Chapter 3. Microbial Source Tracking Methods

The purpose of this chapter is to review and discuss possible methods for detecting and identifying the sources of microbial contamination in surface waters. There are many highly technical methods employed in the source tracking field, with two broad groups that consist of microbial-based methods, called Microbial Source Tracking (MST), and chemical-based methods, or Chemical Source Tracking (CST). These methods are still relatively new procedures being developed and tested in the scientific community and are not necessarily feasible for local communities to conduct at this time. However, as a reference for understanding the state of the science, these techniques are briefly discussed in this chapter, and are fully described in Appendix I for those seeking additional information.

While these technical microbial and chemical source tracking methods may eventually become commonplace in water quality management, there are inexpensive approaches that communities can employ now to help identify the sources of microbial contamination. A shoreline survey is one of these approaches and the North Carolina Shellfish Sanitation Section routinely conducts these surveys. A description of the ordinance the Shellfish Sanitation Section abides by for their shoreline surveys and guidelines for communities interested in conducting this procedure are outlined in this chapter, before the other more sophisticated methods of tracking are discussed.

Section 1. Shoreline Survey Basics

In North Carolina, the Shellfish Sanitation Section, within the Department of Environment and Natural Resources' Division of Environmental Health conducts shoreline surveys every three years, meaning they go door-to-door in the survey area to map the sources of pollution to the local waters[1]. Additionally, annual updates are performed that consist of driving through the surveyed area to determine if development has occurred and if this land use change is a potential pollution source. This identification of potential pollution sources, such as septic systems, marinas and domestic pets, is to ensure the safe and sanitary control of the growing, processing, and shipping of molluscan shellfish for human consumption as well as determine the safety of swimming waters. While Shellfish Sanitation may close waters to shellfishing, they can only release swimming advisories for microbially contaminated waterbodies.

The North Carolina Shellfish Sanitation Section conducts shoreline surveys in accordance with the United States Food and Drug Administration's Office of Seafood's National Shellfish Sanitation Program Model Ordinance[2]. This ordinance is in the form of a guidance document, rather than a binding legal ordinance. However, through participation in the National Shellfish Sanitation Program and membership in the Interstate Shellfish Sanitation Conference, North Carolina has agreed to enforce the requirements of the Model Ordinance, which are minimally necessary for the sanitary control of molluscan shellfish. Conducting shoreline surveys is one requirement of this Model Ordinance's Sanitary Survey (Box 1).

Box 1. Shoreline Survey Requirements[1]

1) In the shoreline survey for each growing area, the Authority shall:
   a) Identify and evaluate all actual and potential sources of pollution which may affect the growing area;
   b) Determine the distance from the pollution sources to the growing area and the impact of each source on the growing area;
   c) Assess the reliability and effectiveness of sewage or other waste treatment systems;
   d) Determine if poisonous or deleterious substances adversely affect the growing area; and
   e) Consider the presence of domestic, wild animal or resident and migrating bird populations for possible adverse effects on growing areas.
2) The Authority shall assure that the shoreline survey meets the following minimum requirements:
   a) The boundaries, based on the area topography, of each shoreline survey area are determined by an in-field investigation which identifies only the properties with the potential to impact the shellfish waters;
   b) Each shoreline survey area is identified by a unique designation which results in identification of all data associated with each shoreline survey by the unique designation;
   c) Each shoreline survey area is investigated and pollution sources evaluated by qualified, trained personnel; and
   d) Documentation for each pollution source identified by the Authority as affecting a growing area includes:
      i) The location of the site on a comprehensive map of the survey area; and
      ii) The determination that the pollution source has a direct or indirect impact on shellfish waters.
   e) A written summary of the survey findings.

Conducting Your Own Shoreline Survey

Within the 20 coastal North Carolina counties, the Division of Environmental Health surveys the immediate shoreline every three years and the data associated with these surveys is available for public use. However, communities may benefit in several ways by conducting their own shoreline surveys, including understanding the area of land that contributes pollutants to coastal waters, the sources of microbial pollution and the types of development within their jurisdictions. All of these benefits may also help with other community projects such as watershed or land use plans. Local communities that are interested in conducting their own shoreline surveys should keep in mind that these surveys can take on many forms, from driving through the survey area to a more complete door-to-door survey. Please keep in mind that a door-to-door survey involves inspecting private property and thus there are legal implications associated with trespassing. An unofficial door-to-door survey conducted by local communities may also hinder the Division of Environmental Health's ability to gain access to these properties during their required surveying periods. Therefore, local communities should respect private property and instead of trespassing, simply walk or drive through the survey area to gain an understanding of the land uses within their jurisdictions. Regardless of the survey type, referring to the following guidelines and tailoring them to the preferred survey style will gather the desired information:

Notify local authorities that you are conducting a survey.

