that they may impact water quality should be collected in the location of study if MST markers have not previously been vetted for the region (Jesser et al., 2025; Schiaffino et al., 2020; Symonds et al., 2017). Sensitivity and specificity testing procedures are outlined in Section 3.2.3.

3.2.3 Performance Testing of Selected Markers

Performance testing on selected markers begins with collection of sewage from one or more wastewater treatment plants, septage from multiple homes, and animal feces from multiple individuals of several species residing in the area. Feces should be collected from both target host species and non-target species (feces that should produce a negative result). The target host group is the phylogenetic group or species for which the marker is designed (e.g. sewage-associated *Bacteroides*), while a non-target host would be determined by amplification of the marker in another species (e.g. HF183 in deer, etc). DNA extracted from reference samples should then be tested for each MST marker of interest.

Sensitivity, a measure of how often an MST marker is present in the feces of a target population, and specificity, the absence of an MST marker from the feces of non-target host species, should be determined using the equations below (See Table 4 for numeric examples and Figures 3 and 4 for a visual representation). We have found that sensitivity and specificity values for a useful MST marker should be at least 80%, a generally agreed-upon criterion in the MST community.

$$Sensitivity = \frac{true\ positives}{(true\ positives\ +\ false\ negatives)}$$

$$Specificity = \frac{true\ negatives}{(true\ negatives\ +\ false\ positives)}$$

As an example, the widely used marker of human fecal and sewage contamination HF183 typically has high sensitivity toward sewage (>95%) and relatively high specificity (>81%) (Ahmed et al., 2012; Boehm et al., 2013; Harwood et al., 2014; Staley et al., 2012). Figures 3 and 4 are graphic demonstrations of how sensitivity and specificity are calculated. Figure 3 demonstrates a useful hypothetical sewage marker, while Figure 4 shows a poorly performing hypothetical sewage marker.

Hypothetical Good Sewage Marker
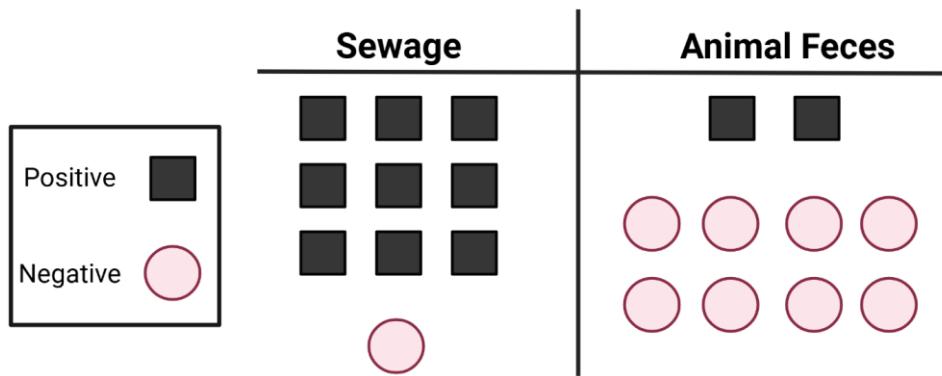
$$Sensitivity = \frac{true\ positives}{(true\ positives\ +\ false\ negatives)} = \frac{9}{(9+1)} = \mathbf{90\%}$$

$$Specificity = \frac{true\ negatives}{(true\ negatives\ +\ false\ positives)} = \frac{8}{(8+2)} = \mathbf{80\%}$$

Figure 3. A representation of sensitivity (left, sewage) and specificity (right, animal feces) parameters for a hypothetical useful sewage marker with 90% sensitivity and 80% specificity. Each shape (square or circle) represents a reference (known source) sample. In this example, ten reference samples are tested for sensitivity (sewage) and specificity (animal feces). Created in https://BioRender.com

Hypothetical Poor Sewage Marker
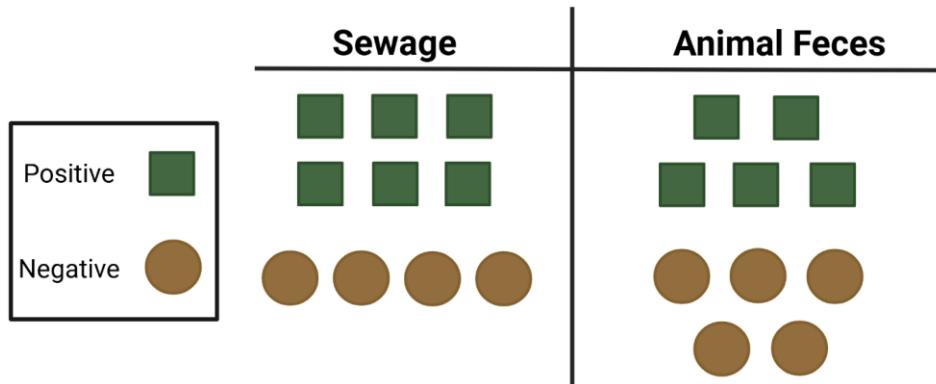
$$Sensitivity \ = \frac{true\ positives}{(true\ positives\ +\ false\ negatives)} = \frac{8}{(8+2)} = \ 60\%$$

$$Specificity \ = \frac{true\ negatives}{(true\ negatives\ +\ false\ positives)} = \frac{5}{(5+5)} = \ 50\%$$

Figure 4. A representation of sensitivity (left, sewage) and specificity (right, animal feces) parameters for a hypothetical poorly performing sewage marker with 60% sensitivity and 50% specificity. Each shape (square or circle) represents a reference (known source) sample. In this example, ten reference samples are tested for sensitivity (sewage) and specificity (animal feces). Created in https://BioRender.com

3.3 Physical methods

The American Society of Civil Engineers rated wastewater infrastructure in the United States D+ in 2021, due in part to the age of many systems (ASCE, 2021). Aging sewer lines may experience leakages due to structural damage (e.g., cracks, collapse, etc.) and blockages (e.g., siltation; consumer products; ASCE, 2021; Gokhale and Graham, 2004). Uncorrected sewer leaks coupled with nearby aged or damaged stormwater conveyance systems can cause sewage exfiltration to stormwater pipes, and ultimately to receiving surface waters (Sercu et al., 2011). Microbial source tracking may localize the source to a given area, but when infrastructure defects are suspected physical assessment may be necessary. Several physical methods exist to

identify infrastructure leaks including closed circuit television, fluorescent dye testing, and smoke testing, among others (Abaya et al., 2018; Gokhale and Graham, 2004). These methods may be used in conjunction with MST results to provide definitive evidence of contamination source (Carson et al., 2024; Gonzalez et al., 2020). In our experience, physical methods are frequently not as sensitive as MST assays.

3.4 Chemical methods

Chemical source tracking is an approach to fecal source identification that relies on detecting human/sewage-associated chemical products in wastewater. The targeted chemicals include household products (e.g., detergents or other chemicals), pharmaceuticals (e.g., acetaminophen), and food additives or metabolites (e.g., sucralose, caffeine, sterols) (Cantwell et al., 2019; Hagedorn and Weisberg, 2009; Liu et al., 2014; Rodríguez-Rodríguez et al., 2024; Staley et al., 2016; Van Stempvoort et al., 2020;). Many of these products, with the exception of fecal sterols and caffeine, are not expected in non-human animal feces or environmental sources (Blanch et al 2006; Hagedorn and Weisberg, 2009).

Chemicals differ in concentration, persistence through wastewater treatment and in the environment, and geographical distribution in wastewater (Hagedorn and Weisberg, 2009; Staley et al., 2016). As such, performance testing is recommended when selecting chemicals for chemical source tracking. The objective(s) and scope of a study informs target chemical selection, just as it does MST marker selection. In a recent microbial source tracking study performed by the Harwood Lab, chemical tracer analysis was performed by the Florida Department of Environmental Protection in a creek where impacts from by a nearby onsite wastewater treatment system were indicated due to elevated HF183 concentrations and cH8 detection (Table 4). Samples were collected upstream and downstream of the impacted area and shipped on ice to the FDEP lab, where the quantities of 33 human-associated chemical tracers were analyzed. Elevated levels of tracers such as acesulfame-K and ibuprofen were detected in the sample downstream of the wastewater treatment system that were not observed at upstream sites or in the treated effluent, supporting the hypothesis that untreated wastewater was impacting the creek.

CHAPTER 4: MST SCENARIOS AND STUDY DESIGN

4.1 Fecal Impairment Scenarios in Surface Water

Many variables (land use, historical data, geography) influence the optimal structure and organization of an MST study. In this chapter, we present four realistic scenarios that represent challenges common to MST studies in many areas around the world. Each hypothetical scenario utilizes the decision tree (Chapter 1) to guide decision making. The scenarios present water bodies with different types of land use, different possible sources of pollution, and different historical trends in FIB levels.

Four hypothetical scenarios are shown below (Table 5), including the historical frequency of exceedance of RWQC. In Scenario 1, the rural area's surrounding land use is very low-density housing and non-agricultural, so septic systems and wild animals are considered to be possible contributors to fecal pollution. In Scenario 2, the rural study area includes agricultural land use, and the possible sources of fecal pollution therefore are identified as septic system leakage, or runoff of fecal material from nearby farms or wooded areas. In Scenario 3, the land use around the water body is primarily urban and high-density residential, therefore sewage as well as dog and bird feces are possible sources. In Scenario 4, urban and rural areas make up the surrounding land use, therefore human fecal pollution may come from municipal sewer systems and septic systems, and both pets and wild animals are considered.

Table 5. Hypothetical scenarios covered in Chapter 4.

Scenarios	FIB Exceedance of RWQC	Historical FIB Seasonal Trends	Surrounding Land Classification	Possible Sources of Fecal Pollution
1	30% (only during dry season)	High only during dry season	Rural	Sewage (septic), deer, and birds
2	50%	Higher during wet season	Rural/agricultural	Sewage (septic), cattle, deer, pigs, and chickens
3	85%	No trend	Urban/residential	Central sewer, dogs, and birds
4	95%	Higher during dry season	Urban/rural	Central sewer & septic, dogs, deer, and birds

4.2 Selecting and Implementing MST Methods

In any pollution scenario, the first steps require analysis of the surrounding land use (2.2.1) and historical data (2.2.4). Appropriate MST markers that are well-vetted, preferably validated in the geographic area, and associated with potential sources determined during the sanitary survey (2.2.5) can be identified from the literature or by the scientific team (3.2.2). Preliminary sites can be chosen based on historical data and sites of interest identified during the walking survey of the water body. Sampling at established regulatory sites allows direct comparison with the historical data. Water body confluences where branches meet (e.g., streams into rivers can serve as hypothetical points of pollution input. Further exploration should be based on the results of the preliminary sampling, with physical methods (3.3) used where appropriate to provide further insight. The scenarios below walk through each of these steps, from assessing the waterbody and potential sources, preliminary sampling and additional exploration, and physical methods if appropriate.

