Microbial Source Tracking (MST): Towards Effective Identification of Fecal Pollution Sources

MST Applications Workshop
Final Report

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- The Division of Research and Graduate Studies, Acadia University
- Synova Diagnostics Incorporated
- Nova Scotia Department of Environment and Labour

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Executive Summary

Microbial source tracking (MST) is an evolving discipline. Since it is a measurement-based technology, MST offers important advantages over source identification practices of the past. By tracking sources of fecal pollution directly, we can better target our remediation efforts; thereby saving time and resources. There are several different phenotype and genotype assessment MST methods currently in use, each with their own advantages and disadvantages. It is therefore likely that a national MST approach in Canada would use a combination of these methods, based on the needs of each project. Despite their present usefulness, there is a need for continued research and development of all MST methods in order to increase their accuracy and expand their applicability. It is anticipated that the methods will be sufficiently improved to make MST a reliable enforcement tool in the future.

The goal of the workshop and this report was to encourage the further development of MST through the creation of several demonstration projects throughout Canada. It is essential that lessons learned from such projects be transferable to future projects. On that note, it is also important to develop and sustain both national and international partnerships to learn from past experiences. In order to achieve these goals, there is a need for an effective coordinating body.

The MST Applications workshop successfully highlighted the importance of this tool as well as the need for further collaborative and financial support in this area of research and development. The workshop provided a forum for information sharing and effectively fostered further cooperation between a number of participants (eg: Environment Canada's Pacific Environmental Science Centre, PESC, in British Columbia and the Department of Fisheries and Oceans in Moncton, and, between PESC and Synova Diagnostics Incorporated in Nova Scotia). A national advising / coordinating working group has been selected. It has initiated collaborations with the National Programme of Action for the Protection of the Marine Environment from Land-Based Activities (NPA), looking first at various options for hosting a MST website. Following on the success of this workshop, there is a strong interest in hosting a similar workshop on Canada's West coast with a view to further MST development in that part of the country. Most importantly however, the workshop recognized the needs of the different user groups, and identified the necessary steps to turn words into action (see Recommendations and Action Plan).

Much work still remains to be done before MST can be used to its full potential. However, by means of public education, effective coordination, and continued support of research and development, MST has the potential to become an important tool in microbial pollution source identification.

Introduction

Microbial contamination of water is becoming an increasingly common problem in most parts of the world. As with most other natural resources, clean water is under mounting pressure from all who use it, including both human and non-human animal populations. Presently, one of the leading concerns regarding water quality is its alteration via the uncontrolled introduction of material of fecal origin. Methods traditionally used for monitoring fecal pollution only provide a quantitative analysis and offer little insight into its sources. Without knowing the sources of pollution, remediation, based on knowledge and assumptions of the surrounding land, use can be very challenging. A major advantage that Microbial Source Tracking (MST) offers to both remediators and regulators is that it is a measurement-based approach and, thus, reduces the subjective nature of remediation practices of the past. This technology offers the potential for identifying, tracking, and characterizing sources and reservoirs, and, therefore, provides a better tool for directing both remediation and enforcement efforts.

The sources of fecal contamination are widespread and the effects, influential. Some of the possible human sources include effluent from sewage treatment plants and faulty on-site septic systems. Of equal importance are the non-human sources of pollution. Animals domesticated by humans can also contribute to the contamination of surfcae water. Surface runoff from animal grazing areas, manure-treated agricultural land, and more recently, golf courses, can find its way into nearby bodies of water. Other domesticated animals, such as cats and dogs, must also be considered possible sources. Finally, wildlife can contribute significant amounts of fecal contamination to otherwise pristine environments that are minimally impacted by humans.

Regardless of its source, fecal pollution of surface water has strong impacts on the environment, the economy, and to human health. The presence of the commonly monitored intestinal microbe *Escherichia coli* indicates the likely presence of ecologically and physiologically related human pathogens such as *Salmonella, Campylobacter,* and *Shigella*, and other dangerous viruses such as hepatitis A. Fecal pollution can also indicate the presence of pathogenic parasites, such as *Giardia* or *Cryptosporidia*. The occurrence of fecal bacteria in fresh and marine water has therefore justifiably led to the closure of beaches and shellfish harvesting areas; both actions having strong social and economic impacts. Because many other organisms must also share this precious resource, the environmental effects of water with such poor microbiologic quality could be far-reaching.

Microbial source tracking could prove to be an essential tool in the struggle to deal with the ever-increasing problem of fecal contamination of water. This report provides a summary of the MST Applications Workshop, including a detailed action plan to further the practical use of this tool by potential users. It examines the state of the available MST methods as well as their respective advantages and disadvantages. It is hoped that this report will lead to the further development of this evolving technology and benefit all those involved with potential application.

Workshop Objectives

The two-day workshop on Microbial Source Tracking Applications brought current and potential users of MST together with environmental regualtors in the hopes of fostering information sharing and increased networking between people with common interests and goals. As the title implies, it focused on 'real world' applications of this technology with an emphasis on land-based sources of marine pollution. As a first step, the workshop explored the various user groups and identified their needs with respect to MST. This was complemented by detailed talks on various methods, including their respective advantages and disadvantages. To conclude, the workshop participants separated into working groups and addressed five key questions in order to draft an action plan for the practical use of MST in Canada.

The workshop had several objectives:

- Look at available MST techniques, including assessing each method's capacity, or what it can and cannot do. Summary presentations from investigators active in the field helped to determine the breath of applicability of the methods and assisted in the understanding and the interpretation of MST results.

- Explore MST's potential application in controlling microbial pollution events, their remediation and regulation and assess the suitability of MST for use in these fields.

- Assess new research insights as well as information and research gaps.

- Foster further collaboration (including the banking, comparing, and sharing of data) and networking between interested parties.

- Develop an action plan for the practical use of MST by government, academia, and community groups. The plan would also detail the delivery of service and further collaboration needed to put the plan into action.

Steering Committee

The workshop was co-sponsored by Environment Canada, Clean Annapolis River Project, Acadia University, and under the umbrella of the National Programme of Action for the Protection of the Marine Environment from Land-Based Activities (NPA), Atlantic Chapter. The steering committee was comprised of a range of representatives from the three main MST groups: users, regulators, and researchers / developers. They are:

- Diane Tremblay (Chair), Environment Canada, Dartmouth, Nova Scotia
- Amar Menon, Environment Canada, Dartmouth, Nova Scotia
- Peter Johnson, Environment Canada, Dartmouth, Nova Scotia
- Elaine McKnight, Environment Canada, Ottawa, Ontario
- Blair Holmes, Environment Canada, Vancouver, British Columbia
- Stephen Hawboldt, Clean Annapolis River Project, Annapolis Royal, Nova Scotia
- Dr. Greg Bezanson, Acadia University, Wolfville, Nova Scotia
- Dr. Graham Daborn, Acadia University, Wolfville, Nova Scotia
- Sophie Bastien-Daigle, Fisheries and Oceans Canada, Moncton, New Brunswick
- Andy Sharpe, Clean Annapolis River Project, Annapolis Royal, Nova Scotia

Workshop Agenda

Wednesday, April 14, 2004 — KC Irving Centre Auditorium	
7:30 – 8:15	**Registration**
8:30	**Chair:** Joe Kozak, Environment Canada **Welcome:** Dr. Cyrus MacLatchy, Vice President Academic, Acadia University
8:35	**Introduction of Workshop Purpose and Objectives** Joe Kozak, Environment Canada
8:45	**Overview of NPA/GPA issues and priorities** Maureen Copley and Sophie Bastien-Daigle, National Programme of Action for the Protection of the Marine Environment from Land-Based Activities
9:10	**Coastal Community Perspectives on Pollution Sources Identification and Remediation** Susan Farquharson, Eastern Charlotte Waterways Inc.
9:30	**The Impact of Water Pollution on the Sustainability of the Shellfish Aquaculture Industry** Marc Ouellette, Fisheries and Oceans Canada
9:50	**Application of the Fisheries Act to the Deposit of Livestock Wastes into Canadian Watercourses** York Friesen, Environment Canada
10:10	Nutrition Break
10:25	**Overview of Microbial Source Tracking and Canadian Research Activities** Dr. Tom Edge, National Water Research Institute
11:00	**Using rep-PCR Fingerprinting to Determine Sources of *Escherichia coli* in the Environment** Dr. Cindy Nakatsu, Purdue University
11:30	**Application of Pulsed-Field Gel Electrophoresis in MST: Experience Gained and Lessons Learned in an Annapolis Basin Clam Bed** Dr. Greg Bezanson, Acadia University
12:00	Lunch