These agencies may be able to help with the project as well as reduce data collection redundancy:

- County health department has jurisdiction over septic systems;
- Division of Coastal Management can help determine Coastal Area Management Act (CAMA) violations; and
- Shellfish Sanitation Section may already have the area surveyed and can help determine gaps in current surveys.

Determine the boundary of the survey area.

Local communities may want to take into account the entire watershed, or a region that drains into

common body of water, when surveying for pollution sources.

Estimate population of the survey area.

This may be simple for a single town or city. For a larger affected area, count the number of houses in the survey area. Then multiply thenumber of houses by what post office determines as the number of people per household (e.g. 3.5 people per household, 2 people per household, etc.). In more rural areas, the number of houses in a particular area may be determined by consulting postal workers who service a particular area.

Drive-through the survey area to get an understanding of the possible pollution sources.

Pay particular attention to the following:

- Percentage of impervious surface in the watershed (see Chapter 4)
- Marinas and docking facilities:
  - The number of slips may change frequently in a marina. In Class SA (see Chapter 2) waters only a certain number of boat slips are allowed.
  - An automatic closure of the water body to shellfishing may result from a marina going over this certain number of slips.
  - Local communities should be aware that in private developments, a 10 slip docking facility is allowed without being classified as a marina. The concentration of these 10 slip docking facilities may increase without restriction, with their cumulative pollution potential equivalent to a full scale marina. However, since these docking facilities are not classified as marinas, no automatic closure of the water body to shellfishing can occur.
- Wastewater treatment plants
- Package plants (small wastewater treatment plants)
- Agriculture with high numbers of livestock, i.e. cattle, chicken, hogs, etc.
- Domestic pets, i.e. cats, dogs, etc.

Expand your inspection by going door-to-door. Pay particular attention to the following:

- Septic systems (Box 2)
- Stormwater drains
- Ditches, canals

Box 2. Signs of Septic System Failure

There are many things to watch for that can indicate septic system failure. Keep in mind that there can also be problems beneath the land surface that may not be visible. Proper maintenance and use of septic systems are essential to preventing problems.

- **Wet spots or standing water** - Areas that remain wet after rain events or appear without precipitation should be investigated, since effluent can seep up from a failing system and puddle on the ground surface.
- **Odd growth patterns** - Green lines on the drainfield that follow the path of the pipes are normal; however, green blobs not in line with pipes, random patches of lush growth, and bulls-eye patterns (dead areas surrounded by green growth) all indicate potential problems in the drainfield.
- **Slow drains or backups** - If wastewater is slow to drain and the plumbing is working properly, there may be a problem with backup in the septic tank.
- **Septic odor** - Easy to detect, odor is one of the best problem indicators.

It is important to realize that a shoreline survey is somewhat of a time consuming process. It took the Shellfish Sanitation Section's experienced staff over six weeks to survey the White Oak River sub-watershed in 2002, which includes parts of Jones, Onslow, Craven and Carteret counties and the municipalities of Maysville, Cape Carteret, Emerald Isle, Swansboro and Cedar Point. Staff of the Shellfish Sanitation Section suggest that local governments may also find it useful to map the community's stormwater conveyance system (i.e. stormwater drains, ditches, canals, etc.) using a Global Positioning System (GPS) unit and mapping software. This information would be invaluable in targeting problem areas and planning for future growth.

For more information on shoreline surveys, contact the Shellfish Sanitation Section:

(252) 726-6827 or http://www.deh.enr.state.nc.us/shellfish/index.html

Section 2. Technical Source Tracking Methods

The contamination of surface waters from human and animal waste is of great concern to coastal communities due to the economic importance of these waterbodies. While nonpoint source pollution is believed to be the biggest contributor of these wastes to waterbodies, it is difficult to identify the ultimate sources of microbial pollution. Knowledge of microbial pollution sources will help local communities restore water quality, decrease the levels of nutrients leaving their watershed, and decrease the danger of infectious disease that results from exposure to microbially contaminated water[9]. Scientists know the benefits of identifying microbial sources for water quality improvement and have begun researching technical methods that determine these sources. These methods use a wide variety of substances to indicate the presence of human and animal waste, including indicator bacteria (see Chapter 1) and chemical substances. Most of these technical source tracking methods are still in development and not practical for local communities to conduct on their own at this time. Additionally, the current methods do not generally allow for researchers to point fingers directly at a specific farmer or home as a source of microbial pollution, but rather gives a general picture of the microbial sources within the watershed. However, this field is evolving and soon methods may become more accurate and available for use at the local level. This section will briefly review these technical source tracking methods. Please note that each method has its plusses when used to answer a specific question or designed for a specific purpose. So there is no one perfect method, they all have their advantages and disadvantages. For a more complete description of the methods mentioned in this section, see Appendix I.