4.2.1 – Scenario 1

<u>*Watershed and probable pollution sources*</u>*.* In Scenario 1, the sampling team are investigating a first-order freshwater stream (Figure 5) in a rural area where there have been sporadic exceedances of the RWQC. The hypothesis, decision making, and conclusions are shown in Figure 6. The investigation is part of a larger study on sources of pollution to a major river in the area. The historical data shows that FIB concentrations in the stream exceed the RWQC in about 30% of samples, and only during the dry season. The seasonal pattern of contamination runs counter to that observed in many areas, where rainfall tends to elevate FIB levels.

The sampling team identify human feces (via septic systems), and wildlife (primarily deer and birds) as possible sources during the sanitary survey. DNA markers (Table 4) are chosen to investigate the prevalence of each source in the stream. HF183 is selected as a sewage/human fecal marker. HF183 has been detected in deer feces, therefore confirmation of sewage contamination is provided by the H8 gene in culturable *E. coli* (cH8). Rum2Bac and GFD are chosen to quantify ruminants (in this case, deer) and bird fecal contributions, respectively. *E. coli* is the regulatory FIB in the freshwater system.

<u>*Preliminary sampling.*</u> Three preliminary sites are chosen (Figure 5), the most downstream of which is the regulatory sampling site where the historical data has been collected, labelled as W1. This site is sampled off a bridge. While walking the water body, the sampling team identify a large wetland area that borders the main stream, which becomes the second preliminary site (W2) at the most downstream segment of the wetland area. They observe a large population of migratory birds in the wetland area. The third preliminary site (W3) is off of a bridge that crosses the stream further north and upstream of the wetland, in a primarily residential area served by municipal sewer.

The sampling campaign begins at the start of the dry season. All sites are tested for *E. coli*, and the selected MST markers (HF183, Rum2Bac, GFD), and cH8 if HF183 is detected. The data from the first sampling event show moderate FIB concentrations (~2.6 $\log_{10}$ CFU/100mL) at the three preliminary sites, with slightly higher concentrations at W1, the regulatory site (2.8 $\log_{10}$ CFU/100mL). FIB levels at W1 and W2, the marsh, exceed the RWQC. In contrast, *E. coli* concentrations (2.6 $\log_{10}$ CFU/100mL) at the upstream site, W3, are below the RWQC. HF183 and Rum2Bac are both detected at low quantities (~1.4 $\log_{10}$ GC/100mL) at all three sites,

though cH8 is not detected at any site. GFD is detected in high concentrations (3-4 $\log_{10}$ GC/100mL) at W1 and W2, and is not detected at W3. The data suggest the influence of deer feces and possibly low-level contamination from sewer. However, prevalence of migratory birds and high GFD concentrations point at birds as major contributors of fecal contamination.

Additional sites and monthly sampling. To explore this possibility, the sampling team add two more sampling sites: one further upstream from the regulatory site but still downstream of the wetland (W1.1), and one immediately upstream from where the wetland area begins (W2.1, Figure 5). They continue sampling at the original sites as well. FIB concentrations remain high (2.5-3.5 $\log_{10}$ CFU/100mL) at the sites downstream of the wetland (W1, W1.1, and W2), and there are occasional detections of HF183 and Rum2Bac throughout the water body. cH8 is never detected. GFD concentrations remain consistently elevated at W1, W1.1, and W2 and are highest at W2, the site immediately bordering the downstream part of the wetland. The site on the upstream segment of the wetland (W2.1) frequently sees detectable but not quantifiable (DNQ) GFD levels, but no elevated levels. The GFD signal is lower at the regulatory site W1 compared to the marsh W2, although it is still consistently detectable at high concentrations (2-3 $\log_{10}$ GC/100mL).

As wet season begins, GFD concentrations begin to decrease at all sites, and FIB concentrations decrease in tandem. The sampling team notes that the bird population in the area has shrunk, especially in the wetlands. HF183 and Rum2Bac detections remain sporadic, and concentrations of these two markers do not correlate with the decrease in FIB concentrations. It is determined instead that HF183 and Rum2Bac concentrations correlate with each other. These trends hold for the wet season, then GFD and FIB concentrations increase as rainfall decreases, and the migratory bird population arrives.

Conclusions. The correlation of HF183 and Rum2Bac indicates that there is some contribution from deer, though this contribution does not have a great impact on the FIB concentrations in the water body. cH8 is not detected for the duration of the project, confirming that the FIB issue is not due to sewage.

FIB and GFD concentrations were significantly correlated, indicating that the source of the fecal pollution are the migratory birds that settle in the marsh in the dry season. As the bird populations are protected and the area is managed for wildlife, the regulatory agency can also

record the data as part of their larger multi-water body study, and shift their focus to other, more polluted streams.
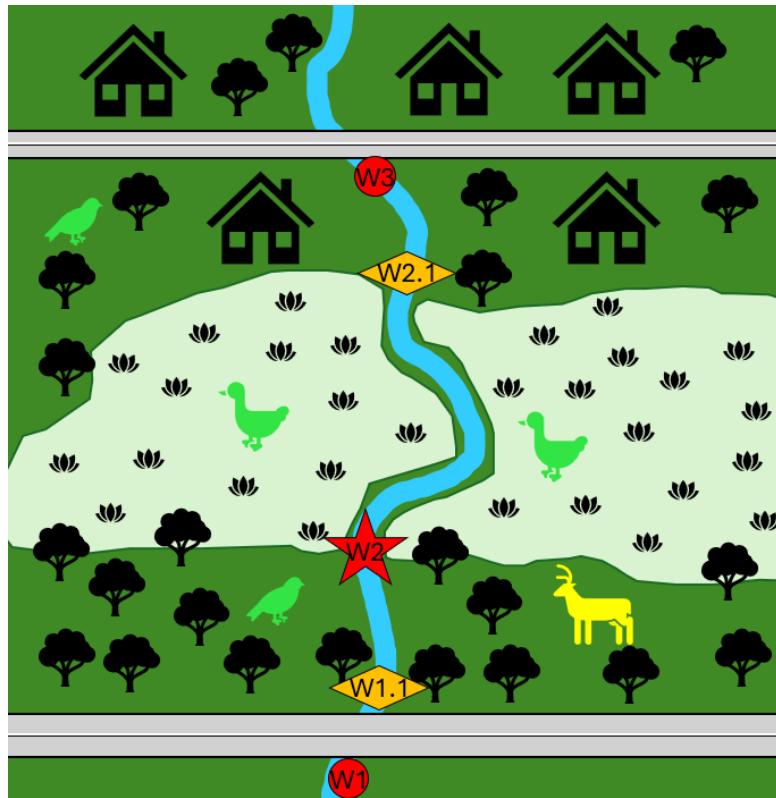
Figure 5. This map represents the stream in Scenario 1. The vertical blue line is the stream, which flows from the top of the map towards the bottom. Gray horizontal lines indicate roads. A wetland area is represented by a light green outlined shape. Red circles/stars are the sites initially selected for the preliminary sampling, while the orange diamonds are additional sites added to the study after the preliminary sampling event. Labels, W1-W3, are site names. The red star indicates the site where the highest FIB and MST marker concentrations were detected. Possible sources are marked in different colors – birds in green and deer in yellow, while the possibility of human contamination comes from the houses along the stream, which are all on septic.
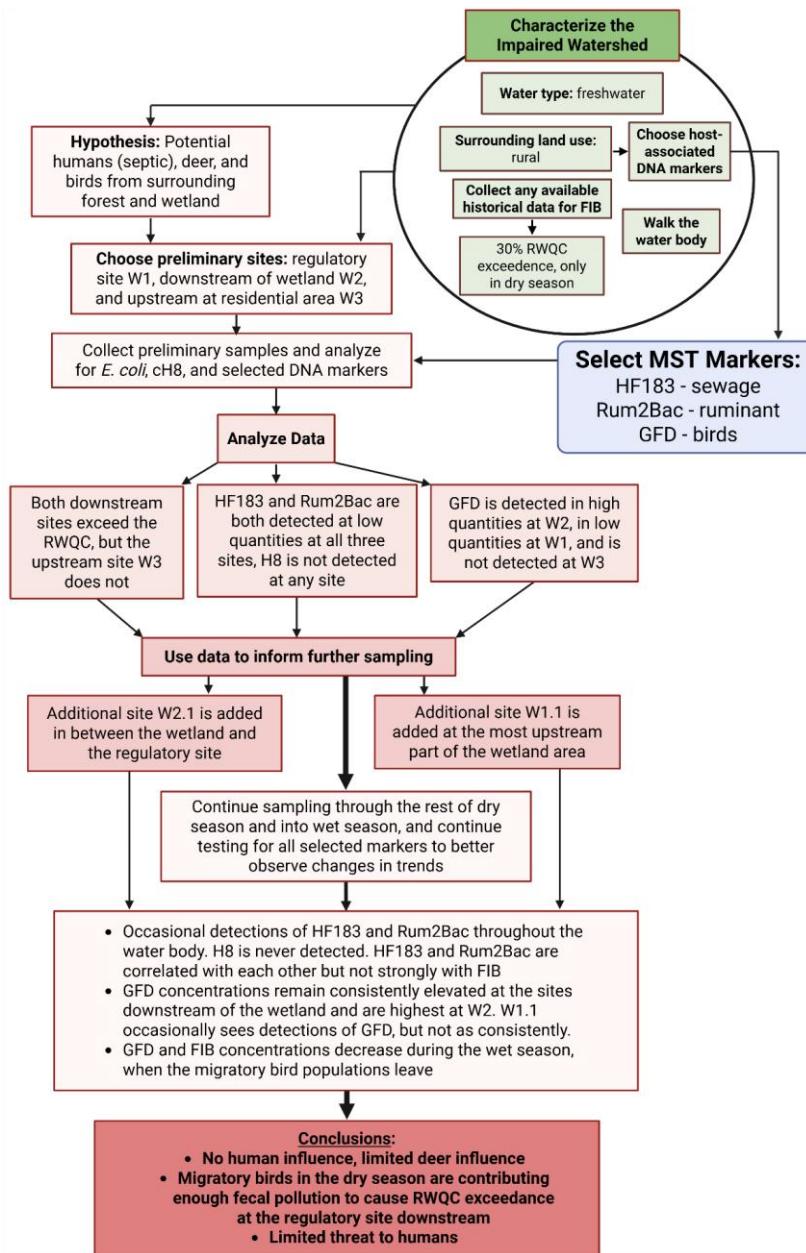
Figure 6. Decision Tree for Scenario 1. Created in https://BioRender.com

4.2.2 – Scenario 2

Watershed and probable pollution sources. Scenario 2 represents a second-order stream (Figure 7) in an agricultural area with low-density housing. The hypothesis, decision making, and conclusions are shown in Figure 8. The stream eventually feeds into a larger river, which is a popular location for boating and fishing. As the stream is entirely freshwater, only *E. coli* concentrations are measured. FIB levels in the stream exceed the RWQC in 50% of samples, and more exceedances occur during the rainy season. The sanitary survey identifies human (septic), domesticated farm animals (cattle, chickens), and wildlife (deer) as possible sources.