Wednesday, April 14, 2004 — KC Irving Centre Auditorium	
1:00	**Chair:** Dr. Colin Bell, Acadia University **Bacteroides-Prevotella MST Genomic Method — Applications in Environment Canada's Pacific and Yukon Region** Heather Osachoff, Environment Canada
1:30	**Use of the Ribotyping Technique for BST/MST in Multiple Use Watersheds Which Are Sources of Drinking Water in the Okanagan Valley, B.C.** Dr. Rick Nordin, University of Victoria
2:00	**Using Microbial Source Tracking in New Hampshire: Applications, Results and Challenges** Natalie Landry, New Hampshire Department of Environmental Services
2:30	Nutrition Break
2:45	**High Throughput Sequencing Projects at Genome Atlantic** Dr. Sharon Bowman, Genome Atlantic
3:15	**Fecal Coliform Source Tracking Methods (Multiple Antibiotic Resistance and Coliphage Typing) and Presumptive Total Maximum Daily Load (TMDL) Modeling to Identify Pollution Sources in Selected Watersheds of the Southeastern US** Dr. Geoffrey Scott, Center for Coastal Environmental Health and Biomolecular Research, National Oceanic and Atmospheric Administration
4:00 — 5:00	Tour of KC Irving Environmental Science Centre
5:30 — 6:30	Networking social hour

Thursday, April 15, 2004 — Wheelock Lounge	
8:30 — 10:00	**Chair:** Peter Johnson, Environment Canada **Breakout sessions: Development of Recommendations for MST** Workshop participants will be divided into 3 groups.
10:00 — 10:15	Nutrition Break
10:15 — 11:00	Working Group reports and recommendations
11:00 — 12:00	**Facilitator:** Peter Johnson, Environment Canada **Plenary: Develop an action plan for the practical use of MST by government, academia and community groups**
12:00 — 1:00	Lunch

Abstracts — Day One

Workshop presentations were divided into two broad categories. The first group consisted of user groups with a direct interest using MST; the second, investigators active in the application and further development of MST techniques. The following is a summary of each presentation, by category.

User Groups

Overview of NPA/GPA Issues and Priorities

Maureen Copley and Sophie Bastien-Daigle, National Programmme of Action for the Protection of the Marine Environment from Land-Based Activities (NPA), Atlantic Chapter

Canada's NPA was released on Oceans Day June 8, 2000. It responds to the UNEP's call to protect the marine environment from land-based activities through co-ordinated actions at the local, regional, national and global levels. It also responds to Canadians who expect clean oceans and sustainable development and it aims to protect the marine environment through co-operative solutions and concrete actions. Implementation of Canada's NPA involves the harmonization of integrated coastal management with river-basin management and land-use planning.

The Atlantic NPA team is comprised of government representatives from the federal and provincial governments of all four Atlantic Provinces. It has been meeting since December 2002 to define a strategic, progressive and results-oriented Action Plan. The presentation will discuss how conducting applied research, assessment and monitoring activities to obtain knowledge needed for appropriate action is encouraged under the NPA and how the MST workshop can contribute to the regional and national process.

Coastal Community Perspectives on Pollution Sources Identification and Remediation

Susan Farquharson, Eastern Charlotte Waterways Inc.

ACAP groups working throughout the Atlantic Region have indicated bacterial contamination a priority for reclaiming polluted coastal and freshwater systems. To date, efforts directed at monitoring, determining and remediating point sources have been extensive. Early and ongoing efforts have been somewhat successful in reducing point source of fecal coliform contaminants but the process has been time consuming and costly. The current investigative system is ineffective when addressing situations where several or no obvious source of fecal coliform contamination can be identified.

The Impact of Water Pollution on the Sustainability of the Shellfish Aquaculture Industry

Marc Ouellette, Fisheries and Oceans Canada

The shellfish aquaculture industry in the Gulf of St. Lawrence has grown from $9.5 M in commercial landings in 1990 to over $35 M in 2002. This was mainly the result of the development and expansion of the blue mussel (*Mytilus edulis*) production on Prince Edward Island. The second species of importance in the region is the common oyster (*Crassostrea virginica*). We are forecasting that, with the development of new culture techniques such as the floating bag system, we should have a very strong growth of this industry in the coming years, particularly in New Brunswick. The shellfish aquaculture industry is different from the fishing industry in many aspects. Aquaculturists need to invest in spat, equipment and labor for several years before they can sell their products. It takes 2 years to produce blue mussels and 3 to 4 years to grow oysters. Good management of these production cycles is crucial to ensure a sustainable and profitable industry.

Shellfish growing activities are carried out mainly in coastal bays and estuaries. Unfortunately, these areas are also vulnerable to contamination produced by many sources including human and industrial waste. This represents an increasing concern among health and environmental agencies, the shellfish aquaculture industry and consumers. The Canadian Shellfish Sanitation Program is responsible for controlling the recreational and commercial harvesting of all shellfish within Canada by monitoring the shellfish growing areas for sanitary and bacterial contaminations. The unpredictability of a sudden closure of shellfish growing areas can seriously jeopardize aquaculture businesses. The ideal solution remains pollution prevention and/or remediation. Finding means to eliminate or control pollution sources is critical in ensuring the health of both the shellfish industry and the marine environment.

Application of the Fisheries Act to the Deposit of Livestock Wastes into Canadian Watercourses

York Friesen and David Aggett, Environment Canada

The Government of Canada has a number of acts, regulations, and policies that protect aquatic organisms and their habitats. Environment Canada administers Section 36 of the federal Fisheries Act, which prohibits the release of deleterious substances into waters frequented by fish. This paper provides an assessment of the application of the federal Fisheries Act to the deposit of livestock wastes into fishery waters and discusses the importance that Environment Canada attaches to this issue.

Investigators Active in MST Development

Overview of Microbial Source Tracking and Canadian Research Activities

Dr. Tom Edge, National Water Research Institute

Many communities across Canada are faced with concerns about fecal contamination of sources of drinking water, recreational waters (e.g. beaches) and shellfish areas. Microbial source tracking (MST) is an emerging field that is providing a variety of techniques for determining the source of fecal contamination. A general overview will be provided of these techniques, which can generally be divided into either library-dependent or library-independent methods. Many MST studies to date have applied library-dependent methods based upon comparing the similarity of *Escherichia coli* in water samples to the *E. coli* in a library of isolates collected from nearby sources of fecal pollution. Their similarity has been determined using phenotypic methods (e.g. antibiotic resistance analysis) or DNA fingerprinting methods (e.g. ribotyping). There is much interest in library-independent methods based upon detecting host-specific fecal indicator microorganisms in water samples, although they have not been as widely tested. At present, there is no widely recognized standard MST method; each method having advantages and disadvantages for specific applications. Additional research is required to test the geographic, temporal, and practical limitations of these techniques in diverse aquatic ecosystems across Canada. There is a growing MST research community in Canada, and the overview will try to identify some of the researchers and activities occurring across this diffuse community. Recent initiatives (e.g. under the Agriculture Policy Framework, and the Canadian Institutes of Health Research) have contributed funding for enhancing MST research in Canada.

