June 10, 2005

Virginia Tech Microbial Source Tracking Program
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Introduction

There are five different Microbial Source Tracking (MST) procedures that have been developed and used in the Virginia Tech Microbial Source Tracking Program. Which methods are selected for any given project depends upon the needs of the specific project and the goals of the sponsors. The five methods are detailed below.

The most stringent of the MST method comparison studies done to date (Stoeckel et al., 2004) concluded that the best approach to using MST was a) perform adequate QA/QC on host origin libraries; b) employ more than one MST method; and c) include an alternate tracer. My approach to research projects follows these USGS recommendations always using at least two MST methods and detection of optical brighteners in detergents as an alternative tracer (an indicator of human-derived pollution). Appropriate QA/QC methods for host-origin libraries were detailed by Kerry et al., 2003, and include use of multiple statistical procedures and library evaluation with both a validation and a challenge set of isolates. These sets are developed new for each project and results are made available to sponsors.

Stoeckel, D. M., M. V. Mathes, K. D. Hyer, C. Hagedorn, H. Kator, J. Lukasik, T. L. O'Brien, M. Samadpour, K. M. Strickler, and B. A. Wiggins. 2004. Direct comparison of seven protocols using *Escherichia coli* to identify fecal contamination sources. Environ. Sci. Tech. 38:6109-6117.

Ritter, K. J., E. Carruthers, C. A. Carson, R. D. Ellender, C. Hagedorn, V. J. Harwood, K. Kingsley, C. Nakatsu, M. Sadowsky , B. Shear, B. West, J. E. Whitlock, B. A. Wiggins and J. D. Wilbur. 2003. Assessment of statistical methods used in microbial source tracking. J. Water Health 01:209-224.

MST Methodologies

1. Phenotypic Expression Systems - Antibiotic Resistance Analysis (ARA)

ARA has been performed on the enterococci, fecal coliforms, and *E. coli* (Booth et al., 2003; Graves et al., 2002; Hagedorn et al., 1999). This method relies on different antibiotic resistance patterns in fecal bacteria that can be related to specific sources of fecal pollution, and is predicated on the rationale that antibiotics exert selective pressure on the fecal flora of the animals that ingest or are treated with the antibiotic(s), and that different types of animals receive differential exposure to antibiotics. Resistance patterns are highest in humans, moderate in livestock, pets, and poultry, and low in birds and wildlife. Benefits of ARA include use of simple laboratory techniques, requiring only basic equipment, and can be performed at a relatively low cost compared to most other MST methods.

To date, ARA has been used in more source tracking projects in the U.S. than any other method. In addition, high levels of separation between known source bacterial isolates have been found comparable to those reported for molecular methods. The ARA procedure detailed in the following four publications and six theses/dissertations is the same procedure used by MapTech. Mrs. Julie McKinney, Lab Manager at MapTech, received her M.S. degree from my program.

Hagedorn Publications using ARA

Hagedorn. C., S. A. Robinson, J. R. Filtz, S. M. Grubbs, T. A. Angier, and R. B. Reneau, Jr. 1999. Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in the fecal streptococci. Applied & Environmental Microbiology. 65:5522-5531.

Graves, A. K., C. Hagedorn, A. Teetor, M. Mahal, A. M. Booth, R. B. Reneau, Jr. 2002. Determining sources of fecal pollution in water for a rural Virginia watershed . J. Environ. Qual. 31:1300-1308.

Booth, A. M., C. Hagedorn, A. K. Graves, S. C. Hagedorn, and K. H. Mentz. 2003. Sources of fecal pollution in Virginia's Blackwater River. J. Environ. Engineering 129:547-552.

Harwood, V. J., B. Wiggins, C. Hagedorn, R. D. Ellender, J. Gooch, J. Kern, M. Samadpour, A. H. Chapman and B. J. Robinson. 2003. Phenotypic library-based microbial source tracking methods: efficacy in the California collaborative study. J. Water & Health 01:153-156.

Hagedorn – Graduate Student Theses and Dissertations using ARA

Graves, A. K. 2000. Determining Sources of Fecal Pollution in Water for a Rural Virginia Community. M.S. Thesis, CSES, May 2000.