DNA markers (Table 4) are chosen to investigate the prevalence of each fecal source in the stream. HF183 is selected for a sewage/human marker, with cH8 as a confirmation. Recycled water (which contains HF183) may be used to water crops, and HF183 has been detected in deer feces, so a confirmation step is necessary. Rum2Bac is selected for ruminant (cattle, deer) fecal detection, while CowM3 serves as a confirmatory assay for cattle. Rum2Bac detection in the absence of the CowM3 marker would indicate deer as a probable contamination source. LA35 is selected to identify chicken fecal contamination.

Preliminary sampling. Several preliminary sites along the stream are chosen (Figure 7), the most downstream of which is at the regulatory sampling site where the historical data has been collected, labelled as FC260 based on its regulatory name. A preliminary site, labelled FC1, is chosen in a forested area with little development roughly a mile upstream, to be a 'clean' site for comparison. During the sanitary survey, the sampling team identified a short, ephemeral ditch that runs alongside the nearby farms. They choose upstream and downstream sites of the ditch input, labelling them FC2 and FC3, respectively (Figure 7). A preliminary site at the end of a residential road upstream of the regulatory site is also selected and labelled FC4, to provide information on any contamination in the creek near the houses.

The data from the preliminary sampling shows high *E. coli* concentrations (~3 $\log_{10}$ CFU/100mL) at FC260, FC4, and FC3. The clean site FC1 has low concentrations (1.2 $\log_{10}$ CFU/100mL) of *E. coli*. At FC260 and FC4 HF183 is detected in high quantities (~3.2 $\log_{10}$ GC/100mL). Additionally, there is an cH8 detection at FC260. Rum2Bac is detected in high quantities (3.4 $\log_{10}$ GC/100mL) at FC3 and at moderate quantities (2.5 $\log_{10}$ GC/100mL) at FC2 and FC4, and then in low concentrations (1.7 $\log_{10}$ GC/100mL) at FC260. Rum2Bac is below the

limit of detection (BDL) at the upstream "clean" site. CowM3 is detected only at FC3, at a moderate concentration (2.1 $\log_{10}$ GC/100mL). LA35 is detected but not quantifiable (DNQ) at both sites near the farms.

Additional sites and monthly sampling. Additional sampling sites are chosen along the lower half of the stream to try and identify the source of the human fecal pollution, and are labelled as FC3.1, FC4.1, and FC4.2 (Figure 7). The new sites (FC3.1, FC4.1, and FC4.2) have similar FIB concentrations as previously observed between FC3 - FC260 (~2.5-3.5 $\log_{10}$ CFU/100mL). FIB concentrations peak in the rainy season, with the highest observed single value of 4.2 $\log_{10}$ CFU/100mL at FC4.1, then decrease as the dry season approaches. The clean site FC1 never exceeds the RWQC.

Rum2Bac similarly peaks in the wet season and then decreases in the dry season. The most upstream new site, FC3.1, sees Rum2Bac concentrations slightly lower than at FC3 (~2.8 $\log_{10}$ GC/100mL). Rum2Bac and CowM3 are always detected at FC3, but in the dry season values are often DNQ at FC3.1, and typically BDL further downstream. Rum2Bac is detected four times at FC2, but in lower quantities (~2 orders of magnitude) than at the FC3 or FC3.1, and CowM3 is only detected at FC3 and FC3.1, likely indicating that any Rum2Bac upstream is from deer.

The highest HF183 value is seen in the wet season at FC4.1 (3.9 $\log_{10}$ GC/100mL). During the wet season, there are several cH8 detections at FC4.1 and FC4.2, but only one at FC3.1. cH8 is also observed three times at FC4 and five times at FC260 through the course of the project. In the dry season, HF183 is still occasionally detectable in low quantities (~1.9 $\log_{10}$ GC/100mL) in the lower half of the stream (between FC4 and FC260), but no more cH8 is detected.

Conclusions. Statistical analysis shows that FIB and HF183 concentrations are strongly correlated with rainfall. One possible explanation is that rain during the wet season helps flush HF183 and H8 from the leaking septic fields into the stream, but the signal diminishes during the dry season. No point source of human fecal pollution is identified, as the HF183 and H8 signal was spread out through the downstream reaches of the stream, and not identifiable as coming from any specific residence or septic system. The regulatory agency could address these issues by working with local agencies to fix the leaking septic tanks or add the residences to the sewer system.

Cow fecal pollution is more concentrated in the stream, likely coming from runoff from the farms. Rum2Bac and CowM3 are also correlated with rainfall, confirming this theory. Some deer contribution is observed upstream, but most Rum2Bac comes from cattle. Chickens are not contributing to the fecal pollution, as LA35 was not quantifiable at any site during the project.
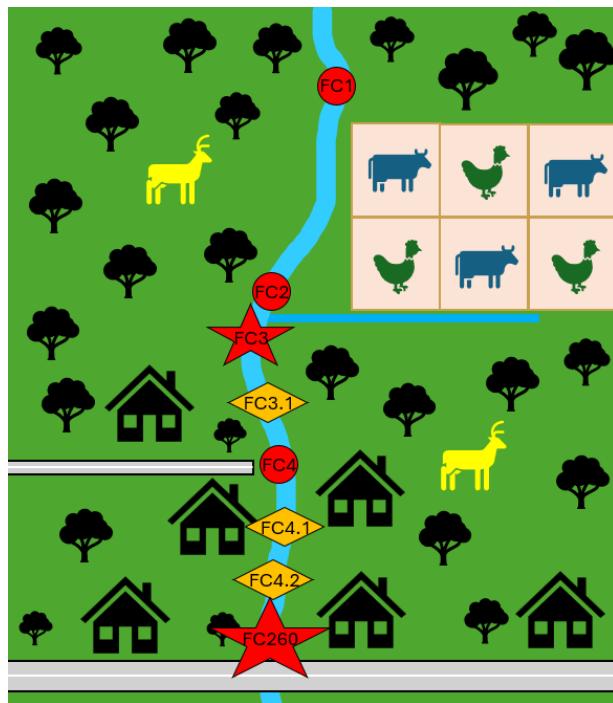
Figure 7. This map represents the stream in Scenario 2. The vertical blue line is the stream, which flows from the top of the map to the bottom, while the horizontal blue line is a ditch that comes from the cattle and poultry farms. Gray horizontal lines indicate roads. Beige squares represent farmland. Red circles/stars are the sites initially selected for the preliminary sampling, while the orange diamonds are additional sites added to the study after the preliminary sampling event. Labels, FC1-FC260, are site names. The red stars indicate the sites where the highest FIB and MST marker concentrations were detected. The houses along the stream are all on septic. Possible sources are marked in different colors – birds in green, cows in blue, deer in yellow, while the possibility of human contamination comes from septic systems servicing homes in the downstream reach of the stream.
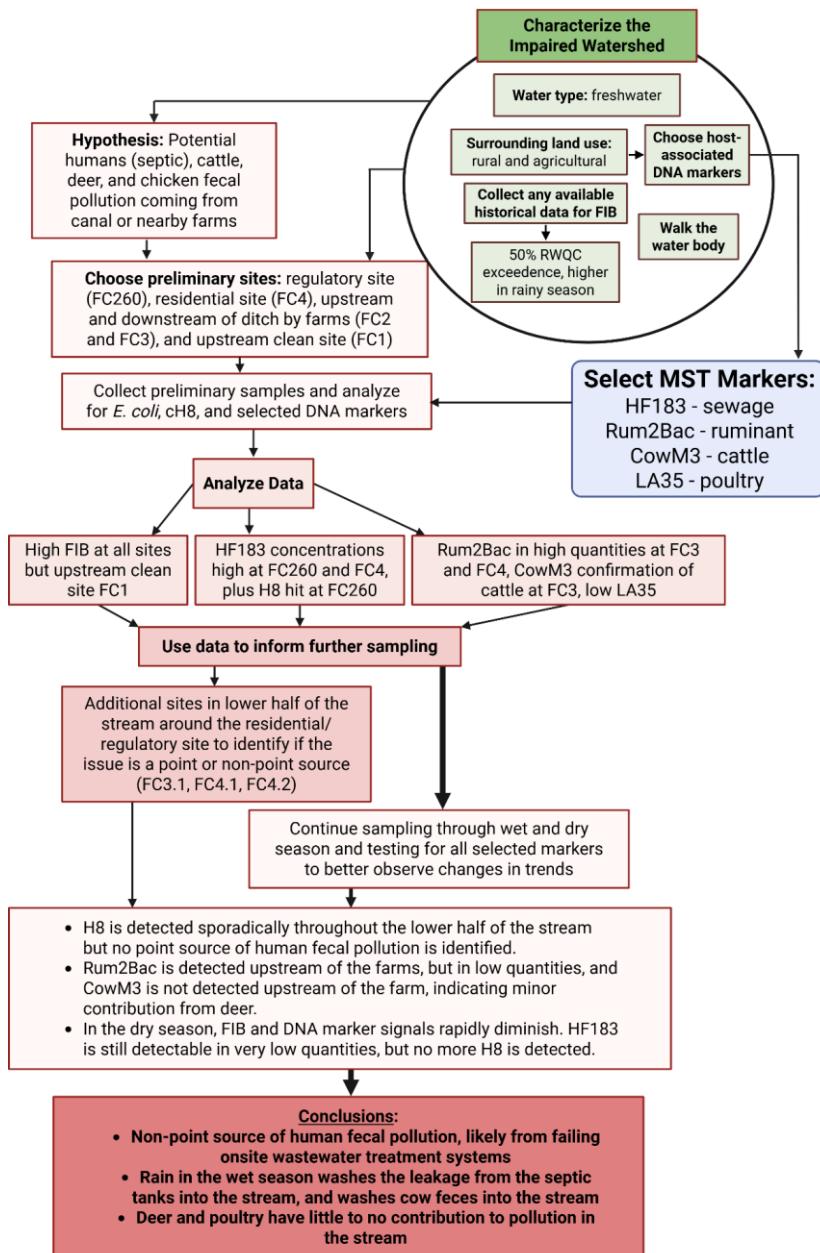
Figure 8. Decision tree process for Scenario 2. Created in https://BioRender.com