Using rep-PCR Fingerprinting to Determine Sources of *Escherichia coli* in the Environment

Dr. Cindy Nakatsu, Purdue University

Molecular (DNA) fingerprinting methods have been shown to be powerful tools for the identification and differentiation of microbial strains. Our objective has been to determine if a DNA fingerprinting method can accurately identify sources of fecal contamination into the environment. In our investigations, we use *Escherichia coli* as our target organism because it is commonly used as a water quality indicator, it is isolated easily in the laboratory, and DNA is easy to extract. An earlier study comparing four different DNA fingerprinting methods, repetitive sequence PCR (rep-PCR), amplified fragment length polymorphism (AFLP), pulse field gel electrophoresis (PFGE) and ribotyping indicated that rep-PCR was the most cost, labour, and time efficient approach. We also confirmed that among the different repeated sequences, Repetitive Extragenic Palindromes (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC), and Box elements, found in *E. coli,* the Box primers typically generated more bands and had better reproducibility than the two others. The specific objective of our current studies is to determine the practicalities of developing a source library for microbial source tracking. All genetic fingerprinting methods require a source library for identification of unknowns. The library size is dependent on the number of potential sources, level of source discrimination needed, and level of accuracy. These factors will directly impact the cost and length of time required to identify sources of contamination. Factors that contributed to increased costs and time were development of a source library, determining accuracy of the library, and optimization and increasing throughput of fingerprints. In conclusion, Box-PCR is a less expensive and less time-consuming method compared to the other common genetic fingerprinting methods but as is the case for all library-based methods library size and accuracy must be included as a factor.

Application of Pulsed-Field Gel Electrophoresis in MST: Experience Gained and Lessons Learned in an Annapolis Basin Clam Bed

Dr. Greg Bezanson, Sanford, S., MacDonald, C. and Scott, C., Department of Biology, Acadia University and Clean Annapolis River Project

In an attempt to identify/track the source(s) of fecal coliform contamination in the soft-shelled clam, *Mya arenaira*, representative *Escherichia coli* recovered from fresh and salt water, marine sediments, clams, and gulls associated with mud flats in Thornes Cove, Annapolis Basin, NS were subjected to DNA typing. Immobilized bacterial DNA was digested with the rare cutter endonuclease, *Sfi*I, and then examined for fragment length polymorphisms (RFLP) in pulsed-field electrophoresis gels. Dice coefficients were used to quantify the similarities of the resultant banding patterns. Both fresh and salt water carried a large number of strain types (pulsotypes) that exhibited diversity indices of 0.90 and 0.74, respectively, and significant temporal and geographic variability. In contrast, sediment, clam and gull isolates displayed a greater degree of population homogeneity (diversity indices: 0.24, 0.18, 0.19, respectively) and less temporal variation. The clonal nature of its isolates suggests that *E. coli* may be able to multiply in marine sediments. Although the sample size is small, clam isolates appear to share a high degree of relatedness with gull and salt water strains. On average, 64% of fresh and salt water, 62% of sediment, 45% of clam and 52% of gull isolates were typable using the PFGE-RFLP procedure. (cf. 98%, 100% and 80% for sewage, ducks and beef cattle respectively). DNA typing via this method is highly discriminatory and reproducible, but appears to be limited by the natural refractivity of environmental isolates of *E. coli*.

Bacteroides-Prevotella MST Genomic Method – Applications in Environment Canada's Pacific and Yukon Region

Heather Osachoff, Environment Canada

The Environmental Toxicology Section and the Shellfish Water Quality Protection Program at the Pacific Environmental Science Centre (PESC) in North Vancouver, B.C. have a Microbial Source Tracking (MST) technique that has been adapted and developed based on the published articles by Dr. Katharine Field from Oregon State University. This MST technique is a genetic assay that detects genomic DNA from the host-specific intestinal bacterial group *Bacteroides-Prevotella* and thereby identifies the organisms responsible for fecal contamination in fresh or marine water samples. Currently at PESC we are able to distinguish between fecal contamination caused by humans, ruminant animals, and/or pigs. The procedure requires one litre of water taken from a potentially contaminated area and after sample processing using molecular biology techniques, the results indicate presence/absence of fecal material from humans, ruminant animals, and/or pigs. A fecal coliform analysis is conducted in parallel because this MST procedure is not quantitative. The method is currently being expanded to work on shellstock and sediment samples.

This technique has been used on marine and fresh water samples from around B.C., including Greater Vancouver, the Sunshine Coast and Vancouver Island, to aid First Nations and government departments (both Provincial and Federal) in identifying sources/organisms causing high fecal coliform counts in water systems.

Use of the Ribotyping Technique for BST/MST in Multiple Use Watersheds Which Are Sources of Drinking Water in the Okanagan Valley B. C.

Dr. Rick Nordin, University of Victoria, Mansour Samadpour, University of Washington, Seattle, Kevin Rieberger and Dennis Einarson, BC Ministry of Water Land and Air Protection, and Burke Phippen, BWP Consulting, Kamloops BC

In many areas of BC, the source of bacterial contamination of drinking water is a contentious issue with recreational users, farmers, wildlife and others using watersheds being suspected of contaminating water supplies. To properly evaluate the true sources of bacteria and to use this information as the basis of watershed management, identification of *E.coli* strains using the ribotyping technique was used as a tool to evaluate the relative contribution from different potential sources. Three multiple use watersheds near Kelowna BC were sampled at water utility intakes for a variety of water quality and microbial indicators. In the subsequent year three additional watersheds were evaluated in the Vernon BC area.

The results of the sampling in all of the watersheds were analyzed together with other water quality indicators and evaluations of land use, which included recreational (camping and hiking), cattle grazing permits and wildlife presence. In the Kelowna study, two of the watersheds indicated the division between the general categories of *E. coli* of human origin, cattle origin and wildlife origin was approximately one-third of each category. In the third watersheds, fingerprinting indicated primarily a wildlife origin of *E.coli* with some contribution from cattle during storm events or when cattle were in proximity to water sources. In the 2001 study in the Vernon area, results showed less influence of cattle in multi-use watersheds and almost all *E.coli* originating from wildlife in a control watershed. The results have been used for management of activities in watersheds to minimize the risk of microbial contamination of drinking water supplies.

Using Microbial Source Tracking in New Hampshire: Applications, Results and Challenges

Natalie Landry, New Hampshire Department of Environmental Services

Traditional investigatory methods are used by state agencies to track sources of fecal-borne microbial contamination that are causing pollution problems for recreational and shellfish growing waters. While methods such as bracketing streams using microbial indicator organisms and shoreline surveys have been successful in identifying various pollution sources in coastal New Hampshire, estuarine and coastal waters still have elevated bacteria levels in some areas. Since 1999, the New Hampshire Department of Environmental Services (NHDES) has worked with University of New Hampshire (UNH) researchers to identify specific source species using a microbial source tracking technique called Ribotyping. NHDES and UNH have applied this MST technique while investigating sources of bacterial contamination in shellfish growing waters, freshwater streams, and tidal rivers. The results, which show the relative contribution of specific source species, have been used in a Total Maximum Daily Load study and to guide remedial actions in both estuarine and fresh waters. In some cases the results were as expected, in others the results indicated unexpected sources, which were eventually verified. Research is continually refining the methodology including a move from manual to automated ribotyping using a RiboPrinter. The cost for ribotyping is an issue that has lead to several studies exploring the potential for using small source species databases that reflect local source species during the time of the study. Other ongoing research and experimental designs seek to expand possible applications of ribotyping for source tracking.

High Throughput Sequencing Projects at Genome Atlantic

Dr. Sharen Bowman, Genome Atlantic

Genome Atlantic is one of five regional genome centers created by Genome Canada. It currently administers four scientific and one technology platform in the Atlantic region. Research projects include: Understanding prokaryotic genome evolution and diversity (Ford Doolittle); The protist EST program (Michael Gray); The Canadian potato genome project (Barry Flinn and Sharon Regan) and Pleurogene - flatfish genomics (Sue Douglas and Mike Reith). The Genome Atlantic technology platform supports all research projects by providing high throughput DNA sequencing and associated technologies.

