

# Bacterial Source Tracking Uncovers Culprits of Contamination in Watersheds



**Criminals may lie to cover up their devious deeds, but their DNA doesn't. Since the late 1980s many criminals have been convicted based upon DNA evidence, while other individuals have been exonerated of crimes due to this same technology.**

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**Now this same concept of DNA "fingerprinting" is being used in many parts of the U.S. to identify the culprits of fecal contamination in watersheds.**

## **Bacterial Source Tracking**

Bacterial Source Tracking (BST) is a relatively new methodology that has been developed and deployed in many parts of the U.S. to identify sources of fecal contamination in watersheds. Fecal bacteria are isolated from water samples, and BST methodology is employed to determine the sources of those bacteria, whether it be human, livestock, or wildlife in origin. The implementation of the Total Maximum Daily Load (TMDL) by the U.S. Environmental Protection Agency (EPA) has been the driving force behind BST development.

A TMDL is a calculation of the maximum amount of a pollutant that a watershed can receive and still meet water quality standards. Under section 303(d) of the 1972 Clean Water Act, states, territories, and tribes are required to develop lists of impaired waters. More than 40 percent of assessed watersheds do not meet the water quality standards these states, territories, and tribes have set for them. These impaired waters include approximately 300,000 miles of rivers and shorelines and approximately 5 million acres of lakes.

In an effort to achieve water quality standards and improve the TMDL program, in 1996 the EPA began a comprehensive evaluation of EPA's

and the states' implementation of their Clean Water Act section 303(d) responsibilities. After this evaluation, new TMDL rules were established requiring states, territories, and tribes to come up with a plan for implementing load allocations for waters impaired solely or primarily by nonpoint sources. Though it was relatively easy to find out if a watershed contained fecal bacteria, in many cases proving who or what were the sources of contamination was much harder, making the implementation of a plan for best management practices (BMPs) difficult. This is where bacterial source tracking has come into play and taken most of the guesswork out of implementation.

## **From Sceptic to Believer**

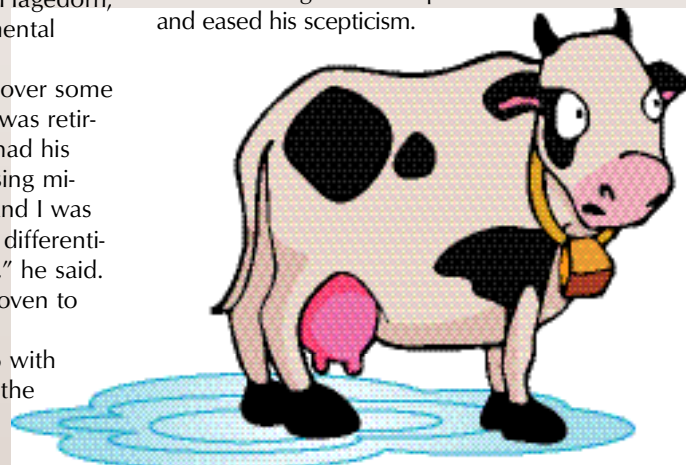
When George Simmons, Ph.D., asked his colleague Charles Hagedorn, Ph.D., professor of Environmental Sciences at Virginia Tech in Blacksburg, Virginia, to take over some of his BST projects since he was retiring, Hagedorn admitted he had his reservations. "I've been chasing microbes for nearly 30 years, and I was just sceptical that you could differentiate fecal bacterial by source," he said. "But I certainly now have proven to myself that you can."

That proof came in 1996 with Hagedorn's first field test of the

Page Brook Watershed in Clarke County, Virginia. The watershed is located in a rural area with an agricultural economy. Walking along the watershed, he observed large numbers of cattle, waterfowl, and horses having unrestricted access to the stream. For Hagedorn, the source of fecal contamination seemed to be a "no-brainer" and just the thing to convince him of the validity of BST. "My goal with this one was to convince myself that source tracking worked," he said.

Even though it appeared that the source of contamination was probably livestock, county officials thought the contamination was instead from failing septic systems and were contemplating an expensive bond issue to hook up 40-some homes in the watershed to public sewer. But the results of the BST moved county officials in a different direction.

After BST was performed, the results confirmed Hagedorn's suspicions and eased his scepticism.