Bowman, A. M. 2001. Determining Sources of fecal Pollution in the Blackwater River Watershed, Franklin County, Va. M.S. Thesis, ESEN, August 2001.

Graves, A. K., Identifying Sources of Fecal Pollution in Water as a Function of Sampling Frequency Under Low and High Stream Flow Conditions. Ph.D. Dissertation, CSES, August 2003.

Szeles, C. L., Determining Sources of Fecal Contamination in Two Rivers of Northumberland County, Virginia. M.S. Thesis, CSES, August 2003.

Porter, K. R., Identifying Sources of Fecal Pollution in Washington D.C. Waterways. M.S. Thesis, ESEN, December 2003.

McKinney, J. M. Identifying Sources of Fecal Pollution in the Appomattox River Watershed. M.S. Thesis, CSES, May 2004.

2. Carbon Utilization Patterns (CUP) and *Enterococcus* species profiles

The CUP system is based on nutrient utilization profiles (or fingerprints). It is a nearly foolproof system because it uses an electronic plate reader that removes judgment decisions by laboratory personnel when evaluating plates (Hagedorn et al., 2003). In the CUP system, each well in a 96-well microplate contains a single nutrient source (one well is a water blank) and a metabolic dye (tetrazolium violet). Each isolate of *E. coli* or *Enterococcus* is grown for 12 to 24 hrs at 37^{o}C on commercial Blood Agar (BBL) and then diluted to a standardized concentration in a liquid medium that contains all nutrients for growth except a carbon source. The isolate from the Blood Agar plate is transferred to a liquid medium with a sterile cotton swab, and enough is transferred to reach an optical density of 0.23 to 0.25 absorbance on a spectrophotometer. Then, 150 µl of the liquid medium is added to each of the 96 wells in a commercial Biolog System plate with an automated 8-row pipettor. After incubation for 12 to 24 hrs at 37^{o}C, a color forms (from the metabolic dye) in any well where the isolate was able to use the carbon compound in that well and grow. The pattern of positive wells, out of a total of 95, is used as a metabolic profile. Positive wells are recorded as growth (1) and clear wells as no growth (0). The results are determined and recorded with an electronic plate reader connected to a computer. The patterns of positive and negative wells is compared against the Biolog library, and identification of *Enterococcus* includes a species designation that is used in the source-related profiles (*Ent. faecalis* and human sources, Wheeler et al., 2002). The positive/negative well patterns are also used to determine sources by comparison against a known-source library.

Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson, and R. B. Reneau, Jr. 2003. Carbon source utilization profiles as a method to identify sources of fecal pollution in water. J. Appl. Microbiol. 94:1-8.

Wheeler, A. L., P. G. Hartel, D. G. Godfrey, J. L. Hill, and W. I. Segars. 2002. Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. J. Environ. Qual., 31:1286-1293.

3. DNA Fingerprint Systems - Pulsed-Field Gel Electrophoresis (PFGE)

The PFGE procedure is the same as that reported by Simmons (Simmons and Herbein, 1998; Simmons et al., 1995; Simmons et al., 2000). Pulsed field gel electrophoresis differentiates closely related isolates of the same species by detection of variations in the position of chromosomal restriction sites. In this technique chromosomal DNA is carefully extracted and cleavage of the DNA is carried out using a "rare cutting" restriction enzyme such as *Not*I. The discrete fragments of DNA are separated using pulsed-field gel electrophoresis, which resolves the fragments into distinct bands. The gel is nonspecifically stained with a dye such as ethidium bromide, allowing comparison of the banding pattern of various isolates. The molecular weight of each DNA band is then determined by comparison with a standard DNA ladder. The banding pattern of a particular isolate is the set of variables that is analyzed by statistical analysis. Dr. George Simmons closed his research laboratory in the Biology Department at Virginia Tech in July 2000. His PFGE library, *E. coli* culture collection (some 6,800 isolates from known sources) and BioRad PFGE equipment is now located in Hagedorn's laboratory. The culture collection contains a large selection of isolates from wildlife sources plus some from humans, pets, and livestock. This collection served as the basis for a much larger *E. coli* PFGE library and a new *Enterococcus* library that has been developed in our laboratory. PFGE has now been used as a cross-validation tool in numerous source tracking projects around the U.S.