Fecal Coliform Source Tracking Methods (Multiple Antibiotic Resistance and Coliphage Typing) and Presumptive Total Maximum Daily Load (TMDL) Modeling to Identify Pollution Sources in Selected Watersheds of the Southeastern US

Dr. Geoffrey Scott, National Oceanic and Atmospheric Administration, National Ocean Service, National Centers for Coastal Ocean Science, Center for Coastal Environmental Health and Biomolecular Research, Charleston, South Carolina

Discharges of wastewater from sewage treatment plants (STPs), septic tanks, farm animal operations, urbanization and wildlife pollution sources may adversely affect estuarine water quality, often closing shellfish beds for harvesting, downgrading water quality classification and potentially affecting the safety of rivers and streams for contact recreation. Development of methods for differentiating human versus wildlife coliform bacterial sources is needed to properly manage bacterial pollution emanating from different sources. Several methods for differentiating human and wildlife coliform bacterial sources were evaluated including Multiple Antibiotic Resistance (MAR) and coliphage typing. Water samples were collected from several river and estuarine watersheds in South Carolina and selected pollution sources (STPs, chicken/hog farms and septic tanks). Samples were enumerated for fecal coliform bacterial densities (MPNs or Membrane Filter) and *E. coli* were isolated by API biotyping. Samples were then analyzed by MAR and coliphage viral typing.

Results indicated that the % of *E. coli* comprising the coliform group and MAR was highest at sewage treatment plants, chicken/hog farms and in urban areas adjoining sites with septic tanks or influenced by waste water treatment plant discharges. Wildlife areas had negative MARs or resistance to a single antibiotic and a lower % of *E. coli*. F^+ RNA coliphage typing results proved useful in differentiating human (groups II and III) versus animal (group IV) pollution sources. GIS provided methods to locate human pollution sources, identify land metrics affecting coliform MPNs, and quantify presumptive Total Maximum Daily Load estimates of fecal coliform sources in shellfish harvesting areas. These findings indicate that these methods may be helpful in identifying different sources of fecal coliform bacteria in shellfish harvesting areas.

Working Groups – Day Two

The second day of the workshop facilitated discussion among the different user groups and provided an opportunity to express the various needs that must be fulfilled in order to have an effective national approach to MST. Workshop participants were divided into 3 groups and asked the following questions:

1. How would this tool be of use to you?
2. What would be the key elements of an effective and practical national MST program?
3. What do we need and who needs to do it (e.g., government, universities, community groups, private sector service providers) to put these elements in place? Please consider sequencing and timelines when formulating your responses to this question.
4. Are there any pilot projects you can think of where MST would be especially suited and which could be used to help build the knowledge base and support for the use of this tool in Canada?
5. Are there any other key questions we should have asked but didn't?

The following is a detailed review of each question and recommendations from each working group.

Group 1

Question 1 - How would this tool be of use to you?

- MST would be most useful for remediation. By discriminating between human and non-human sources, time and money for remediation can be better directed and utilized. The best method to provide this initial distinction would be the *Bacteroides Prevotella* method, as it is the most cost-effective method, which could accommodate the extensive sampling needed for water quality monitoring.
- The use of the technique should begin sooner rather than later, and be done concurrently with research. Less time would then be spent waiting for the 'ideal' method, and its practical use could add to the understanding and development of the method.
- A second way this tool would be of use is in enforcement. However, the level of discrimination needed is much higher than in remediation. The applicability of MST, as it is presently, would therefore be limited. Further research is needed to make MST a practical enforcement tool.

Question 2 - What would be the key elements of an effective and practical national MST program?

- There is no 'silver bullet' method that will be ideal in every scenario. A practical national MST program would use a combination of many methods.
- The national approach may use the *Bacteroides Prevotella* method as an initial screening to discriminate between human and non-human sources. This could be done at 1 or 2 local laboratories. If the sources are found to be non-human, remediators can proceed with the knowledge of land use in the affected area.
- If further information is needed, a national network of laboratories could provide the more detailed source identification needs.

Question 3 - What do we need and who needs to do it to put these elements in place?

- Research and development must be done in parallel to initial trials in order to improve the methods as they are being used. Due to the high number of samples that would inevitably be collected, there is a need for a laboratory with high throughput. One possibility would be Genome Canada.
- It is important to use existing resources and avoid trying to continually create new ones.

Question 4 - Are there any pilot projects you can think of where MST would be especially suited and which could be used to help build the knowledge base and support for the use of this tool in Canada?

Identification of a specific project can help identify the needs with respect to MST. An initial project would preferably be small and in a watershed that is well known, where the pollution problem seems simple.

Some possible pilot projects include:

- The Annapolis River, Nova Scotia. There is 15 years of data available and an infrastructure is already present through the Clean Annapolis River Project (CARP).
- Tracadie, Nova Scotia. There are 3 potential inputs: farm, sewage, and wildlife.
- Two sites, one in the Bay of Fundy, and another in the Gulf of St. Lawrence, using the same technique for both. Such a project could address oyster bags in the Gulf of St. Lawrence where seabirds are a potential source of contamination.

Group 2

Question 1 - How would this tool be of use to you?

- This tool would be of use in shellfish growing areas, freshwater stream, farming areas as well as with outreach groups. Particular examples include fecal coliform studies on rivers with potential human and non-human sources of contamination. Additional research and development would allow presence/absence analyses to be taken a step further. The latter is considered a useful tool by Environment Canada's shellfish section from Newfoundland to British Columbia. MST could provide this service and would be an effective remediation tool that would be cost effective, accurate and done in house.
- MST, at present, would be more effective for remediation, and would need improvement to be an acceptable enforcement tool.

Question 2 - What would be the key elements of an effective and practical national MST program?

The key elements would include:

- Standardization and accuracy of methods
- Use as a remediation tool, and a planning tool
- Educational outreach to both the public and to administrative officials
- Access to information by each region
- Reduced risk by elimination of human sources

Question 3 - What do we need and who needs to do it to put these elements in place?

- The national program must be a coordinated approach with several partnerships.
- The network must extend from government all the way to community groups. Once fully educated, government can take the lead.
- A useful tool that must be developed is a flowchart identifying the various methods and the respective problems they are best suited to solve. The costs of each method as well as their precision, accuracy, and limitations must also be identified. (Dr. Geoffrey Scott plans to develop this flow chart)
- A regulatory body with enough power must be responsible for the MST national program. Environment Canada could possibly fulfill this role. Canada's NPA could also create a coordinating team.
- The reproducibility of the methods, which must be accurate in repeated tests, is of key importance.
- A national program must have practical applications in the field with demonstration projects that are available for public input.
- The MST program needs stable funding, without which any project might breakdown.

Question 4 - Are there any pilot projects you can think of where MST would be especially suited and which could be used to help build the knowledge base and support for the use of this tool in Canada?

- The pilot projects should focus on smaller watersheds with less complex sources.
- They should heavily involve the public and communities, and must involve groups at the working level.
- They should serve as examples for other areas that wish to use MST in the future, and should therefore be called "demonstration projects".
- One possible site is Prince Edward Island, where there is a growing concern for contamination from cattle, pesticides, and golf courses, and where an important aquaculture industry exists.
- Another possibility would be on the Miramichi River, where pollution sources may include Canada Geese, sewage treatment plants, and farms. A partnership between the Komi Republic of Russia and the Miramichi River Environmental Assessment Committee already exists, working collaboratively on a genome project.
- Possible actors in pilot projects include Acadia University, Fisheries and Oceans in Moncton, and Environment Canada.

Group 3

Question 1 - How would this tool be of use to you?

- MST would be most useful as a 2-step process, starting with the *Bacteroides Prevotella* method and followed by ribotyping for more detailed analysis when necessary. There is no silver bullet, and therefore MST would be most effective with a combination of methods. The appropriate method would be determined by the question being asked.
- This tool would be most useful for remedial action, as continual monitoring with no clear idea of sources is cost ineffective.
- MST reduces the 'finger-pointing' at certain groups, and focuses on the real causes.