"We never did find a human signature in the stream. The dominant signature was cattle, with high levels of pollution especially in the summer when the animals are in the creek a lot," he said.

### BST Methods

While BST methods are constantly advancing and changing, the three basic types are molecular, biochemical, and chemical. Molecular (genotype) are all referred to as "DNA fingerprinting" and are based on the unique genetic makeup of different strains, or subspecies, of fecal bacteria. The fecal bacteria in any two animals are very much genetically similar. There are unique differences, but the differences are only in a small percentage of an organism's total DNA. The key to molecular BST is finding these differences against a large background of similarity. Some types of molecular BST include Ribotyping, Pulsed-Fielded Gel Electrophoresis (PFGE), and Randomly Amplified Polymorphic DNA (RAPD). (Refer to the chart on pages 28 and 29.)

One commonly used biochemical (phenotype) BST method is Antibiotic Resistance Analysis (ARA). This method uses fecal *Streptococcus* (*Enterococcus*) and/or *E. coli* and patterns of antibiotic resistance for separation of sources. The premise is that human fecal bacteria will have the greatest resistance to antibiotics and that domestic and wildlife animal fecal bacteria will have significantly less resistance to the battery of antibiotics and concentrations used. Other biochemical methods include F-Specific Coliphage and Sterols or Fatty Acid Analysis, and Fecal Bacteria Ratios (see chart on page 28 and 29).

Chemical BST methods do not detect fecal bacteria; instead these methods are designed to detect chemical compounds that are associated with

humans. These chemicals are often found in wastewater effluent. Some of these methods detect optical brighteners that are in all laundry detergents, and there is a method to detect caffeine, which passes through the human digestive system (see chart on pages 28 and 29).

With all BST methods (except chemical), it is necessary to first build a library or database of isolates taken from known sources such as humans, cattle, deer, dogs, etc. The size of the library is partially determined by the number of potential major sources of pollution in the targeted area.

Many researchers in the BST field do not solely use one particular method, but employ a combination of techniques. For example, in the Page Brook watershed, BST was performed on fecal coliform and streptococcal isolates with a combination of tests that included ARA and PFGE procedures. These isolates were then compared to a known source library of fecal samples from beef cattle, dairy cattle, wildlife, chickens, horses, sheep, the influent of the local municipal sewage plant, and human sewage, which were collected from a variety of locations in Virginia.

### Using BST Wisely

Although BST can be a valuable resource for an impaired watershed, it is not mandatory. However, in part due to Hagedorn's urging, the state of Virginia now is requiring BST be performed on every impaired watershed. "I think source tracking should be used in every impairment that's on the nation's impaired waters list where the impairment is due to fecal contamination," Hagedorn said.

Hagedorn cited a perfect example of what can happen when BST is not used and instead officials guess at the cause of contamination. "If it's not used, then you can have a situation like the one that developed in Cottonwood Creek in Idaho. Based on TMDL modeling, regulatory authorities decided that the Cottonwood Creek watershed was contaminated by livestock," he said. "But farmers raised a ruckus at a public meeting, officials reconsidered, and decided it wasn't livestock; rather, it was elk. It didn't seem to matter that during the summer the grazing range of the elk is way up in the mountains far away from the creek. So this is an example of what can happen when you don't use

the best methods available and other types of agendas creep into the process."

In many cases when BST is conducted and the culprits of the fecal contamination are found, implementation of BMPs is often not followed up, according to Hagedorn. "That's a requirement now when you have a TMDL plan in place that's supposed to prevent pollution source as far as possible."

"I examined some counties further managing their implementation to determine if the source tracking was right," he added.

And one of those few examples Hagedorn noted is Page Brook Creek.

### Cleaning up Page Brook Creek

There are about 18 farms in the vicinity of Page Brook Creek, and of those, roughly a dozen have done some type of management to reduce livestock access to the stream. Hagedorn said he has been monitoring the creek regularly, and in areas where farmers have either fenced the stream or limited livestock access to the stream, the cattle signature has disappeared.

Further downstream, Page Brook Creek combines with another stream to form Spout Run. Spout Run is about 3.5 miles long and empties into the Shenandoah River. Along Spout Run is an unsewered community with





Lisa Smith of Marshall University (maroon tank top), Dr. Miriam Kannan of Northern Kentucky University (gray T-shirt), and David Harris of Thomas More College (in water) launch the Monark at Moundsville, West Virginia. Lisa was part of the group studying antibiotic resistant and fecal indicator bacteria.