4.2.3 – Scenario 3

Watershed and probable pollution sources. In Scenario 3, the stream of interest is a first-order stream in an urban/residential area (Figure 9), with 85% of samples exceeding the enterococci and *E. coli* RWQC. The hypothesis, decision making, and conclusions are shown in Figure 10. The stream flows into the ocean, where recreational activities are common. Due to the tidally-influenced nature of the stream, samples are collected only on an outgoing tide and both *E. coli* and enterococci are measured. A large pond at a nearby park feeds into the stream. Neither *E. coli* nor enterococci levels vary seasonally. The sewer lines in the area are over 50 years old, so sewage is a likely source. HF183 is chosen to investigate this potential source (Table 4), with cH8 as a confirmation. Additionally, dogs and birds are considered as possible sources of pollution, due to many people walking their dogs at the park, and the large population of waterfowl that live in the pond and stream. DG37 and GFD are chosen (Table 4) to quantify pollution from dogs and birds, respectively.

Preliminary sampling. Six preliminary sites are chosen (Figure 9). The sampling team selects the regulatory site (labelled in the historical data as RC13), which is near where the stream meets the ocean. They also choose a site at a bridge upstream of the regulatory site (RC5), two sites just upstream and downstream (RC3 and RC4, respectively) of where the pond input enters the stream, as well as a site within the pond itself (RC2), and a final preliminary site (RC1) is chosen upstream at the headwaters, which is a stormwater pipe. Sampling begins in the wet season, and all samples are tested for FIB and the chosen MST markers.

FIB concentrations are high (~3.6 $\log_{10}$ CFU/100mL) throughout the stream, particularly at RC5, RC4, and RC2, and are lowest (~2.1 $\log_{10}$ CFU/100mL) at RC1, the headwaters. HF183 was detected in high quantities (~2.0-2.9 $\log_{10}$ GC/100mL) in the pond itself (RC2) and gradually diluting down to ~1.3 $\log_{10}$ GC100mL across the sites downstream of the pond (RC4, RC5, and RC13). HF183 was seen in low quantities (~1.5 $\log_{10}$ GC/100mL) at RC3 and was not detected at the headwaters. cH8 was not detected at any site in the preliminary sampling. DG37 and GFD were both detected in high quantities (2.8 and 3.1 $\log_{10}$ GC/100mL, respectively) at RC2, and GFD was also detected in lower quantities (~1.2-1.8 $\log_{10}$ GC/100mL) at RC4 and RC5.

Additional sites and monthly sampling. An additional sampling location is chosen at the pond (RC2.1), closer to the popular dog walking path (Figure 9). Another sampling site is selected

within the small stream that connects the pond and the main stream (RC2.2). Samples are taken at an additional site (RC4.1) upstream of the bridge to try to get a better picture of the gradient from the pond input to the bridge. Although FIB and HF183 were low or not present, the sampling team continues monitoring around the headwaters to monitor for any changes. They also sample a couple of times at an exploratory sampling site (RC1.1) downstream of the headwaters and upstream of the input from the pond (Figure 9), to have a clearer picture of the stream before the input of the pond.

cH8 is never detected in or around the pond (RC2, RC2.1, and RC2.2) throughout the project, despite consistent persistent HF183 concentrations (average of 2.4 $\log_{10}$ GC/100mL). The sampling team learns that recycled water is used for irrigation at the park, which is the likely source of that signal. An increase in HF183 signal (highest observed value 3.1 $\log_{10}$ GC/100mL at RC2.2) during the dry season confirms this theory, as more watering with recycled water occurs. The HF183 signal at RC5 and regulatory site RC13 remains lower (1 order of magnitude less than at RC2) and is most likely from the high HF183 signals at the pond.

RC 2 and RC2.1 have a high average concentration of DG37, ~3.1 $\log_{10}$ GC/100mL, and concentrations of DG37 at both RC2 and RC2.1 are determined to be linked to rainfall, which indicates that the fecal waste from dogs in the park may be washing into the pond. GFD concentrations are consistently high (~2.7 $\log_{10}$ GC/100mL) at RC2 and RC2.1, and slightly lower (2.1 GC/100mL) at RC2.2, and RC4. Moderate quantities of GFD (1.8 $\log_{10}$ GC/100mL) at RC5 and RC13 could be from the pond or from the other waterfowl in the area.

Conclusions. While HF183 was prominent in this scenario, the source of this marker was from recycled water used at the park. HF183 is not significantly correlated to FIB concentrations and increased in the dry season as watering in the park with recycled water increased. H8 was never detected, confirming that this was not a sewage issue.

The real source of the high FIB load was the dogs at the park and the local waterfowl in the pond and in the stream. GFD and DG37 are identified as being correlated with concentrations of both FIB types. The stream soon drains into the ocean, where the fecal pollution will be washed out, although regulators may want to continue monitoring the stream as a source of pollution due to the abundance of recreational water activities that take place there. Park visitors should be

warned to stay out of the pond, and putting up additional dog waste stations may help reduce some of the contamination from dogs.
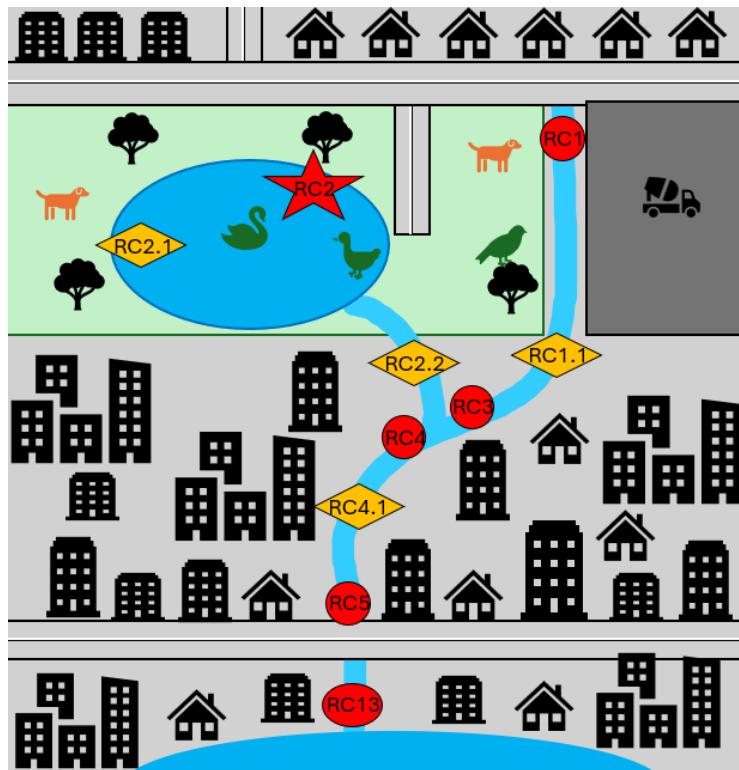
Figure 9. This map represents the stream in Scenario 3. The vertical blue line is the stream, which flows from the top of the map to the bottom, while the blue circle is the pond, which flows into the stream. Gray horizontal and vertical lines indicate roads. The stream begins at a stormwater pipe that is underneath the topmost road. Red circles/stars are the sites initially selected for the preliminary sampling, while the orange diamonds are additional sites added to the study after the preliminary sampling event. Labels, RC1-RC13, are site names. The red star indicates the sites where the highest FIB and MST marker concentrations were detected. Possible sources are marked in different colors – birds in green and dogs in orange, while the possibility of sewage comes from pipes under the roads that cross the stream.
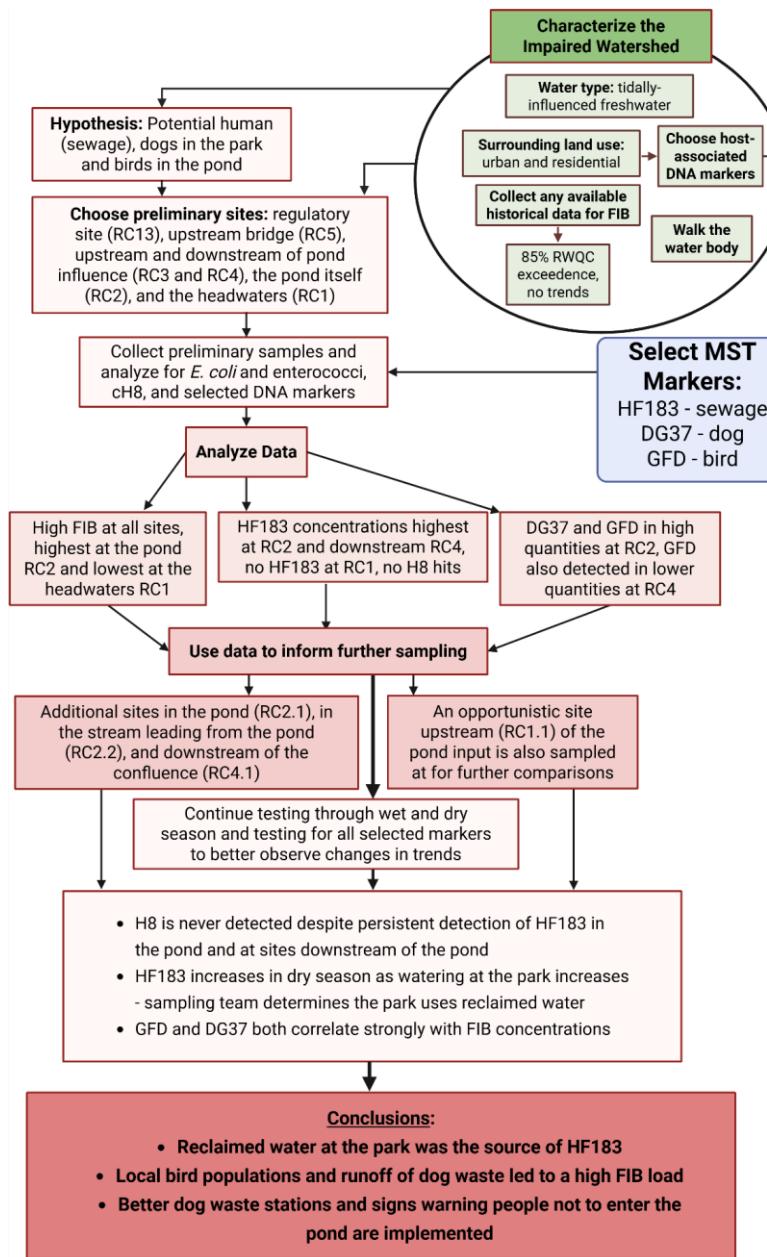
Figure 10. Decision tree process for Scenario 3. Created in https://BioRender.com