Question 2 - What would be the key elements of an effective and practical national MST program?
The key elements would include:

- Standardization with evaluation of the methods
- Standardized statistical analysis (interpretation of results can be a huge variable)
- A national data bank to draw general trends
- A regional data bank for more direct use with MST (could include three regions: Western, Central, and Atlantic Canada)
- A phased-in approach, reacting to lessons learned through pilot projects
- MST information that is readily accessible
- Increased awareness and education

Question 3 - What do we need and who needs to do it to put these elements in place?

- The first step is to articulate the needs (achieved at the MST workshop). The next step is to pass on the information to the appropriate governing body, possibly Environment Canada, who must then act on those needs.
- The needs will be dependant upon different types of users, including (but not exclusive to):
    1. Private users (e.g.: environmental engineers / consultants, such as the Halifax Regional Municipality)
    2. Community groups (e.g.: ACAP groups).

Question 4 - Are there any pilot projects you can think of where MST would be especially suited and which could be used to help build the knowledge base and support for the use of this tool in Canada?

- Pilot projects on the Atlantic Seaboard should include a North / South interaction and focus on shellfish and/or freshwater issues.
- Possible locations include: the Bras d'Or Lakes and the Gulf of Maine.
- Projects should address management questions, not only source questions.
- Projects should also help move toward the standardization of methods and include quality assurance and quality control mechanisms.

Recommendations and Action Plan

Question 1 — How would this tool be of use to you?	
Recommendation	Next Steps
MST seems ideal for use in remediation by regulators, community groups, farmers, and the shellfish industry. In its present state, its usefulness as an enforcement tool is limited by questions related to accuracy (eg: specificity, reproducibility). The various methods must be further developed before MST can be truly effective in this way. A national screening program that uses the *Bacteroides-Prevotella* presence / absence test to distinguish between human and non-human sources would be most effective as a first step. This would be followed by the more complex, supportive MST methods, in the form of a standardized toolbox, when necessary.	**Immediate next steps include:** **Establish a collaboration between private sector laboratories (NS) and Environment Canada, Pacific and Yukon Region (BC).** **Establish a collaboration between Fisheries and Oceans Canada, Moncton, and the Environment Canada laboratories, Atlantic Region.** **These partnerships should lead to the establishment of at least 1 or 2 local laboratories in Eastern Canada capable of conducting the *Bacteroides-Prevotella* presence / absence method, and a smaller network of labs across the country with capacities to perform more complex, detailed analysis when necessary.**

Question 2 — What would be the key elements of an effective and practical national MST program?	
Recommendation	Next Steps
Continued method development (possibly in association with Genome Canada) in parallel with the refinement of those currently being used and tested in the field. Also key to a national MST program is the standardization of methods.	**Explore possible working relationships between universities (e.g.: Acadia University) and Genome Atlantic in order to improve the accuracy and standardization of the methods.** **The Atlantic Innovation Fund could possibly provide funding.**
Standardization of statistical analyses and modeling for greater quality assurance.	**Develop a collaboration with the United States Environmental Protection Agency, Food and Drug Administration statistician (recommendation by Dr. Geoffrey Scott).** **Recommendation by the MST advisory working group to Dr. Geoffrey Scott at the National Oceanic and Atmospheric Administration (NOAA) for a modelling workshop.**

Use of common reference / control material for increased confidence and broad acceptance.	Establish a collaboration with the National Water Research Institute for the development of common reference material. Recommendation by the MST advisory working group of the need for further work with the United States and the US National Institute of Standards and Technology.
National data bank to use for general trends on the status of Canadian surface water. Regional data banks for local use in MST.	Identify and contact possible providers / hosts of a data bank including Genome Canada and the national-scale MST project of Health Canada and Agriculture and Agri-Food Canada.
Outreach and education to both public and administrative officials.	Distribute the results of the workshop and future demonstration projects broadly to both management and grassroots levels. Public education can be accomplished in part through the ACAP coordinators.

Question 3 — What do we need to do and who needs to do it (government, universities, community groups, private sector service providers) to put these elements in place? Please consider sequencing and timelines when formulating your responses to this question.	
Recommendation	Next Steps
Articulate the needs (technical, financial, infrastructure, authority / mandate) to the appropriate decision maker (once determined) and ensure that these needs are acted upon. This should be done through an effective coordinating team, which will focus on using existing resources.	Under the NPA umbrella, set up a core advisory working group to provide coordination of follow-up activities (could include partners from the United States). This group will meet via conference call early May, 2004. Creation / access to a MST website for information sharing. The NPA secretariat will investigate possible options under the NPA website. Another possible contact is Dr. Charles Hagedorn, professor at Virginia Tech University in Virginia, USA. He currently maintains a MST website: (http://soils1.cses.vt.edu/ch/biol_4684/bst/BST.html)
A flowchart (decision tree) with information on MST methods. Useful when deciding which method will best address particular needs.	Flowchart to be prepared by Dr. Geoffrey Scott, NOAA and made available to the MST advisory working group.

Question 4 — Are there any pilot projects you can think of where MST would be especially suited and which could be used to help build the knowledge base and support for the use of this tool in Canada?	
Recommendation	Next Steps
There are several potential sites for demonstration projects. These include: - Annapolis River, Nova Scotia, where there is already a lot of scientific data; - Tracadie, Nova Scotia, with an emphasis on shellfish; - Thornes Cove, Nova Scotia, where an MST study is already in progress; - Gulf of St. Lawrence; - Prince Edwatd Island; - Bras d'Or Lakes, Nova Scotia; - Richibucto, New Brunswick; - Tabusintac, New Brunswick; - ACAP sites, which have special funding; - Sites on the West Coast of Canada. The demonstration projects should be in watersheds where the sources of contamination are less complex. Most importantly, the results and lessons learned from the projects must be transferable to other groups wanting to use MST. They should involve the communities and should also help to move toward the standardization of the MST methods. Finally, they should be oriented towards marine systems and fecal contamination / remediation in shellfish issues to differentiate them from other studies that are presently underway.	**The above-noted MST advisory working group will put together a short list of potential demonstration projects, including possible ACAP sites.**

Conclusion

Water is a finite resource that is in great demand. In order to best protect it, important steps must be taken to reduce the harmful, yet increasing introduction of materials of fecal origin. The sources of fecal contamination are very broad, and are both human and non-human. The effects can be damaging and have strong impacts to society, the environment, and to human health. For pollution remediation and enforcement efforts to be truly effective, the sources of contamination must be tracked directly. The evolving discipline of Microbial Source Tracking (MST) could prove to be the tool necessary to attain the accuracy in source detection that is badly needed. However, much research and development is still required in order to perfect the various MST methods and make them reliable remediation and enforcement tools.

An effective national MST approach will require the commitment of a national advisory working group. This group could be comprised of both Canadian and American counterparts and will be responsible for coordinating follow up activities after the MST Applications Workshop. It is likely that a national approach would be most effective as a screening process using a variety of available MST methods, based on the needs of individual projects. The continuous development of methods in terms of accuracy and reproducibility will be an integral part of the national approach. It is anticipated that the MST Applications Workshop and the recommendations compiled in this report will lead to the development of several demonstration projects. It is expected that these projects will aid in the further development of MST and benefit everyone involved, humans and non-humans alike.

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Appendix B
Service Providers

Service Providers

Present at the workshop were both public and private laboratories and potential service providers of MST. Several expressed a keen interest in participating in the further use and development of MST. These include, but are certainly not limited to:

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Appendix C
Reference Material

Summary of Available Methods for Bacterial Source Tracking

UBC Microbiology Co-op work term report, written for the
Shellfish Waters Quality Protection Program

November, 2003

Authored by Brian McClure

Note: Advantages / Disadvantages of the various methodologies described in this document were taken from the cited references at the time of writing.

Summary of Available Methods for Bacterial Source Tracking

Abstract

In order to aid in remediation of fecal-contaminated waters, methods are being developed to track sources of fecal contamination. This field of methodology has been named Bacterial Source Tracking and is the focus of this summary. As Bacterial Source Tracking is a developing field, and still in the early stages, all methods within have been presented based on the most current literature; new developments are happening continually which will likely out-date this report in a few years.