Veronica Zapata of Northern Kentucky University (left) and Lisa Smith of Marshall University (right) inoculate media in a temporary laboratory (motel room) in Golconda, IL

onsite systems. Hagedorn said that when BST was performed there, a human signature was found. To clean up the watershed, the county has received a block grant through the EPA to expand lines to a nearby sewer plant for the community.

“My hope is that after this treatment plant goes in, we will find that the human signature in the stream has disappeared,” Hagedorn said. “But this is just one of the few cases that I know of where best management practices have gone into play.”

### Other BST Projects

Another Virginia researcher, Bruce Wiggins, Ph.D., professor of Biology at James Madison University in Harrisonburg, developed the ARA technique that several other researchers are currently using. “We analyze the antibiotic resistance patterns of strains of the intestinal bacterium *Enterococcus* to see if different patterns of resistance exist for the bacteria that inhabit humans, cattle, poultry, and wild animals,” Wiggins said. “With this information, we hope to be able to identify the source of fecal pollution in a sample of stream water.”

After Holmans Creek, a tributary of the North Fork of the Shenandoah River in Shenandoah and Rockingham counties in Virginia, was listed as an

impaired watershed in 1996, Wiggins stepped in to conduct an ARA of the watershed. For a year, water samples were taken of the watershed at various times. As Wiggins explained, “Each individual measurement takes around five or six days to get the result. It’s not really that accurate to do a single grab sample; it’s better to do a series of samples over a period of time because there’s variability in the watershed and there’s also variability in our methods. We like to have multiple samples to get a good picture of what’s in there,” he said.

The two primary sources of contamination Wiggins found in the watershed were cattle and human.

Shenandoah County is currently implementing BMPs to alleviate contamination, which includes pumping and repairing septic systems in the vicinity of the creek.

In some ways Virginia is at the forefront when it comes to BST and implementation, but other states are also applying BST. BST has been used in watersheds in Wyoming, New York, Washington, and Maine, just to name a few.

The Department of Forensic Science and Microbiology at Marshall University in Huntington, West Virginia in collaboration with the West Virginia Department of

Agriculture in Moorefield, has been working for the past four years on an *E. coli* database of known sources in a six-county area encompassing 3,490 square miles. The database consists of PFGE-generated DNA fingerprints and contains 3,598 entries.

Pam Staton, Ph.D., associate professor of the Forensic Science Program and co-investigator, said that the project began after several watersheds in West Virginia were placed on the biologically impaired list, including the Potomac River. There was controversy as to whether or not the source of contamination was coming from Moorefield, West Virginia, where 340,000 birds are slaughtered daily in the region’s largest chicken plant. These chickens also produce nearly 155,000 tons of waste a year.

“The project began by collecting scat samples in the Potomac River region where there was an issue about high fecal contamination in the river and whether or not it was due to poultry, wildlife, or humans,” Staton said. “Marshall University’s participation in the BST project was initiated by Terry Finger, who joined forces with West Virginia Department of Agriculture and later the U.S. Department of Agriculture, to gather BST information to provide information needed by administrators and



**Joey Van Skaik, a student at Thomas More College in Kentucky, looks for zebra mussels on rocks gathered from the Ohio River during a survey. Other water quality indicators, such as what types of aquatic life inhabit the body of water, are sometimes used in correlation with the microbiology data.**

legislators to address the origin of fecal pollution in these waterways.”

Staton reported that the Marshall University Department of Agriculture research team has completed two regional databases in West Virginia and nearly ready to analyze water samples in Moorefield using the PFGE technique. Until then, the guilt or innocence of the chickens remains a mystery.

### **Cost and Effectiveness of BST**

There is some controversy over the effectiveness of BST methods, and this has prompted the EPA and U.S. Geological Survey to begin comparison studies of various BST methods to find out which is most effective. The general consensus is that biochemical BST methods are simpler, faster, less expensive, and allow large numbers of samples to be analyzed in a short period of time. Molecular BST methods may offer the most precise identification of specific types of sources, but are limited by high per-isolate cost and detailed, time-consuming procedures.