4.2.4 – Scenario 4

Watershed and probable pollution sources. The water body of interest in Scenario 4 is a large second-order freshwater stream in a mixed urban/rural area (Figure 11). The hypothesis, decision making, and conclusions are shown in Figure 12. It is identified for further study due to both high RWQC exceedances (95%, primarily in the dry season), and due to complaints of the smell from residents in the neighborhood nearby. The residents particularly have complained about the stormwater pond nearby which feeds into the stream. The previous year, a lift station by the stream had leaked and spilled several hundred gallons of sewage into the stream, but this had been fixed and cleanup efforts had been performed. No new indications of leakage had occurred, so the objective is to identify what is causing the high FIB concentrations.

Although FIB concentrations at the regulatory site are highest in the dry season, 95% of samples have exceeded the RWQC, so runoff cannot be excluded as a contributing factor. The sampling team identifies several possible sources, including human (central sewer or septic), dog, and wildlife (deer and birds). To investigate these sources, the sampling team selects HF183 (Table 4) as a sewage/human fecal marker, with cH8 as confirmation. Recycled water is used at the local dog park, and HF183 has been detected in both dogs and deer previously, thus a confirmation is necessary. DG37, Rum2Bac, and GFD (Table 4) are chosen to identify any contribution from dogs, deer, and birds, respectively. Finally, GenBac is chosen as a general fecal marker.

Preliminary sampling. Sampling begins at the start of the rainy season, and the sampling team walks the water body and selects six preliminary sampling sites (Figure 11). They select the regulatory site (AC233) as their most downstream site, which will allow them to compare their data against the historical data. They also choose a site (AC5) just upstream of it, close to a neighborhood. Another preliminary site (AC4) in the stormwater pond is selected to identify what the pond may be contributing to the stream. The sampling team also selects as preliminary sites: the bridge by the lift station (AC3), upstream by the dog park (AC2), and then a clean site further upstream (AC1), about 500 meters upstream of the dog park.

All samples are tested for *E. coli* and the selected MST markers. FIB concentrations are moderately elevated at all sites (~2.5-3.1 $\log_{10}$ CFU/100mL), highest at AC233 (3.1 $\log_{10}$ GC/100mL) and AC2 (2.9 $\log_{10}$ CFU/100mL). HF183 is detected in moderately elevated

concentrations (~2.1-2.4 $\log_{10}$ GC/100mL) at all sites except AC1 (which is BDL), but cH8 is not detected at any site. DG37 is detected in high quantities (2.7-3.1 $\log_{10}$ GC/100mL) at AC2 and AC3, and GFD is found in the stormwater pond, AC4, in low quantities (1.5 $\log_{10}$ GC/100mL). Rum2Bac is detected at the two most downstream sites, including the regulatory site. GenBac is detected at all sites in moderate quantities (~3.8-5.0 $\log_{10}$ GC/100mL), highest at AC4 and lowest at AC1.

Additional sites and monthly sampling. The sampling team adds a couple of additional sites to gain more information (Figure 11). They choose sites upstream and downstream of the input from the pond (labelled AC3.1 and AC5.1, respectively). Although the pond mainly had low levels of HF183 and GFD, they wish to better understand its impact on the stream. Additionally, AC3.1 is close to the lift station, which may help catch any possible leaks.

Sampling continues for the rest of the rainy season. FIB concentrations remain constant and elevated at all sites (~2.5-3.8 $\log_{10}$ CFU/100mL) and are lowest at AC1 (~2.1 $\log_{10}$ CFU/100mL). HF183 detections are consistent, most commonly observed in larger quantities (~2.1-2.6 $\log_{10}$ GC/100mL) at AC5 and AC233 and in lower quantities (~1.8 $\log_{10}$ GC/100mL) at AC2 by the dog park. No cH8 detections occur in this time period. Rum2Bac is detected at AC5 and AC233 as well as at AC5.1 after a large rainfall event. GFD is detected only in the pond, AC4, and at AC5.1 in low quantities (~1.2-1.5 $\log_{10}$ GC/100mL). DG37 continues to be detected in high quantities at AC2 and AC3 (~2.5-3.0 $\log_{10}$ GC/100mL), and lower at AC3.1 (~1.9 $\log_{10}$ GC/100mL). After the rainfall event DG37 is detected at AC5.1 as well, it is not quantifiable. FIB concentrations remain high (~2.9-3.5 $\log_{10}$ CFU/100mL) at all sites down to AC233, with the exception still of the clean site AC1.

The sampling continues into the dry season, and FIB concentrations increase (now ~2.9-4.1 $\log_{10}$ CFU/100mL) even as the pond turns into a marsh and the water level in the stream drops. GFD concentrations increase to ~2.5-3.0 $\log_{10}$ GC/100mL at AC4 and AC5.1 as migratory birds settle into the forest and the marsh. DG37 concentrations decrease dramatically, now only quantifiable at AC2 and detectable but not quantifiable (DNQ) at AC3. HF183 is not frequently detected in the dry season, although Rum2Bac is detected a few times in low concentrations (~1.2-1.5 $\log_{10}$ GC/100mL) at AC5.1, AC5, and AC233. There continue to be no detections of cH8 at any site.

GenBac concentrations decrease by an order of magnitude at all sites in the stream but increase (now ~5.4 $\log_{10}$ GC/100mL) in the pond in parallel with GFD.

Performing some preliminary statistical analysis, the sampling team are surprised to see that GenBac concentrations are only weakly correlated with FIB concentrations in the stream. GenBac typically correlates strongly and positively with FIB concentrations. GenBac is a general fecal marker and is found in any recent fecal pollution. The sampling team theorizes that perhaps what they are observing is not recent fecal pollution, but instead a naturalized FIB population.

Sediment sampling. The sampling team continues to sample in the stream for three more months and also collect sediment samples, to look for a potential naturalized population. They observe that FIB concentrations within the sediments are almost an order of magnitude higher than in the water at all sites, with the exception of AC1, where the water and sediment concentrations are similar. FIB levels are highest at the three most downstream sites: AC5.1, AC5, and AC233. MST markers HF183, and Rum2Bac are observed in the sediment at AC5.1, AC5, and AC233, but infrequently and usually DNQ. GenBac is also observed in the sediment, but an order of magnitude less than in the water samples.

Paying more attention to the sediment, the sampling team observes deer and other animal tracks in the stream and also notice local children playing in the shallow stream, stirring up the sediment. As the wet season approaches once more, the water level in the stream rises, and the FIB concentrations decrease (to an average of 2.8 $\log_{10}$ CFU/100mL), although this is still exceeding the RWQC. The sampling team theorizes that the higher water level means that there is less disturbance of the sediment and the naturalized bacteria.

Conclusions. Statistical analysis shows that FIB and MST markers are significantly correlated with large rain events. The sampling team presumes that the MST markers increase during rain events due to runoff from the surrounding area. The correlation between FIB and rainfall is likely due in part to runoff, especially with the dog park upstream, but may also be influenced by the disturbance of the sediment during large rain events, stirring up the naturalized population within.

FIB concentrations are correlated to DG37, and weakly to GFD, but no correlation is observed to HF183 or Rum2Bac. High DG37 concentrations were observed during the rainy season, and

especially during large rain events, when runoff from the dog park would impact the stream. The pond had high FIB levels when the bird population was present, but this impact was seasonal and limited to the pond. The influence from migratory birds cannot be mitigated, but a local agency could put in dog waste bins and fixing the drainage problem at the dog park to reduce the impact of dog fecal waste on the stream.

HF183 and Rum2Bac are found to be correlated to each other, indicating deer as the source of HF183. HF183 is also correlated, though more weakly, with DG37. The lack of cH8 detection throughout the study confirms that deer and dogs are the source of HF183, not the lift station.