Methods were grouped based on the type of method (microbial, phenotypic, genotypic, chemical or other) and presented in a fundamental manner. Microbial methods use the culturing of host-specific organisms and their presence or absence indicates fecal sources. Phenotypic methods rely on analyzing the specific characteristics of strains of fecal indicators which show host tropism. Genotypic methods are nucleic acid-based (DNA or RNA) and rely either on detecting specific sequences of nucleic acid in contaminated water, or on patterns generated by processed DNA or RNA when separated (by gel electrophoresis) and then visualized, a technique called DNA fingerprinting. Chemical methods do not rely on growth of microbes or analysis of microbial components but on chemicals released into the environment from the guts of polluting animals or humans. The most obvious strengths and weaknesses of each method were highlighted.

Methods presented within include Bifidobacterial Association with Human Waste, F-specific RNA Coliphage, Multiple Antibiotic Resistance Analysis, Ribotyping, Rep-PCR, Pulse-field Gel Electrophoresis, *Bacteroides-Prevotella* Host Specific Biomarkers, Amplified Fragment Length Polymorphisms, Caffeine Assaying, Fecal Stanol Assaying, and sIgA ELISA.

Three methods currently stand out from the rest. The *Bacteroides-Prevotella* Host Specific Biomarkers method stands out because of its lack of dependence on a library, and its high reliability. sIgA ELISA is notable due to its superior resolution of fecal sources, extreme specificity, and future potential. Ribotyping has been used in many case studies and appears to have the most solid background of any other method presented here. Although some other methods such as Rep-PCR, Pulse-field Gel Electrophoresis, and Amplified Fragment Length Polymorphisms, promise greater results than Ribotyping, until more case studies have been completed with those methods they cannot be considered.

Currently, at Environment Canada's Pacific Environmental Science Centre, the *Bacteroides-Prevotella* Host Specific Biomarkers method is being developed and evaluated, but progress is slow without a large workforce devoted to the research.

Introduction

Fecal pollution is responsible for the closure of streams and lakes to fishing and swimming, as well as contamination of our drinking water and shellfish harvesting waters. Besides, posing a health risk, the contamination of some of these waters (e.g. shellfish harvesting waters) can result in closures that harm the economy.

In order to prevent or remove shellfish sanitary closures, contaminated waters must be remediated. This involves first identifying sources of fecal pollution so that they can be reduced or eliminated. Potential sources of fecal pollution are most easily separated into three categories: human, domesticated animals, or wildlife. Common domesticated animal sources of pollution include cows, horses, pigs, goats, sheep, chickens, cats, and dogs. Common wildlife sources include raccoons, birds (especially waterfowl), bears, canines, and markedly in shellfish waters, seals, otters, and sea lions.

Bacterial Source Tracking (BST, also called Microbial Source Tracking, MST) tools are intended to identify the actual contributing factors to fecal contamination out of a list of potential sources. Once contributing sources of fecal contamination have been identified, the efficient use of remediation strategies can be applied.

BST, however, is a very young field and many of the techniques are either still in development, or inadequate at resolving the sources of contamination to a suitable degree. For any BST technique to be viable, it has a daunting array of challenges to address. Among the most prominent are the following:

- Inability to detect *direct* indicators (substances originating directly from animal cells and are associated only with fecal matter) of fecal pollution sources; direct indicators are uncommon, and currently the only BST technique able to detect direct indicators is the *sIgA ELISA* technique. Indirect indicators of sources are much more prevalent and include bacteria, viruses, protists, molecular markers, and chemicals.
- Many indirect indicators are non-endemic; that is, a given indirect indicator may be indicative of more than one source, limiting its usefulness. However, if an indicator is more commonly associated with a particular source than another, it may still prove useful.
- Persistence of indicators in the environment: an indicator may be too volatile to detect or may be too persistent to be a functional indicator of recent, nearby pollution. Factors which may affect persistence are temperature, light-penetration, predators, or matrix[1]
- Geographic instability of indicator: the prevalence of different indicators – of the same pollution source-group – may vary among watersheds. Factors which may affect this are temperature, light-penetration, predators, matrix, and sediment-type.
- Temporal instability of indicator: the prevalence of different indicators – of the same pollution source-group – may shift over time. This may be due to changes in the environment of a watershed, or more often, seasonal changes. Or, it may simply be due to the process of ascendance.
- Quantifying fecal pollution from different sources is important to determine the profile of contamination and assist in directing remediation efforts to the most prevalent sources of pollution. Many BST methods cannot quantify source input, only confirm that a particular source of fecal pollution is contributing, but not how much it may be contributing.

A review of the more relevant BST technologies follows. Evaluations are made on the basis of accuracy, efficiency and cost estimates, and also based on information from recent studies of individual methods and cross-comparisons, where applicable.

Summary of Technologies Available

Microbial Methods

Bifidobacterial Association with Human Waste

Bifidobacterium spp. have been found to be widely associated with human feces (1,2). Although rarely found in animals, ratios between frequencies of isolation of different species of bifida from various animals have been observed and may prove to be useful in discriminating between certain animal sources (3, 4). However, bifidobacteria found to ferment sorbitol appear to be almost exclusive to humans and this property is being explored as an indicator of human fecal pollution (4).

Human Bifid Sorbitol Agar (HBSA) was developed for the purpose of isolating sorbitol fermenting bifidobacteria (1). Water samples are filtered and the filters are incubated on HBSA media from four to six days. Colonies arising on the HBSA plates are examined for typical bifidobacterial colony morphology, and qualifying colonies are then microscopically examined for characteristic bifidobacterial cellular morphology.

Using this particular technique in testing for *diffuse* pollution sources, M. W. Rhodes and H. Kator (4) observed high background colony counts that interfered with the growth of some sorbitol-fermenting bifidobacteria colonies. Selective media was tested but did not enhance recovery; exposure to sea water injures bifidobacteria enough that they cannot recover when incubated with stress-inducing selective media (5). However, a recovery phase was tested and was not found to improve recovery of bifidobacteria. Rhodes and Kator concluded that this technique could not be used as a primary BST tool.

[1] Types of matricies include fresh water, marine water, turbid water, humic acid water, sediment, or fauna.

Sorbitol-fermenting bifidobacteria may yet still prove to be valuable for BST as a number of new methods are being investigated. Among these, new selective media (6), gene probing (7,8) and PCR-based methods (9) may prove to be useful for BST.

Advantages.

- Relatively simple; can be performed by lab personnel with little training.
- Low sample-processing cost.
- Accurately discerns between human or animal sources of fecal pollution.

Disadvantages.

- Low source-resolving power; cannot yet discriminate between *specific* sources of pollution.
- Long sample turnaround time (4-6 days for preliminary results, more for verification).
- Survival of organisms in environment is highly variable likely due to temperature and matrix.
- High background hampers technique.

F-specific RNA Coliphage

F+ coliphages of the *Leviviridae* group are comprised of four subgroups: group I, group II, group III, and group IV. Members of:

- Group I have been associated with both human and non-human feces.
- Group IV have been found to be largely associated with animal wastes.
- Group II and III appear to be largely associated with human fecal contamination and domestic sewage.

These host tropisms can be used to differentiate between human and non-human sources of fecal pollution.

In order to assay for phage types, large sample volumes must be processed. Three prominent processing methods are currently used to concentrate and enumerate phage, each with specific advantages and disadvantages. As the F-specific RNA coliphage method for BST is not currently advantageous, these methods will not be explained in detail. Please refer to references 8, 9 and 10 for full detail, if desired.

Advantages.

- None, as of this writing

Disadvantages.

- Low source-resolving power; cannot yet discriminate between specific sources of pollution.
- Expensive due to a combination of materials and labour required to process samples.
- Presently, can not determine relative contributions of human and animal fecal pollution when both are significantly present.
- Large sample volumes must be processed to capture sufficient numbers of viral particles for assaying.