Charles Somerville, Ph.D., associate professor of Biological Sciences at Marshall University, has been applying an alternative form of ARA to the Ohio River. He said that with this form of ARA, the cells are enumerated from

the total cultivable bacterial community that are resistant to a set of antibiotics. Somerville said that the cost of testing a site is \$15, and the results of the test are usually completed in a week. “It’s a quick and relatively cheap survey of a water body to determine where the hot spots are, and since we are testing against different antibiotics, we think we can get a very crude idea of the contamination,” he said. Since ARA may only give a crude indication of what the contamination is, Somerville said that Fecal Bacteria Ratios are taken and then molecular BST techniques are also applied through Marshall laboratories.

Hagedorn believes that no one technique is clearly the best in every situation. “I think it’s going to be a toolbox approach, and you’re going to reach into that toolbox and pull out a variety of methods depending upon the question you need answered,” he said.

As for cost, from his experience, Hagedorn reported that in Virginia, depending on the size of the watershed, the cost can run anywhere from \$20,000 to \$30,000 per watershed BST. He said that many of these projects are funded through EPA 319 (h) funds.

### **The Future of BST**

As with any technology, BST will evolve, change, and be refined. But as

for the future use of the BST technique, Hagedorn thinks that remains to be seen. “I think the future is going to depend on federal regulatory agencies and whether or not they are going to continue the TMDL process with phase two, which would be implementation, because that is when source tracking is really going to be essential.”

When it comes to cleaning up a watershed, it is important to know the source of contamination, as Hagedorn explained. “When you go back to communities and tell them they are going to have to start spending big money to deal with livestock or to reduce the impact of waste from dogs or to upgrade a wastewater treatment plant or to fix failing septic systems—now that’s when you’re talking big ticket, and you’re going to want to use source tracking to make absolutely sure that you’re spending money on the right sources and that is when BST is really going to come to the forefront.”

For more information on BST contact Hagedorn at (540) 231-4895 or [chagedor@vt.edu](mailto:chagedor@vt.edu), Wiggins at (540) 568-6196 or [wigginba@jmu.edu](mailto:wigginba@jmu.edu), Staton at (304) 696-7343 or [staton1@marshall.edu](mailto:staton1@marshall.edu), and Somerville at (304) 696-2424 or [somervil@marshall.edu](mailto:somervil@marshall.edu). ■

# ***BST Methods***

## ***Molecular BST Methods (Genotype)***

### **Ribotyping**

Ribotyping involves the bacterial genes that code for ribosomal RNA. With this method, the total genomic bacterial DNA are cut with different restriction enzymes, followed by gel electrophoresis. Following electrophoresis, Southern blotting is performed to blot the DNA bands onto nylon membranes from the gels. Membrane hybridization is then performed to hybridize the probes with the appropriate DNA bands on the nylon filter. Differences in the size and location of the ribosomal RNA bands on the filters can then be used to differentiate between the sources that the fecal bacteria came from.

### **Pulse Field Gel Electrophoresis (PFGE)**

The PFGE method, developed commercially by BioRad, uses a gel apparatus where electric current is passed through the gel in different directions at low voltage for 10 to 12 hours to achieve the best level of band separation. Another modification of PFGE involves embedding the bacterial DNA in an agarose plug. The DNA is digested while in the plug, and the plugs are placed in hollow gel combs and become part of the gel as the gel is cast around the combs. Gels are then stained and photographed after electrophoresis.

### **Randomly Amplified Polymorphic DNA (RAPD)**

The RAPD method involves identifying unique polymorphisms within the DNA of fecal bacteria. Arbitrary primers are used to identify randomly selected polymorphisms, and amplification occurs via polymerase chain reaction (PCR). Performing RAPD first involves DNA isolation, purification, and quantification; followed by addition of primers to the DNA, then amplification with PCR followed by gel electrophoresis. This method requires screening primers to find sets of polymorphisms that are either unique to fecal bacteria from a given source or occur in a given source to a large and predictable degree. Once such sets of polymorphisms have been found, fecal bacteria can be "sourced" by comparison.

## ***Chemical BST Methods***

### **Optical Brighteners**

This method detects the optical brighteners that are in all laundry detergents. They are persistent in the environment and are detected using low-tech black lights or mass spectroscopy. Sample collection is accomplished by placing optical brightener-free cotton in a wire mesh trap and placing the trap in the stream for a few days. After the trap is recovered the cotton is examined with a black light to see if it glows. The fluorescent cotton can then be examined with mass spectroscopy to verify the presence of the compounds. It is postulated that if these chemicals can be detected, there must be a human source. The problem with persistent chemicals is that they may not reflect recent pollution.