The main source of FIB in the stream is the naturalized population in the sediment. The lack of a strong correlation between FIB and GenBac, a general fecal marker, as well as the high population in the sediments indicate the majority of the FIB were from a naturalized population in the sediment of the stream. This population was stirred up by wild animals, children, and large rain events.
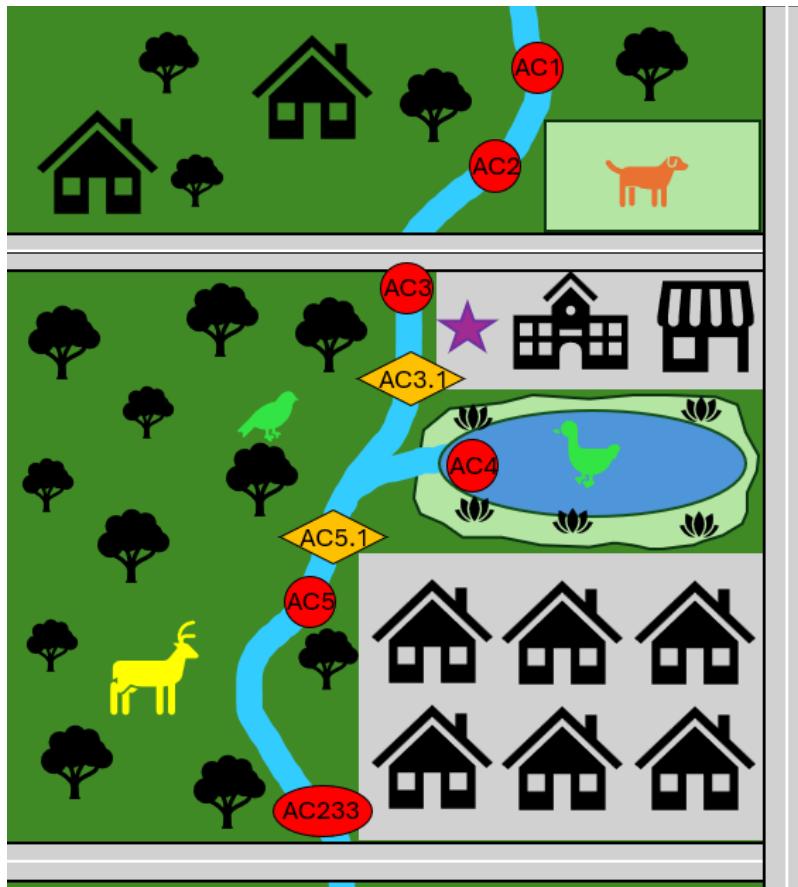
Figure 11. This map represents the stream in Scenario 4. The vertical blue line is the stream, which flows from the top of the map to the bottom, while the blue circle is a stormwater pond surrounded by a marshland (light green). The pond flows into the stream, as shown by a connecting blue line. Gray horizontal and vertical lines indicate roads. A dog park is shown as a green rectangle. The location of a sewage lift station is indicated by a purple star. Red circles are the sites initially selected for the preliminary sampling, while the orange circles are sites added to the study after the preliminary sampling event. Possible sources are marked in different colors – birds in green, dogs in orange, deer in yellow, and human in purple.
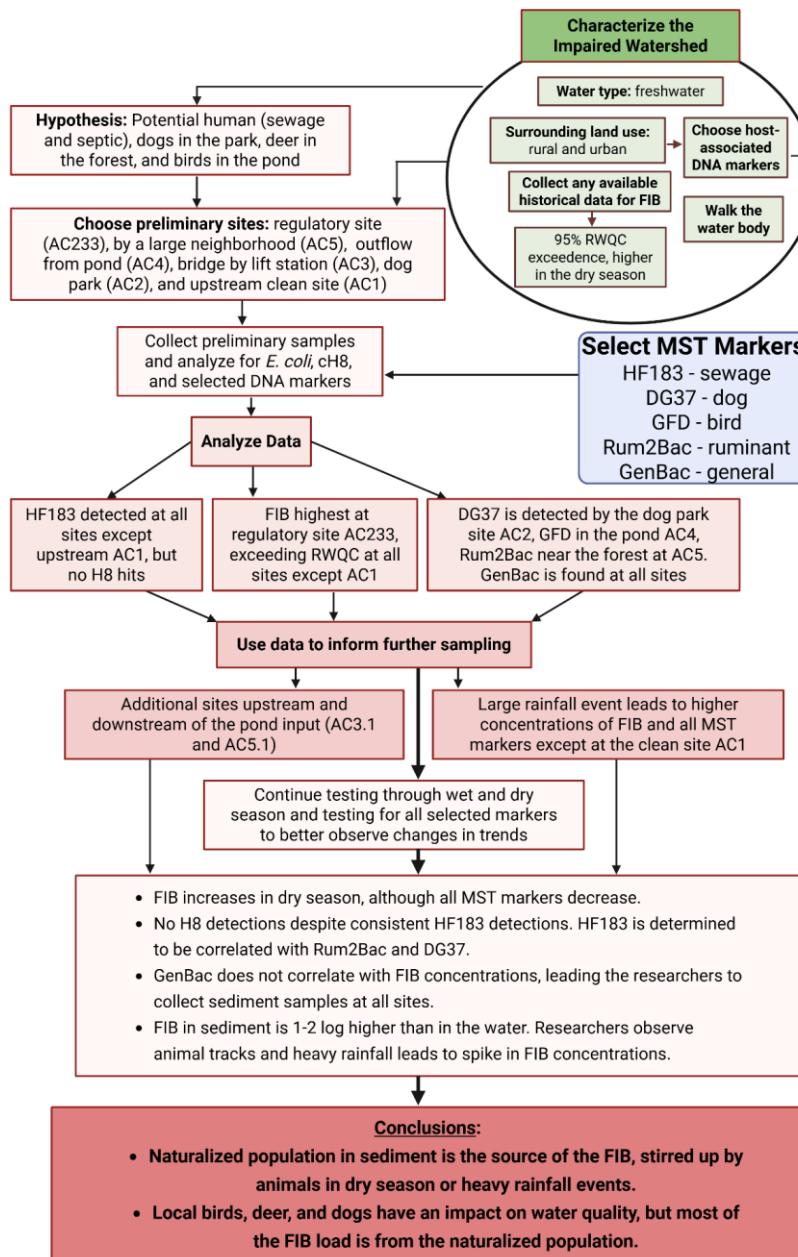
Figure 12. Decision tree for Scenario 4. Created in https://BioRender.com

CHAPTER 5: MST STUDY EXAMPLES FROM THE LITERATURE

The purpose of this chapter is to highlight the methodology and key findings from several MST studies, which are intended to give readers a sense of the breadth of questions that can be answered by MST. The specific approaches utilized in the studies are also emphasized. The sections below include the rationale for each case study, methods used to identify sources of fecal pollution including MST markers selected, and recommended actions from each study.

5.1 Case Studies Highlighting the Impact of Animal Sources on Water Quality

5.1.1 – The Importance of Sample Matrices and the Influence of Wild Bird Populations on FIB Concentrations

A case study conducted in freshwater Reedy Creek in central Florida investigated a watershed that is also managed for wildlife conservation (Figure 13). A segment of Reedy Creek was added to Florida's Department of Environmental Protection 303(d) (impaired water body) list in 2010 (Nguyen et al., 2018) due to elevated "pathogen" (fecal coliform) levels. Six sites were sampled monthly over a two-year period to determine whether birds or sewage were major contributors to FIB levels and whether site, temporal factors, and sample matrix (sediment, vegetation, and water) affected FIB and MST marker concentrations.

Study Conclusions*:*

- The random effects (variables) of years and months accounted for 43% and 50% of the variation in GFD and HF183 measurements, respectively, indicating temporal variations in the MST variables.
- FIB and GFD were significantly more concentrated in sediment and vegetation compared to water, demonstrating that these matrices can harbor fecal contaminants.
- The hypothesis that high FIB levels were primarily influenced by bird feces was supported in this study by the infrequent detection of HF183 at low concentrations, and GFD levels whose peak coincided with migratory bird populations.
- Sewage-associated HF183 was cross-reactive with deer feces collected locally, and the large deer population around the segment of Reedy Creek is one possible explanation for the sporadic detection of HF183.

- Identification of a natural source (i.e., birds) influencing FIB concentrations helped inform watershed managers and prevented an unnecessary total maximum daily load program from being implemented.
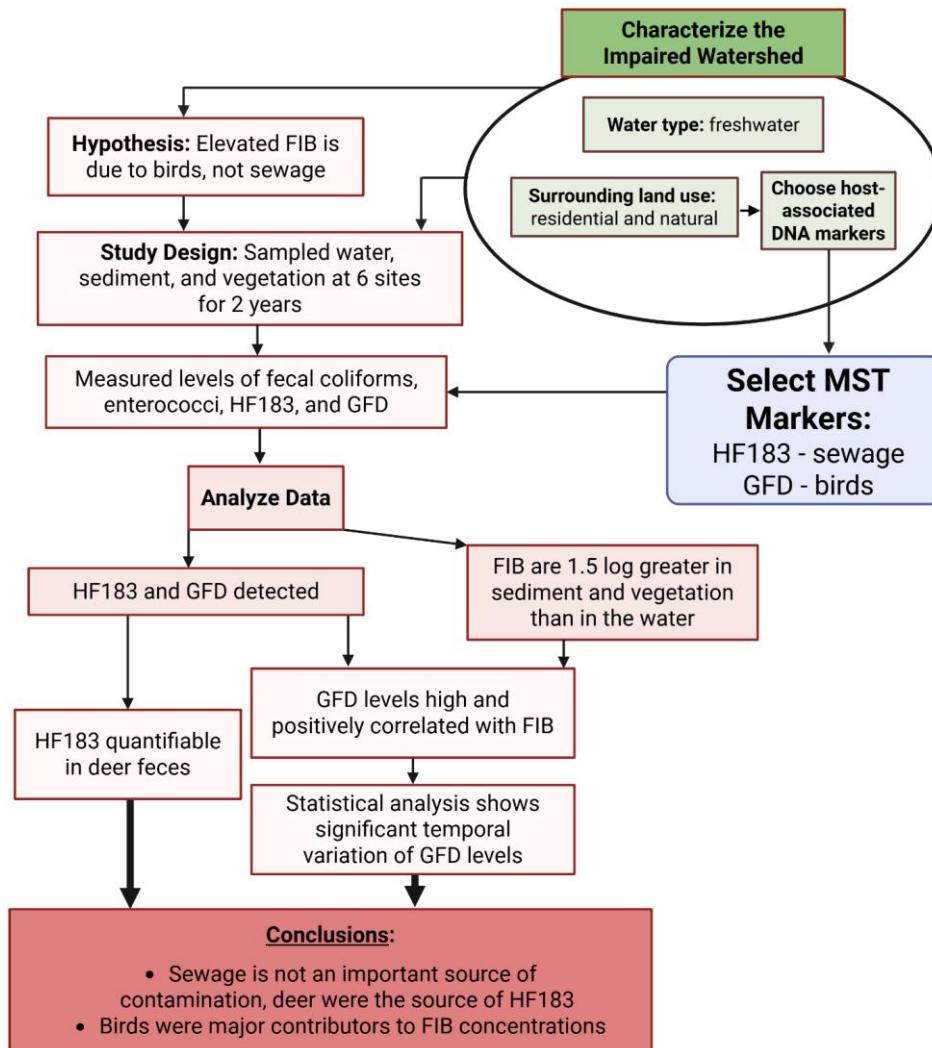

Figure 13. Flow chart of study design and key findings from case study (Nguyen et al., 2018). Created in https://BioRender.com

5.1.2 – Contribution of Runoff from Poultry Farms to Fecal Pollution

A case study conducted in Oklahoma (Weidhaas et al., 2011) examined the covariance between levels of the LA35 poultry marker and other water quality parameters in the Illinois River watershed, which experienced extensive land application of poultry litter (the used bedding from industrial-scale poultry operations). The sampling plan was designed to test the hypothesis that LA35 positive samples would also have higher concentrations of FIB (fecal coliforms, *E. coli*, and enterococci) and heavy metals (As, Cu, P, and Zn) as these pollutants are abundant in poultry litter (Figure 14). Field work and sample collection were completed over a two-year period. Samples included poultry litter from active houses (birds present), soil in areas where poultry litter was spread, runoff from soil where litter was applied (edge-of-field runoff), and streams, rivers, Lake Tenkiller, and groundwater including wells and springs.