For source tracking purposes, the gene expression and PFGE profiles are analyzed by discriminant analysis (DA) using JMP-In™ statistical software (version 5.1, SAS Inc). First, known source isolates are analyzed and placed in various categories such as human, livestock, wildlife, or cow, deer, horse, waterfowl, etc., depending on the level of classification desired when developing the known source library. Discriminant analysis (DA) assigns a predicted source to each isolate: human and animal in a 2-way split, and human, livestock, urban (dogs and cats), or wildlife in a 4-way split, or a larger split based on specific sources (deer, cow, goose, etc.) as a result of the fingerprints of the isolates in the library. Unknown source isolates (isolates from water samples) are then compared against the library to classify them by source, with the match probability set at 80% or greater correct rate. PFGE was determined to be the top method in the USGS comparison study (Stoeckel et al., 2004), and was one of the two top methods (with ribotyping) in the SCCWRP study (Myoda et al., 2003).

Simmons, G. M., Jr., S. A. Herbein, and C. M. James. 1995. Managing nonpoint fecal coliform sources to tidal inlets. Water Resource Update 100:64-74.

Simmons, G. M., Jr., and S. A. Herbein. 1998. Shellfish and water column comparison of fecal coiform diversity using *NotI* DNA fingerprints of *Escherichia coli* generated by puLse field gel electrophoresis. Final Report for the Virginia Coastal Resource Management Program Department of Environmental Quality, Richmond, VA.

Simmons, G. D. Waye, S. Herbein, S. Myers, E. Walker. 2000. Estimating nonpoint fecal coliform sources in northern Virginia's Four Mile Run Watershed. Presented at Virginia Water Research Symposium 2000, Blacksburg, VA, Younos, T., Poff, J., Eds.; VWRRC Special Report SR-19-2000.

Stoeckel, D. M., M. V. Mathes, K. D. Hyer, C. Hagedorn, H. Kator, J. Lukasik, T. L. O'Brien, M. Samadpour, K. M. Strickler, and B. A. Wiggins. 2004. Direct comparison of seven protocols using *Escherichia coli* to identify fecal contamination sources. Environ. Sci. Tech. 38:6109-6117.

Myoda, S. P., C. A. Carson, J. J. Fuhrmann, Byoung-Kwon Hahm, P. G. Hartel, H. Yampara-Iquise, LeeAnn Johnson, R. L. Kuntz, C. H. Nakatsu, M. J. Sadowsky and M. Samadpour. 2003.

Comparison of genotypic-based microbial source tracking methods requiring a host origin database. J. Water Health 01:167-180.

4. Chemical Detection Systems - Fluorometry

A separate project (sponsored by NOAA) evaluated the use of a fluorometer in estuarine and coastal zone environments to determine if the equipment could detect a human waste signature. The fluorometer detects compounds that fluoresce under ultraviolet light such as fecal sterols, detergent surfactants and optical brighteners. Optical brighteners in laundry and dishwashing detergents fluoresce when exposed to certain ultraviolet wavelengths, so water samples that fluoresce under those same wavelengths are contaminated by residues from laundry and dishwashing detergents (human sources). There are at least two major potential human sources of contamination that could contain optical brighteners, and these include leachates from improperly functioning on-site wastewater systems (OWS) and leaking pipes from community wastewater treatment systems. In rural areas where the majority of homes are served by on-site systems, optical brighteners in water samples indicate failing conditions within OWS in close proximity to the sampled bodies of water.

Detectors from different manufacturers were evaluated, and the portable fluorometer from Turner Designs, Inc., performed the best in both laboratory and field tests. The detector located fluorescent plumes in water samples taken in coastal rivers where a human signature was known to exist based on microbial source-tracking results. Additionally, the detector correctly identified samples in controlled laboratory tests that had been spiked with detergents and/or septage. Samples without septage or detergents (or containing detergents without optical brighteners) all failed to fluoresce. The instrument was then used successfully on a variety of waterways (both salt and fresh) where human sources of pollution were suspected or could be confirmed with microbial source tracking technology. In larger bodies of water, fluorescent plumes could be identified and mapped with the fluorometer, and then traced back to the shore and directly to homes that appeared to be the source of the pollution. The fluorescent signals appeared to be stable over seasons, storage in refrigeration for at least four months, and over different water conditions. Whenever fluorescent plumes were found, microbial source tracking tests demonstrated a human signature in every case where source tracking was performed.