Microbial Source Tracking Methods

Of the technical source tracking methods currently in development, microbial (bacterial and viral) methods are by far the most useful and advanced methods. Most microbial source tracking (MST) methods utilize intestinal bacterial and viral indicator organisms that are often associated with pathogens to assess the level of microbial contamination at a given site. The current MST field generally consists of two distinct approaches that use either the molecular structure (genotypic) of the microbial indicator or non-molecular/physical characteristics (phenotypic) of microbial indicators. Physical characteristics may include degree of antibiotic resistance, chemical secretions, and growth requirements.

Most non-molecular methods described below offer certain advantages over molecular methods. The advantages include less training for lab personnel, lower per isolate cost (in terms of time and materials), and the potential to process hundreds of isolates per week (a few dozen isolates per week is typical for molecular-based methods). Highly contaminated sites may need hundreds of isolates analyzed so that the results will be representative of all possible microbial contaminants. Thus, non-molecular methods have some advantages over molecular methods in that large volumes of samples can be processed in less time, and for lower cost.

Box 3. Microbial Source Tracking Methods Classification

Non-molecular Library-dependent Methods
- Antibiotic Resistance Methods
- Carbon Source Profiling (Nutritional Analysis)

Non-molecular Library-independent Methods
- Fecal Bacteria Ratios
- Non-molecular Species-Specific (Host-Specific) Indicators
- F+ coliphage serotyping
- Enterotoxin Biomarkers

Molecular Library-dependent Methods
- Rep-PCR (Polymerase Chain Reaction)
- PFGE (Pulsed-Field Gel Electrophoresis)
- Ribotyping
- RAPD (Randomly Amplified Polymorphic DNA)

Molecular Library-independent Methods
- Bacteriophage Indicators
- Virus (Human Pathogen) Indicators
- Molecular Species-Specific (Host-Specific) Indicators
- Bacterial Endemism and Co-Speciation
- tRFLP (Terminal Restriction Fragment Length Polymorphism)

Many MST methods require the development of a background library or database of known microbial sources. Libraries are used to compare samples of unknown origins to a sample of known sources, such as water samples collected from a contaminated site to fecal matter collected from a nearby cow pasture[3].

Recently, top scientists and managers in the field of MST combined their efforts at the first annual US EPA Workshop on MST in Irvine, California[3] to review the latest techniques and innovations in the field, plan future research, and come up with a basic MST classification system. This system divides most MST techniques first among their main identifying characteristics (non-molecular vs. molecular) and second among their requirement for a comparison library (library-dependent vs. library-independent). Box 3 lists these various MST methods. For a complete review of these methods, see Appendix I.

Chemical-Based Source Tracking Methods

There are several chemical-based methods used to signal the presence of human waste. These methods are generally not applicable to animal waste because they detect by-products of human activities. Chemical indicators can either be human metabolic substances such as caffeine or coprostanol (by-product of cholesterol breakdown), or biochemical substances associated with household water (or grey water) discharge such as detergents and optical brighteners. Chemical-based source tracking is often highly limited because the detection of chemical indicators only occurs when samples are collected in close proximity to pollution sources and when there is a considerable amount of sewage present. These methods are capable of providing presence/absence data in highly urbanized areas only and do not work very well in rural areas where the amount human sewage is generally not very high[4].

Whitening Agents & Optical Brighteners: Whitening agents and optical brighteners are chemicals used in laundry detergents. They are often a large component of grey water discharge and have been used as indicators of sewage fallout[5].

Caffeine: Caffeine is generally found in highest concentrations in highly urbanized areas. Caffeine levels must be present in very high levels (<200:1) to be detected by current methods[6].

Coprostanol: Coprostanol is a byproduct of the breakdown of cholesterol and is present in human and some other mammal species. Human secretion of coprostanol can be inconsistent but in general it is a good indicator of human fecal pollution[7].

FAME (Sterols/Fatty Acid Methyl Ester Analysis): Sterols are biochemical components of fatty acids that make up cell walls and membranes of bacteria. The fatty acid analysis method is proposed to differentiate between the types and amount of sterols found both in human and animal *E. coli*[4].