Study Conclusions:

- Poultry-associated LA35 was consistently present in surface waters sampled near large scale poultry production and where poultry litter was applied to land as fertilizer.
- Concentrations of fecal coliforms, *E. coli*, enterococci, As, Cu, P, and Zn were significantly and positively correlated with levels of LA35 in surface water samples.
- The data presented in this study indicate that the Illinois River watershed was contaminated by land application of poultry litter and that the LA35 marker was useful for identifying poultry litter contamination.
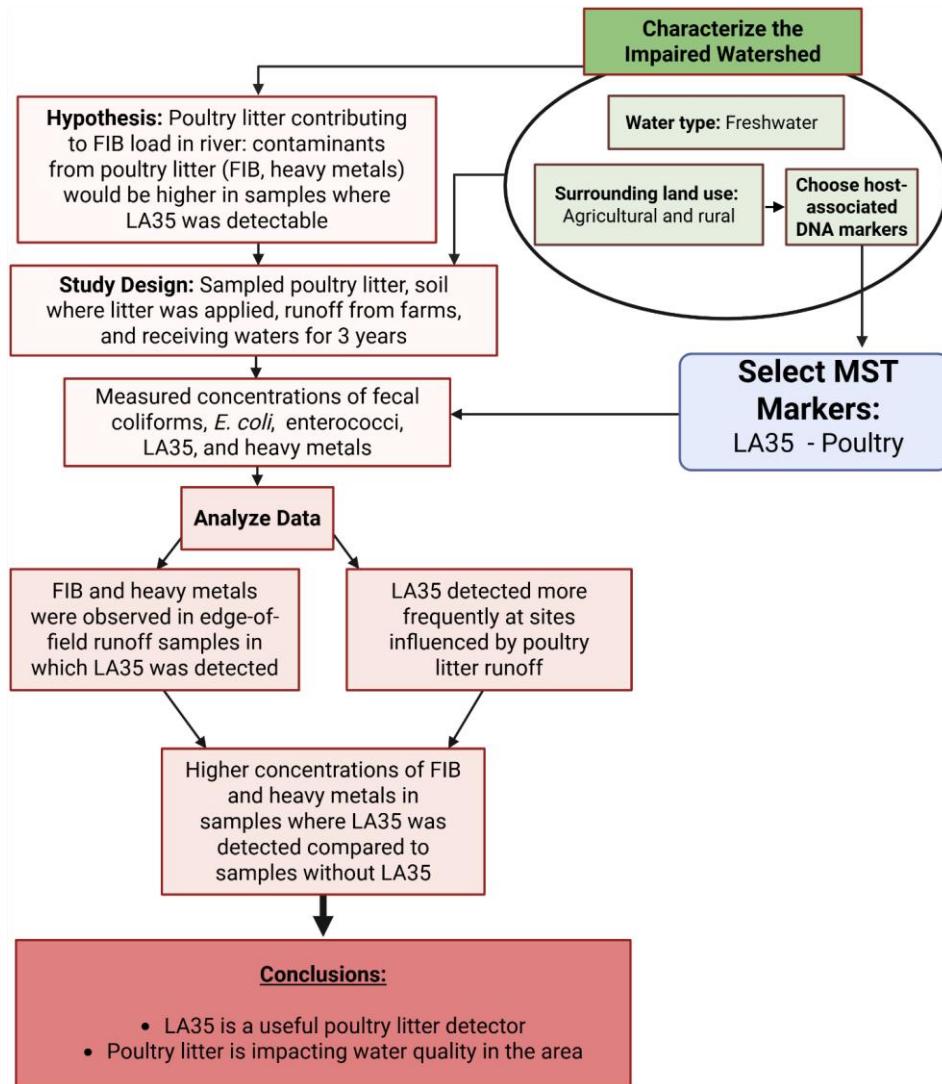
Figure 14. Flow chart of study design and key findings from case study (Weidhaas et al., 2011). Created in https://BioRender.com

5.2 Sewage Infrastructure Failures Identified by MST

5.2.1 – Identification of Sewage Pollution at a Florida Beach is Followed by Successful Remediation

A study in Tampa Bay utilized the sewage-associated MST markers *Enterococcus faecium esp$_{fm}$* and human polyomaviruses (HPyVs) to identify sewage contamination as a major contributor to FIB concentrations and to measure the effect of remediation efforts at a recreational beach (Korajkic et al., 2011). This study targeted two beaches in Hillsborough County, Florida with contrasting water quality. Recreational water quality criteria were exceeded in >50% of water samples from Ben T. Davis Beach. Possible sources of fecal pollution included stormwater runoff, functional restrooms that were temporarily closed to the public, and portable toilets (Figure 15). In contrast, the RWQC values for fecal coliforms and enterococci were infrequently exceeded at Bahia Beach (13.9%), which was not directly impacted by stormwater or other anthropogenic factors. Note: HPyVs and *esp$_{fm}$* are infrequently used in current MST studies because (1) HPyVs are less concentrated than other markers in sewage, contributing to the potential for false-positive findings and (2) the need to pick and analyze *Enterococcus* colonies makes the method relatively labor-intensive.

Study Conclusions*:*

- At Ben T. Davis Beach, frequent detection of sewage-associated HPyVs and *esp$_{fm}$* identified an ongoing leak in the wastewater collection system, resulting in the repair of a sewer main near the polluted beach.
- Sewage impacts were also evident at a second Ben T. Davis Beach site. In this case, demolition of the closed restrooms was expedited, and portable toilets were moved away from the shoreline.
- Remediation efforts at the impacted beach significantly mitigated the sewage contamination from faulty infrastructure and portable toilets, supported by fewer exceedances of FIB criteria and a lower frequency of detection for sewage-associated MST markers.
- RWQC at Bahia Beach were infrequently exceeded and each sewage-associated MST marker was detected only once over the study duration, leading to the conclusion that water quality at this beach was not significantly impacted by sewage.
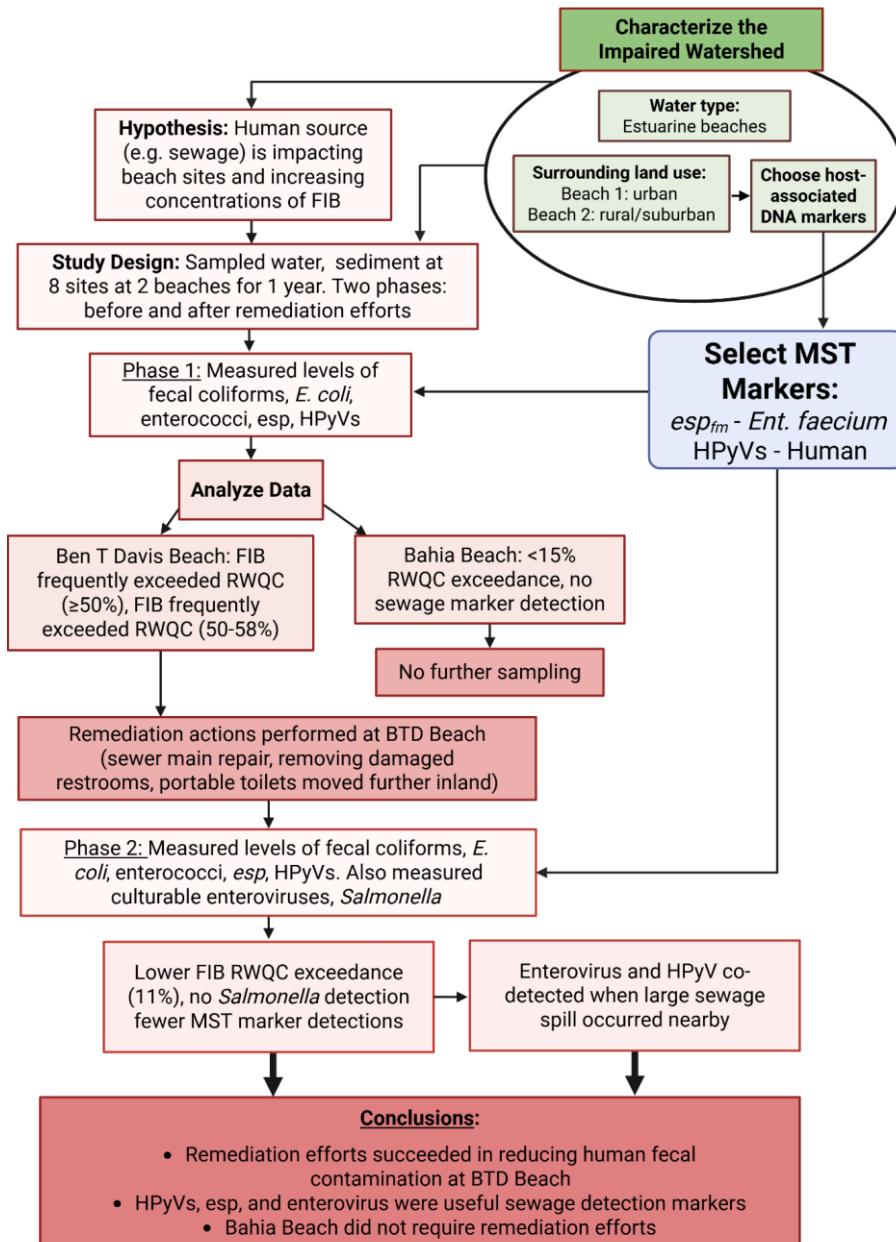
Figure 15. Flow chart of study design and key findings from case study (Korajkic et al., 2011). Created in https://BioRender.com

5.2.2 – Sewage Contamination Identified with MST in Stormwater Conveyance Systems

A study in Virginia utilized an adaptive sampling approach and measured HF183 to find "hot spots" of sewage influence in the pipes making up stormwater conveyance systems (Gonzalez et al., 2020). Sampling effort was focused on areas with relatively high frequency of detection and levels of HF183. Routine sampling of surface waters was conducted to monitor FIB levels, while focused, adaptive sampling was used to trace sewage signals from stormwater outfall to upstream stormwater systems (Figure 16). A second set of markers (HumM2, crAssphage, human polyomaviruses) was used to confirm successful remediation of areas where infrastructure failures were found using HF183.