### **Caffeine Detection**

This method is currently being developed to identify areas where a human source is suspected, which can result in a low-cost test that can be used to detect caffeine in a water sample. Caffeine passes through the human digestive system and could be used as an indicator chemical. The major problem with this method now is that it is expensive, running approximately \$100 per sample. Also, there are some other plants that have significant levels of caffeine (e.g., watermelon), and could confuse results. Lastly, caffeine is easily degraded by soil microbes, so it is not known what proportion of human sources actually contain detectable levels of caffeine.

## **Biochemical BST Methods (Phenotype)**

### **Antibiotic Resistance Analysis (ARA)**

This method uses fecal *Streptococcus* (including the *Enterococci*) and/or *E. coli* and patterns of antibiotic resistance for separation of sources. The premise is that human fecal bacteria will have the greatest resistance to antibiotics and that domestic and wildlife animal fecal bacteria will have significantly less resistance (but still different) to the battery of antibiotics and concentrations used. Most investigators are testing each isolate on 30 to 70+ antibiotic concentrations. Fecal bacteria are grown in wells in microtiter trays and then replica-plated onto a series of agar plates, each containing one specific antibiotic concentration. Forty-eight cultures can be transferred to each agar plate simultaneously with a stainless steel replicator. After incubation, each isolate is scored for growth or no growth on each plate, and a resistance pattern emerges that can be used in source differentiation.

### **F-Specific (F+ or FRNA) Coliphage**

Considered perhaps more indicative of viral contamination, the FRNA coliphages are pathogens of *E. coli* and infect the pilus of male *E. coli* strains. These coliphages can be differentiated using serology. There are four antigenically distinct serogroups of FRNA coliphages, and those predominating in humans (groups II and III) differ from those predominating in animals (groups I and IV). Hence, it may be possible to distinguish between human and animal wastes by serotyping FRNA coliphage isolates. However, there is a problem with separation between human serotypes and serotypes associated with pigs, which also contain group II. Additionally not all animals have FRNA coliphage associated with their respective *E. coli*. The coliphage is persistent in the environment for less than a week and survival is a function of sunlight and water temperature. Ultra-violet light denatures the virus, and below 25 degrees C, F-pilus synthesis ceases. The coliphage does not replicate in the environment, only in the presence of F-pilus *E. coli*, and is not found in sediments, just in the water column. DNA fingerprinting of FRNA coliphages may be able to resolve some of the problems with serological typing.

### **Sterols or Fatty Acid Analysis**

Sterols are constituents of the fatty acids in cell walls and membranes. This method is designed to differentiate between the types and quantities of sterols in human *E. coli* cell walls and membranes versus those in other animals. This method is currently under development and there are no published reports of its use in fecal sourcing. Access to gas and/or HPLC chromatography equipment is required to perform fatty acid analysis. Fatty acids are first converted to fatty acid methyl esters (FAMES) by chemical methods prior to performing gas chromatography.

### **Nutritional Patterns**

This technique is based on differences among bacteria in their use of a wide range of carbon and nitrogen sources for energy and growth. This method works well in the laboratory. However, there are many environmental factors in a watershed that can affect bacterial nutrient requirements that may make this method impractical for field determination. The BIOLOG system allows the user to rapidly perform, score, and tabulate 96 carbon source utilization tests per isolate and is widely used in the medical field for microbial identification. Another modification of the nutritional pattern concept is the use of human-specific (sorbitol-fermenting) bifidiobacteria as indicators of nonpoint source human fecal pollution.

### **Fecal Bacteria Ratios**

This procedure is based on the ratios (presence and numbers) of many different types of stomach and intestinal bacteria, not just fecal coliform bacteria, to develop a ratio coefficient that could be useful in source identification. While the traditional fecal coliform-fecal streptococcus ratio is no longer considered reliable for accurate source identification, ratios may still be useful as a general indicator of human versus nonhuman fecal bacterial contamination, and the ratio concept could perhaps be found more reliable if other microbes were used in developing the ratios (e.g. *Bacteroides*, *Prevotella*, and *Clostridium*).