Checks, Balances, and Trade-offs in Source Tracking Studies

This section briefly reviews some important issues associated with selecting source tracking methods and describes some issues researchers need to consider when deciding which are the best source tracking methods to use for a particular study design.

Importance of Local Knowledge[6]

Before any source tracking study is undertaken, it is vital to gather land use information that identifies potential contamination sources within the watershed. A good deal of useful information for determining the sources of microbial contamination can be gathered based on prior knowledge of watershed characteristics. For instance, if it is known that the only local fecal sources are a hog farm and some wildlife, there would be little need to monitor for urban pet waste. This local knowledge, also known as a shoreline survey, includes:

- Identification of all possible sources of microbial contamination (deer, pig farms, etc.);
- Location of all input sources (septic systems, sources of water onto roadways, level of ditching, etc.); and
- Understanding of how water flows over and through the local landscape (dye tracing studies may be helpful).

Knowledge of land use can be very helpful for identifying potential sources to track. In addition, knowledge of individual watershed characteristics (e.g. impact of storm events) can help tracking studies capture site-specific differences that occur over time and space.

Library Development[4]

Some MST methodologies (except chemical methods) require building extensive libraries or databases of either molecular or non-molecular information (fingerprints) from isolates of known sources of fecal contamination (human, cow, deer, etc.). Fecal isolates of unknown origins sampled during a study are then compared to these source libraries, and identified by comparison of the 'fingerprint' of the unknown as compared to the library of 'fingerprints'. One major problem for researchers and managers is the need to determine how big source libraries need to be to obtain meaningful, interpretable results as well as statistical significance. Considerations in the development of each MST library include the size and characteristics of the watershed, the diversity of animal species present, the natural changes in the bacterial populations over time, and the number and type of human sources that may impact water quality. In addition, while the size of each source library will be largely determined by the number of potential major sources of microbial pollution in the target area, it is still unknown at this point how large a library has to be or how similar libraries from different geographical areas must be. Therefore, at this time building new libraries for each individual study (at least between watersheds) is often required and at least a few hundred isolates from all major known sources are needed. It is necessary to continually maintain the library with new known source isolates to ensure continued correct source identification.

The Toolbox Approach[4]

There is some controversy over the use of molecular (genotypic) vs. non-molecular (phenotypic) methods within the MST field. In reality, these methods should be viewed as complimentary, rather than opposing one another or in competition with one another. Molecular methods, in general, are more precise, yet often complex, costly, and time consuming. Most non-molecular methods are often less precise in identifying microbial contamination sources but are generally simpler and less costly, thus many more samples (offering greater spatial resolution in some cases) can often be analyzed. Researchers and managers should keep in mind that there is not one "best" or "perfect" source tracking method and that a better approach is to combine several molecular and non-molecular methods. By using this "toolbox" approach, researchers can:

- Tailor source tracking methodologies to individual study needs and limitations;
- Increase statistical confidence by obtaining similar results from different methods;
- Expand research results by identifying sources from one method that the other was unable to identify; and
- Supplement source tracking methods that give only qualitative or quantitative information.

Using the "toolbox" approach to select appropriate source tracking methods for a particular monitoring study can be highly effective. In addition, utilizing multiple microbial indicators in a monitoring study can help to provide a much more complete picture of impaired sites. Currently, *E. coli* and fecal streptococcus are the focus of the majority of bacterial source tracking methods with many regulatory agencies preferring to use only *E. coli* (now a widespread indicator standard). But *E. coli* is often problematic, being difficult to detect under certain growing conditions and many times too cosmopolitan (occurring in all hosts) for all source tracking applications. *E. coli* is also known to regenerate in the environment under some conditions but natural regrowth and resuspension are currently unquantifiable. In these cases, additional indicator organisms such as bifidobacteria, Bacteroides-Prevotella group, or coliphages (viruses that infect coliform bacteria) may provide better source tracking of microbial pollution. Again, no one "best" indicator is available because most microbial indicators are not optimal for every situation.

Methodology Considerations: Cross-Comparability, Repeatability, Accuracy[3]

One major limitation to the "toolbox" approach is interpretation of the results of very different research methods. Often, comparing MST methods is analogous to comparing apples and oranges, particularly so for statistical comparisons. Because MST techniques are still in a rapid phase of development, there is a lack of extensive studies comparing these various methods to each other.

Additionally, the MST field suffers from a lack of uniform standards. Methodological protocol for sampling and measurement are routinely constructed for almost every new study. This is largely due to new and constantly evolving methodologies within the source tracking field and not necessarily any lack of rigor on the part of investigators. Because there is a general lack of knowledge of field and laboratory protocols, it is necessary that MST studies, at the very least, provide, accurate, repeatable results. Researchers and managers need to be keenly aware of their own collection protocols, laboratory methods, quality assurance and quality control measures, and statistical analyses to ensure that they provide results that are of sound, scientific basis.