Study Conclusions:

- In-pipe sampling is essential for success of this approach, termed "collection system investigation."
- Gaining useful regional infrastructure information for tracking possible sources of contamination requires effective collaboration with stakeholders.
- This approach identified pipe locations with consistently high HF183 levels. Sampling infrastructure around the hot spots narrowed the possibilities for the location of infrastructure failure, and increased the probability of finding and fixing issues in the stormwater conveyance system.

Characterize the Impaired Watershed

Objective: Identify sewage infrastructure failure by MST testing in stormwater conveyance systems

Study Design: Surveyed stormwater outfalls and upstream pipes adjacent to and upstream of locations with elevated FIB concentrations, sampling before and after repairs were enacted

Measured concentrations of enterococci and HF183 (pre- and post-repair), and HumM2, crAssphage, HPyVs (post-repair only)

Analyze Data

Detected HF183 at stormwater outfall upstream of site with elevated FIB

Sampled the stormwater conveyance system and narrowed down the possible source of the sewage signal to a specific segment suspected of infrastructure failure

Stormwater pipes positive for sewage contamination were repaired, with physical methods (smoke testing, CCTV, dye testing, etc.) to identify the failure point

Sampling one month after repair, enterococci was significantly reduced and no detection of sewage associated markers in the stormwater conveyance system

Water type: Tidally-influenced river

Surrounding land use: Residential and commercial

Choose host-associated DNA markers

Elevated FIB levels during dry and wet weather events

Select MST Markers:
HF183 - sewage
(Pre- and post-repair)

HumM2 - sewage
crAssphage - human
HPyVs - human
(Post-repair)

Conclusions:

- Sewage marker and physical methods identified infrastructure failure
- After the sewer line was repaired, sewage MST markers were not detected in the stormwater system nor in Wayne Creek

Figure 16. Study design and key findings from the case study (Gonzalez et al., 2020). Created in https://BioRender.com

5.2.3 –Investigation of Septic System Failure at a Campground following Norovirus Outbreak

A study that coupled epidemiology and MST with hydrogeology explored the transport of sewage from a septic system to a potable water well and recreational water (Figure 17) (Mattioli et al., 2021). The Norovirus outbreak occurred at a campground/outdoor event space, in Pennsylvania, USA, causing 179 illnesses; however, multiple exposure routes were suspected, including a freshwater creek used for swimming, two groundwater wells used for potable water supply, communal kitchen and restroom/shower facilities. A site evaluation identified the septic system as a possible primary contamination source for the creek and wells Sampling was completed over two consecutive days to investigate potential hydrological connectivity through the septic leach field, the potable groundwater well, and the adjoining creek with recreational waters. HF183, F-specific and somatic coliphages, and FIB were measured over a two-day study.

Study Conclusions*:*

- MST can identify sewage pollution in the environment and help identify a point source for outbreak events.
- Geological analysis proved useful for identification of an unexpected connection between the septic system leach field and both potable water wells and surface water.
- MST can help to develop and implement evidence-based, adaptive intervention strategies to reduce human exposure to pathogens and avoid subsequent outbreaks associated with sewage or septic pollution in recreational waters.
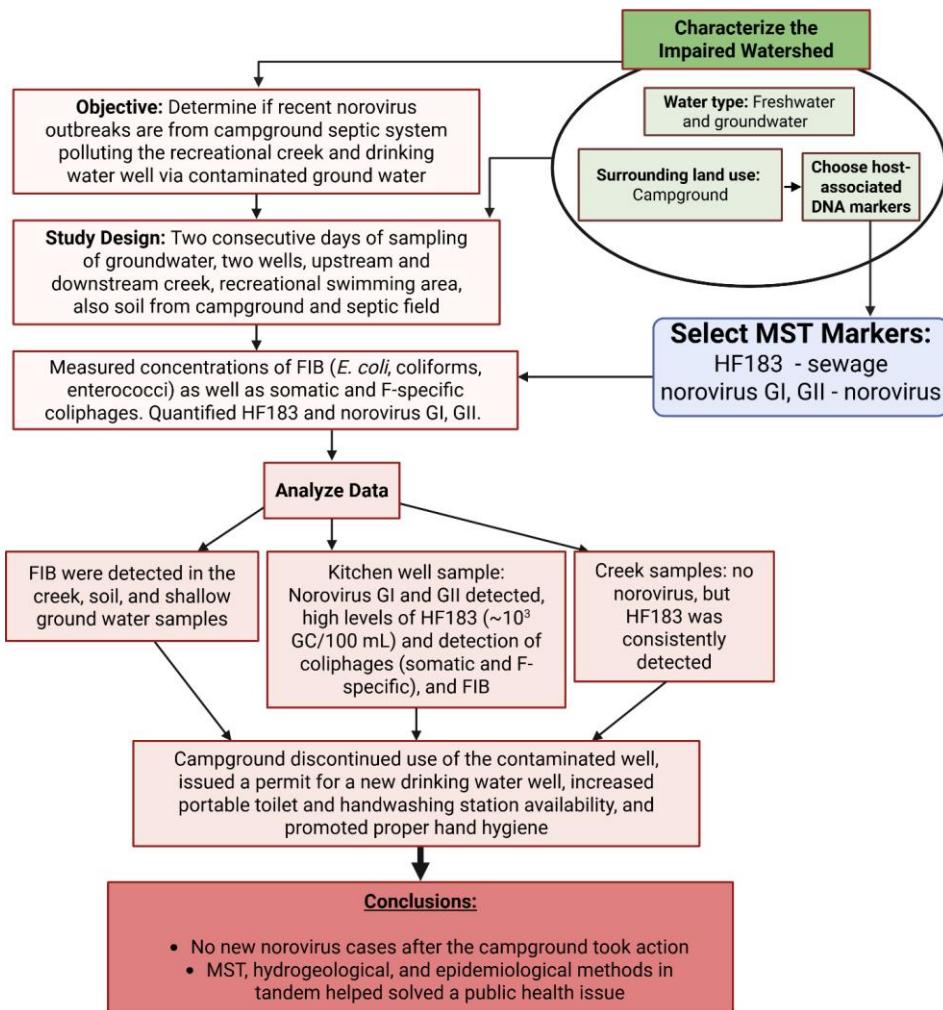
Figure 17. Flow chart of study design and key findings from case study Decision Tree (Mattioli et al., 2021). Created in https://BioRender.com

CONCLUSIONS

In this guide document, we present a comprehensive overview of microbial source tracking. We describe the framework and decision-making process involved in MST projects and translate them into both hypothetical scenarios and real-life examples. We introduce the various processes that can be utilized by managers, regulatory agencies, and researchers, including FIB culturing, MST marker usage and validation, and chemical and physical methods.

The benefits and limitations of FIB culturing and MST methods are presented in this guide document. Culturing and marker quantification are essential tools for MST researchers; both provide data that will allow for the identification of sources of contamination. When conducting your project, it is essential to remember that:

- FIB are useful, but have limitations:
    - FIB data alone constitute a regulatory tool and a categorizing tool by which we can compare gross contamination levels among sites or water bodies.
    - FIB provide imperfect information about human health risk and no information about source.
- MST provides information about sources of FIB and/or fecal pollution:
    - Validation of MST markers (e.g. assess sensitivity and specificity) in the local environment before implementing them in a project will ensure that useful methods that provide accurate results are chosen.
    - MST tools that are based on nucleic acid (DNA or RNA), without culture, should be used with the caveat that treated wastewater, including recycled (reuse) water, contains nucleic acid that can be detected by methods such as qPCR. This can provide a false-positive signal of sewage contamination. Understanding of treated wastewater discharges and recycled water use in the study area is important to minimize errors.
    - In locations with high potential for impact from treated wastewater, use of a secondary marker in a cultured organism is very useful.
    - The marker-based assays described in this document are limited, in that one assay is needed per putative pollution source. Useful markers have not been developed for all host types and, if many different animals may impact a given water body, it can be quite expensive to test for many probable sources.
- FIB and MST data are synergistic:
    - Using FIB and MST together allows better risk assessment and allows prioritization of impaired waters for mitigation strategies.
    - Correlation of FIB and MST markers is an indication that a particular source is contributing to fecal contamination at that location. This can be useful for discriminating between marker contributions from treated wastewater vs sewage.
- MST is an evolving tool, therefore it is important to keep abreast of the literature for new assays and methodologies that may be useful.

We hope that this guide document has provided a road map for better understanding of the myriad factors involved in planning and executing a study of the sources of fecal pollution in

water. Interpretation of the data should also be facilitated by this document. We end with advice for managers and regulators who plan to embark on a MST study: find a scientist with experience in environmental water quality and MST. Work closely with them on every aspect of the study. Understand that technology and capabilities are constantly improving, so that if your study raises more questions than answers, as sometimes happens, you can move forward armed with better questions, and more advanced methods.

ACKNOWLEDGEMENTS

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