Hagedorn, C., and R. B. Reneau, Jr. 2002. Identifying Sources of Fecal Pollution Based on Detection of Optical Brighteners. Progress Report to the National Oceanic and Atmospheric Administration (available upon request).

5. DNA Fingerprint Systems - *Escherichia coli fim* Gene and *Enterococcus esp* Gene Polymorphisms

Approximately 2,000 base pairs (bp) of bacterial DNA encompassing several *fim* genes, genes responsible for the production of fimbriae which allow attachment of the bacterium to a host, have been sequenced. Several unique DNA polymorphisms have been found and are being exploited as a new and novel MST tool. DNA primers were developed that amplify an approximately 257bp region of DNA encompassing several polymorphisms, recognized by specific restriction enzymes. While it has been determined that the polymorphisms assessed thus far are not entirely animal specific, they may be useful in the pursuit of a 'cassette' or 'tool box' of sequence-based MST methods, and one polymorphism with a high degree of human specificity has been located and is under evaluation at present. Due to its simplicity and reproducibility, this sequence-based tool, or one like it, may help with the precision of current or future MST methods. The results to date show that *fim* genes are an important region of genetic variation, but also that the differences present within them may yield a faster, more efficient sequence-based bacterial source tracking method that will eliminate the need for host-origin libraries. Provided that the T*aiI* gene site yields results, this combination of source-specific DNA polymorphisms in one PCR product could assist with current methods to yield better correct classification rates. Sequence-based methods which are rapid and cost-effective will undoubtedly assist with or even overshadow library-based methods in the near future.

For the *Enterococcus esp* gene, it is under development as a genetic sequence that is capable of identifying sources of human fecal pollution in the environment. We have worked with a company in Florida to develop this approach (see following publication), and this approach should be considered **confidential** until we have completed testing this summer.

Scott, T., T. M. Jenkins, J. Lukasik, and J. B. Rose. 2005. Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. Environ. Sci. Technol. 39:283-287.

We have made one presentation to document our progress as we develop this new technology.

Crozier, B., M. Salama, A. Hinlicky, C. Hagedorn, and A. Strand. 2005. Use of *Escherichia coli fim* Gene Polymorphisms as a Bacterial Source Tracking Tool. 105th Annual Meeting of the American Society for Microbiology, Atlanta, GA, ASM Abstracts.

Brief Vitae of the Program Director

Dr. Charles Hagedorn
Professor of Environmental Microbiology
Department of Crop and Soil Environmental Sciences, Virginia Tech (VPI & SU)

Education

Ph.D. Degree, Microbiology, Iowa State University, 1974
M.S. Degree, Microbiology, Iowa State University, 1972
B.S. Degree, Biology, Kansas State University, 1970

Professional Experience

Professor of Crop and Soil Environmental Sciences, VPI & SU, Blacksburg, VA. 1987 – present.
Manager, Crop Biotechnology Program, Allied-Signal Corporation, Syracuse, NY, 1983-87.
Associate Professor of Agronomy, Mississippi State University, Starkville, MS, 1979-83.
Assistant Professor of Microbiology, Oregon State University, Corvallis, OR, 1974-79.

Honors Received

Distinguished Service Award, USDA, for service to the National Biological Impact Assessment Program (NBIAP).
Outstanding Service Award, U.S. EPA, for service on the Biotechnology Science Advisory Committee (BSAC).
Distinguished Service Award, American Society for Microbiology, for service on Editorial Board of *Applied and Environmental Microbiology*.
Outstanding Service Award, National Onsite Wastewater Recycling Association, for service on the Board of Directors.