State of Research Methods, Applications, and Summary

Source Tracking Methodology – State of the Science

Out of the current technical MST methods, there appears to be one non-molecular method (multiple antibiotic resistance) and three molecular methods (ribotyping, pulsed-field gel electrophoresis, and polymerase chain reaction) that are sufficiently developed and capable of determining sources of fecal contamination (see Appendix I). Most studies that have used antibiotic resistance have obtained average rates of correct classification (ARCC) for fecal coliform and enterococcal isolates in the range of 50% to 90% or higher and it has been demonstrated that the ARCC could be increased even more by using a larger number of antibiotics and concentrations[8]. Other non-molecular methods such as carbon source profiling and fatty acid analyses (FAME) are promising but still in the developmental phase and may be available in the near future. Reported results for molecular techniques are widely varied and highly dependent on the method used. Currently, all molecular techniques are costly, labor-intensive, and tedious; molecular procedures inherently require many delicate procedures where minute errors can produce different results. However, molecular techniques are highly sensitive methods, and future automation should decrease the potential for human error, increase sample volumes processed, and reduce per isolate costs[4, 10]. Chemical methods may have some use as low-cost, low-resolution indicators of human presence. Applications are limited largely by the inability to quantify the level of chemical presence with a given microbial load. Coprostanols appear the most promising and future research may eventually make this a widely applicable source tracking method.

Source Tracking Application in TMDL[4]

Source tracking methodologies are developing rapidly and regulatory agencies are beginning to incorporate MST into Total Maximum Daily Load (TMDL) programs to identify sources of microbial pollution. MST methods currently represent the best tools available for developing TMDL implementation plans, determining TMDL load allocations, and restoring watersheds (see Chapter 2 for its application in NC's TMDL Program). The use of MST technologies can also be effective in directing best management practices (BMPs) to remove impaired watersheds from the State's List of Impaired Waterbodies. One useful approach in implementing TMDL projects is to classify isolates based on broad categories such as human vs. wildlife vs. livestock, largely because landowner cooperation can be a major obstacle to obtaining property access to collect known source samples[4, 11,12]. A broad classification system is non-confrontational, and does not "point fingers" at individual property owners which is an important consideration as landowner participation in TMDL projects is largely voluntary. In addition, the EPA will soon require reasonable assurances of TMDL implementation and MST can be useful in helping states meet this requirement.

Summary[6]

Source tracking is a rapidly developing field that can provide valuable information to watershed managers; however, there is no one simple, low-cost method for differentiating between human and non-human sources of microbial contamination. Currently, the most widely used method is genetic fingerprinting (see Appendix I)[6]. Multiple antibiotic resistance (see Appendix I) can provide comparable source resolution to genetic methods, and is less costly and labor-intensive and therefore may be more useful to managers

looking for TMDL applications. However, multiple antibiotic resistance methods rely on development of large libraries, so are relatively time-consuming in their implementation. Other promising methods on the horizon include bacteriophages and quantitative detection of source-specific viral indicators, especially because they do not require library development.Since there is not any one MST technique that will work for every situation, the "toolbox" method appears to be the most effective use of these methodologies at present. ***The best approach at this time is to evaluate land uses and sources under investigation, and tailor the research methods to fit each individual situation or question.*** Some considerations in choosing source tracking methods are[6]:

- Source types (human, non-human, livestock, domestic pets, or wildlife);
- Pollutant loading sources and delivery vehicles (point sources, nonpoint sources);
- Sample medium (marine, freshwater, groundwater, sediments, shellfish tissue);
- Level of source resolution needed (human vs. non-human or individual categories for all types of fecal contamination);
- Cost for each method (usually estimated on a cost per isolate basis);
- Library building needs and capabilities; and
- Level of training of laboratory personnel.

Due to the recent focus on nonpoint source pollution, there is a great deal of research being done on a wide variety of methods to determine sources of microbial pollution. Location and routes of nonpoint source pollution can be extremely difficult to identify, and superior techniques such as MST can identify the source of microbial pollution at the site of contamination. Microbial indicators and MST can be particularly useful in identifying both sources of microbial contaminants to control violators and reduce source loads in a cost-effective manner. Because technologies are constantly developing, managers are urged to stay current on evolving methods, so that they have the best information on hand for both point source and nonpoint source management of microbial contamination[6].

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