Phenotypic Methods

Multiple Antibiotic Resistances Analysis (MAR, ARA)

This method relies on the assumption that different animals will have gut bacteria with dissimilar antibiotic resistance patterns. Presence of antibiotic resistances can largely be attributed to human antibiotic usage and administration of specific antibiotics to certain domesticated animals. Wild animals have digestive floras which exhibit less antibiotic resistances, due to low selective pressures for resistance.

Contaminated waters are collected and fecal indicators such as *E. coli* or *Streptococcus* spp. are isolated and replica plated on agar medium plates containing different antibiotics, and also plates with the same antibiotic at different concentrations. Antibiotic resistances are scored based on presence or absence of growth. Profiles are compiled and compared to a library constructed in the same manner, from known sources.

This technique has been successfully used to identify sources of fecal contamination in Holman's Creek, Virginia (16), Stevenson Creek, Florida (17) and Apalachicola National Estuarine Reserve, Apalachicola Bay (18).

In one study of fecal contamination in shellfish harvesting areas, misclassification rates for humans were found to be high, with many human isolates being mistakenly classified as chicken isolates (14). This may have been due to the particular antibiotics selected to construct resistance profiles. Nonetheless, this method has performed well in limited studies and further studies would do well to hone the technology.

Variations on the MAR technique can affect its reliability and accuracy; different indicator organisms may be used and/or different antibiotics. As more studies are completed using different variations, the MAR technique may come to be much more valuable as a BST technique.

A particular variation of the technology which uses enterococci as indicator organisms generally gave poor rates of correct classification (RCC) in a study by Wiggins, et al., signifying that enterococci may not be appropriate for use with MAR (22).

Advantages.

- Good resolution: able to discriminate between certain sources of fecal contamination.
- High throughput.
- Rapid.
- Relatively inexpensive.
- Proven technique; many field studies have been completed to date with successful results.

Disadvantages.

- Isolates with little or no antibiotic resistances cannot be typed (may not be suitable for identifying wildlife sources).
- Requires a large reference library which may show geographic and temporal instability.
- Plasmids are often a source of antibiotic resistance and acquiring or losing plasmids can affect MAR profiles.

Genotypic Methods

Ribotyping

Ribosomal RNA genes have highly conserved sequences between closely related strains of bacteria. Using this knowledge, a technique to quickly identify polymorphisms in rRNA gene sequences by DNA fingerprinting was developed for use as a BST technology.

Bacteria must first be isolated and cultured, and genomic DNA extractions performed on pure cultures. The genomic DNA is then digested with restriction endonucleases and probed with labeled oligonucleotides. Banding patterns are generated by visualizing electrophoresed, Southern blotted samples. These patterns can be discriminately analyzed using software and then compared to a database of isolates. This technique is highly labour intensive and expensive (both material and labour costs).

Different bacteria can be utilized in this technique, different restriction endonucleases employed, and different methods of analysis can be applied to give this technique a great degree of flexibility. This also means that a great deal of optimization can be carried out to improve results and RCC values.

It has been shown that animal diets affect their ribotype profiles, indicating that there may be significant geographical and temporal instability (23). This also suggests that reproducibility is in question, despite claims that the technique is highly reproducible (24, 25).

Multiple field studies have been performed using ribotyping and indicate that pollution sources can be successfully tracked using this technique (24, 25, 26, 27). One study demonstrated that the "two-enzyme" method (whereby DNA is digested by two restriction endonucleases) gives much more reliable results than the "one-enzyme" method (27).

Dr. Mansour Samadpour, with the University of Washington Department of Environmental and Occupational Health Sciences, has been developing a ribotyping method over the last decade which claims greater than 96% accuracy. There is a lack of literature published on his particular methodology, or on field-studies performed using the methodology but Dr. Samadpour is a reputable character in the field of BST. It is known that Dr. Samadpour's method uses a "two-enzyme" method of analysis.

Source Molecular Corporation, Florida, USA, provides a commercial fecal coliform-ribotyping service called E.col.I.D. For a considerable fee ($585 USD for a 5-10 days turnaround, or $390 USD for a 8-21 days turnaround, per sample) ribotyping analyses will be performed on five E. coli isolates (per sample), and each isolate will be classified as having human or non-human origin. Source Molecular will provide species-specific analysis if they are provided with "suspected" comparison samples (i.e. cow dung) and each comparison sample is charged separately.

Advantages.

- Methods of analysis are flexible; can tweak methods to give best possible average rate of correct classification (ARCC). This can also be considered a disadvantage when attempting to compare studies using different methodologies.

Disadvantages.

- Expensive due to a combination of materials and labour required to process samples.
- Labour intensive.
- Requires a large reference library, which may show temporal and geographical instability.

Rep-PCR

Rep-PCR is a DNA fingerprinting method based on palindromic sequences of DNA which are highly conserved and present across multiple species. There are 3 currently targeted elements: BOX, ERIC and REP[2]. Oligonucleotide primers have been designed which amplify outwards from the targeted elements, effectively amplifying DNA between replica elements. The polymorphic lengths of amplified DNA are electrophoresed and the visual pattern is scanned into special software (e.g. AMBIS, GelCompare, or BioNumerics), which compare the generated fingerprints with those in a stored database. The software scores matches based on similarity co-efficients.

Studies using rep-PCR to identify sources of fecal contamination have indicated that primers which target BOX elements seem to work best, giving high RCC values for humans, geese, sheep, pigs, chickens and cows, with an acceptable RCC for ducks (28). When samples were incorrectly classified, they were found to be incorrectly classified more often with certain groups than others, but the trend did not appear to be reciprocal (e.g. whereas 6.9% of human isolates were incorrectly classified as being duck isolates, 0% of duck isolates were incorrectly classified as being human). These ratios of incorrect classification could be used as a tool for more accurate classification in themselves. A larger library size or different algorithm for analysis might provide even more accurate RCC values. Overall, this appears to be a powerful tool for analysis.

[2] BOX, ERIC, and REP elements are 3 different forms of repetitive palindromic sequences, each with slightly different features than the others.

A study comparing ribotyping and rep-PCR for source identification of *E. coli* revealed that rep-PCR was more accurate, efficient and reproducible than ribotyping (29).

Advantages.

- Methods of analysis are flexible; can tweak methods to give best possible rates of correct classification. This could also be considered a disadvantage when attempting to compare studies using different methodologies.
- High resolution; discriminates very well between source groups.
- Rapid
- Ease of application.

Disadvantages.

- Requires cells to be cultured.
- Requires a reference library which may show geographic instability and temporal instability.
- Reproducibility in question.

Pulse-field Gel Electrophoresis (PFGE)

Pure bacterial cultures are embedded in agarose plugs containing a series of rare-cutting restriction enzymes for DNA digestion and the genomic DNA is digested under special conditions. The plugs containing the digested genomic DNA are then placed into wells in a special electrophoretic gel and electrophoresed for a lengthy period of time (~30-50 hours). During electrophoresis, the current alternates direction and strength periodically in order to allow for greatest separation and resolution of digested genomic DNA, producing a DNA fingerprint.

PFGE is used in determining bacterial relatedness and is also used extensively in clinical microbiology as a critical epidemiological tool (30). Thus, there exists a great deal of background literature on the technique in general, although literature on its use for microbial source tracking is currently limited.

Studies that have been completed suggest that PFGE may be a useful tool in determining sources of fecal pollution (31). Just how useful in comparison to some of the superior techniques will be revealed as more detailed studies are performed.

Advantages.

- Greater discrimination than any other method to date; able to visualize minute genetic differences.
- Reproducible.
- Applicable to broad range of bacterial strains.

Disadvantages.

- Possibly too discriminatory to detect broader differences between host specific strains, meaning that a very large database of isolates may be necessary for meaningful results.
- Low throughput; limited by number of lanes in a gel and number of gel apparatuses available.
- Lengthy assay time.
- Requires a large reference library which may show geographic and temporal instability.