Qualifications

My research and scientific expertise has been recognized by awards of 51 state, private, and federal competitive research grants; publication of 93 refereed journal articles; 16 invited review articles; 7 invited book chapters; co-editor of one book; 34 invited presentations at international, national, and state conferences; 17 invited memberships on proposal review panels; 12 refereed bulletins; and 112 abstracts and presentation papers. Eleven Ph.D. and eighteen M.S. students have completed degrees under my direction and I have generated in excess of $3,245,000 in external grants and contracts to support my research program that covers environmental microbiology, including public health, microbial pathogens

in the environment, waste management, the impact of releasing genetically modified organisms into the environment, and determining sources of fecal pollution in water.

Over the past twelve years, I have been involved in the development of microbial source tracking methods, and have deployed these methods to determine sources of fecal pollution in 40+ projects in Virginia and 14 in other states. My research program on source tracking has been supported by competitive awards from USDA-NRI, EPA, NOAA, and USGS.

Teaching

CSES/BIOL 4684, Environmental Microbiology
CSES 4644, Land-Based Waste Treatment Systems

Patents

Water Sample Viral Contamination Detection System. U.S. Patent No. 5,527,667, Issued 06/96.

Related Publications, 93 total, most recent from 1999:

Hagedorn. C., S. A. Robinson, J. R. Filtz, S. M. Grubbs, T. A. Angier, and R. B. Reneau, Jr. 1999. Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in the fecal streptococci. Applied & Environmental Microbiology. 65:5522-5531.

Huang, J., R. B. Reneau, Jr., and C. Hagedorn. 2000. Nitrogen removal in constructed wetlands employed to treat domestic wastewater. Water Research. 34:2582-2588.

Graves, A. K., C. Hagedorn, A. Teetor, M. Mahal, A. M. Booth, R. B. Reneau, Jr. 2002. Determining sources of fecal pollution in water for a rural Virginia watershed . J. Environ. Qual. 31:1300-1308.

Hagedorn, C., J. B. Crozier, K. A. Mentz, A. M. Booth, A. K. Graves, N. J. Nelson, and R. B. Reneau, Jr. 2003. Carbon source utilization profiles as a method to identify sources of fecal pollution in water. J. Appl. Microbiol. 94:1-8.

Booth, A. M., C. Hagedorn, A. K. Graves, S. C. Hagedorn, and K. H. Mentz. 2003. Sources of fecal pollution in Virginia's Blackwater River. J. Environ. Engineering 129:547-552.

Harwood, V. J., B. Wiggins, C. Hagedorn, R. D. Ellender, J. Gooch, J. Kern, M. Samadpour, A. H. Chapman and B. J. Robinson. 2003. Phenotypic library-based microbial source tracking methods: efficacy in the California collaborative study. J. Water & Health 01:153-156.

Stoeckel, D.M., Mathes, M.V., Hyer, K.E., Hagedorn, C., Kator, H., Lukasik, J., O'Brien, T., Fenger, T.W., Samadpour, M., Strickler, K.M., and Wiggins, B.A., 2004, Comparison of seven protocols to identify fecal contamination sources using *Escherichia coli*. Environmental Science and Technology, v. 38, no. 22, p. 6109-6117.

Recent Presentations

Hassall, A., and C. Hagedorn. 2004. Sources of fecal pollution in four mixed-use watersheds in Prince William County, Va. 104th Annual Meeting of the American Society for Microbiology, New Orleans, La. ASM Abstracts.

McKinney, J., and C. Hagedorn. 2004. Identifying sources of fecal pollution in the Appotomattox River Watershed. 104th Annual Meeting of the American Society for Microbiology, New Orleans, La. ASM Abstracts.

Herbein, S., and C. Hagedorn. 2004. Fecal *E. coli* and *Enterococcus* isolated from two humans and three companion animals compared by pulsed-field gel electrophoresis. 104th Annual Meeting of the American Society for Microbiology, New Orleans, La. ASM Abstracts.

Hagedorn, C., A. H. Chapman, S. Herbein, M. Saluta, and P. McClellan. 2004. Microbial source tracking as a technology for identifying sources of fecal pollution in water. 4th National Conference on Science, Policy, and the Envrionment, National Council for Science and the Environment. Washington, D.C.