Bacteroides-Prevotella Host Specific Biomarkers

Bacteroides, and close relatives from the genus *Prevotella* are non-spore-forming, obligate anaerobes (32). These two qualities limit spread of the bacteria and have allowed certain strains to evolve over time to be more specific to certain animal hosts independent of geographic variation. Once released into the environment, they cannot thrive and survive only for a short period of time (up to 5 days in 14ºC water), ideal for detecting recent fecal pollution (15). *Bacteroides-Prevotella* account for close to one-third of all intestinal flora, and are associated exclusively with fecal waste.

Dr. Katherine Field at the University of Oregon has developed oligonucleotide PCR primers (single stranded DNA complementary to a specific target sequence used in detecting and amplifying sequences of DNA) which may be used to quickly and accurately discriminate between human and ruminant sources of fecal pollution, and is in the process of developing primers for other common, suspect species. Identification of fecal pollution sources is determined based on the presence or absence of distinct bands of PCR products observed after electrophoresis of the amplified DNA.

New primer pairs are being designed to allow for identification of more sources of fecal pollution. However, actions on Dr. Field's part suggest that new primer-pairs may become patented. While new primer-pairs may allow for better identification of pollution sources, they would also multiply the labour involved with testing samples greatly; more amplification reactions would have to be performed, and those extra samples electrophoresed through agarose gels.

This method is currently being used at Environment Canada's Pacific Environmental Science Centre in a joint project between the Shellfish Water Quality Protection Program and Environmental Toxicology sections. Attempts are being made to adapt the technique to a variety of matrices, besides fresh and marine water, like sediment and shellfish-stock.

Advantages.

- Highly reproducible.
- Sensitive.
- Biomarkers appear to be widely distributed.
- Potential for excellent discrimination between species as primer-pairs are designed.
- None or little requirement for a database.
- No requirement for cultivation of fecal indicator.

Disadvantages.

- Presently, can only discriminate between human and ruminant sources of fecal pollution.
- Doesn't give accurate information on level of contribution to fecal pollution. Modification of technique to include quantitative-PCR is a potential, but expensive solution to this problem.
- Labour intensive.
- Low throughput.
- Difficult to optimize: individual labs need to optimize the technique for use with their own equipment before sensitivity is achieved.

AFLP

Amplified Fragment Length Polymorphism analysis is a method of generating DNA fingerprints by analysing arbitrary portions of an entire bacterial genome, whereas many other molecular methods of BST produce DNA fingerprints by analysing a single portion or specific portions (33).

A total genomic DNA preparation is made of each isolate, and digested with restriction endonucleases. To the digested DNA is then ligated oligonucleotide adapters. PCR amplification of the restriction fragments is performed with oligonucleotide primers that anneal to sequences which contain the oligonucleotide adapter, the restriction site, and a small arbitrary length of sequence past the restriction site. It is this small length of sequence which gives specificity to the PCR while circumventing the need for knowledge of the sequence of the restriction fragment which is to be amplified. Patterns generated by the PCR are analyzed using computer software and compared to a database of isolates and identified.

Guan et al. (34) recently performed a comparison study of AFLP, MAR, and 16S rRNA gene sequencing and reported that AFLP was the most effective of the three methods. However, only a small number of isolates were analyzed. Further studies are required to accurately gauge and compare the effectiveness of the AFLP method as a BST tool.

Advantages.

- Highly sensitive to genetic polymorphism.
- Highly reproducible.

- Highly automatable.

Disadvantages.

- Requires a large reference library which may show geographic instability.
- Few field studies have been published using this technique, and unapparent weaknesses in the technology may yet be discovered.
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Chemical Methods

Caffeine Assaying

Caffeine is contained in many beverages such as soft drinks, tea, and coffee, and is also found in some pharmaceuticals like migraine medication. Ingested caffeine exits the body through the urinary tract, and can be found in human sewage. Caffeine in domestic sewage has been found to have a concentration between 20 to 300 μg/L (16). Levels of caffeine in freshwaters and marine waters contaminated by human fecal pollution would be substantially lower due to dilution of the pollution; assays sensitive enough to detect and quantify such low levels of caffeine can be expensive.

Analysis of caffeine directly cannot determine whether caffeine came from urine or gray water sources. However, when caffeine enters the body it can be partially metabolized and these metabolites are present only in urine, or in urine-receiving waters. Since urine and fecal pollution are highly correlated, analysis of water samples for caffeine metabolites may be able to allow for better quantification of water pollution resulting from fecal contamination.

Advantages.

- A quantifiable indicator of human pollution.

Disadvantages.

- Expensive assays required for detection of minute levels in environment.
- Low resolution; only indicates presence of human waste (caffeine metabolites may be able to improve resolution). Coliform counts could be performed in tandem, which would allow for resolution of human and non-human sources with this technique, at the cost of added labour.
- Little is known of the persistence of caffeine (or metabolites) in different matrices.
- Difficult to distinguish between caffeine from urine, and caffeine poured down the sink. Analyses of caffeine metabolites may allow for clarification.

Fecal Stanols

Coprostanol is a catabolic product of cholesterol formed by gut bacteria in higher animals. It is the most abundant stanol detected in human sewage and although it can be found in other animals beside humans (i.e. pigs and cats) it is detected at ten-fold lower levels than in humans (35, 36). Other fecal stanols may prove to be more specific to other sources or source groupings. One such stanol is 24-ethyl-coprostanol, and was found to be highly associated with herbivores such as cows, horses and sheep (36).

Fecal stanols are found naturally in soils and in cases where these sedimentary stanols wash into a water body, interfering background may lower the sensitivity of an assay substantially. Because sensitivity is often already an issue, this interference can limit the usefulness of the technique. Gas Chromatographic-Mass Spectroscopy is used to identify and quantify sterols and stanols and may benefit from improvements to sensitivity and accuracy as more research into the fecal stanol technique is performed.

Advantages.

- Rapid.
- Good resolution of sources; currently able to resolve human and herbivore pollution sources. Further research may improve resolution.
- A quantifiable indicator of fecal pollution.

Disadvantages.

- Low concentrations of stanols are an issue when assaying samples.
- Sterols and stanols naturally present in soil threaten sensitivity in some cases.
- Expensive assays required for detection of minute levels in environment.

Other Methods

Animal Source Tracking (sIgA ELISA)

Currently, the only method to directly detect the source of fecal pollution is to assay for secreted immunoglobulin A (sIgA) antibodies present in contaminated waters (37). sIgA is an antibody class found in mucosal secretions and in the guts of higher animals. Part of its function is to bind microbes in the intestinal lumen and prevent their attachment to the epithelial lining. Thus it can be detected attached to particulate matter, such as bacterial cells, in affected waters.

sIgA is present in very low concentrations in all but the most seriously affected water and must be purified and concentrated. Water samples are centrifuged to pellet particulates to which the sIgA are bound, followed by elution of the sIgA from the particulate matter and recentrifugation to pellet the clear the supernatant of all particulate matter leaving the sIgA in solution. Further concentration is achieved by filtration through a low nominal molecular weight limit filter which traps the sIgA on its surface; washing the filter with a small volume of ELISA incubation buffer dissolves the sIgA once more and readies it for immediate assaying. Using this method, sIgA can be concentrated 500,000-fold, depending on the initial volume of the sample.

A study using the aforementioned method was able to detect sIgA in water samples at concentrations as low as 0.5 femtograms per mL, after taking the concentration factor into effect. That is, the ELISA technique applied had a 1 nanogram per mL sensitivity threshold.

Advantages.

- Sensitive.
- Quantifies contribution of sources in waters affected by multiple types of fecal pollution.
- Very high resolution; can discriminated between sources at the species level.
- Direct and undisputable indicator of exact source of fecal pollution.

Disadvatages.

- High cost per sample due to labour intensity, equipment, and reagent costs.
- Low throughput.
- Reagents used to yield a highly sensitive ELISA appear to be unstable and produce inconsistent sensitivities. Further refinement of the ELISA is necessary to increase precision of technique.

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